ROLE OF NRF2 IN DISEASE: NOVEL MOLECULAR MECHANISMS AND THERAPEUTIC APPROACHES

EDITED BY: Javier Egea, Carmen Gomez-Guerrero,
Águeda González Rodríguez and Juan Antonio Moreno

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Topic Editors:
Javier Egea, Institute of Health Research of the University Hospital of La Princesa, Spain
Carmen Gomez-Guerrero, Autonomous University of Madrid, Spain
Águeda González Rodríguez, Institute of Health Research of the University Hospital of La Princesa, Spain
Juan Antonio Moreno, Universidad de Córdoba, Spain

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Editorial: Role of Nrf2 in Disease: Novel Molecular Mechanisms and Therapeutic Approaches

Javier Egea[1,2†], *Águeda González-Rodríguez*[3,4†], *Carmen Gómez-Guerrero*[5,6†] *and Juan Antonio Moreno*[7,8,9,10*†]

[1] Molecular Neuroinflammation and Neuronal Plasticity Research Laboratory, Hospital Universitario Santa Cristina, Instituto de Investigación Sanitaria-Hospital Universitario de la Princesa, Madrid, Spain, [2] Instituto Teófilo Hernando, Departamento de Farmacología y Terapéutica, Facultad de Medicina, UAM, Madrid, Spain, [3] Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Instituto de Salud Carlos IIII, Madrid, Spain, [4] Unidad de Investigación Hepática, Hospital Universitario Santa Cristina, Instituto de Investigación Sanitaria del Hospital Universitario de La Princesa, Madrid, Spain, [5] Renal and Vascular Inflammation Lab, Health Research Institute-Fundacion Jimenez Diaz (IIS-FJD), Autonoma University of Madrid (UAM), Madrid, Spain, [6] Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain, [7] Department of Cell Biology, Physiology and Immunology, University of Cordoba, Cordoba, Spain, [8] Maimonides Biomedical Research Institute of Cordoba (IMIBIC), University of Cordoba, Cordoba, Spain, [9] Hospital Universitario Reina Sofia, Cordoba, Spain, [10] Centre of Biomedical Research in Network of Cardiovascular Disease (CIBERCV), Madrid, Spain

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Edited by:
Paola Patrignani,
Università degli Studi G. d'Annunzio Chieti e Pescara, Italy

Reviewed by:
Annalisa Trenti,
University of Padova, Italy

***Correspondence:**
Juan Antonio Moreno
juan.moreno@uco.es

†These authors have contributed equally to this work

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Editorial on the Research Topic

Role of Nrf2 in Disease: Novel Molecular Mechanisms and Therapeutic Approaches

Oxidative stress and inflammation are pathological processes involved in the genesis and development of numerous disorders. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that regulates the expression of multiple antioxidant and cytoprotective proteins and enzymes, thus playing a key role in protection against oxidative damage. There is a growing interest in understanding the specific role of Nrf2 in the development and progression of diseases as well as design novel approaches targeting Nrf2 to prevent and/or retard tissue injury.

This special issue contains 16 papers, including 7 original research articles and 9 reviews, reporting important data about the key role of Nrf2 in different pathological conditions.

In the article entitled "Nrf2 as a potential mediator of cardiovascular risk in metabolic diseases" by da Costa et al., a reduction in Nrf2 activity in diabetes, obesity, and atherosclerosis, thus increasing cardiovascular risk, was described. Importantly, systemic administration of specific Nrf2 inducers provided beneficial effects in cardiovascular diseases (da Costa et al.). In this sense, a wide range of natural products are promising therapeutic approaches to reduce atherogenesis progression by targeting the Nrf2 signaling pathway. These compounds activate Nrf2 by multiple mechanisms, such as targeting cysteine residues of Keap-1, disturbance of Nrf2/Keap1 interaction, epigenetic modification, and activation of protein kinases pathways. The beneficial effects of bioactive compounds are related to a reduction in reactive oxygen species (ROS) production, augmentation of glutathione levels, inhibition of nuclear factor kappa B inflammatory signaling pathway, and lowering foam cell production (Ooi et al.). Nrf2 activation endows antioxidant defense and reduces atherosclerosis risk in diabetes, where vascular lesions are a common complication of this disease. Tert-butyl hydroquinone (tBHQ) increased Nrf2 activation in vascular smooth cells and macrophages. Treatment with tBHQ

decrease the size and extension of the atheroma plaque, besides lipid content, inflammation, foam cell size, and chemokine expression. An increase in the autophagic flux in the aorta and an upregulation of autophagy-related molecules after tBHQ treatment were also observed (Lazaro et al.). Another complication of chronic hyperglycemia is related to cognitive dysfunction. Nrf2 is involved in cellular homeostasis during diabetes mellitus. Astaxanthin, a potent antioxidant, exerted a protective effect on cognitive function through the inhibition of oxidative stress and inflammatory responses by the elevation of the Nrf2 antioxidant responsive element signaling pathway in the brains of chronic diabetic rats (Feng et al.). Nrf2 activation has been proposed as an antidiabetic strategy. In this context, naringenin, a bioactive flavonoid found in citrus fruits, protected pancreatic beta cells from oxidative damage *in vitro* and in streptozotocin-induced diabetic mice by activating Nrf2. Naringenin treatment decreased blood glucose levels, normalized lipid profile, restored insulin expression, promoted glycolysis, and inhibited gluconeogenesis in streptozotocin-induced diabetic mice (Rajappa et al.).

Oxidative stress and inflammation play a key role in the central nervous system physiology and pathophysiology of different diseases. The brain is highly susceptible to oxidative damage, so modulation of Nrf2 is important to provide beneficial effects in cerebrovascular diseases like ischemic stroke (Liu et al.). Free radical accumulation, due to impaired cellular antioxidant defenses or excessive production, results in neurotoxicity and cell death. Aging leads to a gradual increase in brain oxidative stress, which is accompanied by reduced antioxidant defenses, and a reduction in Nrf2 activity. Nrf2 could be modulated by dietary interventions (dietary energy restriction and high energy consumption) in animal models of neurological disorders, which leads to improving cognitive function, metabolic health, and longevity (Vasconcelos et al.). As oxidative stress is an important contributor of age-related macular degeneration, an increase in the antioxidant defenses can provide novel therapeutic strategies for this disease. Beneficial effects of Nrf2 activation have been described in retinal cells exposed to different types of oxidative stress inductors, so several natural and synthetic activators of Nrf2 have been tested as effective therapeutic agents for age-related macular degeneration (Bellezza).

Depression is a heterogeneous mood disorder characterized by mood alterations, social withdrawal, feelings of guilt, low self-esteem, anhedonia, idiopathic pain, loss of interest in enjoyable activities, and suicidal tendencies. Clinical and preclinical studies have revealed that oxidative stress and increased activity of immune factor cascades play significant roles in the pathophysiology of depression. Different studies published in this special issue have outlined the participation of Keap1-Nrf2 axis in depression (Hashimoto) and the role of quinolinic acid, an N-methyl-D-aspartate agonist, a pro-oxidant factor that reduce Nrf2 activity in depression (Bansal et al.).

Oxidative stress and inflammation are also a common feature of painful diseases. ROS are generated by enzymes of immune and nonimmune cells as part of protective actions. In this context, Staurengo-Ferrari et al. reviewed the involvement of Nrf2 in the mechanisms of action of classic analgesic and anti-inflammatory drugs, as well as natural products and other molecules that modulate Nrf2, in preclinical and clinical stages (Staurengo-Ferrari et al.). Chronic neuropathic pain is associated with anxiety- and depressive-like disorders. Interestingly, administration of sulforaphane (SFN), a Nrf2 inductor, had antinociceptive, anxiolytic, and antidepressant effects after the induction of persistent neuropathic pain (chronic constriction injury of the sciatic nerve) by normalizing oxidative stress and inhibiting microglial activation, which goes along with by the normalization of several biochemical parameters in the spinal cord. Moreover, SFN potentiated the antiallodynic effect of morphine (Ferreira-Chamorro et al.). In another article, administration of SFN 30 min before spared nerve injury surgery significantly reduced mechanical withdrawal threshold scores and sucrose preference, and restored tissue Keap1 and Nrf2 levels. This study suggests that decreased Keap1-Nrf2 signaling in the medial prefrontal cortex, hippocampus, and muscle may contribute to anhedonia susceptibility and that SFN exerts beneficial effects by normalization of decreased Keap1-Nrf2 signaling (Li et al.).

In another review submitted to this special issue, different therapeutic approaches for improving the prognosis of liver diseases, including acute liver failure, alcoholic and nonalcoholic fatty liver disease, viral hepatitis, and hepatocellular carcinoma by targeting hepatic Nrf2 are extensively described (Xu et al.). Besides liver diseases, Nrf2 activators can be used as potential therapies for other inflammatory disorders. For example, dinardokanshone C, a new compound isolated from the roots of *Nardostachys chinensis*, suppressed the induction of inflammatory markers such as cyclooxygenase 2, prostaglandin E2, tumor necrosis factor alpha, and interleukin 6 in lipopolysaccharide-stimulated macrophages, inhibiting M1 phenotype and ROS production, through the activation of Nrf2 pathway (Luo et al.).

Finally, the article by Rubio-Navarro et al. described that Nrf2 also plays an important role in renal protection against oxidative stress in renal diseases (Rubio-Navarro et al.). Massive intravascular hemolysis induces acute kidney injury. In this context, hemoglobin is accumulated in the kidney, inducing oxidative stress, inflammation, and tubular cell death. It has been demonstrated that Nrf2 plays an important role against hemoglobin-mediated renal damage. Thus, Nrf2 deficiency produces an increase in renal injury, loss of kidney function, oxidative and reticulum endoplasmic stress, and cell death. Moreover, Nrf2 activation by SFN treatment protected both *in vivo* and *in vitro* against hemoglobin toxicity.

In summary, this special issue will help to understand the important role of Nrf2 in disease. The articles published in this special issue show novel molecular mechanisms involved in

Nrf2-mediated tissue protection as well as possible therapeutic approaches to decrease organ injury.

AUTHOR CONTRIBUTIONS

JE, AG-R, CG-G and JM wrote the manuscript. All authors critically revised the manuscript for important intellectual content.

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The Protective Effect of Astaxanthin on Cognitive Function via Inhibition of Oxidative Stress and Inflammation in the Brains of Chronic T2DM Rats

*Yonghao Feng[1], Aiqun Chu[2], Qiong Luo[3], Men Wu[1], Xiaohong Shi[1] and Yinghui Chen[3]**

[1] Department of Endocrinology, Jinshan Hospital, Fudan University, Shanghai, China, [2] Department of General Medicine, Shihua Community Health Service Center, Shanghai, China, [3] Department of Neurology, Huashan Hospital North, Fudan University, Shanghai, China

Currently, there are no effective treatments for diabetes-related cognitive dysfunction. Astaxanthin (AST), the most powerful antioxidant in nature, exhibits diverse biological functions. In this study, we tried to explore whether AST would ameliorate cognitive dysfunction in chronic type 2 diabetes mellitus (T2DM) rats. The T2DM rat model was induced via intraperitoneal injection of streptozotocin. Forty Wistar rats were divided into a normal control group, an acute T2DM group, a chronic T2DM group, and an AST group (treated with AST at a dose of 25 mg/kg three times a week). The Morris water maze test showed that the percentage of time spent in the target quadrant of the AST group was identical to that of the chronic T2DM group, while the escape latency of the AST group was decreased in comparison to that of the chronic T2DM group. Histology of the hippocampus revealed that AST ameliorated the impairment in the neurons of diabetic rats. Western blot showed that AST could upregulate nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1) expression and inhibit nuclear transcription factor kappa B (NF-κB) p65 activation in the hippocampus. We found that AST increased the level of superoxide dismutase (SOD) and decreased the level of malondialdehyde (MDA) in the hippocampus. In addition, the levels of interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) were reduced in the AST group compared with those in the chronic T2DM group. The findings of this research imply that AST might inhibit oxidative stress and inflammatory responses by activating the Nrf2-ARE signaling pathway.

Keywords: astaxanthin, type 2 diabetes mellitus, oxidative stress, inflammatory response, Nrf2, cytokines

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Edited by:
Águeda González Rodríguez, Instituto de Investigación Sanitaria del Hospital Universitario de La Princesa, Spain

Reviewed by:
Adolfo Andrade-Cetto, Universidad Nacional Autónoma de México, Mexico
Giustino Orlando, Università degli Studi G. d'Annunzio Chieti e Pescara, Italy

****Correspondence:***
Yinghui Chen
yinghuichen@fudan.edu.cn

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INTRODUCTION

Diabetes mellitus (DM) has become the most common chronic metabolic disease that threatens global health. The most common form of DM is type 2 diabetes mellitus (T2DM), characterized by insulin resistance and relative insulin insufficiency (Chatterjee et al., 2017). T2DM is associated with long-term complications that affect the eyes, kidneys, heart, blood vessels, and brain. Increasing evidence has indicated that patients with chronic T2DM can exhibit various intracranial neuropathies and neurobehavioral changes (McCrimmon et al., 2012). Lately, the incidence

of diabetes-related cognitive dysfunction has increased with the aging of the global population. This diabetes-related cognitive dysfunction is connected to the duration of diabetes and to the level of blood glucose; it can also be partially prevented by strict blood glucose control (Munshi et al., 2006).

The multifactorial process of cognitive dysfunction in DM is not yet completely understood (Gaspar et al., 2016), however, classical factors, such as oxidative stress and inflammatory responses, may contribute to the pathogenesis of diabetes-related cognitive dysfunction, leading to abnormal brain structure and dysfunction in diabetic rats (Mastrocola et al., 2005; Wang et al., 2015a). Nuclear factor erythroid 2-related factor 2 (Nrf2) plays an anti-oxidative role by binding with the antioxidant responsive element (ARE) in the nucleus (Nguyen et al., 2009). In addition, Nrf2 signaling is involved in inflammatory responses (Ahmed et al., 2017).

Astaxanthin (AST) is the most potent antioxidant found in nature (Ambati et al., 2014). It is one of the carotenoids, and the hydroxyl and keto endings located on ionone ring at both ends. Due to the optical rotation of hydroxyl at both ends, AST mainly has 3 isomers of 3S-3′S, 3R-3′S, and 3R-3′R (Guerin et al., 2003). Among them, the 3S-3′S has stronger antioxidant properties than ordinary carotenoids, which makes it easily penetrate the cell membrane and maintain the integrity of the membrane structure. The unique chemical structure of AST enables it to cross the blood-brain barrier easily and plays an important role in the treatment of central nervous system diseases (Zhang et al., 2014b; Ying et al., 2015). AST is capable of various biological characteristics, such as anti-inflammation, anti-oxidation, anti-apoptosis, and other biological characteristics (Zhang et al., 2014a,b; Ying et al., 2015). It has been demonstrated that AST can inhibit oxidative stress in human vascular endothelial cells exposed to glucose fluctuations (Regnier et al., 2015). Meanwhile, AST is considered able to alleviate cognitive dysfunction in diabetic mice by inhibiting inflammatory responses (Zhou et al., 2015). Whether AST inhibits oxidative stress and inflammatory responses in T2DM rats with cognitive dysfunction via Nrf2/ARE signaling is unclear. To investigate the therapeutic effect and potential mechanism of AST in chronic T2DM rats with cognitive dysfunction, we performed this study.

MATERIALS AND METHODS

Reagents

Astaxanthin (purity $\geq$ 98%) was purchased from Shanghai Kaimaishu Biotechnology Co., Ltd. Streptozocin (STZ) was purchased from Sigma company. Chloral hydrate was purchased from Shanghai Engineering Show Biological Technology Co., Ltd. The MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead Panel was purchased from Merck Millipore.

Experimental Grouping and Establishment of the T2DM Rat Model

A total of 40 male Wistar rats (140–160 g) were purchased from Shanghai Slack Laboratory Animal Co., Ltd. These rats were utilized in conformity with the regulations of the Shanghai Animal Management Commission. The experimental procedures were approved by Ethics Committee of Jinshan Hospital, Fudan University, and the authorization number was Jin Medical Ethics 2016(21). These animals were kept under a 12 h light/dark cycle at 20 $\pm$ 2°C and a relative humidity of 60%. The rats were divided into a control group, an acute T2DM (aT2DM) group, a chronic T2DM (cT2DM) group, and an AST group, with 12 rats in each group. The control group was fed a common diet for 12 weeks. The aT2DM group was fed a common diet for 6 weeks and then was given a high-fat, high-sugar diet containing 10% lard, 20% sucrose, and 70% regular feed for another 6 weeks. Then, the rats were injected with STZ intraperitoneally at a dose of 35 mg/kg. Fasting blood glucose levels (fasted for 12 h, with water) were determined 72 h later. The T2DM rats were considered successfully established with blood glucose levels $>$16.7 mmol/L. The cT2DM group and the AST group were directly fed a high-fat, high-sugar diet for 6 weeks. After, the T2DM rats were produced in the same manner. AST, dissolved in polyethylene glycol 400 (PEG400) (Choi et al., 2011), was administered intraperitoneally at a dose of 25 mg/kg (Tripathi and Jena, 2010), three times a week for 6 weeks. The cT2DM group was injected intraperitoneally with the same amount of PEG400. During this period, the two groups of rats were still fed a high-fat, high-sugar diet (**Figure 1**).

Blood Glucose Measurement

Abbott blood glucose meter and test strips were used to measure fasting blood glucose in rats. At first, the test strip was inserted into the groove in the Abbott blood glucose meter. Then about 0.05 mL blood samples of the rats were taken from the tail vein 72 h after the intraperitoneal injections. At last, the obtained venous blood was transferred to the detection area of test strip. Before the rats were sacrificed, the FBG level of each rat was measured again in the same way.

Morris Water Maze Test

The Morris water maze test is used to detect spatial learning and memory in rodents that rely on distal cues to navigate from start locations around the perimeter of an open swimming tank to locate a submerged platform. Spatial learning is evaluated via repeated place navigation, and reference memory is assessed by a preference for the platform area when the platform is absent. The spatial learning and memory of the rats were assessed using the Morris water maze test system (Shanghai Xin Soft Information Technology Co., Ltd.). The place navigation test was performed four times a day for 4 consecutive days with intervals of 15–20 min between each test, and a probe trial was conducted on the fifth day. In each test, a rat was released into the water from each quadrant, in turn, facing the pool wall. The rats were allowed to swim for a maximum of 60 s until they found the platform. If the rat failed to find the platform in 60 s, it was assigned a score of 60 s and was gently guided to the platform and allowed to stay on it for 15 s. On the probe trial day, the platform was removed, and each rat was released into the pool from the position opposite the target quadrant. The swimming paths of the rats were recorded for 60 s. The room was maintained at 20 $\pm$ 2°C, and the water in the pool was changed every day.

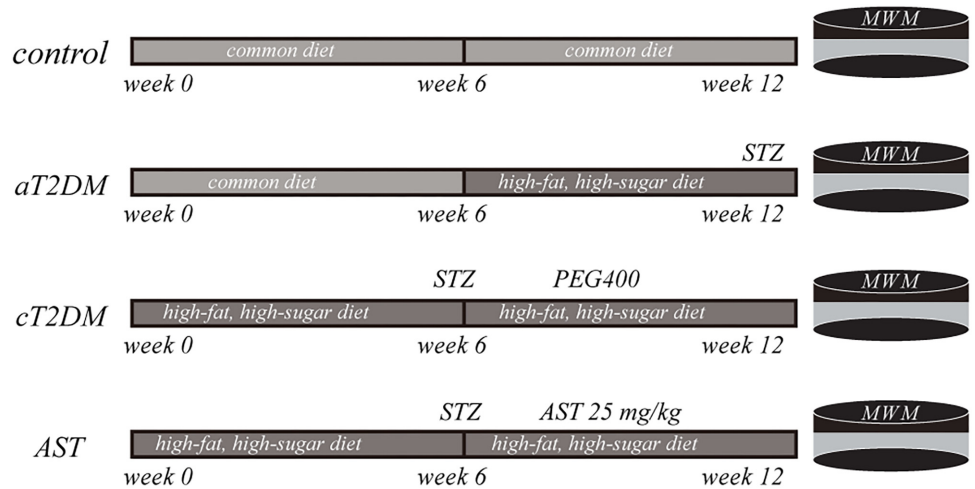

FIGURE 1 | A schematic flow chart exhibiting the experimental paradigm of the present study. aT2DM, acute type 2 diabetes mellitus; cT2DM, chronic type 2 diabetes mellitus; AST, astaxanthin. WMM: Morris water maze; STZ: streptozocin; PEG400: polyethylene glycol 400.

Histology Analysis

The four groups of rats were deeply anesthetized via intraperitoneal injections with 10% chloral hydrate at a dose of 30–35 mg/kg. The rats were first perfused with 100 mL of physiological saline in the left ventricle and then with 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. Brain tissue was obtained and dissected into four small blocks. After the brain blocks were soaked in 4% paraformaldehyde for 24 h, they were dehydrated and embedded in paraffin. Coronal sections (5 μm thick) were stained with hematoxylin-eosin (H-E). The stained sections were then viewed and photographed with an Olympus BX53 light microscope (Tokyo, Japan).

Western Blot Assay

After the rats were deeply anesthetized, they were sacrificed to directly obtain hippocampal tissue. Total protein was obtained from the hippocampal tissue with sodium dodecyl sulfate lysis buffer (Beyotime, Shanghai, China) and mixed with 1 mM phenylmethylsulfonyl fluoride (Beyotime). Equal amounts of protein were examined with 10% SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, United States). The membrane was blocked with 5% bovine serum albumin at room temperature for 1 h and then incubated with primary antibodies at 4°C for 12 h. The primary antibodies were Nrf2 antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, United States), HO-1 (1:500 dilution; Abcam, Cambridge, MA, United States), NF-κB p65 antibody (1: 1,000 dilution; Cell Signaling Technology, Danvers, MA, United States), anti-tubulin and anti-β-actin primary antibody (1:5,000 dilution; Proteintech Group Inc., Chicago, IL, United States). After washing, suitable horseradish peroxidase-conjugated secondary antibodies (1: 5,000 dilution) were incubated with the membranes at room temperature for at least 2 h. The bound antibody was visualized using enhanced chemiluminescence solution (Merck Millipore), and the signals were detected using ECL-Plus (Merck Millipore). The relative density of each group was quantified using the ImageJ Software (National Institutes of Health, Bethesda, MD, United States).

Determination of MDA and SOD

The determination of superoxide dismutase (SOD) and malondialdehyde (MDA) in the hippocampal tissue was quantified using a SOD kit (Fujian Fuyuan Biological Technology Co., Ltd., Fujian, China) and an MDA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A 25 mg sample of hippocampal tissue was weighed and added to 475 μl of physiological saline. The mixture was homogenized using a vortex mixer. Supernatants were obtained after centrifugation at 4,000 RPM for 5 min. The SOD was determined by pyrogallol autoxidation method. Reagent 1 and reagent 2 in the SOD reagent kit are formulated in a ratio of 5:1. Then 2 mL of working solution and 100 ul of sample were added to each detection well and were mixed. Then the mixed liquor was detected by automatic biochemical analyzer at a wavelength of 420 nm and the temperature of 37°C. At last the SOD level of each sample were calculated by automatic biochemical analyzer. The level of MDA was quantified by thiobarbituric acid (TBA) method. In the process of measuring, standard tube, standard blank tube, and measuring tube were used. The 4 mL of working solution was added to the three kinds of tube respectively. Besides, 100 μL of standard sample was added to standard tube. At the same time, 100 μL of anhydrous alcohol was added to standard blank tube and 100 μL of sample was added to measuring tube. The mixed solution of each tube was homogenized using a vortex mixer, and then were incubated for 80 min at 95° water temperature. When they were cool down by running water and then were centrifuged at 3,500 RPM for 10 min. The 200 μL of supernatants were obtained from each tube. The absorbance of each sample was measured using a microplate reader at a wavelength of 532 nm. Each sample was calculated according to the conversion formula.

Determination of Inflammatory Cytokines

When the rats were deeply anesthetized, the blood was collected from the heart. The blood samples were centrifuged at 4,000 RPM for 5 min, and then plasma samples were obtained. The plasma samples were stored at −80°C when not in use. The levels of interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) in the serum were quantified using the MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead Panel as per the manufacturer's instructions (Merck Millipore, Billerica, MA, United States). The IL-6 and IL-1β cytokines were determined. Premixed magnetic beads conjugated to specific antibodies for 12 analytes were mixed with 25 μL of plasma samples in 96-well plates. Plates were preserved to avoid light and were incubated on a shaker at 4°C for less than 12 h, and then magnetic beads were washed for three times with 200 μL of wash buffer. Then detection antibodies were added to each well, and the mixtures were incubated at room temperature for 1 h. Streptavidin-phycoerythrin was added to each well, and the mixtures were incubated at room temperature for 30 min. The magnetic beads were resuspended in sheath fluid, and plates were assayed on a Luminex® 200™ system with xPONENT® software. The experimental data are presented in pg/mL as a unit.

Statistical Analysis

All the measurement data are expressed as the mean ± standard deviation (SD). The SPSS 23.0 (IBM Corp., Armonk, NY, United States) statistical software was used for the analyses. Two sets of data collected from the same sample at different times were analyzed using paired *t*-tests. One-way ANOVA was used to compare the differences among multiple samples. Repeated-measures ANOVA was used to analyze continual measurement data for the same subject among multiple samples. Pearson correlation analyses were carried out to clarify the relationship between Nrf2 and SOD and between MDA and inflammatory cytokines. Differences were statistically significant at $P < 0.05$.

RESULTS

Effect of AST on the Blood Glucose Levels

As shown in **Table 1**, the fasting blood glucose level of each group was different at the 6th week ($F = 941.6$, $P < 0.01$). The blood glucose levels of the cT2DM group and of the AST group were significantly higher than that of the control group at the 6th week ($P < 0.01$). There was no significant difference between the AST group and the cT2DM group ($P = 0.785$, $P > 0.05$). The fasting blood glucose levels of the control, cT2DM, and AST groups were not different at the 12th week ($F = 0.388$, $P > 0.05$). The blood glucose level of the AST group was slightly lower than that before AST treatment, but there was no significant difference between the two groups ($t = 0.464$, $P > 0.05$). This finding implied that AST had no obvious effect on the blood glucose levels of the diabetic rats in this study.

Effects of AST on Cognitive Dysfunction

It has been considered that cognitive function is correlated with hippocampal bioenergetics. Thus, we performed the place navigation and probe trials to explore whether AST would successfully preserve the cognitive dysfunction of diabetic rats. Repeated-measures ANOVA proved that the escape latencies were significantly different between each group ($F = 5.893$, $P < 0.01$, **Figure 2A**) and among the different periods ($F = 55.568$, $P < 0.01$, **Figure 2A**). Compared with the control group, the escape latency in the aT2DM group was unchanged ($P = 0.201$, $P > 0.05$), while the escape latency in the cT2DM group was increased ($P = 0.002$, $P < 0.01$). The escape latency in the AST group was shorter than that in the cT2DM group after the treatment with AST ($P = 0.001$, $P < 0.01$). In the probe trial session, the percentage of time spent in the target quadrant was different for each group ($F = 6.706$, $P < 0.01$). The percentage of time spent in the target quadrant was not significantly different between the control group and the aT2DM group ($P = 0.058$, $P > 0.05$). The percentage of time spent in the target quadrant in the cT2DM group was less than that of the control group ($P < 0.01$). Under treatment with AST, the percentage of time spent in the target quadrant in the AST group was identical to that in the cT2DM group ($P = 0.072$, $P < 0.05$). The behavioral data indicated that the cT2DM group rats displayed severe cognitive dysfunction in terms of both damaged spatial learning (**Figure 2A**) and spatial memory (**Figure 2B**). In addition, there existed no serious cognitive impairments in the early stages of diabetes. Under the treatment with AST, the spatial learning of the diabetic rats was evidently improved (**Figure 2A**). However, the spatial memory deficits were not obviously alleviated (**Figures 2B,C**). The data acquired from the Morris water maze test revealed that AST could ameliorate the cognitive impairment of chronic diabetic rats to a certain extent.

Histological Examination

The dentate gyrus has been considered to be closely related to cognitive function. To observe the morphological changes in the dentate gyrus, we performed HE staining. No obvious histological changes were observed among the control group, the aT2DM group, or the AST group (**Figures 3A–D,G,H**). Meanwhile, karyopyknosis was observed in the dentate gyrus of the cT2DM group hippocampus compared with those of the control group and the AST group (**Figures 3E,F**). This

TABLE 1 | Effect of AST on the blood glucose level in each group (unit: mmol/l).

Time	Control	aT2DM	cT2DM	AST
6th week	2.45 ± 0.59	2.97 ± 0.63	19.21 ± 1.20**	19.09 ± 1.28**
12th week	2.82 ± 0.35	18.58 ± 1.32	18.27 ± 1.13	18.09 ± 1.29

*Data are shown as the mean ± SD. **P < 0.01. aT2DM, acute type 2 diabetes mellitus; cT2DM, chronic type 2 diabetes mellitus; AST, astaxanthin.*

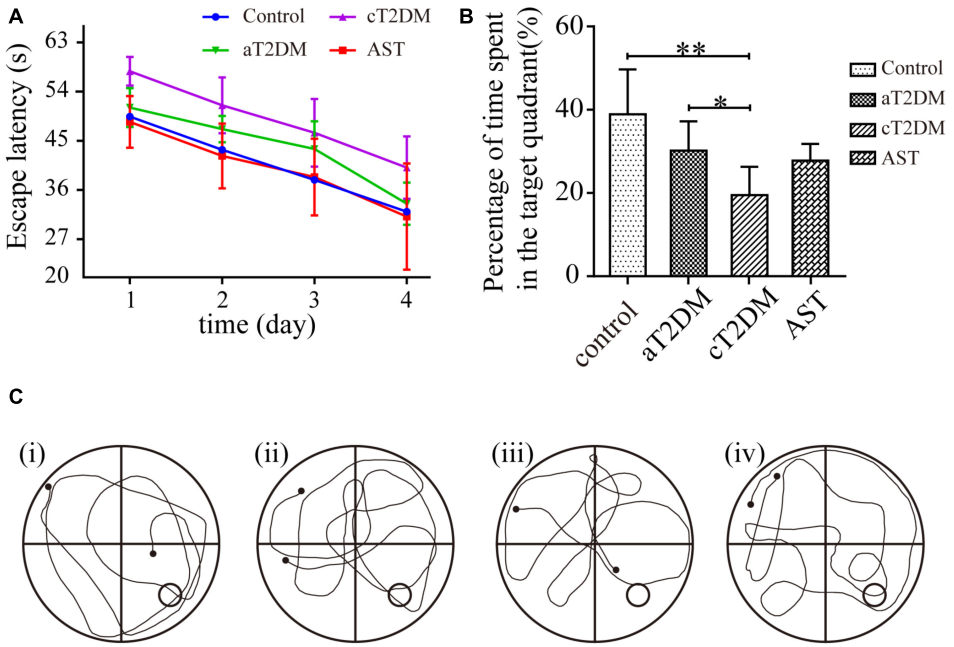

FIGURE 2 | Effect of AST on the spatial learning and memory of each group. This figure exhibits the cognitive function (as measured by the Morris water maze test) of the control, aT2DM, cT2DM, and AST groups. The line chart shows the average escape latency **(A)** in the place navigation test over 4 consecutive days. In the probe trial, the percentage of time spent in the target quadrant **(B)**, and the typical swimming traces **(C)** were recorded. (i) control group; (ii) aT2DM group; (iii) cT2DM group; (iv) AST group. Data are shown as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. aT2DM, acute type 2 diabetes mellitus; cT2DM, chronic type 2 diabetes mellitus; AST, astaxanthin.

finding indicated that the morphological impairment was clearly distinguished in chronic diabetic rats, and AST could ameliorate this morphological impairment.

The Expression Levels of Nrf2, HO-1, SOD, and MDA in Each Group

Western blot (**Figures 4A,B**) analysis showed that the expression levels of Nrf2 were significantly different among the groups ($F = 44.858$, $P < 0.01$). Compared with the control group, the expression of Nrf2 in the aT2DM group was statistically unvaried ($P = 0.109$, $P > 0.05$), while the expression of Nrf2 in the cT2DM group was significantly reduced ($P = 0.003$, $P < 0.01$). Meanwhile, the expression of Nrf2 in the cT2DM group was decreased compared with that in the aT2DM group ($P < 0.01$). The expression of Nrf2 in the AST group was higher than that in the cT2DM group ($P < 0.01$). The expression levels of HO-1 were also significantly different between each group ($F = 46.616$, $P < 0.01$) (**Figures 4A,B**). The expression of HO-1 in the aT2DM group was lower than that in the control group ($P < 0.01$) but significantly higher than that in the cT2DM group ($P = 0.008$, $P < 0.01$). The expression of HO-1 in the cT2DM group was visibly increased in comparison to that in the control group ($P < 0.01$). The expression of HO-1 in the AST group was higher than that in the cT2DM group ($P = 0.001$, $P < 0.01$). This finding revealed that AST could upregulate the expression of Nrf2 and HO-1 and that Nrf2 and HO-1 were slightly changed in the early periods of diabetes. The levels of SOD were significantly different in each group ($F = 7.496$, $P < 0.01$, **Figure 4C**). Compared with the control group, the level of SOD in the aT2DM group was slightly decreased ($P = 0.044$, $P < 0.05$) but was higher than that in the cT2DM group ($P = 0.033$, $P < 0.05$). The level of SOD in the cT2DM group was decreased in comparison to that in the control group ($P < 0.01$). The level of SOD in the AST group was higher than that in the cT2DM group under treatment with AST ($P = 0.003$, $P < 0.01$). This result revealed that the ability to resist oxidative stress in the early stages of diabetes was diminished and that AST elevated the ability to resist oxidative stress in the diabetic rats. The levels of MDA were significantly different between each group ($F = 91.727$, $P < 0.01$, **Figure 4D**). Compared to the control group, the level of MDA in the aT2DM group was unchanged ($P = 0.113$, $P > 0.05$), while the level of MDA in the cT2DM group was increased ($P < 0.01$). Meanwhile, the level of MDA in the cT2DM group was higher than that in the aT2DM group ($P < 0.01$). The level of MDA in the AST group was lower than that in the cT2DM group after treatment with AST ($P < 0.01$). This finding demonstrated that AST could clearly inhibit lipid peroxidation and that there was no obvious lipid peroxidation in early periods of diabetes. According to the above results, it was implied that the oxidation and anti-oxidation systems are slightly unbalanced in the early stage of diabetes and that AST inhibits the level of oxidative stress.

The Expression Levels of NF-κB p65 and Inflammatory Cytokines in Each Group

The immunoblots (**Figures 5A,B**) indicated that the expression levels of NF-κB p65 were significantly different between each

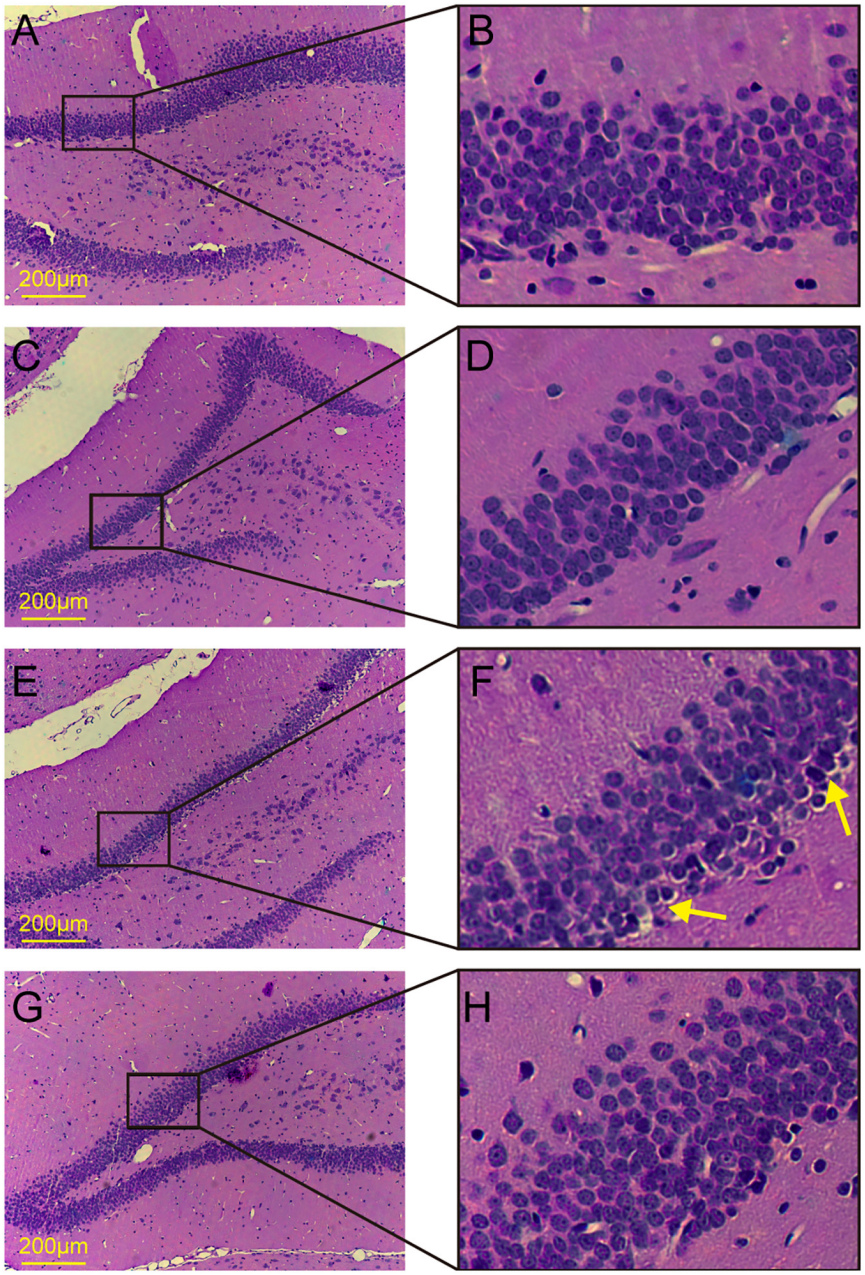

FIGURE 3 | HE staining of the dentate gyrus in the hippocampus. **(A,B)** Control group; **(C,D)** acute type 2 diabetes group; **(E,F)** chronic type 2 diabetes group; **(G,H)** astaxanthin group.

group ($F = 64.459$, $P < 0.01$). Compared with the control group, the expression levels of NF-κB p65 in the aT2DM and cT2DM groups were increased ($P < 0.01$). However, the expression of NF-κB p65 in the aT2DM group was lower than that in the cT2DM group ($P < 0.01$). The expression level of NF-κB p65 in the AST group was significantly lower than that in the cT2DM group ($P = 0.003$, $P < 0.01$). This result indicated that AST could down-regulate the expression of NF-κB p65 to a certain extent and that NF-κB p65 was wildly activated in the early periods of diabetes. The results of the multiplex immunoassay (**Figures 5C,D**) showed that the expression levels of IL-β and IL-6 in each group were significantly different ($F = 62.072$, $P < 0.01$; $F = 218.975$, $P < 0.01$). The expression of IL-1β in the aT2DM group was significantly higher than that in the control group ($P < 0.01$) but lower than that in the cT2DM group ($P < 0.01$). Meanwhile, the expression of IL-1β in the cT2DM group was increased in comparison to the control group ($P < 0.01$). The expression of IL-1β in the AST group was lower than that in the cT2DM group ($P < 0.01$). Compared with the control group, the expression of IL-6 in the aT2DM group was significantly

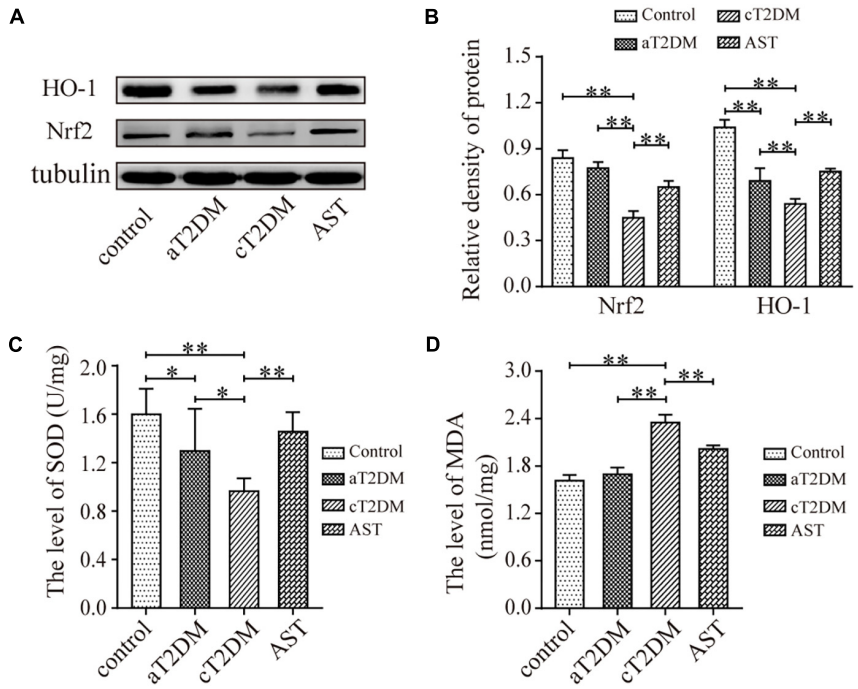

FIGURE 4 | The expression levels of Nrf2, HO-1, SOD, and MDA in each group. Representative protein bands of Nrf2 and HO-1 **(A)**; Nrf2/tubulin and HO-1/tubulin ratio according to band density **(B)**; The average level of SOD in each group (**C**, units: U/mg); The average level of MDA in each group (**D**, units: nmol/mg). Data are shown as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. aT2DM, acute type 2 diabetes mellitus; cT2DM, chronic type 2 diabetes mellitus; AST, astaxanthin.

increased ($P < 0.01$) but was lower than that in the cT2DM group ($P < 0.01$). The expression level of IL-6 in the cT2DM group was increased in comparison to the control group ($P < 0.01$). The expression level of IL-6 in the AST group was lower than that in the cT2DM group ($P < 0.01$). This finding indicated that a mild inflammatory reaction arose in the early stages of diabetes and that AST could down-regulate the expression of inflammatory cytokines in chronic diabetic rats.

The Correlation Between Nrf2 and SOD, MDA, and Inflammatory Cytokines

Pearson correlation analyses showed that Nrf2 was positively correlated with SOD (**Figure 6A**). There was a negative correlation between Nrf2 and MDA, IL-1β, and IL-6 (**Figures 6B–D**). This demonstrated that MDA, IL-6, and IL-1β were decreased, but SOD was increased when Nrf2 was activated. This finding implied that activated Nrf2 inhibited oxidative stress and inflammatory responses.

DISCUSSION

Diabetes mellitus is a common chronic metabolic disease that can cause a variety of complications. As a complication of DM, cognitive dysfunction manifests as impairments in learning and memory, space-time orientation dysfunction, and even in the formation of dementia. Large-scale cross-sectional studies have found that diabetes and dementia are positively correlated (Ahtiluoto et al., 2010). A prospective population-based study found that baseline scores on cognitive function tests in elderly patients with T2DM were lower than those without diabetes (Logroscino et al., 2004). Several clinical studies confirmed that T2DM was associated with cognitive decline (Nooyens et al., 2010; Gao et al., 2016). The diabetes-related cognitive dysfunction appears mild to moderate but can significantly impede daily functioning and seriously decrease the quality of life (Brands et al., 2005). At present, most of the research regarding the diabetes-related cognitive dysfunction is based on STZ-induced diabetic animal models. STZ at low doses partly impairs the function of islet beta cells, resulting in decreased insulin sensitivity in peripheral tissues. A high-fat, high-sugar diet with low doses of STZ can induce T2DM rats (Srinivasan et al., 2005). In this study, we produced T2DM rats using a high-fat, high-sugar diet with STZ intraperitoneally injected at a dose of 35 mg/kg.

Due to the complicated pathogenesis of diabetes-related cognitive dysfunction, there is currently no effective treatment. Therefore, looking for specific and effective drugs for treatment is necessary. AST is a keto carotenoid that is widely found in algae, shrimp, crabs, shellfish, fish and other foods (Higuera-Ciapara et al., 2006). Recent studies have shown that AST elicits the benefits of anti-inflammation, anti-apoptosis, and anti-oxidation, anti-aging, anti-tumor, and immunity enhancement (Wang et al., 2015b). It has been widely applied in the areas of intellectual nourishment, healthcare products, and commercialism (Guerin et al., 2003). The linear portion of the AST molecule contains

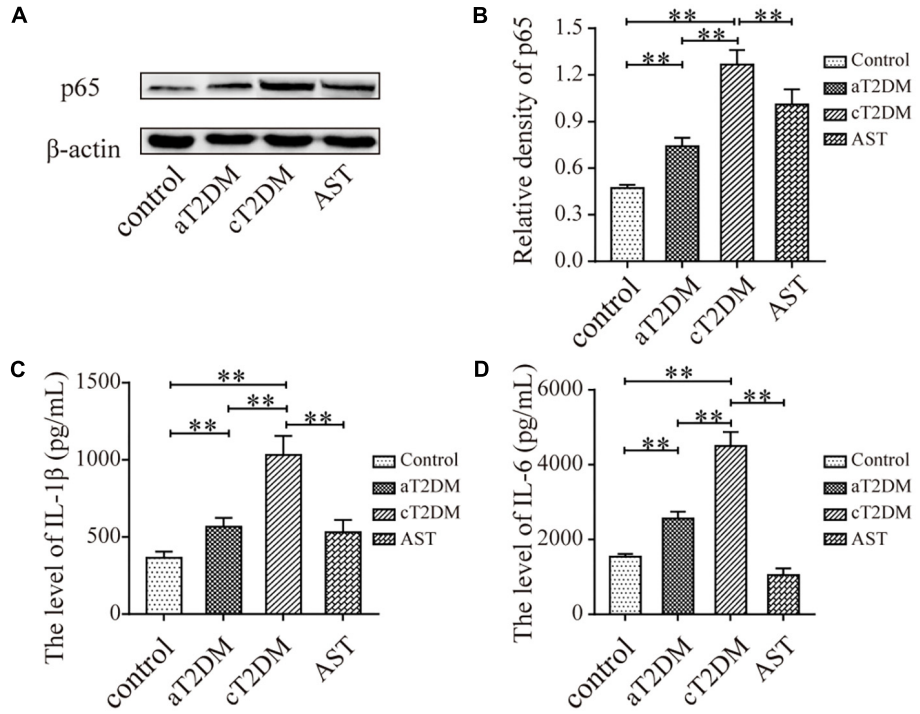

FIGURE 5 | The expression levels of NF-kB p65, IL-1β, and IL-6 in each group. Immunoblots **(A)** and histogram **(B)** showing the expression of NF-kB p65. The levels of IL-6 **(C)** and IL-1β **(D)** are also displayed in the histogram. Data are shown as the mean ± SD. $^{**}P < 0.01$. aT2DM, acute type 2 diabetes mellitus; cT2DM, chronic type 2 diabetes mellitus; AST, astaxanthin.

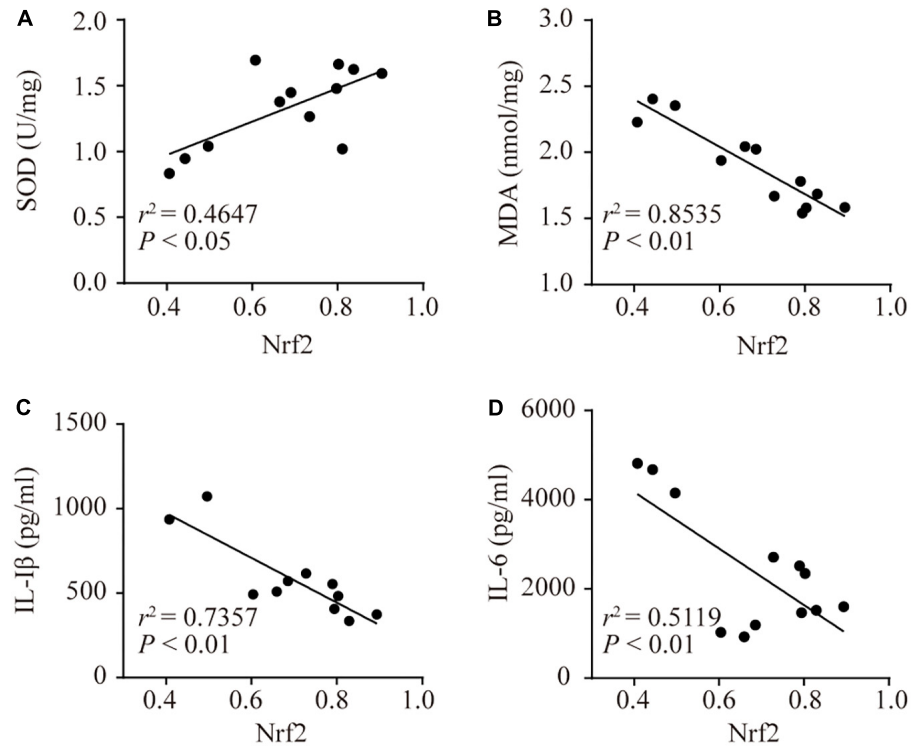

FIGURE 6 | The correlations between Nrf2 and SOD, MDA, IL-1β, and IL-6. Associativity between Nrf2 and SOD (**A**, $r^2 = 0.4647$, $P < 0.05$), MDA (**B**, $r^2 = 0.8535$, $P < 0.01$), IL-1β (**C**, $r^2 = 0.7357$, $P < 0.01$), and IL-6 (**D**, $r^2 = 0.5119$, $P < 0.01$).

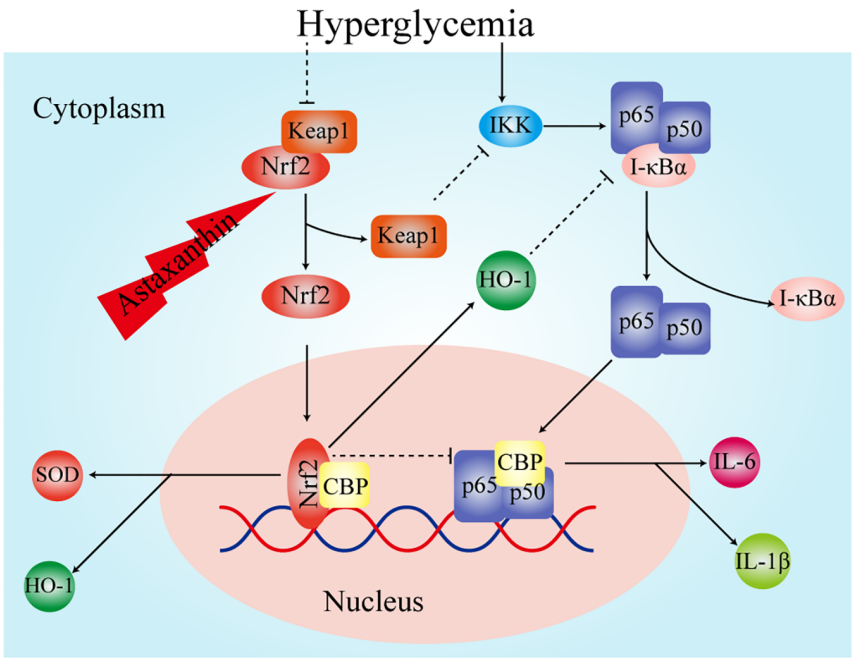

FIGURE 7 | The potential pathway of Nrf2 inhibiting oxidative stress and inflammatory response.

multiple double bonds, each of which can be either *cis* or *trans*. This enables AST to generate many isomers, of which all-*trans* structures are almost stable and widely disseminated in nature. AST has been proven to exert potent protective effects on diabetic nephropathy in rat models of T2DM by inhibiting expression of oxidative stress and inflammatory mediators (Naito et al., 2004). In addition, it is also effective as an anticoagulant and an anti-inflammatory in diabetic rats (Chan et al., 2012). In T2DM mice, AST ameliorates the apoptosis of retinal ganglion cells by inhibiting oxidative stress (Dong et al., 2013). In the present study, AST alleviated cognitive dysfunction and the structural damage to the hippocampus to some extent, suggesting that AST has a certain therapeutic effect on diabetic cognitive dysfunction.

The role of oxidative stress and inflammatory responses in diabetes-related cognitive dysfunction has gradually attracted the attention of researchers (Zhou et al., 2015; Tian et al., 2016). Under normal and unstressed conditions, the levels of oxidation and of antioxidants are in a relatively balanced state. Under harmful external stress, the oxidation and anti-oxidation systems of an organism lose balance, resulting in the mass production of reactive oxygen species (ROS) and antioxidants that are relatively inadequate (Mittler, 2002). Subsequently, substantial ROS accumulate in the organism, causing lipid peroxidation of polyunsaturated fatty acids to form MDA. Nrf2, a key transcription factor, regulates the expression of cytoprotective and antioxidant genes when it is activated (Nguyen et al., 2009). Under normal conditions, Nrf2 forms a complex with Kelch-like ECH-related protein 1 (Keap1), resulting in the degradation and inactivation of Nrf2 via ubiquitination. When the cell is stimulated by harmful stimuli, Nrf2 separates from Keap1, translocates to the nucleus and binds to the ARE to induce the expression of glutathione-S-transferase, HO-1, NADPH quinine oxidoreductase 1 and other genes (Ma, 2013). This study revealed that AST promotes the expression of Nrf2, HO-1, and SOD, while inhibiting the generation of MDA. From this viewpoint, the protective effects of AST on cognitive dysfunction may be partially due to the recovery of anti-oxidative enzyme levels through Nrf2 activating.

In recent years, a large number of experiments have confirmed that the inflammatory reaction is also closely related to diabetes-related cognitive dysfunction. It has been experimentally confirmed that IL-6, tumor necrosis factor-α, and cyclooxygenase-2 are elevated in the brain tissue of T2DM rats with cognitive dysfunction (Miao et al., 2015). The above studies showed that inflammatory cytokines are involved in the pathogenesis of diabetes-related cognitive dysfunction. In present study we also found the level of pro-inflammatory cytokines IL-1β and IL-6 in chronic diabetic rats were elevated, which was consistent with the opinion that inflammatory cytokines are involved in the pathogenesis of diabetes-related cognitive dysfunction. NF-κB, as one of the regulators of inflammatory responses, plays a key role in the inflammatory response and could promote lipid peroxidation (Menghini et al., 2016; Mitchell et al., 2016). It consists of five protein subunits: RelA (p65), RelB, C-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p50 and its precursor p105). There is growing evidence that NF-κB is closely linked to synaptic plasticity, memory, and cognitive dysfunction (Kaltschmidt et al., 2006; Ahn et al., 2008). The activation of NF-κB in microglia plays a major role in inflammatory processes. NF-κB is also involved in long-term memory consolidation and fear memory recovery (Boccia et al., 2007). Studies have confirmed that the NF-κB

signaling pathway is activated in diabetic rats with cognitive dysfunction (Kuhad et al., 2009). This is consistent with the results of this experiment. At the same time, we found that AST reduced the level of IL-1β and IL-6 in chronic diabetic rats, which implied that AST inhibited the level of inflammatory response. Some experiments confirmed that AST plays a role in lowering blood glucose (Uchiyama et al., 2002), whereas we found that the blood glucose levels were not significantly different between the AST group and the cT2DM group. This might have been due to fact that the treatment time of AST was not long enough. Therefore, the decreased inflammatory responses in the diabetic rats was not caused by hypoglycemia and might be achieved by other pathways. Nrf2 is thought to inhibit the expression of inflammatory cytokines (Ahmed et al., 2017). Previous studies have shown that the expression of inflammatory cytokines is higher in knockout Nrf2 mice than in non-knockout mice (Kong et al., 2015). Experiments have demonstrated that when Nrf2 is activated, Keap1 is isolated from it and then can inhibit the activity of IKK kinase, resulting in NF-κB inactivation (Kim et al., 2010). The carbon monoxide and biliverdin, catalytic products of HO-1, have been shown to suppress the activation of NF-κB (Soares et al., 2004). The transcriptional coactivator, CREB binding protein (CBP), performs its role by activating the transcription process, wherein interaction with transcription factors is managed by its domains (Gerritsen et al., 1997; Wang et al., 2012). The Nrf2 of a free state in the nucleus competitively binds CBP with NF-κB, resulting in impedance of NF-κB activation when the level of Nrf2 increases (Liu et al., 2008; Kim et al., 2013). We surmise that AST improves the expression of Nrf2, and Nrf2 and its intermediate metabolites exert an inhibitory effect on the inflammatory response by inhibiting the activation of NF-κB (**Figure 7**).

In summary, with the assistance of behavioral approaches, morphology, and molecular bioenergetic, this study clarified that AST can effectively alleviate cognitive dysfunction. Although the comprehensive mechanism related to the protective effects of AST on ameliorating the cognitive impairment remains to be fully understood, inhibiting oxidative stress and inflammatory response by the elevation of the Nrf2-ARE signaling may be effective for diabetic cognitive dysfunction.

AUTHOR CONTRIBUTIONS

XS and YC contributed conception and design of the study. YF and QL accomplished the experiments. MW and AC performed the statistical analysis. YF and AC wrote the manuscript. XS and YC contributed to English editing. All authors contributed to manuscript revision, read and approved the submitted version.

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REFERENCES

Ahmed, S. M., Luo, L., Namani, A., Wang, X. J., and Tang, X. (2017). Nrf2 signaling pathway: pivotal roles in inflammation. *Biochim. Biophys. Acta* 1863, 585–597. doi: 10.1016/j.bbadis.2016.11.005

Ahn, H. J., Hernandez, C. M., Levenson, J. M., Lubin, F. D., Liou, H. C., and Sweatt, J. D. (2008). c-Rel, an NF-kappaB family transcription factor, is required for hippocampal long-term synaptic plasticity and memory formation. *Learn. Mem.* 15, 539–549. doi: 10.1101/lm.866408

Ahtiluoto, S., Polvikoski, T., Peltonen, M., Solomon, A., Tuomilehto, J., Winblad, B., et al. (2010). Diabetes, Alzheimer disease, and vascular dementia: a population-based neuropathologic study. *Neurology* 75, 1195–1202. doi: 10.1212/WNL.0b013e3181f4d7f8

Ambati, R. R., Phang, S. M., Ravi, S., and Aswathanarayana, R. G. (2014). Astaxanthin: sources, extraction, stability, biological activities and its commercial applications-a review. *Mar. Drugs* 12, 128–152. doi: 10.3390/md12010128

Boccia, M., Freudenthal, R., Blake, M., De La Fuente, V., Acosta, G., Baratti, C., et al. (2007). Activation of hippocampal nuclear factor-kappa B by retrieval is required for memory reconsolidation. *J. Neurosci.* 27, 13436–13445. doi: 10.1523/JNEUROSCI.4430-07.2007

Brands, A. M., Biessels, G. J., De Haan, E. H., Kappelle, L. J., and Kessels, R. P. (2005). The effects of type 1 diabetes on cognitive performance: a meta-analysis. *Diabetes Care* 28, 726–735. doi: 10.2337/diacare.28.3.726

Chan, K. C., Pen, P. J., and Yin, M. C. (2012). Anticoagulatory and antiinflammatory effects of astaxanthin in diabetic rats. *J. Food Sci.* 77, H76–H80. doi: 10.1111/j.1750-3841.2011.02558.x

Chatterjee, S., Khunti, K., and Davies, M. J. (2017). Type 2 diabetes. *Lancet* 389, 2239–2251. doi: 10.1016/S0140-6736(17)30058-2

Choi, H. D., Kang, H. E., Yang, S. H., Lee, M. G., and Shin, W. G. (2011). Pharmacokinetics and first-pass metabolism of astaxanthin in rats. *Br. J. Nutr.* 105, 220–227. doi: 10.1017/S0007114510003454

Dong, L. Y., Jin, J., Lu, G., and Kang, X. L. (2013). Astaxanthin attenuates the apoptosis of retinal ganglion cells in db/db mice by inhibition of oxidative stress. *Mar. Drugs* 11, 960–974. doi: 10.3390/md11030960

Gao, Y., Xiao, Y., Miao, R., Zhao, J., Cui, M., Huang, G., et al. (2016). The prevalence of mild cognitive impairment with type 2 diabetes mellitus among elderly people in China: a cross-sectional study. *Arch. Gerontol. Geriatr.* 62, 138–142. doi: 10.1016/j.archger.2015.09.003

Gaspar, J. M., Baptista, F. I., Macedo, M. P., and Ambrosio, A. F. (2016). Inside the diabetic brain: role of different players involved in cognitive decline. *ACS Chem. Neurosci.* 7, 131–142. doi: 10.1021/acschemneuro.5b00240

Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2927–2932. doi: 10.1073/pnas.94.7.2927

Guerin, M., Huntley, M. E., and Olaizola, M. (2003). *Haematococcus astaxanthin*: applications for human health and nutrition. *Trends Biotechnol.* 21, 210–216. doi: 10.1016/S0167-7799(03)00078-7

Higuera-Ciapara, I., Felix-Valenzuela, L., and Goycoolea, F. M. (2006). Astaxanthin: a review of its chemistry and applications. *Crit. Rev. Food Sci. Nutr.* 46, 185–196. doi: 10.1080/10408690590957188

Kaltschmidt, B., Ndiaye, D., Korte, M., Pothion, S., Arbibe, L., Prullage, M., et al. (2006). NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling. *Mol. Cell. Biol.* 26, 2936–2946. doi: 10.1128/MCB.26.8.2936-2946.2006

Kim, J. E., You, D. J., Lee, C., Ahn, C., Seong, J. Y., and Hwang, J. I. (2010). Suppression of NF-kappaB signaling by KEAP1 regulation of IKKbeta activity through autophagic degradation and inhibition of phosphorylation. *Cell. Signal.* 22, 1645–1654. doi: 10.1016/j.cellsig.2010.06.004

Kim, S. W., Lee, H. K., Shin, J. H., and Lee, J. K. (2013). Up-down regulation of HO-1 and iNOS gene expressions by ethyl pyruvate via recruiting p300 to Nrf2 and depriving It from p65. *Free Radic. Biol. Med.* 65, 468–476. doi: 10.1016/j.freeradbiomed.2013.07.028

Kong, M., Mao, J., Luo, B., and Qin, Z. (2015). Role of transcriptional factor Nrf2 in the acute lung injury of mice. *Int. J. Clin. Exp. Pathol.* 8, 10929–10934.

Kuhad, A., Bishnoi, M., Tiwari, V., and Chopra, K. (2009). Suppression of NF-kappabeta signaling pathway by tocotrienol can prevent diabetes associated cognitive deficits. *Pharmacol. Biochem. Behav.* 92, 251–259. doi: 10.1016/j.pbb.2008.12.012

Liu, G. H., Qu, J., and Shen, X. (2008). NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim. Biophys. Acta* 1783, 713–727. doi: 10.1016/j.bbamcr.2008.01.002

Logroscino, G., Kang, J. H., and Grodstein, F. (2004). Prospective study of type 2 diabetes and cognitive decline in women aged 70-81 years. *BMJ* 328:548. doi: 10.1136/bmj.37977.495729.EE

Ma, Q. (2013). Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Mastrocola, R., Restivo, F., Vercellinatto, I., Danni, O., Brignardello, E., Aragno, M., et al. (2005). Oxidative and nitrosative stress in brain mitochondria of diabetic rats. *J. Endocrinol.* 187, 37–44. doi: 10.1677/joe.1.06269

McCrimmon, R. J., Ryan, C. M., and Frier, B. M. (2012). Diabetes and cognitive dysfunction. *Lancet* 379, 2291–2299. doi: 10.1016/S0140-6736(12)60360-2

Menghini, L., Ferrante, C., Leporini, L., Recinella, L., Chiavaroli, A., Leone, S., et al. (2016). An hydroalcoholic chamomile extract modulates inflammatory and immune response in ht29 cells and isolated rat colon. *Phytother. Res.* 30, 1513–1518. doi: 10.1002/ptr.5655

Miao, Y., He, T., Zhu, Y., Li, W., Wang, B., and Zhong, Y. (2015). Activation of hippocampal creb by rolipram partially recovers balance between tnf-alpha and il-10 levels and improves cognitive deficits in diabetic rats. *Cell. Mol. Neurobiol.* 35, 1157–1164. doi: 10.1007/s10571-015-0209-3

Mitchell, S., Vargas, J., and Hoffmann, A. (2016). Signaling via the NFkappaB system. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 8, 227–241. doi: 10.1002/wsbm.1331

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405–410. doi: 10.1016/S1360-1385(02)02312-9

Munshi, M., Grande, L., Hayes, M., Ayres, D., Suhl, E., Capelson, R., et al. (2006). Cognitive dysfunction is associated with poor diabetes control in older adults. *Diabetes Care* 29, 1794–1799. doi: 10.2337/dc06-0506

Naito, Y., Uchiyama, K., Aoi, W., Hasegawa, G., Nakamura, N., Yoshida, N., et al. (2004). Prevention of diabetic nephropathy by treatment with astaxanthin in diabetic db/db mice. *Biofactors* 20, 49–59. doi: 10.1002/biof.5520200105

Nguyen, T., Nioi, P., and Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem.* 284, 13291–13295. doi: 10.1074/jbc.R900010200

Nooyens, A. C., Baan, C. A., Spijkerman, A. M., and Verschuren, W. M. (2010). Type 2 diabetes and cognitive decline in middle-aged men and women: the Doetinchem Cohort Study. *Diabetes Care* 33, 1964–1969. doi: 10.2337/dc09-2038

Regnier, P., Bastias, J., Rodriguez-Ruiz, V., Caballero-Casero, N., Caballo, C., Sicilia, D., et al. (2015). Astaxanthin from *Haematococcus pluvialis* prevents oxidative stress on human endothelial cells without toxicity. *Mar. Drugs* 13, 2857–2874. doi: 10.3390/md13052857

Soares, M. P., Seldon, M. P., Gregoire, I. P., Vassilevskaia, T., Berberat, P. O., Yu, J., et al. (2004). Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. *J. Immunol.* 172, 3553–3563. doi: 10.4049/jimmunol.172.6.3553

Srinivasan, K., Viswanad, B., Asrat, L., Kaul, C. L., and Ramarao, P. (2005). Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol. Res.* 52, 313–320. doi: 10.1016/j.phrs.2005.05.004

Tian, X., Liu, Y., Ren, G., Yin, L., Liang, X., Geng, T., et al. (2016). Resveratrol limits diabetes-associated cognitive decline in rats by preventing oxidative stress and inflammation and modulating hippocampal structural synaptic plasticity. *Brain Res.* 1650, 1–9. doi: 10.1016/j.brainres.2016.08.032

Tripathi, D. N., and Jena, G. B. (2010). Astaxanthin intervention ameliorates cyclophosphamide-induced oxidative stress, DNA damage and early hepatocarcinogenesis in rat: role of Nrf2, p53, p38 and phase-II enzymes. *Mutat. Res.* 696, 69–80. doi: 10.1016/j.mrgentox.2009.12.014

Uchiyama, K., Naito, Y., Hasegawa, G., Nakamura, N., Takahashi, J., and Yoshikawa, T. (2002). Astaxanthin protects beta-cells against glucose toxicity in diabetic db/db mice. *Redox Rep.* 7, 290–293. doi: 10.1179/135100002125000811

Wang, B., Miao, Y., Zhao, Z., and Zhong, Y. (2015a). Inflammatory macrophages promotes development of diabetic encephalopathy. *Cell. Physiol. Biochem.* 36, 1142–1150. doi: 10.1159/000430285

Wang, B., Waters, A. L., Valeriote, F. A., and Hamann, M. T. (2015b). An efficient and cost-effective approach to kahalalide F N-terminal modifications using a nuisance algal bloom of *Bryopsis pennata*. *Biochim. Biophys. Acta* 1850, 1849–1854. doi: 10.1016/j.bbagen.2015.05.004

Wang, B., Zhu, X., Kim, Y., Li, J., Huang, S., Saleem, S., et al. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Radic. Biol. Med.* 52, 928–936. doi: 10.1016/j.freeradbiomed.2011.12.006

Ying, C. J., Zhang, F., Zhou, X. Y., Hu, X. T., Chen, J., Wen, X. R., et al. (2015). Anti-inflammatory effect of astaxanthin on the sickness behavior induced by diabetes mellitus. *Cell. Mol. Neurobiol.* 35, 1027–1037. doi: 10.1007/s10571-015-0197-3

Zhang, X. S., Zhang, X., Wu, Q., Li, W., Zhang, Q. R., Wang, C. X., et al. (2014a). Astaxanthin alleviates early brain injury following subarachnoid hemorrhage in rats: possible involvement of Akt/bad signaling. *Mar. Drugs* 12, 4291–4310. doi: 10.3390/md12084291

Zhang, X. S., Zhang, X., Zhou, M. L., Zhou, X. M., Li, N., Li, W., et al. (2014b). Amelioration of oxidative stress and protection against early brain injury by astaxanthin after experimental subarachnoid hemorrhage. *J. Neurosurg.* 121, 42–54. doi: 10.3171/2014.2.JNS13730

Zhou, X., Zhang, F., Hu, X., Chen, J., Wen, X., Sun, Y., et al. (2015). Inhibition of inflammation by astaxanthin alleviates cognition deficits in diabetic mice. *Physiol. Behav.* 151, 412–420. doi: 10.1016/j.physbeh.2015.08.015

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Nrf2 Activation Provides Atheroprotection in Diabetic Mice Through Concerted Upregulation of Antioxidant, Anti-inflammatory, and Autophagy Mechanisms

Iolanda Lazaro[1], Laura Lopez-Sanz[1,2], Susana Bernal[1,2], Ainhoa Oguiza[1], Carlota Recio[1], Ana Melgar[1], Luna Jimenez-Castilla[1], Jesus Egido[1,2], Julio Madrigal-Matute[3] and Carmen Gomez-Guerrero[1,2]*

[1] Renal, Vascular and Diabetes Research Lab, IIS-Fundacion Jimenez Diaz, Autonoma University of Madrid, Madrid, Spain, [2] Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders, Madrid, Spain, [3] Department of Developmental and Molecular Biology, Institute for Aging Studies, Albert Einstein College of Medicine, New York City, NY, United States

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***Correspondence:**
Iolanda Lazaro
iolazaro@clinic.cat;
iolanda.lazaro@fjd.es

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Interactive relationships between metabolism, inflammation, oxidative stress, and autophagy in the vascular system play a key role in the pathogenesis of diabetic cardiovascular disease. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a stress-sensitive guarantor of cellular homeostasis, which cytoprotective contributions extend beyond the antioxidant defense. We investigated the beneficial effects and underlying mechanisms of the Nrf2 inducer tert-butyl hydroquinone (tBHQ) on diabetes-driven atherosclerosis. In the experimental model of streptozotocin-induced diabetes in apolipoprotein E-deficient mice, treatment with tBHQ increased Nrf2 activity in macrophages and vascular smooth muscle cells within atherosclerotic lesions. Moreover, tBHQ significantly decreased the size, extension and lipid content of atheroma plaques, and attenuated inflammation by reducing lesional macrophages (total number and M1/M2 phenotype balance), foam cell size and chemokine expression. Atheroprotection was accompanied by both systemic and local antioxidant effects, characterized by lower levels of superoxide anion and oxidative DNA marker 8-hydroxy-2′-deoxyguanosine, reduced expression of NADPH oxidase subunits, and increased antioxidant capacity. Interestingly, tBHQ treatment upregulated the gene and protein expression of autophagy-related molecules and also enhanced autophagic flux in diabetic mouse aorta. *In vitro*, Nrf2 activation by tBHQ suppressed cytokine-induced expression of pro-inflammatory and oxidative stress genes, altered macrophage phenotypes, and promoted autophagic activity. Our results reinforce pharmacological Nrf2 activation as a promising atheroprotective approach in diabetes, according to the plethora of cytoprotective mechanisms involved in the resolution of inflammation and oxidative stress, and restoring autophagy.

Keywords: nuclear factor (erythroid-derived 2)-like 2, redox balance, autophagy, inflammation, diabetes complications

INTRODUCTION

Diabetes is a major risk factor for atherosclerosis (Eckel et al., 2002). Vascular complications are the leading cause of disability and mortality in diabetic patients (Rask-Madsen and King, 2013). Chronic hyperglycemia has been linked to a low-grade inflammatory state, in which excessive production of pro-inflammatory cytokines and ROS by mitochondria and NADPH oxidase (Nox), and impaired antioxidant and autophagic mechanisms contribute to the pathology and complications of diabetes (Jung et al., 2008; Rask-Madsen and King, 2013). Therefore, in addition to intensive glycemic control, there is an urgent need for novel therapies to limit vascular inflammation and restore redox balance in diabetic patients.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a redox-sensitive transcription factor and a master regulator of cytoprotective and antioxidant genes including heme oxygenase 1 (HMOX1), superoxide dismutase 1 (SOD1) and catalase (Itoh et al., 1997). Beyond the resolution of oxidative stress, additional roles of Nrf2 include the inhibition of inflammation by directly impeding pro-inflammatory cytokine gene transcription (Kobayashi et al., 2016), the regulation of genes involved in lipid metabolism, apoptosis and cell death, and the induction of autophagy markers such as sequestosome 1 (SQTSM1/p62) and autophagy-related protein 5 (ATG5) (Pajares et al., 2016). Nrf2 under the canonical pathway remains bound to Kelch-like ECH-associated protein 1 (KEAP1) which facilitates the ubiquitination and constant proteasomal degradation of Nrf2 (Itoh et al., 1997; Ma et al., 2011). Upon cellular stress, KEAP1 inhibitory effect is abolished, and subsequently stabilized Nrf2 translocates into the nucleus and binds to the AREs to activate transcription of detoxifying and antioxidant genes (Itoh et al., 1997). Furthermore, the non-canonical pathway tightly links Nrf2 and autophagy. In this cysteine-independent mechanism, SQSTM1/p62 sequesters KEAP1 to autophagic degradation that ultimately leads to Nrf2 stabilization and transactivation of Nrf2-dependent genes (Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010). Autophagy is a highly conserved lysosomal degradation pathway that removes protein aggregates and damaged organelles to maintain metabolic processes and cellular homeostasis (Singh et al., 2009; Levine and Klionsky, 2017).

Nrf2-mediated pathway is increasingly proposed as a way to prevent or treat disease. In preclinical models, pharmacological Nrf2 activators including 1,2-mercapto-3-sulfur ketone derivatives (oltipraz), isopropyl sulfur cyanogen compounds (sulforaphane), selenium-containing drugs (ebselen), natural products (resveratrol and curcumin) and phenolic compounds tBHQ, have been used as treatments for cancer, cardiovascular, metabolic and neurodegenerative diseases (Jiang et al., 2010; Ma, 2013; Tan and de Haan, 2014; Tan et al., 2014; Chen et al., 2015; Lazaro et al., 2017). Several *in vivo* studies have described tBHQ cytoprotective actions under pathological conditions. Indeed, tBHQ treatment suppresses ischemia and reperfusion injury in brain (Shih et al., 2005; Hou et al., 2018; Xu et al., 2018) and kidney (Guerrero-Beltrán et al., 2012). Neuroprotective actions have been also reported in experimental models of traumatic brain injury (Saykally et al., 2012; Chandran et al., 2017), Alzheimer's disease (Akhter et al., 2011), and neonatal hypoxic-ischemic brain damage (Zhang et al., 2018). Moreover, tBHQ ameliorates overload-induced cardiac dysfunction by suppressing apoptosis and promoting autophagy (Lin et al., 2014; Zhang et al., 2015), and also improves angiogenesis and heart function in a model of myocardial infarction (Zhou et al., 2017). tBHQ results from butylated hydroxyanlisole biotransformation, and readily auto-oxidizes to an electrophilic metabolite, tert-butylbenzoquinone (tBQ). It has been described that tBQ chemically modifies KEAP1 protein by covalent binding through its reactive cysteines (Cys23, Cys151, Cys226, and Cys368). Due to the resultant profound conformational change in KEAP1, Nrf2 escapes from KEAP1-mediated proteasomal degradation and is able to trigger the antioxidant response (Abiko et al., 2011).

Because uncontrolled inflammation, oxidative stress, and defective autophagy all concur in the pathogenesis of diabetic micro- and macrovascular complications (Schrijvers et al., 2011; Tabas et al., 2015), we hypothesize that targeting Nrf2 in the diabetic milieu could lead to a concerted upregulation of cytoprotective pathways in the vasculature to mitigate atherosclerosis progression. This study aims to explore *in vivo* and *in vitro* the effects and underlying mechanisms of the Nrf2 activator tBHQ, on preventing diabetes-associated atherosclerosis. To that end, *in vivo* studies were performed in streptozotocin (STZ)-induced diabetic apolipoprotein E-deficient (apoE$^{-/-}$) mice, an insulin-deficient model that combines hyperglycemia and hyperlipidemia and develops accelerated vascular injury with similarities to human atherosclerosis (Hsueh et al., 2007; Chew et al., 2010). And finally, *in vivo* results were further mechanistically characterized *in vitro* in vascular cell cultures.

Abbreviations: apoE$^{-/-}$, apolipoprotein E-deficient; ARE, antioxidant response element; ARG, arginase; ATG5, autophagy-related gene 5; ATG7, autophagy-related gene 7; BECN1, beclin 1; CAT, catalase; CCL C-C motif, chemokine ligand; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HMOX, heme oxygenase 1; IL-6, interleukin 6; IFNγ, interferon γ; KEAP1, Kelch-like ECH-associated protein 1; MAP1LC3B, microtubule-associated protein 1A/1B light chain B; MRC1, mannose receptor C-type 1; NF-κB, nuclear factor κB; NOS2, inducible nitric oxide synthase; Nox, NADPH oxidase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; O_2^-, superoxide anion; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; SMA, smooth muscle actin; SOD1, superoxide dismutase 1; SQSTM1/p62, sequestosome 1; STZ, streptozotocin; tBHQ, tert-butyl hydroquinone; tBQ, tert-butylbenzoquinone; VSMC, vascular smooth muscle cell.

MATERIALS AND METHODS

Diabetes Model and Treatments

The housing and care of animals and all the procedures performed in this study were strictly in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committee of IIS-Fundacion Jimenez Diaz (Reference No. PROEX 116/16).

Study I: Atherogenesis Model (Figure 1A)

Experimental diabetes was induced in 8-week old male apoE$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME, United States) by intraperitoneal injection of STZ (125 mg/kg/day in 10 mmol/L citrate buffer, pH 4.5; S0130, Sigma-Aldrich, St. Louis, MO, United States) once a day for two consecutive days (Recio et al., 2014; Lazaro et al., 2015). Animals were maintained on standard diet and monitored every 2–3 days (always at the same time of day) for body weight and non-fasting blood glucose. Mice with overt diabetes (glucose >19.4 mmol/L) were randomized to receive 6 weeks of treatment with tBHQ (50 mg/kg/day; 112941, Sigma-Aldrich, St. Louis, MO, United States; n = 15) or vehicle (5% ethanol in saline; n = 15), via intraperitoneal injection every second day. Age-matched non-diabetic mice (tBHQ and vehicle; n = 6 each group) were used as controls. At the study endpoint, 16 h-fasted mice were anesthetized (100 mg/kg ketamine and 15 mg/kg xylazine), saline-perfused and euthanized. Blood, urine, and tissue (aorta and liver) samples were collected. Serum levels of glucose, cholesterol (total, HDL, and LDL), triglycerides, and transaminases were measured by automated methods. Serum and urine concentrations of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were assessed by ELISA (SKT-120, StressMarq Biosciences, Inc., Victoria, BC, Canada); total antioxidant capacity by colorimetric assay (STA-360, OxiSelect TAC Assay Kit, Cell Biolabs, Inc., San Diego, CA, United States).

Study II: *In Vivo* Blockage of Autophagic Flux (Figure 1B)

Diabetic apoE$^{-/-}$ mice were treated with tBHQ and vehicle (n = 6/group) for 3 weeks (same protocol as described for Study I). Circadian and nutritional regulation of autophagy was considered in experimental design for autophagic flux measurement (Haspel et al., 2011; Ma et al., 2011). We injected intraperitoneally a single dose of leupeptin 20 mg/kg b.w. (BP2662, Fisher Scientific, Waltham, MA, United States) or saline into 6 h starved mice during dark cycle, and harvested tissues (aorta and liver) 2 h later.

Atherosclerotic Lesion Analysis

To analyze plaque area and composition, the upper aortic root was embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Alphen aan den Rijn, Netherlands) and cryosectioned. Atherosclerotic lesion area (μm^2) and neutral lipid content were quantified in serial 8 μm aortic sections (covering about 1,000 μm from valve leaflets) after oil-red-O/hematoxylin staining and averages were calculated from 2 to 3 sections. The average foam cell size was calculated by counting the number of nuclei (hematoxylin staining) in lipid-rich areas (oil-red-O positive staining) of sections from 10 randomly selected mice per group. Total macrophages (CD68; ab53444, Abcam, Cambridge, United Kingdom), macrophage phenotypes [M1 marker arginase (ARG) 2, sc-20151; M2 marker ARG1, sc-18354; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States], VSMCs (α-smooth muscle actin; ab5694, Abcam) and HMOX1 protein (ADI-SPA-895, Enzo Life Sciences, Farmingdale, NY, United States) were detected by immunoperoxidase (HRP-conjugated secondary antibodies: anti-rabbit, 711-035-152; anti-mouse, 715-035-150; anti-rat, 712-035-150; Jackson ImmunoResearch, West Grove, PA, United States) or immunofluorescence (Alexa Fluor 488/568 antibodies: anti-rabbit, A-11011; anti-goat, A-11055; Thermo

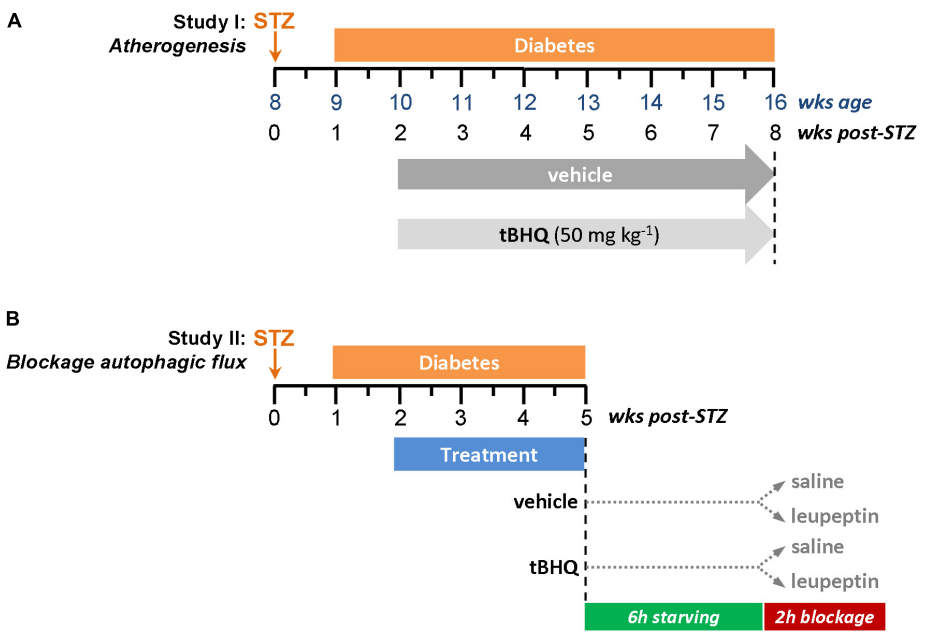

FIGURE 1 | Experimental protocols for diabetes induction in apoE$^{-/-}$ mice. **(A)** Study I: Atherogenesis model. **(B)** Study II: *In vivo* blockage of autophagic flux model.

Fisher Scientific, Waltham, MA, United States). Activated Nrf2 was detected by *in situ* Southwestern histochemistry using digoxigenin-labeled probe (Mallavia et al., 2013; Lazaro et al., 2015, 2017). Intracellular superoxide anion (O_2^-) in atherosclerotic lesions was assessed by microscopy using the O_2^--sensitive fluorescent dye dihydroethidium (DHE; D1168, Life Technologies, Carlsbad, CA, United States), followed by nuclear counterstain (DAPI; D9542, Sigma-Aldrich, St. Louis, MO, United States) (Mallavia et al., 2013). As negative control, adjacent sections were treated with polyethylene glycol-superoxide dismutase (PEG-SOD 500 U/mL; Sigma, S9549) for 2 h before DHE to determine the specificity of the fluorescence signal. All the histological evaluations were conducted in a blinded fashion. Positive staining was quantified in at least two sections per mice using Image Pro-Plus (Media Cybernetics, Bethesda, MD, United States) and expressed as percentage or number of positive cells per lesion area.

Cell Cultures

Vascular smooth muscle cell (VSMC) were isolated from mouse aorta by enzymatic digestion with collagenase type II (C6885, Sigma-Aldrich, St. Louis, MO, United States), cultured in DMEM (D6546, Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% FBS (F7524, Sigma-Aldrich, St. Louis, MO, United States), and used between 2nd and 8th passages (Recio et al., 2014; Lazaro et al., 2015). Murine bone marrow-derived macrophages were obtained after 7 days in DMEM containing 10% FBS and 10% L929 cell-conditioned medium as a source of macrophage colony stimulating factor (Recio et al., 2014; Lazaro et al., 2015). All culture media were supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (G7513 and P0781, Sigma-Aldrich, St. Louis, MO, United States). Cell viability was assessed by the 1-(4,5-dimethylthiazol2-yl)-3,5-diphenylformazan thiazolyl blue formazan (MTT; M5655, Sigma-Aldrich, St. Louis, MO, United States) method. Quiescent cells pretreated with tBHQ (5–25 μmol/L for 1–3 h) were exposed to the combination of inflammatory cytokines [10^2 units/mL interleukin (IL)-6 plus 10^3 units/mL interferon-γ (IFNγ); 216-16 and 315-05, PeproTech, Rocky Hill, NJ, United States], then processed and analyzed for gene and protein expressions. For autophagy experiments, cells in serum-deprived (0% FBS) or serum-supplemented (10% FBS) medium were treated with tBHQ for 90 min prior to incubation with autophagy inhibitors (100 μmol/L leupeptin plus 20 mmol/L NH_4Cl; L2884 and A9434, Sigma-Aldrich, St. Louis, MO, United States) for additional 2 h (Bejarano et al., 2014).

mRNA Expression Analysis

Total RNA from mouse tissues and cultured cells was extracted with TRIzol (Life Technologies). The resulting total RNA was quantified using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). For each RNA sample, 1.5 μg of total RNA was reversely transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). Real-time PCR was performed using a 7,500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, United States) with TaqMan or SYBR Green Gene Expression detection assays. Expression levels of target genes were normalized to 18S housekeeping gene. The relative expression was determined using the formula $2^{-\Delta Ct}$.

Protein Expression Analysis

For total protein extraction, mouse tissues and cells were homogenized in 0.25 M sucrose buffer containing 0.2 mmol/L Na_3VO_4, 10 mmol/L NaF, 0.2 mmol/L PMSF, and protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, United States). Nuclear fractions were obtained by cellular homogenization in 10 mmol/L HEPES (pH 7.8), 15 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L PMSF, and protease inhibitors. Proteins were resolved on SDS-PAGE gels, transferred and immunoblotted for Nrf2 (sc-722, Santa Cruz Biotechnology, Beverly, MA, United States; orb6544, Biorbyt, Ltd., Cambridge, United Kingdom), HMOX1, microtubule-associated protein 1 light chain 3 (MAP1LC3B; 2,775, Cell Signaling), SQSTM1/p62 (sc-28359, Santa Cruz Biotechnology) and beclin-1 (BECN1; sc-11427, Santa Cruz Biotechnology), using histone H3 (4499, Cell Signaling), β-actin (sc-47778, Santa Cruz Biotechnology) and α-tubulin (T5168, Sigma-Aldrich) as loading controls. Peroxidase-conjugated secondary antibodies (anti-rabbit 711-035-152, anti-mouse 715-035-150, Jackson ImmunoResearch) were used for chemiluminescence detection. Blots were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, United States). For monitoring autophagic flux blockage *in vivo* (Study II) and *in vitro*, tissue and cell lysates were immunoblotted for MAP1LC3B or SQSTM1/p62 and the autophagic flux rate was determined as the amount of accumulated proteins (normalized to loading control) in the presence of lysosomal protease inhibitors vs. protease inhibitor-free conditions (Bejarano et al., 2014; Klionsky et al., 2016).

Statistical Analysis

Results are presented as individual data points and mean ± SEM of duplicate/triplicate determinations from separate animals and cell experiments. Statistical analyses were performed using Prism 5 (GraphPad Software, Inc., La Joya, CA, United States). Differences across groups were considered significant at $P < 0.05$ using either non-parametric Mann–Whitney *U*-test, or one-way ANOVA with *post hoc* Bonferroni pairwise comparison test when appropriate.

RESULTS

In Vivo and *in Vitro* Induction of Nrf2 Activity by tBHQ

To explore *in vivo* whether Nrf2 induction protects against development of diabetes-driven atherosclerosis, we set up an experimental model of accelerated vascular injury alike to human atherosclerotic lesions, resulting from the combination of hyperglycemia and hyperlipidemia (Hsueh et al., 2007;

Chew et al., 2010; Recio et al., 2014; Lazaro et al., 2015). In this study, streptozotocin-induced diabetic apoE$^{-/-}$ mice were treated with either vehicle or tBHQ for 6 weeks. *In situ* southwestern histochemistry revealed a 2.2-fold increase in the number of Nrf2-activated cells within atherosclerotic lesions of tBHQ-treated mice (**Figure 2A**). Activated Nrf2 colocalized with both macrophages and VSMC (**Figure 2B**) which are main cellular constituents of atherosclerotic lesions with an active role in atherogenesis. In addition, tBHQ treatment induced Nrf2 expression at both mRNA and protein levels in the aorta of non-diabetic and diabetic mice, as showed by real-time PCR (**Figure 2C**) and Western blot (**Figure 2D**). The induction of Nrf2 gene was also confirmed in liver tissue from tBHQ-treated mice (**Figure 2C**). According to these *in vivo* observations, we next tested *in vitro* the ability of tBHQ to bolster intrinsic Nrf2 pathway in cultures of VSMC and macrophages. Western blot analysis revealed increased levels of Nrf2 protein in nuclear fractions from both VSMC and macrophages incubated with tBHQ (**Figure 2E**). Real-time PCR analysis confirmed a dose-dependent induction of Nrf2 gene by tBHQ in primary macrophages (**Figure 2F**). Consistently, tBHQ dose-dependently induced the gene and protein expression of HMOX1, which is a Nrf2-downstream target gene, in both cell types (**Figures 2G,H**).

Atheroprotective Effect of tBHQ Treatment in Non-diabetic and Diabetic Mice

Quantitative assessment of aortic root sections after oil-red-O/hematoxylin staining evidenced the role of diabetes as a driving force in the early progression of atherosclerosis (twofold increase in lesion area in diabetic vs. non-diabetic mice; $P = 0.0004$) (**Figures 3A,B**). Interestingly, tBHQ treatment significantly reduced the size (**Figure 3B**) and extension (**Figure 3C**) of atherosclerotic lesions both in non-diabetic and diabetic mice compared to their respective age-matched vehicle controls (% of decrease: non-diabetic, 66 $\pm$ 6; diabetic, 46 $\pm$ 8). In addition, plaques from tBHQ-treated mice displayed lower neutral lipid content (% of decrease: non-diabetic, 64 $\pm$ 5; diabetic, 28 $\pm$ 5; **Figure 3D**). Throughout the study, non-fasting blood glucose levels remained similar between vehicle and tBHQ in both non-diabetic and diabetic groups (**Figure 3E**). At the end of the study, there were no significant changes in body weights and serum lipid levels (**Table 1**) after tBHQ treatment. Serum transaminase activities kept similar between vehicle and tBHQ groups, indicating preserved liver function (**Table 1**).

Impact of Nrf2 Activation on Inflammation and Redox Balance

Histological assessment of diabetic mouse aorta showed that tBHQ treatment limited the accumulation of macrophages (CD68 positive staining) within atherosclerotic plaques (**Figure 4A**) and also altered the phenotypic distribution of macrophages in atheroma by reducing M1 marker (ARG2) and increasing M2 marker (ARG1) (**Figure 4B**). Furthermore, calculation of average foam cell size in atherosclerotic plaques demonstrated that lesional macrophages in tBHQ-treated mice were smaller than in vehicle group (**Figure 4C**), indicating a reduced lipid accumulation in these cells. Likewise, real-time PCR analysis in aortic samples evidenced that tBHQ significantly reduced the chemokine gene pattern both in non-diabetic and diabetic mice (**Figure 4D**), thus confirming its anti-inflammatory function.

Next, we explored *in vitro* the Nrf2-dependent anti-inflammatory effects of tBHQ in macrophages stimulated with cytokines (IL-6 plus IFNγ), in an attempt to mimic the inflammatory environment of the atheroma plaque. As shown in **Figure 4E**, tBHQ prevented cytokine-induced expression of chemokines C-C motif chemokine ligand (CCL) 2 and CCL5. In addition, tBHQ primed macrophages toward the anti-inflammatory M2 phenotype, as evidenced by decreased expression of inducible nitric oxide synthase (NOS2, marker of pro-inflammatory M1 phenotype), modest increase in mannose receptor C-type 1 (MRC1, M2 marker), and lower ratio of ARG isoforms [ARG2 (M1) to ARG1 (M2)].

To assess the impact of tBHQ on redox balance, we analyzed oxidative stress markers in atherosclerotic plaques. *In situ* detection of O_2^- production by PEG-SOD-inhibitable DHE fluorescence in aortic sections revealed that tBHQ treatment markedly reduced the number of DHE$^+$ cells in atherosclerotic plaques compared to vehicle-treated group (**Figure 5A**). Concomitantly, tBHQ almost reversed the increase of serum and urinary levels of 8-OHdG (a marker of oxidative DNA damage) to control levels (**Figure 5B**). Next, we examined the tBHQ effectiveness on antioxidant defense by assessing Nrf2-driven antioxidant genes not only in aorta but also in hepatic tissue, due to the important role of liver in atherogenesis as an essential hub of lipid metabolism. Immunodetection of HMOX1 protein revealed higher expression levels in atherosclerotic plaques (**Figure 5C**) and also in protein extracts from aortic and hepatic tissues (**Figure 5D**) of tBHQ-treated mice. Real-time PCR analysis also showed upregulated gene expression of the antioxidant enzymes HMOX1, SOD1, and CAT in both diabetic and non-diabetic mice receiving tBHQ when compared with respective vehicle-treated groups (**Figure 5E**). A parallel analysis of the total antioxidant status revealed that tBHQ recovered the impaired capacity of serum and urine to resist oxidation (**Table 1**). Complementary *in vitro* studies in primary macrophages confirmed that tBHQ altered the macrophage redox balance, with reduced gene expression of Nox components (membrane-associated Nox2 protein and cytosolic p47^{phox} and p67^{phox} regulatory subunits), alongside increased expression of antioxidant enzymes HMOX1, SOD1, and CAT (**Figure 5F**).

Effect of tBHQ Treatment on Autophagy Mechanisms *in Vivo* and *in Vitro*

To investigate the involvement of autophagy in Nrf2-mediated atheroprotection we analyzed the expression of genes involved in key steps of autophagy, from initiation (BECN1), to autophagosome formation (ATG7, ATG5, and MAP1LC3B) and targeting polyubiquitinated proteins for degradation (SQSTM1/p62). Real-time PCR analysis in diabetic mouse aorta showed that tBHQ treatment significantly increased the mRNA expression of autophagy genes, except for MAP1LC3B

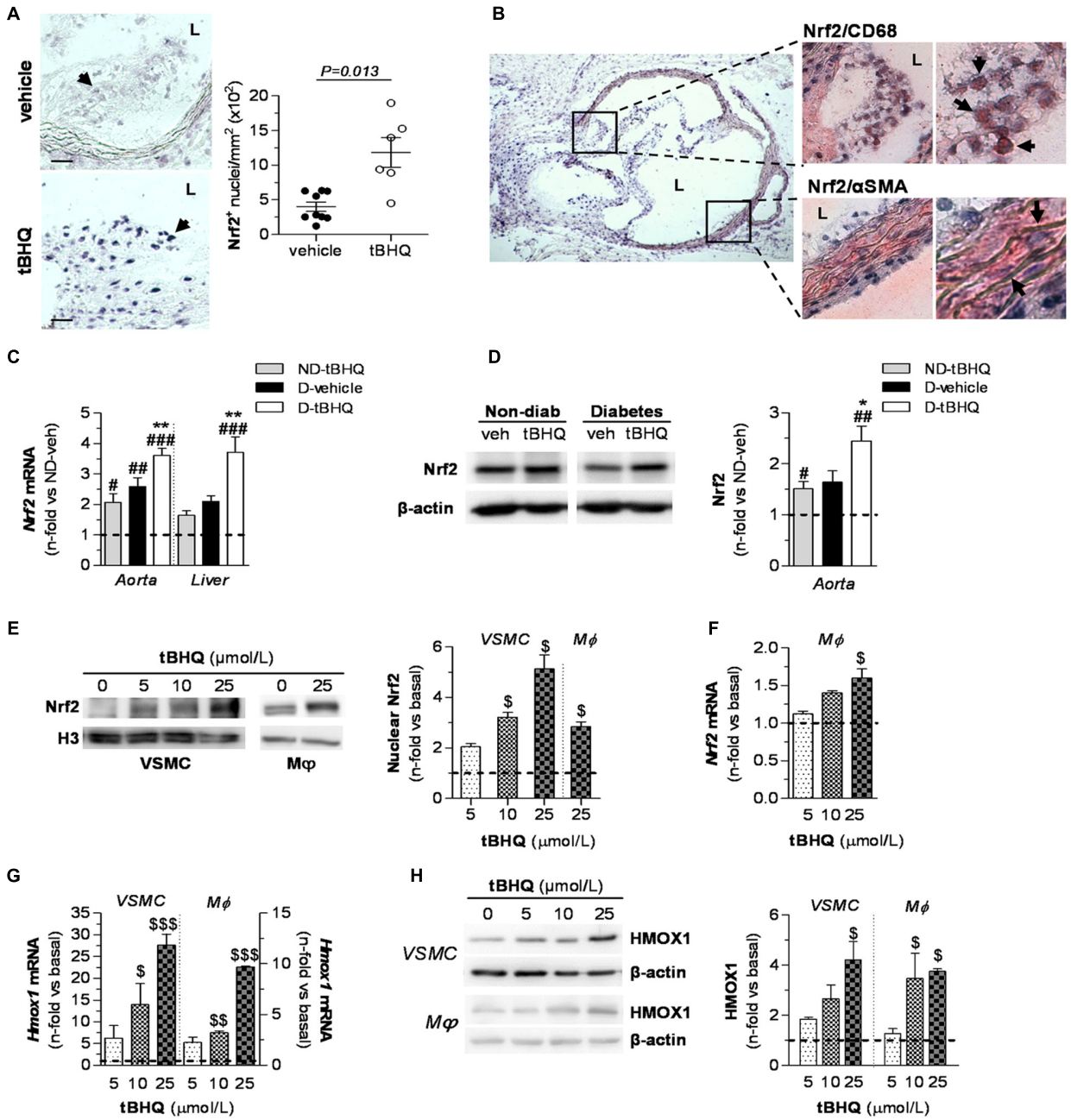

FIGURE 2 | Nrf2 activation by tBHQ *in vivo* and *in vitro*. **(A,B)** *In situ* detection of activated Nrf2 by Southwestern histochemistry in aorta of diabetic apoE$^{-/-}$ mice (2 sections per mice) after 6 weeks of treatment with either vehicle (n = 9) or tBHQ (n = 6). **(A)** Representative images (magnification ×200, scale bars: 40 μm) and quantification of positive cells per lesion area are shown. Arrows indicate Nrf2 positive cells (blue–purple). Results are presented as individual data points and mean ± SEM (P-value as indicated). **(B)** Representative micrographs (magnification ×40) and higher-magnifications (×200 and ×400) of the boxed regions showing colocalization of Nrf2 with macrophages (CD68) and VSMC (α-SMA). Arrows indicate double positive staining (blue–purple, Nrf2; red-brown, cell type marker). L, lumen. **(C)** Real-time PCR analysis of *Nrf2* gene expression in aorta and liver from non-diabetic and diabetic mice treated with vehicle or tBHQ. The mRNA values were normalized to 18S rRNA. **(D)** Western blot analysis of Nrf2 levels in aortic protein extracts (β-actin as loading control). Shown are representative blots and the summary of normalized densitometric quantification. Data in **(C,D)** are presented as mean ± SEM [non-diabetic (ND)-vehicle, n = 4; ND-tBHQ, n = 4; diabetic **(D)**-vehicle, n = 6; D-tBHQ, n = 9] of the relative (fold change) expression over control group (ND-vehicle; indicated by horizontal dashed lines). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. ND-vehicle; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. D-vehicle. **(E)** Representative Western blot images and quantification of Nrf2 in nuclear extracts from cultured VSMC and macrophages (Mϕ) incubated with increasing concentrations of tBHQ (0–25 μmol/L) for 3 h. Histone H3 was used as nuclear loading control. Real-time PCR analysis of *Nrf2* **(F)** and *Hmox1* **(G)** gene expression at 6 h of treatment with tBHQ. Data are normalized to 18S. **(H)** Western blot analysis of HMOX1 (β-actin as loading control) in total cell lysates from VSMC and macrophages incubated for 24 h with tBHQ. Representative immunoblots and summary of normalized densitometry values are shown. Data in **(E–H)** are expressed as fold increases over basal conditions (indicated by horizontal dashed lines) and are the mean ± SEM of n = 3 independent experiments analyzed in duplicate. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, and $^{\$\$\$}P < 0.001$ vs. basal.

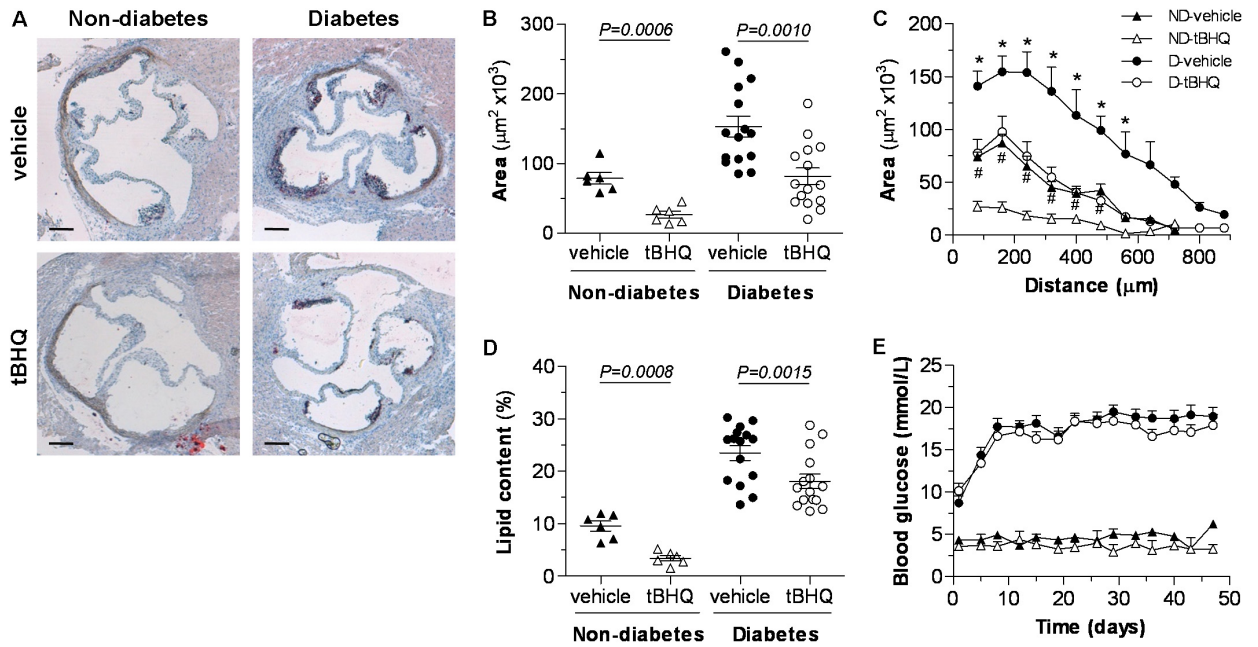

FIGURE 3 | tBHQ treatment lessens the development of atherosclerosis in mice. **(A)** Representative images (magnification ×40, scale bars: 200 μm) of oil-red-O/hematoxylin staining in aortic root sections from non-diabetic and diabetic mice treated with either vehicle or tBHQ. **(B)** Average of individual maximal lesion area in each group (calculated from 2 to 3 sections per mice). **(C)** Quantification of the extent of atherosclerotic lesions within the aorta (9–11 sections per mice). **(D)** Quantification of intraplaque lipid content (% oil-red-O per lesion area, calculated from 2 to 3 sections per mice in individual maximal lesion area). **(E)** Evolution of mean blood glucose values in non-fasted mice along the 6-week follow-up. Results are expressed as individual data points and mean ± SEM [non-diabetic (ND)-vehicle, $n = 6$; ND-tBHQ, $n = 6$; diabetic (D)-vehicle, $n = 15$; D-tBHQ, $n = 15$]. $^{*}P < 0.05$ vs. D-vehicle; $^{\#}P < 0.05$ vs. ND-vehicle.

TABLE 1 | Biochemical data of apoE$^{-/-}$ mice after 6 weeks of treatment.

	Non-diabetic		Diabetic	
	Vehicle	tBHQ	Vehicle	tBHQ
	(*n* = 6)	(*n* = 6)	(*n* = 15)	(*n* = 15)
Body weight change (final–initial) (g)	3.2 ± 0.0	0.0 ± 3.0	−2.8 ± 1.0	−3.0 ± 1.0
Total cholesterol (mg/dL)	330 ± 19	263 ± 18	852 ± 47$^{\#\#\#}$	935 ± 69$^{\#\#\#}$
LDL cholesterol (mg/dL)	293 ± 18	227 ± 13	819 ± 42$^{\#\#\#}$	901 ± 66$^{\#\#\#}$
HDL cholesterol (mg/dL)	15.3 ± 0.6	15.5 ± 0.8	10.3 ± 0.7$^{\#\#}$	11.2 ± 0.7$^{\#}$
Triglycerides (mg/dL)	111 ± 10	124 ± 16	109 ± 15	112 ± 15
Aspartate aminotransferase (units/L)	169 ± 4	169 ± 25	188 ± 21	184 ± 14
Alanine aminotransferase (units/L)	51 ± 7	52 ± 7	86 ± 11	88 ± 8
Serum total antioxidant capacity (mmol/L)	0.34 ± 0.04	0.62 ± 0.06$^{\#\#}$	0.19 ± 0.02	0.42 ± 0.03*
Urine total antioxidant capacity (mmol/L)	1.5 ± 0.1	2.4 ± 0.1$^{\#\#\#}$	1.2 ± 0.1	2.0 ± 0.1$^{**,\#}$

Data are mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. non-diabetic vehicle group. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. diabetic vehicle group.

(**Figure 6A**). Western blot further confirmed upregulated levels of BECN1 and SQSTM1/p62 proteins, and conversion of MAP1LC3B-I to MAP1LC3B-II form in tBHQ-treated mice (**Figure 6B**). Similar results were observed in liver (**Figures 6A,B**), thus corroborating the beneficial systemic effects of tBHQ.

In addition to the steady-state changes of autophagy markers, we also studied the effect of tBHQ on autophagic flux by assessing MAP1LC3B-II levels in the presence or absence of lysosomal protease inhibitors. In these experiments, blockage of lysosomal proteolysis *in vivo* was assessed by intraperitoneal injection of leupeptin in 6 h starved diabetic mice. Under these conditions, Western blot analysis revealed that tBHQ increases autophagic flux in the vasculature, as evidenced by higher accumulation of MAP1LC3B-II protein in tBHQ-treated mice compared with vehicle group (**Figure 7A**). However, no accumulation of autophagic adaptor SQSTM1/p62 was found in the aortic tissue upon tBHQ treatment (**Figure 7A**). Finally, we investigated the *in vitro* effects of tBHQ on autophagy machinery. Real-time PCR

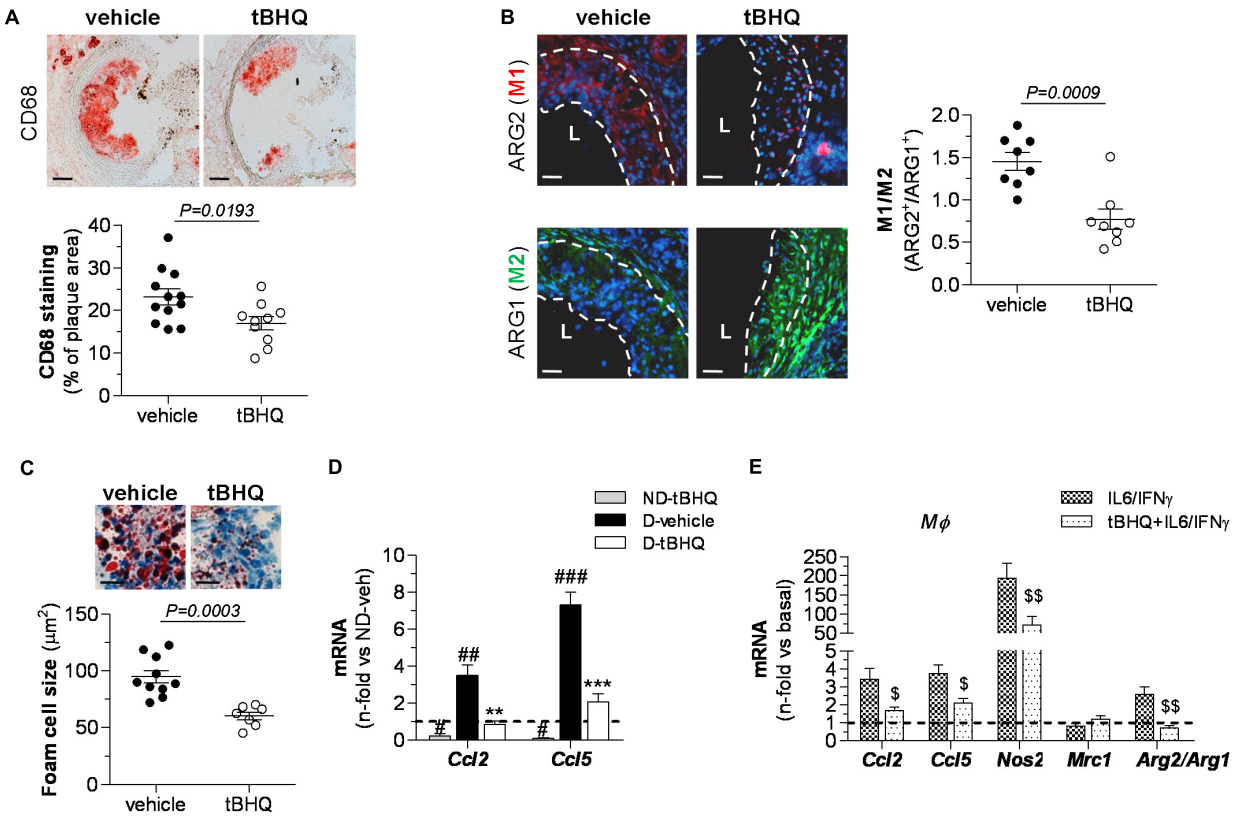

FIGURE 4 | Reduced inflammation in atherosclerotic plaques of tBHQ-treated mice. **(A)** Immunoperoxidase detection of total macrophages (magnification ×100, scale bars: 80 μm) and quantitative analysis of CD68+ cells in aortic root sections of diabetic mice (vehicle, *n* = 12; tBHQ, *n* = 10; 2 sections per mice). **(B)** Immunofluorescence analysis of macrophage phenotypes (*red*, ARG2 (M1); *green*, ARG1 (M2); *blue,* DAPI (nuclear staining); magnification ×200; scale bars: 20 μm; L, lumen) and quantification of M1/M2 ratio in aorta of diabetic mouse (vehicle, *n* = 8; tBHQ, *n* = 8; 2 sections per mice). **(C)** Representative images (magnification x200, scale bars: 40 μm) of foam cell-rich areas in atherosclerotic lesions of diabetic mice (vehicle, *n* = 10, and tBHQ, *n* = 7; 2–3 sections per mice) and quantitative assessment of foam cell size. **(D)** Real-time PCR analysis of inflammatory markers [chemokines *Ccl2* and *Ccl5*) in aortic tissue from non-diabetic (ND)-vehicle (*n* = 4), ND-tBHQ (*n* = 4), diabetic (D)-vehicle (*n* = 10), and D-tBHQ (*n* = 10) mice]. Data normalized by 18S are expressed as fold increases over control group (ND-vehicle, represented by horizontal dashed line). **(E)** Real-time PCR analysis of *Ccl2* and *Ccl5*, macrophage phenotype markers *Nos2* (M1), *Mrc1* (M2), and *Arg2/Arg1* (ratio M1/M2) in primary macrophages pretreated with tBHQ (25 μmol/L, 90 min) or vehicle prior to stimulation with cytokines (IL-6 10^2 units/mL plus IFNγ 10^3 units/mL) for 6 h. Data normalized by 18S are expressed as fold increases over basal condition (vehicle-treated cells, represented by horizontal dashed line; *n* = 6 independent experiments). Results are expressed as individual data points and mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. D-vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. ND-vehicle; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ vs. cytokine stimulation.

analysis showed upregulated BECN1, ATG5/7, MAP1LC3B, and SQSTM1/p62 gene expression by tBHQ in VSMC under both pro-autophagic (serum-deprived medium) and basal (serum-supplemented medium) conditions (**Figure 7B**). Moreover, increased autophagic flux rate by tBHQ was underscored by the higher accumulation of MAP1LC3B-II protein, but not SQSTM1/p62, in VSMC incubated with lysosomal inhibitors (**Figure 7C**).

DISCUSSION

The present study provides mechanistic evidence of the atheroprotective effects of Nrf2 pathway beyond its role in commanding the antioxidant response. We report that Nrf2 activation by tBHQ counteracts diabetes-accelerated atherosclerosis through a multiple cytoprotective action based on the attenuation of vascular inflammation and oxidative stress, and bolstering autophagy.

Previous reports demonstrated that myeloid-specific Nrf2 deletion in mice aggravates early and advanced stages of atherosclerosis (Ruotsalainen et al., 2013), and controversially, total gene deficiency protects against atherogenesis by affecting both systemic and local mechanisms (Barajas et al., 2011; Freigang et al., 2011). Our work indicates that administration of tBHQ in diabetic mice further activated Nrf2 in macrophages and VSMC, two main cellular components of atherosclerotic lesions with a key role in atherogenesis (Moore and Tabas, 2011; Razani et al., 2012; Salabei and Hill, 2013). Associated with Nrf2 induction in diabetic mouse aorta, tBHQ treatment reduced the area, extension, lipid content and inflammatory milieu of atherosclerotic plaques, independently of changes in blood glycemia and lipids. Our results are in agreement with the previously reported effect of tBHQ in ischemic

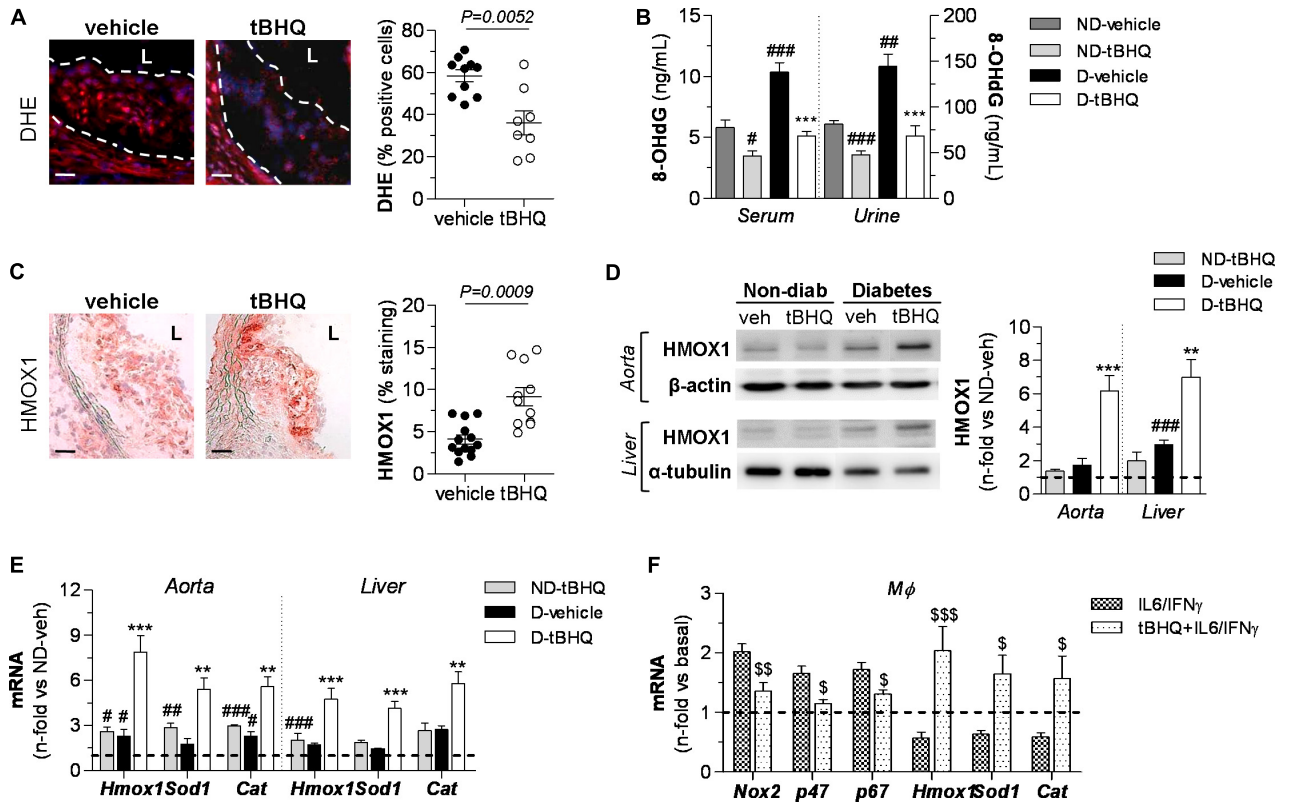

FIGURE 5 | tBHQ treatment attenuates oxidative stress and promotes antioxidant response in mice. **(A)** *In situ* detection of O_2^- production by DHE fluorescent probe labeling in aortic sections of diabetic mice (vehicle, $n = 10$; tBHQ, $n = 8$; 2 sections per mice). Representative images (magnification x200, scale bars: 40 μm; L, lumen) and quantification of percentage of positive DHE red-cells vs. total DAPI blue-nuclei. **(B)** Concentrations of 8-OHdG in serum and urine samples from non-diabetic (ND)-vehicle ($n = 4$), ND-tBHQ ($n = 6$), diabetic (D)-vehicle ($n = 3$) and D-tBHQ ($n = 6$) mice. **(C)** Immunoperoxidase images (magnification ×100, scale bars: 80 μm; L, lumen) of HMOX1 protein in aortic sections of diabetic mice (vehicle, $n = 13$; tBHQ, $n = 11$; 2 sections per mice) and summary of quantification. **(D)** Western blot analysis of HMOX1 in aorta and liver extracts from ND-vehicle ($n = 4$), ND-tBHQ ($n = 4$), D-vehicle ($n = 11$) and D-tBHQ ($n = 11$) mice (β-actin and α-tubulin as loading controls). Shown are representative blots and the summary of normalized densitometry expressed as fold increases over control group (ND-vehicle, represented by horizontal dashed line). **(E)** Real-time PCR analysis of antioxidant defense genes (*Hmox1*, *Sod1*, and *Cat*) in aorta and liver from ND-vehicle ($n = 4$), ND-tBHQ ($n = 4$), D-vehicle ($n = 12$) and D-tBHQ ($n = 12$) groups. Data normalized by 18S are expressed as fold increases over control group (ND-vehicle, represented by horizontal dashed line). **(F)** Real-time PCR analysis of Nox subunits and antioxidant enzymes in primary macrophages pretreated with tBHQ (25 μmol/L, 90 min) prior to stimulation with cytokines (IL-6 10^2 units/mL plus IFNγ 10^3 units/mL) for 6 h. Data are normalized to 18S and expressed as fold increases over basal condition (vehicle-treated cells, represented by horizontal dashed line; $n = 6$ independent experiments). Results are expressed as individual data points and mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. D-vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. ND-vehicle; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, and $^{\$\$\$}P < 0.001$ vs. cytokine stimulation.

stroke (Shih et al., 2005) and cardiac dysfunction (Zhang et al., 2015) and support the use of Nrf2 activators as potential therapy for diabetic complications (Xue et al., 2008; Jiang et al., 2010; Tan and de Haan, 2014; Tan et al., 2014).

Macrophage accumulation in the subendothelial space contributes to atherogenesis by promoting inflammatory response and plaque instability (Moore and Tabas, 2011). Our results showed that tBHQ treatment impaired the accumulation of macrophages within atherosclerotic lesions, but also affected the macrophage inflammatory state by favoring the alternatively activated M2 phenotype. Previous studies have determined a linkage of M2 macrophages with fatty acid oxidation (Vats et al., 2006), a process regulated by Nrf2 (Dinkova-Kostova and Abramov, 2015). In this sense, tBHQ might modulate macrophage capacity to catabolize extravased modified lipoproteins, leading to smaller-sized foam cells. Additionally, tBHQ treatment attenuated the aortic expression of CCL2 and CCL5, two prototypical chemokines of fundamental importance in the atherosclerotic process (Zernecke et al., 2008; van der Vorst et al., 2015). Either gene deficiency or pharmacological inhibitions of CCL2, CCL5, and their cognate receptors have been demonstrated to reduce lesion size and macrophage infiltration and to promote plaque stability in mice. In vascular cells and leukocytes, CCL2 and CCL5 expression by many atherogenic stimuli is transcriptionally regulated by nuclear factor κB (NF-κB) (Monaco and Paleolog, 2004). Evidence indicates a functional crosstalk between Nrf2 and NF-κB pathways. In macrophages, Nrf2 opposes transcriptional upregulation of proinflammatory cytokine

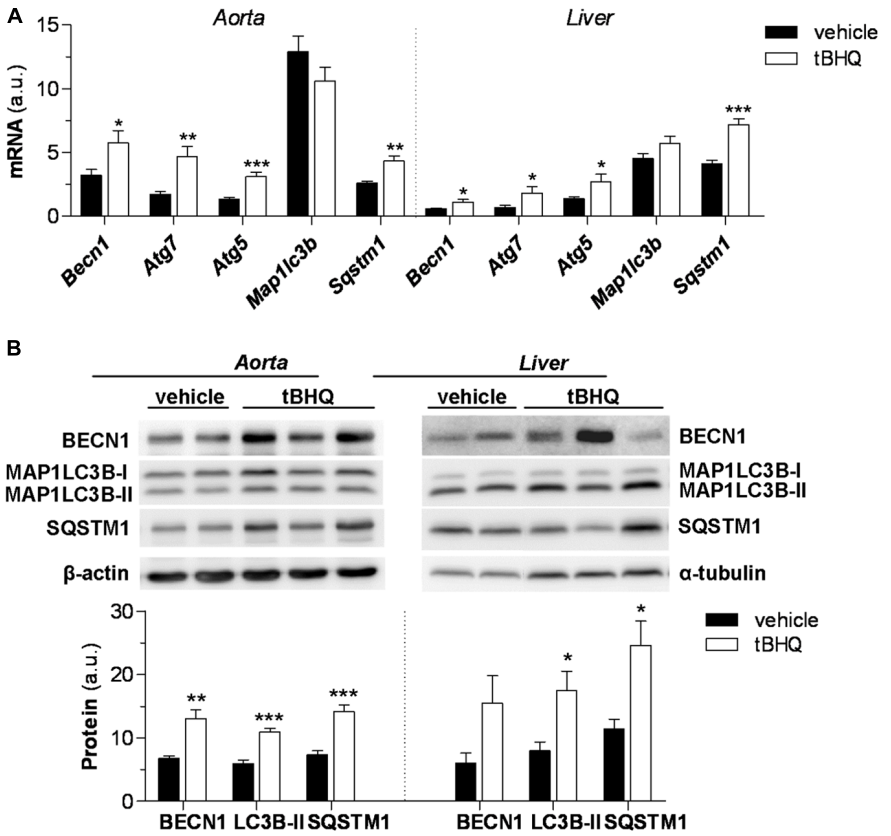

FIGURE 6 | tBHQ promotes autophagy in diabetic mouse. **(A)** Real-time PCR analysis of autophagy genes in aortic and hepatic tissues from diabetic mice treated with either vehicle (n = 15) or tBHQ (n = 15). Values normalized by 18S are expressed as arbitrary units. **(B)** Western blot analysis of autophagy markers BECN1, MAP1LC3B-II, and SQSTM1/p62 in aorta and liver of diabetic mice (D-vehicle, n = 13; D-tBHQ, n = 14). Values normalized by loading controls (β-actin and α-tubulin) are expressed as arbitrary units. Results are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. D-vehicle.

genes (Kobayashi et al., 2016) and its absence exacerbates NF-κB activity and promotes atherogenesis (Ruotsalainen et al., 2013). Conversely, NF-κB represses Nrf2 by a physical interaction with KEAP1 (Yu et al., 2011). We have reported opposite regulation of NF-κB and Nrf2 by heat shock protein 90 inhibitor in diabetic atherosclerosis (Lazaro et al., 2017). In consonance, our results reinforce Nrf2 as direct regulator of inflammation, and help reconsider the concept of Nrf2-mediated anti-inflammation as an off-target effect of ROS suppression.

Hyperglycemia-induced ROS generation by Nox family promotes cellular damage and contributes to development and progression of vascular diabetic complications (Giacco and Brownlee, 2010; Jiang et al., 2011; Rask-Madsen and King, 2013). In atherosclerotic lesions, macrophages and VSMC contribute to Nox-derived ROS production (Vendrov et al., 2007), which is exacerbated in the absence of Nrf2 (Kong et al., 2010; Ashino et al., 2013). In line with this, our study demonstrates that tBHQ treatment attenuated oxidative stress both locally (reduced O_2^- production in aorta) and systemically (lower circulating levels of oxidative DNA stress marker 8-OHdG) in diabetic mice. Moreover, tBHQ suppressed the cytokine-induced expression of Nox subunits in macrophages. It is well-known that diabetes itself promotes antioxidant defense in aortas, as an adaptative response to overcome injury due to the chronic exposure to hyperglycemia-driven oxidative stress (Giacco and Brownlee, 2010; Rask-Madsen and King, 2013). Remarkably, we observed that tBHQ treatment further boosted Nrf2 system for the expression of antioxidant enzymes (HMOX1, SOD1, and CAT). Besides the positive effects on the aortic tissue, we also showed that tBHQ reinforced antioxidant machinery in liver and restored systemic total antioxidant capacity in diabetic mice.

Autophagy is activated in response to several stressors such as starvation, lipids, ROS and cytokines to maintain cellular integrity, but chronic insults can result in autophagic impairment and loss of vascular cellular homeostasis thus contributing to atherogenesis (Schrijvers et al., 2011; Salabei and Hill, 2013). Autophagy related gene deficiency in macrophages promotes atherosclerosis by triggering inflammasome hyperactivation, Nox-mediated oxidative stress, apoptosis, defective efferocytosis and necrosis (Liao et al., 2012; Razani et al., 2012), whereas VSMC-specific deletion promotes a phenotype switch with attenuated proliferative capacity, ensuing migration and premature senescence (Salabei and Hill, 2013; Grootaert et al., 2015). Moreover, defective lipid management due to a blunted

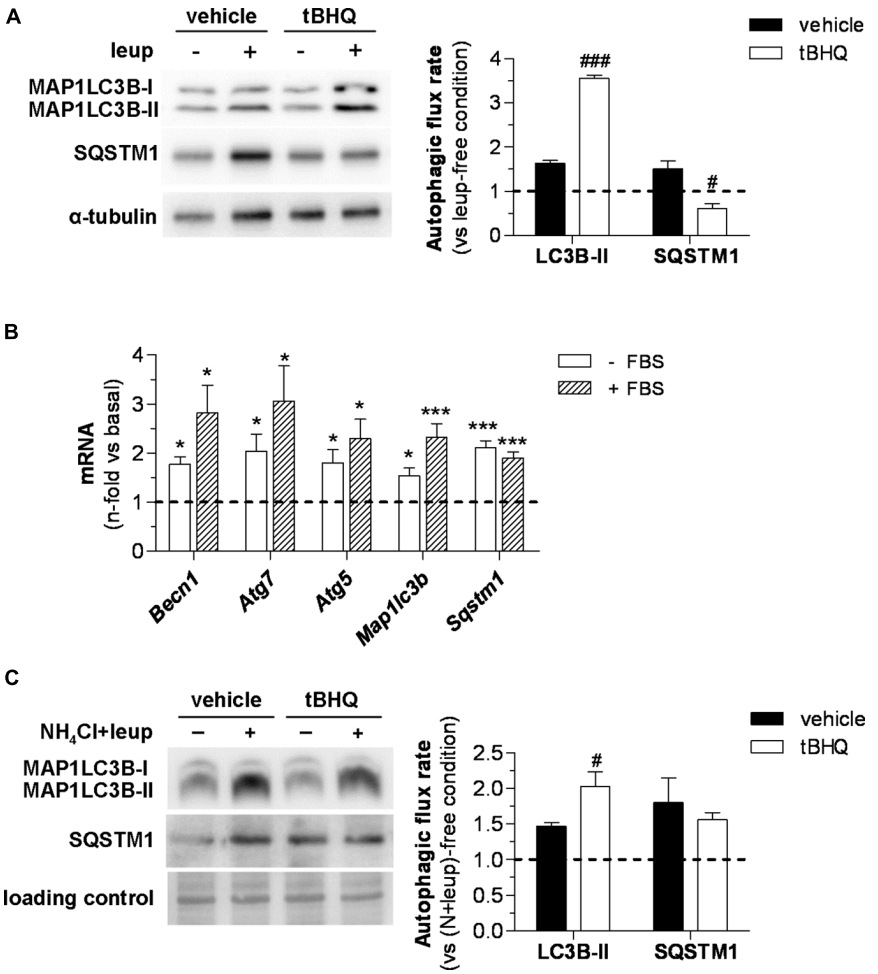

FIGURE 7 | *In vivo* and *in vitro* effect of tBHQ on autophagic activity. **(A)** *In vivo* 2 h blockage of autophagic flux in 6 h starved diabetic mice treated with either vehicle or tBHQ for 3 weeks by intraperitoneal injection of leupeptin (vehicle-group, $n = 3$; tBHQ, $n = 3$) or saline (vehicle-group, $n = 3$; tBHQ, $n = 3$). Shown are representative blots of MAP1LC3B-II, SQSTM1/p62 and α-tubulin (loading control) and quantification of autophagic flux rate in aortic tissue. **(B)** Real-time PCR analysis of autophagy genes in VSMC treated with tBHQ 25 μmol/L for 6 h in serum-deprived (-FBS) or serum-supplemented (+FBS) conditions. Data normalized by 18S are expressed as fold increase over respective basal conditions ($n = 9$ independent experiments). **(C)** *In vitro* blockage of autophagic flux in starved VSMC pretreated with either tBHQ (25 μmol/L, 90 min) or vehicle prior to the addition of lysosomal inhibitors (20 mmol/L NH_4Cl plus 100 μmol/L leupeptin; N-leup) for 2 h. Shown are representative blots and quantification of autophagic flux rate in VSMC ($n = 7$ independent experiments). The rate of autophagic flux is expressed as lysosomal protease inhibitor (leup or N-leup) induced protein accumulation (MAP1LC3B-II or SQSTM1/p62) vs. respective lysosomal protease inhibitor-free condition. Results are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. basal; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. respective lysosomal protease inhibitor-free condition.

autophagy impedes cholesterol efflux and contributes to foam cell formation (Singh et al., 2009; Koga et al., 2010). In line with this, the atheroprotection found in diabetic mice after tBHQ treatment can be linked to the restoration of cellular autophagy and the subsequent attenuation of macrophage infiltration, intracellular lipid content and foam cell formation.

Nrf2-KEAP1-ARE axis and autophagy are linked by SQSTM1/p62 protein. Indeed, direct interaction between SQSTM1/p62 and KEAP1 favors its autophagy-mediated degradation with subsequent activation of Nrf2-driven genes (Komatsu et al., 2010; Lau et al., 2010). Nrf2 upregulates SQSTM1/p62 and ATG5 genes thus creating a positive feedback loop (Jain et al., 2010; Pajares et al., 2016). In agreement, our results showed that Nrf2 activation by tBHQ upregulated gene and protein expression of components of the autophagy machinery in diabetic mice. Remarkably, tBHQ promoted autophagic activity, as measured by MAP1LC3B-II accumulation, the gold standard method to monitor autophagic activity (Klionsky et al., 2016). The data *in vivo* (aorta and liver of diabetic mice) were consistent with the data *in vitro* (primary VSMC), and together they demonstrate structural and functional modulation of autophagy by Nrf2 activation. It has previously been reported that SQSTM1/p62 protein plays a pivotal role between autophagy and proteasome degradation pathways in a MAP1LC3B-II-independent manner (Itakura and Mizushima, 2011; Liebl and Hoppe, 2016). Therefore, and according to

previous reports in other systems (Jain et al., 2010; Xu et al., 2016), it is likely that the protective role of tBHQ involves an increase of SQSTM1/p62 and an activation in proteasomal activity, and be related with a switch in the shuttling of SQSTM1/p62 observed in diabetic mouse aorta and cultured VSMC.

Collectively, this study proposes pharmacological Nrf2 activation as a useful therapeutic strategy to restrain diabetes-driven atherosclerosis, by attenuating oxidative stress and inflammation, and bolstering antioxidant defense and autophagy.

AUTHOR CONTRIBUTIONS

IL designed experiments, researched and analyzed data, and wrote the manuscript. LL-S researched, analyzed or interpreted data, and revised the manuscript. SB, AO, CR, AM, and LJ-C performed *in vivo* studies and revised the manuscript. JE critically revised the manuscript for important intellectual content. JM-M designed experiments and critically revised the manuscript. CG-G designed the study, analyzed data, and wrote the manuscript.

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REFERENCES

Abiko, Y., Miura, T., Phuc, B. H., Shinkai, Y., and Kumagai, Y. (2011). Participation of covalent modification of Keap1 in the activation of Nrf2 by tert-butylbenzoquinone, an electrophilic metabolite of butylated hydroxyanisole. *Toxicol. Appl. Pharmacol.* 255, 32–39. doi: 10.1016/j.taap.2011.05.013

Akhter, H., Katre, A., Li, L., Liu, X., and Liu, R. M. (2011). Therapeutic potential and anti-amyloidosis mechanisms of tert-butylhydroquinone for Alzheimer's disease. *J. Alzheimers Dis.* 26, 767–778. doi: 10.3233/JAD-2011-110512

Ashino, T., Yamamoto, M., Yoshida, T., and Numazawa, S. (2013). Redox-sensitive transcription factor Nrf2 regulates vascular smooth muscle cell migration and neointimal hyperplasia. *Arterioscler. Thromb. Vasc. Biol.* 33, 760–768. doi: 10.1161/ATVBAHA.112.300614

Barajas, B., Che, N., Yin, F., Rowshanrad, A., Orozco, L. D., Gong, K. W., et al. (2011). NF-E2-related factor 2 promotes atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow antioxidant protection. *Arterioscler. Thromb. Vasc. Biol.* 31, 58–66. doi: 10.1161/ATVBAHA.110.210906

Bejarano, E., Yuste, A., Patel, B., Stout, R. F. Jr., Spray, D. C., and Cuervo, A. M. (2014). Connexins modulate autophagosome biogenesis. *Nat. Cell Biol.* 16, 401–414. doi: 10.1038/ncb2934

Chandran, R., Kim, T., Mehta, S. L., Udho, E., Chanana, V., Cengiz, P., et al. (2017). A combination antioxidant therapy to inhibit NOX2 and activate Nrf2 decreases secondary brain damage and improves functional recovery after traumatic brain injury. *J. Cereb. Blood Flow Metab* doi: 10.1177/0271678X17738701 [Epub ahead of print].

Chen, B., Lu, Y., Chen, Y., and Cheng, J. (2015). The role of Nrf2 in oxidative stress-induced endothelial injuries. *J. Endocrinol.* 225, R83–R99. doi: 10.1530/JOE-14-0662

Chew, P., Yuen, D. Y., Stefanovic, N., Pete, J., Coughlan, M. T., Jandeleit-Dahm, K. A., et al. (2010). Antiatherosclerotic and renoprotective effects of ebselen in the diabetic apolipoprotein E/GPx1-double knockout mouse. *Diabetes Metab. Res. Rev.* 59, 3198–3207. doi: 10.2337/db10-0195

Dinkova-Kostova, A. T., and Abramov, A. Y. (2015). The emerging role of Nrf2 in mitochondrial function. *Free Radic. Biol. Med.* 88, 179–188. doi: 10.1016/j.freeradbiomed.2015.04.036

Eckel, R. H., Wassef, M., Chait, A., Sobel, B., Barrett, E., King, G., et al. (2002). Prevention conference VI: diabetes and cardiovascular disease: writing group II: pathogenesis of atherosclerosis in diabetes. *Circulation* 105, e138–e143. doi: 10.1161/01.CIR.0000013954.65303.C5

Freigang, S., Ampenberger, F., Spohn, G., Heer, S., Shamshiev, A. T., Kisielow, J., et al. (2011). Nrf2 is essential for cholesterol crystal-induced inflammasome activation and exacerbation of atherosclerosis. *Eur. J. Immunol.* 41, 2040–2051. doi: 10.1002/eji.201041316

Giacco, F., and Brownlee, M. (2010). Oxidative stress and diabetic complications. *Circ. Res.* 107, 1058–1070. doi: 10.1161/CIRCRESAHA.110.223545

Grootaert, M. O., da Costa Martins, P. A., Bitsch, N., Pintelon, I., De Meyer, G. R., Martinet, W., et al. (2015). Defective autophagy in vascular smooth muscle cells accelerates senescence and promotes neointima formation and atherogenesis. *Autophagy* 11, 2014–2032. doi: 10.1080/15548627.2015.1096485

Guerrero-Beltrán, C. E., Tapia, E., Sánchez-González, D. J., Martínez-Martínez, C. M., Cristobal-García, M., and Pedraza-Chaverri, J. (2012). Tert-Butylhydroquinone pretreatment protects kidney from ischemia-reperfusion injury. *J. Nephrol.* 25, 84–89. doi: 10.5301/JN.2011.8345

Haspel, J., Shaik, R. S., Ifedigbo, E., Nakahira, K., Dolinay, T., Englert, J. A., et al. (2011). Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy* 7, 629–642. doi: 10.4161/auto.7.6.15100

Hou, Y., Wang, Y., He, Q., Li, L., Xie, H., Zhao, Y., et al. (2018). Nrf2 inhibits NLRP3 inflammasome activation through regulating Trx1/TXNIP complex in cerebral ischemia reperfusion injury. *Behav. Brain Res.* 336, 32–39. doi: 10.1016/j.bbr.2017.06.027

Hsueh, W., Abel, E. D., Breslow, J. L., Maeda, N., Davis, R. C., Fisher, E. A., et al. (2007). Recipes for creating animal models of diabetic cardiovascular disease. *Circ. Res.* 100, 1415–1427. doi: 10.1161/01.RES.0000266449.37396.1f

Itakura, E., and Mizushima, N. (2011). p62 Targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. *J. Cell Biol.* 192, 17–27. doi: 10.1083/jcb.201009067

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* 236, 313–322. doi: 10.1006/bbrc.1997.6943

Jain, A., Lamark, T., Sjottem, E., Larsen, K. B., Awuh, J. A., Overvatn, A., et al. (2010). *p62/SQSTM1* is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.* 285, 22576–22591. doi: 10.1074/jbc.M110.118976

Jiang, F., Zhang, Y., and Dusting, G. J. (2011). NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacol. Rev.* 63, 218–242. doi: 10.1124/pr.110.002980

Jiang, T., Huang, Z., Lin, Y., Zhang, Z., Fang, D., and Zhang, D. D. (2010). The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. *Diabetes Metab. Res. Rev.* 59, 850–860. doi: 10.2337/db09-1342

Jung, H. S., Chung, K. W., Won, K. J., Kim, J., Komatsu, M., Tanaka, K., et al. (2008). Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab.* 8, 318–324. doi: 10.1016/j.cmet.2008.08.013

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo, A. A., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12, 1–222. doi: 10.1080/15548627.2015.1100356

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., et al. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat. Commun.* 7:11624. doi: 10.1038/ncomms11624

Koga, H., Kaushik, S., and Cuervo, A. M. (2010). Altered lipid content inhibits autophagic vesicular fusion. *FASEB J.* 24, 3052–3065. doi: 10.1096/fj.09-144519

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., et al. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat. Cell Biol.* 12, 213–223. doi: 10.1038/ncb2021

Kong, X., Thimmulappa, R., Kombairaju, P., and Biswal, S. (2010). NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. *J. Immunol.* 185, 569–577. doi: 10.4049/jimmunol.0902315

Lau, A., Wang, X. J., Zhao, F., Villeneuve, N. F., Wu, T., Jiang, T., et al. (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol. Cell. Biol.* 30, 3275–3285. doi: 10.1128/MCB.00248-10

Lazaro, I., Oguiza, A., Recio, C., Lopez-Sanz, L., Bernal, S., Egido, J., et al. (2017). Interplay between HSP90 and Nrf2 pathways in diabetes-associated atherosclerosis. *Clin. Investig. Arterioscler.* 29, 51–59. doi: 10.1016/j.arteri.2016.10.003

Lazaro, I., Oguiza, A., Recio, C., Mallavia, B., Madrigal-Matute, J., Blanco, J., et al. (2015). Targeting HSP90 ameliorates nephropathy and atherosclerosis through suppression of NF-kappaB and STAT signaling pathways in diabetic mice. *Diabetes Metab. Res. Rev.* 64, 3600–3613. doi: 10.2337/db14-1926

Levine, B., and Klionsky, D. J. (2017). Autophagy wins the 2016 nobel prize in physiology or medicine: breakthroughs in baker's yeast fuel advances in biomedical research. *Proc. Natl. Acad. Sci. U.S.A.* 114, 201–205. doi: 10.1073/pnas.1619876114

Liao, X., Sluimer, J. C., Wang, Y., Subramanian, M., Brown, K., Pattison, J. S., et al. (2012). Macrophage autophagy plays a protective role in advanced atherosclerosis. *Cell Metab.* 15, 545–553. doi: 10.1016/j.cmet.2012.01.022

Liebl, M. P., and Hoppe, T. (2016). It's all about talking: two-way communication between proteasomal and lysosomal degradation pathways via ubiquitin. *Am. J. Physiol. Cell Physiol.* 311, C166–C178. doi: 10.1152/ajpcell.00074.2016

Lin, H., Li, H. F., Chen, H. H., Lai, P. F., Juan, S. H., Chen, J. J., et al. (2014). Activating transcription factor 3 protects against pressure-overload heart failure via the autophagy molecule Beclin-1 pathway. *Mol. Pharmacol.* 85, 682–691. doi: 10.1124/mol.113.090092

Ma, D., Panda, S., and Lin, J. D. (2011). Temporal orchestration of circadian autophagy rhythm by C/EBPβ. *EMBO J.* 30, 4642–4651. doi: 10.1038/emboj.2011.322

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Mallavia, B., Recio, C., Oguiza, A., Ortiz-Munoz, G., Lazaro, I., Lopez-Parra, V., et al. (2013). Peptide inhibitor of NF-kappaB translocation ameliorates experimental atherosclerosis. *Am. J. Pathol.* 182, 1910–1921. doi: 10.1016/j.ajpath.2013.01.022

Monaco, C., and Paleolog, E. (2004). Nuclear factor kappaB: a potential therapeutic target in atherosclerosis and thrombosis. *Cardiovasc. Res.* 61, 671–682. doi: 10.1016/j.cardiores.2003.11.038

Moore, K. J., and Tabas, I. (2011). Macrophages in the pathogenesis of atherosclerosis. *Cell* 145, 341–355. doi: 10.1016/j.cell.2011.04.005

Pajares, M., Jimenez-Moreno, N., Garcia-Yague, A. J., Escoll, M., de Ceballos, M. L., Van , L. F., et al. (2016). Transcription factor NFE2L2/NRF2 is a regulator of macroautophagy genes. *Autophagy* 12, 1902–1916. doi: 10.1080/15548627.2016.1208889

Rask-Madsen, C., and King, G. L. (2013). Vascular complications of diabetes: mechanisms of injury and protective factors. *Cell Metab.* 17, 20–33. doi: 10.1016/j.cmet.2012.11.012

Razani, B., Feng, C., Coleman, T., Emanuel, R., Wen, H., Hwang, S., et al. (2012). Autophagy links inflammasomes to atherosclerotic progression. *Cell Metab.* 15, 534–544. doi: 10.1016/j.cmet.2012.02.011

Recio, C., Oguiza, A., Lazaro, I., Mallavia, B., Egido, J., and Gomez-Guerrero, C. (2014). Suppressor of cytokine signaling 1-derived peptide inhibits Janus kinase/signal transducers and activators of transcription pathway and improves inflammation and atherosclerosis in diabetic mice. *Arterioscler. Thromb. Vasc. Biol.* 34, 1953–1960. doi: 10.1161/ATVBAHA.114.304144

Ruotsalainen, A. K., Inkala, M., Partanen, M. E., Lappalainen, J. P., Kansanen, E., Makinen, P. I., et al. (2013). The absence of macrophage Nrf2 promotes early atherogenesis. *Cardiovasc. Res.* 98, 107–115. doi: 10.1093/cvr/cvt008

Salabei, J. K., and Hill, B. G. (2013). Implications of autophagy for vascular smooth muscle cell function and plasticity. *Free Radic. Biol. Med.* 65, 693–703. doi: 10.1016/j.freeradbiomed.2013.08.003

Saykally, J. N., Rachmany, L., Hatic, H., Shaer, A., Rubovitch, V., Pick, C. G., et al. (2012). The nuclear factor erythroid 2-like 2 activator, tert-butylhydroquinone, improves cognitive performance in mice after mild traumatic brain injury. *Neuroscience* 223, 305–314. doi: 10.1016/j.neuroscience.2012.07.070

Schrijvers, D. M., De Meyer, G. R., and Martinet, W. (2011). Autophagy in atherosclerosis: a potential drug target for plaque stabilization. *Arterioscler. Thromb. Vasc. Biol.* 31, 2787–2791. doi: 10.1161/ATVBAHA.111.224899

Shih, A. Y., Li, P., and Murphy, T. H. (2005). A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J. Neurosci.* 25, 10321–10335. doi: 10.1523/JNEUROSCI.4014-05.2005

Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., et al. (2009). Autophagy regulates lipid metabolism. *Nature* 458, 1131–1135. doi: 10.1038/nature07976

Tabas, I., Garcia-Cardena, G., and Owens, G. K. (2015). Recent insights into the cellular biology of atherosclerosis. *J. Cell Biol.* 209, 13–22. doi: 10.1083/jcb.201412052

Tan, S. M., and de Haan, J. B. (2014). Combating oxidative stress in diabetic complications with Nrf2 activators: how much is too much? *Redox Rep.* 19, 107–117. doi: 10.1179/1351000214Y.0000000087

Tan, S. M., Sharma, A., Stefanovic, N., Yuen, D. Y., Karagiannis, T. C., Meyer, C., et al. (2014). Derivative of bardoxolone methyl, dh404, in an inverse dose-dependent manner lessens diabetes-associated atherosclerosis and improves diabetic kidney disease. *Diabetes Metab. Res. Rev.* 63, 3091–3103. doi: 10.2337/db13-1743

van der Vorst, E. P., Döring, Y., and Weber, C. (2015). Chemokines. *Arterioscler. Thromb. Vasc. Biol.* 35, e52–e56. doi: 10.1161/ATVBAHA.115.306359

Vats, D., Mukundan, L., Odegaard, J. I., Zhang, L., Smith, K. L., Morel, C. R., et al. (2006). Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell Metab.* 4, 13–24. doi: 10.1016/j.cmet.2006.05.011

Vendrov, A. E., Hakim, Z. S., Madamanchi, N. R., Rojas, M., Madamanchi, C., and Runge, M. S. (2007). Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells. *Arterioscler. Thromb. Vasc. Biol.* 27, 2714–2721. doi: 10.1161/ATVBAHA.107.152629

Xu, B. C., Long, H. B., and Luo, K. Q. (2016). Tert-butylhydroquinone lowers blood pressure in AngII-induced hypertension in mice via proteasome-PTEN-Akt-eNOS pathway. *Sci. Rep.* 6:29589. doi: 10.1038/srep29589

Xu, X., Zhang, L., Ye, X., Hao, Q., Zhang, T., Cui, G., et al. (2018). Nrf2/ARE pathway inhibits ROS-induced NLRP3 inflammasome activation in BV2 cells after cerebral ischemia reperfusion. *Inflamm. Res.* 67, 57–65. doi: 10.1007/s00011-017-1095-6

Xue, M., Qian, Q., Adaikalakoteswari, A., Rabbani, N., Babaei-Jadidi, R., and Thornalley, P. J. (2008). Activation of NF-E2-related factor-2 reverses

biochemical dysfunction of endothelial cells induced by hyperglycemia linked to vascular disease. *Diabetes Metab. Res. Rev.* 57, 2809–2817. doi: 10.2337/db06-1003

Yu, M., Li, H., Liu, Q., Liu, F., Tang, L., Li, C., et al. (2011). Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cell. Signal.* 23, 883–892. doi: 10.1016/j.cellsig.2011.01.014

Zernecke, A., Shagdarsuren, E., and Weber, C. (2008). Chemokines in atherosclerosis: an update. *Arterioscler. Thromb. Vasc. Biol.* 28, 1897–1908. doi: 10.1161/ATVBAHA.107.161174

Zhang, J., Tucker, L. D., DongYan, Lu, Y., Yang, L., Wu, C., et al. (2018). Tert-butylhydroquinone post-treatment attenuates neonatal hypoxic-ischemic brain damage in rats. *Neurochem. Int.* 116, 1–12. doi: 10.1016/j.neuint.2018.03.004

Zhang, Y., Fang, L. F., Bi, X., Wang, S., Wu, X., and Jiang, F. (2015). The antioxidant compound tert-butylhydroquinone activates Akt in myocardium, suppresses apoptosis and ameliorates pressure overload-induced cardiac dysfunction. *Sci. Rep.* 5:13005. doi: 10.1038/srep13005

Zhou, N. Q., Liu, N., Li, P., Ping, S., Peng, Q. S., and Shi, W. D. (2017). Tert-butylhydroquinone promotes angiogenesis and improves heart functions in rats after myocardial infarction. *Clin. Exp. Hypertens.* 39, 402–408. doi: 10.1080/10641963.2016.1259322

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Role of Keap1-Nrf2 Signaling in Anhedonia Symptoms in a Rat Model of Chronic Neuropathic Pain: Improvement With Sulforaphane

Shan Li[1†], Chun Yang[1†], Xi Fang[1], Gaofeng Zhan[1], Niannian Huang[1], Jie Gao[1], Hui Xu[1], Kenji Hashimoto[2] and Ailin Luo[1*]*

[1] Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Division of Clinical Neuroscience, Center for Forensic Mental Health, Chiba University, Chiba, Japan

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***Correspondence:**
Kenji Hashimoto
hashimoto@faculty.chiba-u.jp
Ailin Luo
alluo@tjh.tjmu.edu.cn

† These authors have contributed equally to this work

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Patients with chronic neuropathic pain frequently suffer from symptoms of anhedonia (loss of pleasure), which is a core clinical manifestation of depression. Accumulating studies have shown the beneficial effects of the natural compound sulforaphane (SFN), an activator of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), on depression-like phenotype through a potent anti-inflammatory effect. However, it is unknown whether SFN confers beneficial effects in neuropathic pain-associated anhedonia. Spared nerve injury (SNI) is classical rodent model of chronic neuropathic pain. We here used a rat model of SNI. Hierarchical cluster analysis of sucrose preference test (SPT) results was used to classify the SNI rats with or without an anhedonia phenotype. Nrf2 protein expression was significantly decreased in the medial prefrontal cortex (mPFC), hippocampus, spinal cord, and skeletal muscle, but not in the nucleus accumbens, in anhedonia-susceptible rats compared with sham or anhedonia-resistant rats. The expression of Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), a partner of Nrf2, in mPFC, hippocampus, and muscle of anhedonia-susceptible rats was also significantly lower than that in sham or anhedonia-resilient rats. Subsequent SFN administration after SNI surgery exerted therapeutic effects on reduced mechanical withdrawal threshold (MWT) scores, but not on sucrose preference, through the normalization of Keap1-Nrf2 signaling in the spinal cords of anhedonia-susceptible rats. Interestingly, treatment with SFN 30 min prior to SNI surgery significantly attenuated reduced MWT scores and sucrose preference, and restored tissue Keap1 and Nrf2 levels. In conclusion, this study suggests that decreased Keap1-Nrf2 signaling in mPFC, hippocampus, and muscle may contribute to anhedonia susceptibility post-SNI surgery, and that SFN exerts beneficial effects in SNI rats by normalization of decreased Keap1-Nrf2 signaling.

Keywords: pain, anhedonia, sulforaphane, Keap1-Nrf2, medial prefrontal cortex, hippocampus, spinal cord, skeletal muscle

INTRODUCTION

Patients with chronic pain often suffer with depressive symptoms. Previous clinical studies have demonstrated that the incidence of comorbid chronic pain and depression is approximately 30 to 50% (Bair et al., 2003; Gustorff et al., 2008; Radat et al., 2013). Thus, comorbid pain and depression are a serious clinical, social, and economic issue that needs to be resolved. However, the underlying mechanisms and therapeutic strategies for managing this comorbidity remain undetermined.

Non-steroidal anti-inflammatory drugs (NSAIDs) and opioid drugs are widely used for pain relief. However, several studies have shown that NSAIDs are not effective in approximately half of patients with chronic pain (White, 2005; Vanegas et al., 2010; Shah and Mehta, 2012). Furthermore, although opioids have powerful analgesic effects, their considerable side effects limit their widespread use when administered in larger doses (Sehgal et al., 2013). Opioid crisis and drug overdose-related deaths are a serious problem in the United States (Volkow and Collins, 2017). New treatment options for opioid-use disorders are sorely needed. Although analgesics and antidepressant agents are currently prescribed for depression in patients with somatic symptoms or chronic pain, drugs without significant side effects are needed for treating the comorbidity of pain and depression.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that plays a central role in cellular defense against oxidative and electrophilic insults (Kobayashi et al., 2013; Ma, 2013; Suzuki et al., 2013; Suzuki and Yamamoto, 2015; Cuadrado et al., 2018; Yamamoto et al., 2018). Under normal conditions, Nrf2 is repressed by Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), which is an adaptor protein for Nrf2 degradation (Suzuki et al., 2013; Suzuki and Yamamoto, 2015; Yamamoto et al., 2018). During oxidative stress, including inflammation, Nrf2 is derepressed, which activates the transcription of cytoprotective genes. In addition, the Keap1-Nrf2 system is also involved in attenuating inflammation-associated pathogenesis (Kobayashi et al., 2013; Suzuki et al., 2013; O'Connell and Hayes, 2015; Suzuki and Yamamoto, 2015; Yamamoto et al., 2018). In the learned helplessness (LH) paradigm, Keap1 and Nrf2 protein levels in the prefrontal cortex and dentate gyrus of the hippocampus in LH (susceptible) rats were lower than those in control and non-LH (resilient) rats (Zhang et al., 2018). Furthermore, the expression of Keap1 and Nrf2 proteins in the parietal cortex of depressed patients was lower than that in controls, suggesting that Keap1-Nrf2 signaling contributes to stress resilience, which plays a key role in the pathophysiology of depression (Zhang et al., 2018).

Nrf2 signaling pathway has been commonly reported to be activated by food supplements and natural compounds (Bhaskaran et al., 2013; Menghini et al., 2016, 2018; Efentakis et al., 2017). Sulforaphane (SFN), a natural potent anti-inflammatory compound, is an organosulfur compound derived from glucoraphanin (a glucosinolate precursor of SFN) which is fully enriched in cruciferous vegetables (Zhang et al., 1992; Fahey et al., 1997; Dinkova-Kostova et al., 2017; Fahey et al., 2017). SFN is reported to have potent anti-inflammatory effects through the activation of Nrf2. Previously, we reported that pretreatment with SFN has prophylactic effects on depression-like phenotype and dendritic spine changes in an inflammation-induced model of depression (Zhang et al., 2017), and that novel Nrf2 activators (TBE-31 and MCE-1) demonstrate antidepressant effects in an inflammation model of depression (Yao et al., 2016b). Furthermore, dietary intake of glucoraphanin during juvenile and adolescent stages confers resilience to chronic social defeat stress in adulthood (Yao et al., 2016b). Previous reports have shown that intrathecal administration of SFN attenuated mechanical allodynia and thermal hyperalgesia in spinal nerve transfection-injured mice (Kim et al., 2010), and that SFN inhibited complete Freund's adjuvant-induced allodynia and hyperalgesia (Redondo et al., 2017). Taken together, it is likely that SFN is a potential natural compound for treating comorbid pain and depression.

The purpose of the present study was to examine the role of the Keap1-Nrf2 signaling system in selected tissues following surgery for spared nerve injury (SNI). Furthermore, we investigated whether abnormalities in behaviors and Keap1-Nrf2 levels in selected tissues following SNI are attenuated with subsequent administration of SFN. Finally, we examined whether pretreatment with SFN could prevent pain and anhedonia-like symptoms and alter Keap1-Nrf2 protein expression in selected tissues following SNI.

MATERIALS AND METHODS

Animals

Male Sprague Dawley (SD) rats (weighing 180–230 g) were purchased from the Laboratory Animal Centre of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). A total of 98 rats were enrolled and were divided into groups in accordance with the random number table. The animals were housed under 12 h light/dark cycle with free access to food and water. Procedures of this animal experiment were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Experimental Animal Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (No. 34724466).

Experimental Design

As shown in **Figure 1A**, rats were acclimated to environment for 6 days. Then the mechanical withdrawal threshold (MWT) was performed 1 day before the SNI surgery for baseline measurement. MWT and sucrose preference test (SPT) were implemented from day 2 to 5, 9 to 12, and 16 to 19 after surgery, respectively. Twenty-three days after SNI surgery, medial prefrontal cortex (mPFC), hippocampus, and nucleus accumbens (NAc) of brain, L2-5 spinal cord, skeletal muscle, and liver were collected. Tissue samples were stored at −80°C before Western blot analysis. A single dose of SFN (30 mg/kg, Absin Bioscience Inc., Shanghai, China) was

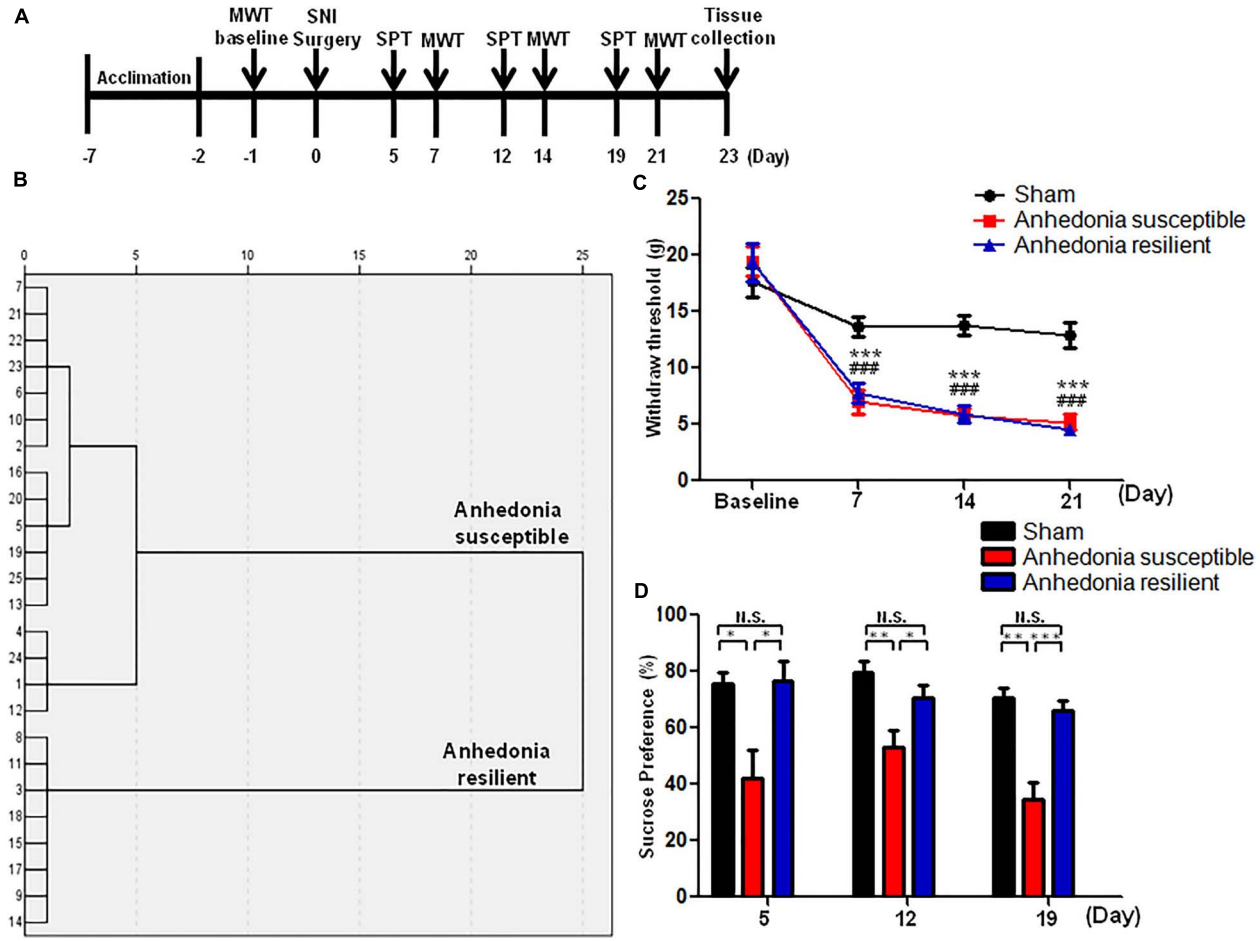

FIGURE 1 | The results of MWT and SPT in sham, anhedonia-susceptible, and anhedonia-resilient rats. **(A)** The schedule of the experiment. SNI surgery was performed on day 0 after acclimation. MWT was measured on day 7, 14, and 21 after SNI, respectively. SPT was performed on day 5, 12, and 19 after SNI, respectively. **(B)** Dendrogram of hierarchical clustering analysis. A total of 25 SNI rats were divided into anhedonia-susceptible and anhedonia-resilient groups by SPT results. **(C)** MWT [Time: $F_{(3,21)} = 38.352$, $P < 0.001$; Group: $F_{(2,14)} = 66.232$, $P < 0.001$; Interaction: $F_{(6,42)} = 9.711$, $P < 0.001$] was measured on day 7, 14, and 21 in the sham, anhedonia-resilient, and anhedonia-susceptible groups after SNI, respectively. Data are shown as mean ± SEM ($n = 8$). $^{***}P < 0.001$, susceptible group vs. sham group; $^{###}P < 0.001$, resilient group vs. sham group. **(D)** SPT [Time: $F_{(2,14)} = 2.523$, $P = 0.13$; Group: $F_{(2,14)} = 30.388$, $P < 0.001$; Interaction: $F_{(4,28)} = 0.687$, $P = 0.609$] was measured in the sham, anhedonia-resilient, and anhedonia-susceptible groups on day 5, 12, and 19 after SNI, respectively. Data are shown as mean ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. MWT, mechanical withdrawal test; N.S., not significant; SNI, spared nerve injury; SPT, sucrose preference test.

intraperitoneally injected before or after SNI surgery to investigate its effects on pain and anhedonia symptoms (**Figures 3A**, **5A**). The dose (30 mg/kg) of SFN was used as previously reported (Shirai et al., 2012, 2015; Zhang et al., 2017).

SNI

The SNI surgery was performed as previously described (Fang et al., 2018). Rats were anesthetized with 10% chloral hydrate (3 ml/kg) and then the skin of left thigh was incised. The sciatic nerve and its three terminal branches after bluntly dissecting biceps femoris muscle were totally exposed. The common peroneal and tibial nerves were ligated with a 4-0 silk and cut off the distal to the ligation. The muscle and skin were sutured with a 4-0 silk. Rats in the sham group were exposed to the sciatic nerve and its three terminal branches but without ligated and cut off the common peroneal and tibial nerves.

MWT

Before MWT, rats were placed in plexiglass chambers with a wire net floor for 30 min avoiding the stress resulting from the test conditions. The Electronic Von Frey (UGO BASILE S.R.L., Italy) filaments were applied to the lateral 1/3 of right paws. The paws quick withdrawal or flinching was considered as a positive response. Every filament stimuli were applied 4 times with a period of 30 s interval (Fang et al., 2018).

SPT

Rats were exposed to water and 1% sucrose solution for 48 h, followed by 24 h of water and food deprivation and a 24 h

exposure to two identical bottles, one is water, and another is 1% sucrose solution. The bottles containing water and sucrose were weighed before and at the end of this period and the sucrose preference was determined (Fang et al., 2018).

Western Blot

Samples were homogenized with RIPA buffer (150 mM sodium chloride, Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) at 4°C for 30 min, then were centrifuged for 15 min at 4°C. BCA protein assay kit (Boster, Wuhan, China) was used to determine the protein levels in supernatant. The samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States). Bands were blocked with 5% BSA in TBST (0.1% Tween 20 in Tris-buffered saline) for 1 h at room temperature. Relative primary antibodies were incubated at 4°C overnight: rabbit Keap1 (1:1,000, Affinity, Cincinnati, OH, United States), rabbit Nrf2 (1:1,000; Affinity, Cincinnati, OH, United States), and mouse GAPDH (1:1,000, Qidongzi, Wuhan, China). Then bands were washed with TBST and incubated second antibody for 2 h at room temperature: goat anti-rabbit IgG horseradish peroxidase or goat anti-mouse IgG horseradish peroxidase (1:5,000, Qidongzi, Wuhan, China). Finally, these bands were detected by enhanced chemiluminescence reagents (Qidongzi, Wuhan, China) with the ChemiDoc XRS chemiluminescence imaging system (Bio-Rad, Hercules, CA, United States).

Statistical Analyses

The data show as the mean ± standard error of the mean (SEM). Analysis was performed using PASW Statistics 20 (formerly SPSS Statistics; SPSS). Comparisons between groups were performed using the one-way analysis of variance (ANOVA) or two-way ANOVA, followed by *post hoc* Tukey test. In Hierarchical cluster analysis, the data were firstly standardized by z-scores. Then, hierarchical cluster analysis of SPT results was performed using Ward's method and applying squared Euclidean distance as the distance measure, and mice were classified as anhedonia-susceptible rats or anhedonia-resilient rats (Fang et al., 2018). The P-values of less than 0.05 were considered statistically significant.

RESULTS

Comparison of MWT and SPT Among the Sham, Anhedonia-Susceptible and Anhedonia-Resilient Rats

A total of 25 SNI rats were divided into anhedonia-susceptible and anhedonia-resilient groups by hierarchical clustering analysis of SPT results (**Figure 1B**). MWT was significantly decreased in both anhedonia-susceptible and anhedonia-resilient rats as compared with that of sham on day 7, 14, and 21 after SNI surgery (**Figure 1C**). However, there was no any change in the MWT between anhedonia-susceptible and anhedonia-resilient rats (**Figure 1C**). Furthermore, the sucrose preference in the anhedonia-susceptible rats was significantly lower than those in the sham or anhedonia-resilient rats on day 5, 12, and 19 after SNI (**Figure 1D**).

Altered Expression of Keap1 and Nrf2 in Selected Tissues in Sham, Anhedonia-Susceptible, and Anhedonia-Resilient Rats

There were significant alterations in the levels of Keap1 protein in the mPFC, hippocampus, L2-5 spinal cord, muscle, and liver in the SNI-treated rats. *Post hoc* test showed a significant decrease of Keap1 protein in the mPFC, hippocampus and muscle in anhedonia-susceptible rats than that of sham and anhedonia-resilient rats (**Figures 2A,C,E**). Interestingly, there was a significant decrease of Keap1 protein in the L2-5 spinal cord and liver in anhedonia-susceptible rats than that of sham rats although there were no changes in the spinal cord and liver between anhedonia-susceptible and anhedonia-resilient rats (**Figures 2D,F**). In contrast, there were no changes of Keap1 in the NAc among the three groups (**Figure 2B**).

Anhedonia-susceptible rats showed a significant decrease of Nrf2 protein in the mPFC, hippocampus, spinal cord, and muscle compared to sham or anhedonia-resilient rats (**Figures 2A,C**). In contrast, the expression of Nrf2 protein in the NAc from anhedonia-susceptible rats was no difference among the three groups (**Figure 2B**). In the liver, both anhedonia-susceptible and anhedonia-resilient rats significantly decreased levels of Nrf2 compared to sham rats, but there was no significant change between the two groups (**Figure 2F**).

Effects of Subsequent SFN Treatment on the Results of MWT and SPT After SNI Surgery

Seventeen anhedonia-susceptible rats from 35 SNI rats were selected by hierarchical clustering analysis of SPT results (**Figures 3A,B**). The reduction of scores of MWT in anhedonia-susceptible rats after SNI surgery was attenuated significantly after subsequent single administration of SFN (30 mg/kg) (**Figure 3C**). In contrast, the reduced sucrose preference in anhedonia-susceptible rats after SNI surgery was not improved after subsequent single administration of SFN (**Figure 3D**).

Effects of Subsequent SFN Treatment on the Levels of Keap1-Nrf2 Signaling in Selected Tissues After SNI Surgery

A single administration of SFN (30 mg/kg) into anhedonia-susceptible rats after SNI surgery did not alter the levels of Keap1 and Nrf2 in the mPFC, NAc, hippocampus, muscle, and liver (**Figures 4A–C,E,F**). In contrast, SFN significantly increased the reduced levels of Keap1 and Nrf2 proteins in the spinal cord of anhedonia-susceptible rats (**Figure 4D**). Collectively, it is likely that activation of Keap1-Nrf2 system in the spinal cord by SFN may play a role in the SFN-induced beneficial effects for reduced MWT scores in anhedonia-susceptible rats after SNI.

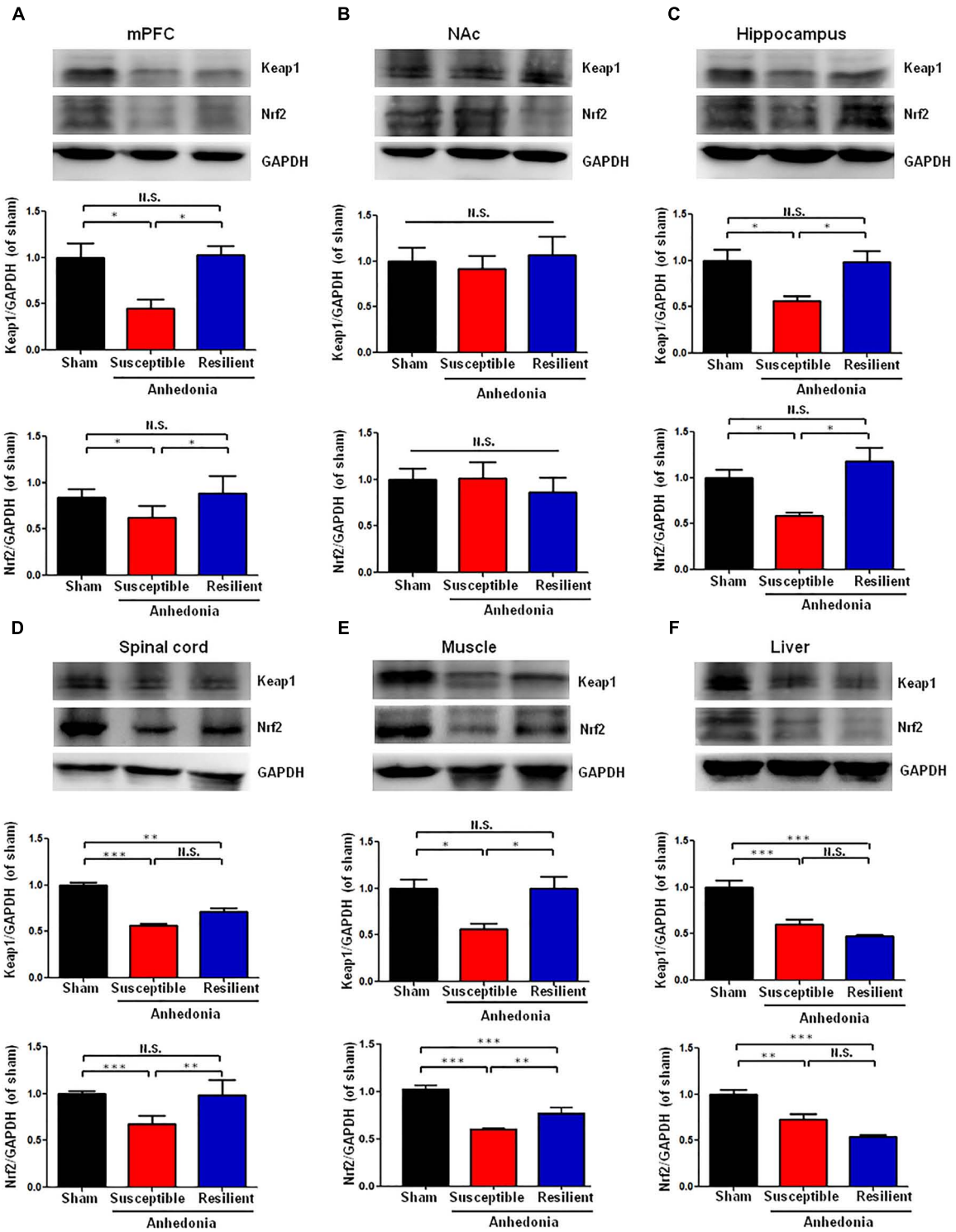

FIGURE 2 | Expression of Keap1 and Nrf2 proteins in selected tissues among the sham, anhedonia-susceptible, and anhedonia-resilient groups. **(A)** Keap1 [$F_{(2,15)} = 9.477$, $P = 0.02$] and Nrf2 [$F_{(2,15)} = 11.497$, $P = 0.013$] levels in the mPFC. **(B)** Keap1 [$F_{(2,15)} = 1.811$, $P = 0.243$] and Nrf2 [$F_{(2,15)} = 0.289$, $P = 0.759$] levels in the NAc. **(C)** Keap1 [$F_{(2,15)} = 10.833$, $P = 0.01$] and Nrf2 [$F_{(2,15)} = 9.248$, $P = 0.015$] levels in the hippocampus. **(D)** Keap1 [$F_{(2,15)} = 62.237$, $P = 0.011$] and Nrf2 [$F_{(2,15)} = 31.095$, $P < 0.001$] levels in the L2-5 spinal cord. **(E)** Keap1 [$F_{(2,15)} = 6.35$, $P = 0.033$] and Nrf2 [$F_{(2,15)} = 144.8$, $P < 0.001$] levels in the muscle. **(F)** Keap1 [$F_{(2,15)} = 25.886$, $P = 0.001$] and Nrf2 [$F_{(2,15)} = 22.201$, $P = 0.002$] levels in the liver. Data are shown as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.01$. Keap1, kelch-like ECH-associated protein 1; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; Nrf2, nuclear factor (erythroid 2-derived)-like 2; N.S., not significant.

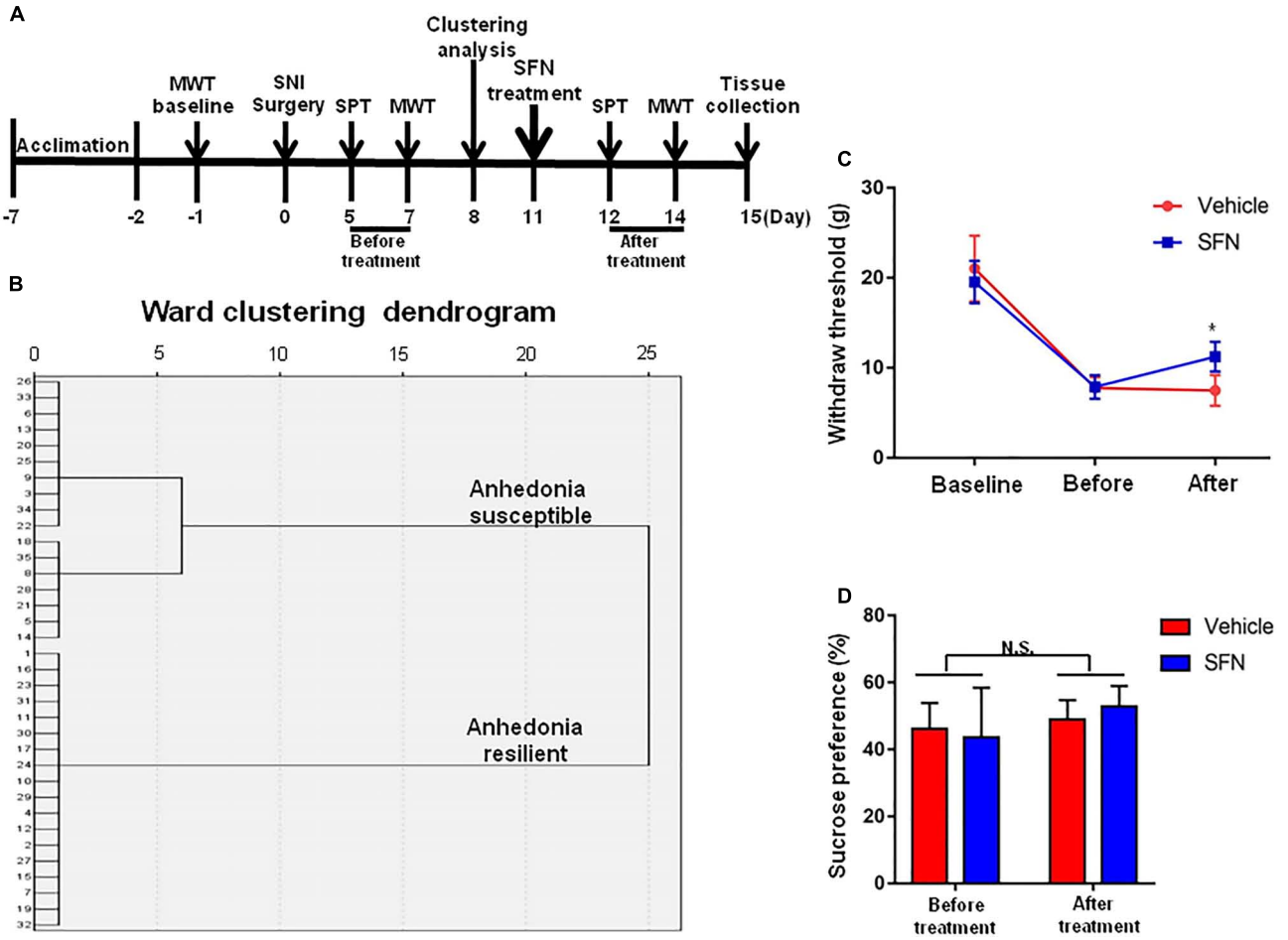

FIGURE 3 | Effects of SFN treatment after SNI surgery on the results of MWT and SPT. **(A)** The schedule of the experiment. SNI surgery was performed on day 0 after acclimation. SFN (30 mg/kg, i.p.) was administered on day 11. SPT was measured on day 5 and 12 after SNI, respectively. MWT was performed on day 7 and 14 after SNI, respectively. **(B)** Dendrogram of hierarchical clustering analysis. A total of 35 SNI rats were divided into anhedonia-susceptible and anhedonia-resilient groups by SPT results. Thirteen-seven of 35 SNI rats were anhedonia-susceptible phenotype. **(C)** MWT [Time: $F_{(2,42)} = 160.9$, $P < 0.001$; SFN: $F_{(1,42)} = 1.635$, $P = 0.208$; Interaction: $F_{(2,42)} = 6.284$, $P = 0.0041$]. Data are shown as mean ± SEM ($n = 8$). *$P < 0.05$, SFN group vs. vehicle group. **(D)** SPT [Time: $F_{(1,28)} = 3.361$, $P = 0.0774$; SFN: $F_{(1,28)} = 0.04322$, $P = 0.8368$; Interaction: $F_{(1,28)} = 0.9291$, $P = 0.3434$]. Data are shown as mean ± SEM ($n = 8$). MWT, mechanical withdrawal test; N.S., not significant; SFN, sulforaphane; SNI, spared nerve injury; SPT, sucrose preference test.

Effects of Pretreatment With SFN on the Results of MWT and SPT After SNI Surgery

Spared nerve injury surgery was performed 30 min after a single administration of SFN (30 mg/kg) or vehicle (**Figure 4A**). The ratio of anhedonia-susceptible rats to total SNI rats in vehicle-treated group and SFN-treated group were 50 and 31%, respectively (**Figures 5B–D**). Thus, pretreatment with SFN significantly increased the number of anhedonia-resilient rats after SNI surgery (**Figure 5D**).

On day 7, SFN (30 mg/kg) significantly increased the scores of MWT in both anhedonia-susceptible and anhedonia-resilient rats (**Figure 5E**). On day 14, SFN-treated rats showed a significant increase in the MWT scores in both anhedonia-susceptible and anhedonia-resilient rats as compared with that of vehicle-treated rats. Furthermore, the MWT scores of SFN-treated anhedonia-resilient rats were significantly higher than those of SFN-treated anhedonia-susceptible rats (**Figure 5E**).

There was a significant change in the results of SPT between anhedonia-susceptible rats and anhedonia-resilient rats in both vehicle- and SFN-treated groups on day 5 and 12 (**Figure 5F**). On day 5, SFN significantly increased the sucrose preference of anhedonia-resilient rats, but not anhedonia-susceptible rats. On day 12, SFN significantly increased the sucrose preference of susceptible rats, but not resilient rats (**Figure 5F**).

Prophylactic Effects of SFN on the Altered Levels of Keap1-Nrf2 Signaling in Selected Tissues After SNI Surgery

Pretreatment with SFN (30 mg/kg) significantly increased the Keap1 and Nrf2 levels in the mPFC, hippocampus,

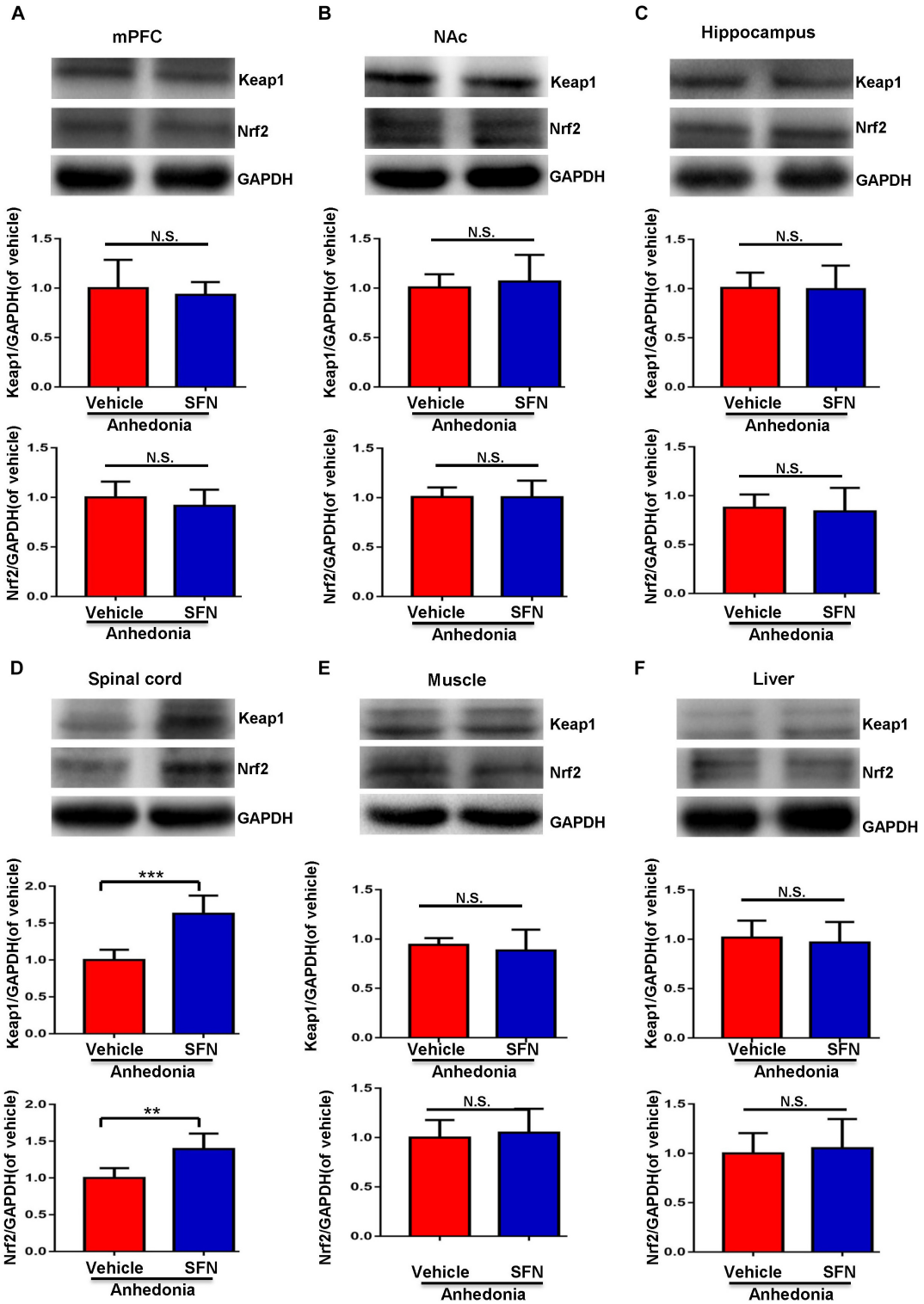

FIGURE 4 | Effects of SFN treatment after SNI surgery on the expression of Keap1 and Nrf2 proteins in selected tissues. **(A)** Keap1 ($t = 0.5248$, $P = 0.6112$) and Nrf2 ($t = 0.9042$, $P = 0.3872$) levels in the mPFC. **(B)** Keap1 ($t = 0.4853$, $P = 0.6379$) and Nrf2 ($t = 0.01363$, $P = 0.9894$) levels in the NAc. **(C)** Keap1 ($t = 0.1111$, $P = 0.9137$) and Nrf2 ($t = 0.3427$, $P = 0.7389$) levels in the hippocampus. **(D)** Keap1 ($t = 5.45$, $P < 0.001$) and Nrf2 ($t = 3.861$, $P = 0.0032$) levels in the L2-5 spinal cord. **(E)** Keap1 ($t = 0.6394$, $P = 0.5369$) and Nrf2 ($t = 0.4178$, $P = 0.6850$) levels in the muscle. **(F)** Keap1 ($t = 0.463$, $P = 0.6533$) and Nrf2 ($t = 0.3559$, $P = 0.7293$) levels in the liver. Data are shown as mean ± SEM ($n = 6$). $^{**}P < 0.01$ or $^{***}P < 0.001$. Keap1, kelch-like ECH-associated protein 1; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; Nrf2, nuclear factor (erythroid 2-derived)-like 2; N.S., not significant; SFN, sulforaphane.

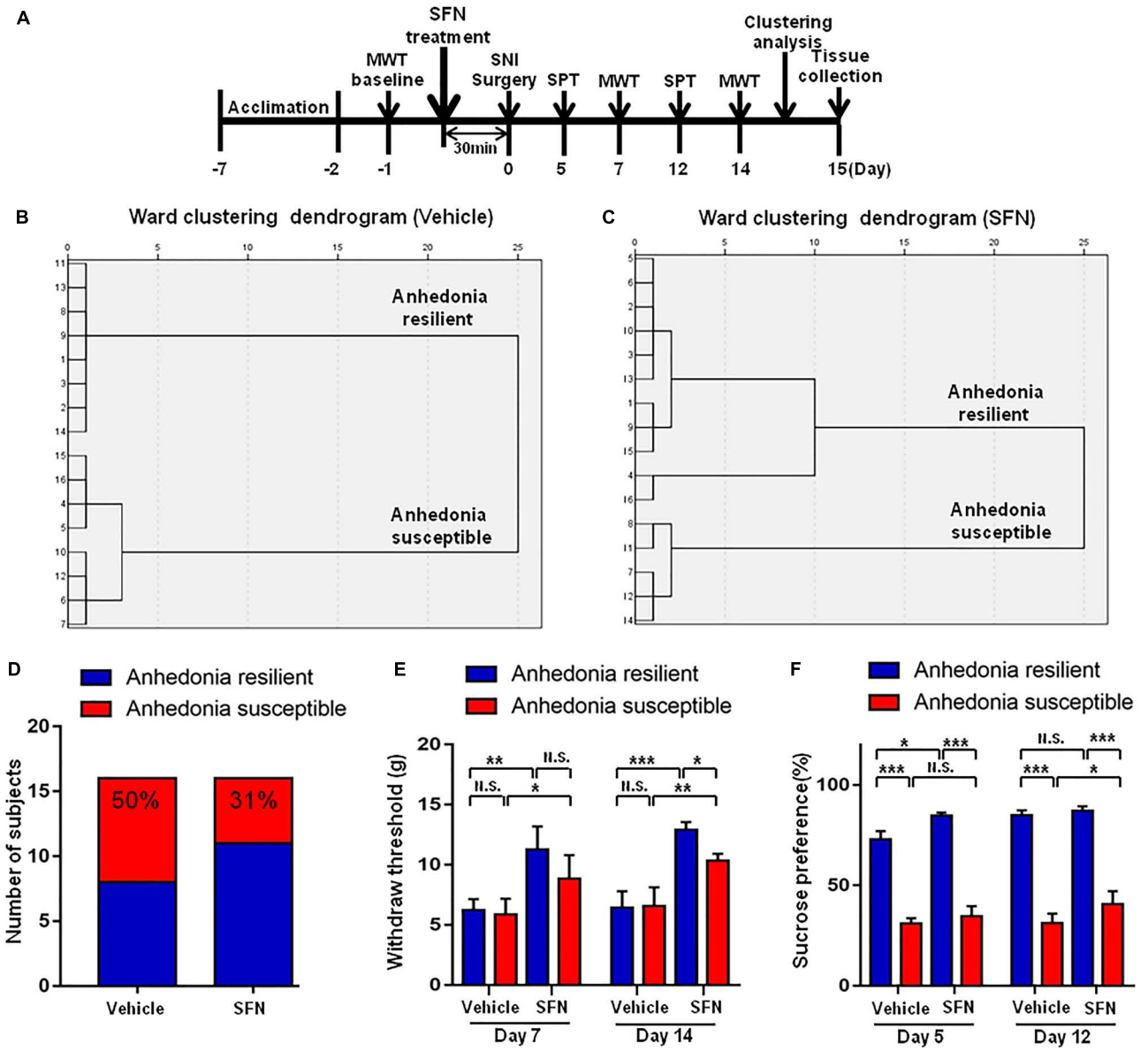

FIGURE 5 | Effects of SFN treatment before SNI surgery on the results of MWT and SPT. **(A)** The schedule of this study. SNI was performed on day 0 after acclimation. SFN at a single dose of 30 mg/kg was administered 30 min before SNI surgery. MWT was measured on day 5 and 12 after SNI, respectively. SPT was performed on day 7 and 14 after SNI, respectively. **(B)** Dendrogram of hierarchical clustering analysis. A total of 16 SNI rats were divided into anhedonia-susceptible and anhedonia-resilient groups by SPT results. Eight of 16 SNI rats were anhedonia susceptible phenotype. **(C)** Dendrogram of hierarchical clustering analysis. A total of 16 SNI rats were divided into anhedonia-susceptible and anhedonia-resilient groups by SPT results. Five of 16 SNI rats were anhedonia-susceptible phenotype. **(D)** Ratio of anhedonia-susceptible rats to total SNI rats. The ratio of anhedonia-susceptible rats in vehicle and SFN groups were 50 and 31%, respectively. **(E)** MWT [Time: $F_{(1,16)} = 8.851$, $P = 0.0089$; SFN: $F_{(3,16)} = 30.58$, $P < 0.001$; Interaction: $F_{(3,16)} = 0.9991$, $P = 0.4186$]. Data are shown as mean ± SEM ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{**}P < 0.001$. **(D)** SPT [Time: $F_{(1,16)} = 32.05$, $P < 0.001$; SFN: $F_{(3,16)} = 361.7$, $P < 0.001$; Interaction: $F_{(3,16)} = 7.569$, $P = 0.0023$]. Data are shown as mean ± SEM ($n = 5$). MWT, mechanical withdrawal test; N.S., not significant; SFN, sulforaphane; SNI, spared nerve injury; SPT, sucrose preference test.

spinal cord, muscle, and liver of anhedonia-resilient rats compared to vehicle-treated anhedonia-resilient rats or SFN-treated anhedonia-susceptible rats (**Figures 6A,C–F**). However, SFN did not induce any change in the levels of Keap1 and Nrf2 protein in the NAc from anhedonia-resilient rats and anhedonia-susceptible rats (**Figure 6B**).

DISCUSSION

The present study demonstrated that although SNI rats suffered almost identical nociceptive damage, some rats exhibited anhedonia-like phenotypes. Tissue levels of Keap1 and Nrf2 in mPFC, hippocampus, and muscle of rats with anhedonia-like phenotypes were lower than those in rats without anhedonia-like

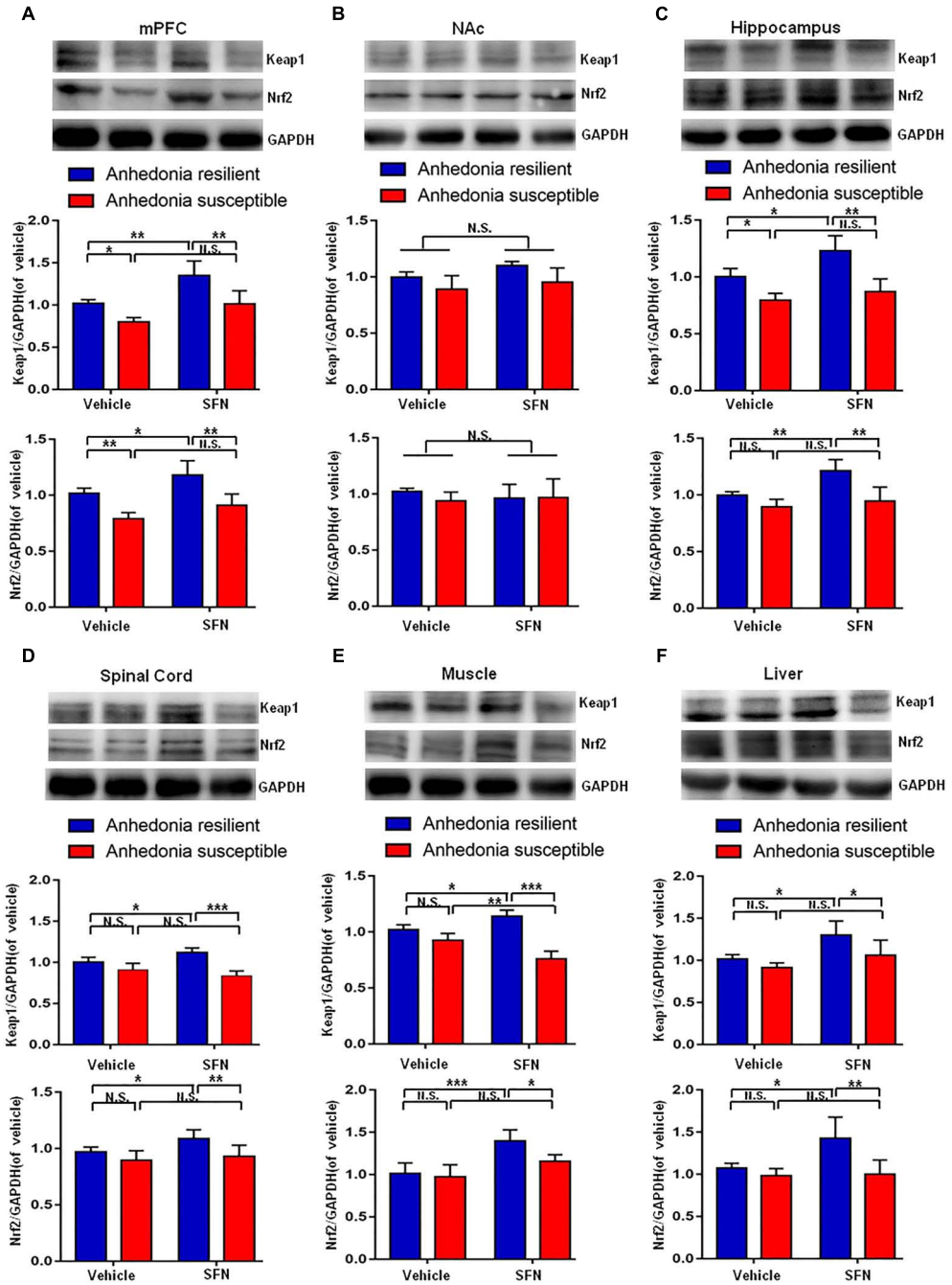

FIGURE 6 | Effects of SFN treatment before SNI surgery on Keap1-Nrf2 signaling in selected tissues. **(A)** Keap1 [SFN: $F_{(1,16)} = 25.1$, $P = 0.001$; Phenotype: $F_{(1,16)} = 26.36$, $P < 0.001$; Interaction: $F_{(1,16)} = 1.184$, $P = 0.2926$] and Nrf2 [SFN: $F_{(1,16)} = 12.9$, $P = 0.0024$; Phenotype: $F_{(1,16)} = 40.27$, $P < 0.001$; Interaction: $F_{(1,16)} = 0.2745$, $P = 0.6075$] levels in the mPFC. **(B)** Keap1 [SFN: $F_{(1,16)} = 3.965$, $P = 0.0638$; Phenotype: $F_{(1,16)} = 9.697$, $P = 0.0067$; Interaction: $F_{(1,16)} = 0.2142$, $P = 0.6497$] and Nrf2 [SFN: $F_{(1,16)} = 0.088$, $P = 0.7706$; Phenotype: $F_{(1,16)} = 0.6729$, $P = 0.4241$; Interaction: $F_{(1,16)} = 0.8057$, $P = 0.3827$] levels in the NAc. **(C)** Keap1 [SFN: $F_{(1,16)} = 11.79$, $P = 0.0034$; Phenotype: $F_{(1,16)} = 41.42$, $P < 0.001$; Interaction: $F_{(1,16)} = 2.94$, $P = 0.1057$] and Nrf2 [SFN: $F_{(1,16)} = 12.01$, $P = 0.0032$; Phenotype: $F_{(1,16)} = 23.12$, $P < 0.001$; Interaction: $F_{(1,16)} = 4.539$, $P = 0.049$] levels in the hippocampus. **(D)** Keap1 [SFN: $F_{(1,16)} = 20.69$, $P < 0.001$; Phenotype: $F_{(1,16)} = 49.2$, $P < 0.001$; Interaction: $F_{(1,16)} = 0.3084$, $P = 0.5863$] and Nrf2 [SFN: $F_{(1,16)} = 13.11$, $P = 0.0023$; Phenotype: $F_{(1,16)} = 34.32$, $P < 0.001$; Interaction: $F_{(1,16)} = 1.179$, $P = 0.2936$] levels in the L2-5 spinal cord. **(E)** Keap1 [SFN: $F_{(1,16)} = 4.55$, $P = 0.0488$; Phenotype: $F_{(1,16)} = 291.3$, $P < 0.001$; Interaction: $F_{(1,16)} = 149.2$, $P < 0.001$] and Nrf2 [SFN: $F_{(1,16)} = 27.85$, $P < 0.001$; Phenotype: $F_{(1,16)} = 6.673$, $P = 0.02$; Interaction: $F_{(1,16)} = 3.469$, $P = 0.081$] levels in the muscle. **(F)** Keap1 [SFN: $F_{(1,16)} = 14.27$, $P = 0.0017$; Phenotype: $F_{(1,16)} = 9.022$, $P = 0.0084$; Interaction: $F_{(1,16)} = 1.393$, $P = 0.2551$] and Nrf2 [SFN: $F_{(1,16)} = 6.814$, $P = 0.0189$; Phenotype: $F_{(1,16)} = 13.18$, $P = 0.0023$; Interaction: $F_{(1,16)} = 5.598$, $P = 0.0309$] levels in the liver. Data are shown as mean ± SEM ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. Keap1, kelch-like ECH-associated protein 1; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; Nrf2, nuclear factor (erythroid 2-derived)-like 2; N.S., not significant; SFN, sulforaphane.

phenotypes and sham-operated rats. Furthermore, tissue levels of Keap1 and Nrf2 in the spinal cord and liver of rats with anhedonia-like phenotypes were lower than those of sham rats. Decreased MWT scores, but not decreased sucrose preference, in susceptible rats following SNI surgery were attenuated with subsequent single administration of SFN (30 mg/kg). Furthermore, SFN significantly improved the decreased Keap1 and Nrf2 expression levels in the spinal cord of susceptible rats following SNI. Moreover, SFN treatment (30 mg/kg) prior to SNI surgery significantly ameliorated the reduced MWT scores, anhedonia-like behavior, and decreased Keap1-Nrf2 signaling in mPFC, hippocampus, and spinal cord of rats with anhedonia-like phenotypes. To the best of our knowledge, this is the first study demonstrating the role of the Keap1-Nrf2 system in individual differences of anhedonia after neuropathic pain. In addition, this is the first study to establish the role of Keap1-Nrf2 signaling in the prophylactic and therapeutic effects of SFN in the comorbidity of neuropathic pain and depression in rodents.

In preclinical studies, depression-related behaviors of hyperalgesic rats were highly diverse. Using resident-intruder social interaction and sleep–wake analyses, it is reported that chronic constriction injury induced a subgroup (approximately 30%) of rats with altered dominant behavior (Monassi et al., 2003) and sleep–wake cycle (Austin and Moalem-Taylor, 2010). In the present study, hierarchical cluster analysis was used to divide SNI rats into two clusters: one group (approximately 68%; anhedonia-like phenotype) with reduced sucrose preference in the SPT and the other (approximately 32%; without anhedonia-like phenotype) with sucrose preference similar to that in sham-operated rats, consistent with our previous report (Fang et al., 2018). In the current study, we also found that rats, regardless of the presence or absence of anhedonia-like phenotype, exhibited similar MWT scores, suggesting that alterations in mood-related behaviors were independent of the degree of nociceptive damage, which was consistent with previous studies (Monassi et al., 2003; Austin and Moalem-Taylor, 2010; Austin et al., 2015; Gui et al., 2016; Xie et al., 2017; Fang et al., 2018).

We identified decreased Keap1-Nrf2 signaling in mPFC and hippocampus of rats with anhedonia-like phenotypes on day 23 following SNI. Low levels of Keap1-Nrf2 in mPFC and hippocampus are reportedly associated with the development of depression-like phenotypes, including anhedonia, in rodents (Yao et al., 2016a; Zhang et al., 2017). Furthermore, *Nrf2* knockout mice demonstrated depression-like phenotype, including anhedonia (Yao et al., 2016b). A recent study using postmortem brain samples exhibited reduced Keap1 and Nrf2 expression levels in the parietal cortex of depressed patients (Zhang et al., 2018), suggesting that decreased Keap1-Nrf2 signaling plays a role in depression. Interestingly, SFN did not improve the decreased sucrose preference in rats with anhedonia-like phenotypes, although it significantly attenuated decreased MWT scores through the improvement of the Keap1-Nrf2 system in the spinal cord of rats with anhedonia-like phenotype. Thus, it appears that Keap1-Nrf2 signaling in the spinal cord plays a role in pain following SNI. Overall, it is likely that decreased Keap1-Nrf2 signaling in mPFC and hippocampus is associated with anhedonia-like phenotype in rats with neuropathic pain, and that Nrf2 activators, including SFN, have therapeutic potential in patients with neuropathic pain-associated anhedonia.

In this study, we found that SNI rats with anhedonia-like phenotype had lower tissue Keap1 and Nrf2 levels in the spinal cord than those in sham-operated rats. Given the role of Keap1-Nrf2 signaling in pain (Kim et al., 2010; Redondo et al., 2017), reduced Keap1-Nrf2 signaling in the spinal cord may play a role in neuropathic pain, although not in anhedonia-like phenotypes.

Skeletal muscle, which consumes two-third of the body's energy supply, comprises approximately 40% of the body mass in a healthy individual with normal body weight (Rolfe and Brown, 1997). Following skeletal muscle injury, Nrf2 activity is required for muscle regeneration and effective healing by regulating satellite cell proliferation (Shelar et al., 2016) and preventing inflammation-induced muscle wasting and fibrosis (Al-Sawaf et al., 2014). In the present study, we also found decreased Keap1 and Nrf2 expression levels in the muscle of anhedonia-susceptible rats. Furthermore, these levels were significantly lower than those in the muscle of anhedonia-resilient rats. Thus, decreased Keap1-Nrf2 signaling in the muscle may contribute to anhedonia susceptibility to SNI surgery, although further detailed studies are warranted.

In addition, we found decreased Keap1 and Nrf2 expression levels in the liver of rats with or without anhedonia-like phenotype following SNI surgery. Hence, it is unlikely that Keap1 and Nrf2 signaling alterations in the liver contribute to anhedonia susceptibility to SNI surgery. Given the role of the Keap1-Nrf2 system in the host cell defense against oxidative stress (Kobayashi et al., 2013; Suzuki et al., 2013; O'Connell and Hayes, 2015; Suzuki and Yamamoto, 2015; Wardyn et al., 2015; Cuadrado et al., 2018; Yamamoto et al., 2018), decreased Keap1-Nrf2 signaling in the liver may play a role in oxidative stress in the liver of SNI rats. Nonetheless, further detailed studies regarding the underlying role of Keap1-Nrf2 signaling in the brain and peripheral tissues (spinal cord, muscle, and liver) are needed.

In the present study, we used a single injection of SFN (30 mg/kg) in a rat SNI model. Our previous studies demonstrated that dietary intake of glucoraphanin (a precursor of SFN) can prevent the onset of behavioral abnormalities and biochemical changes in the brain following phencyclidine administration (Shirai et al., 2015), inflammation (Zhang et al., 2017), chronic social defeat stress (Yao et al., 2016a), and maternal immune activation (Matsuura et al., 2018). Collectively, dietary intake of glucoraphanin during the experimental period may show prophylactic effects in reducing MWT and SPT scores following SNI surgery. Therefore, further study on the long-term administration of SFN (or glucoraphanin) is of great interest. These results suggested that SFN or its precursor glucoraphanin could potentially be used for the treatment of anhedonia in patients with neuropathic pain, because they are naturally occurring compounds found in cruciferous vegetables.

Finally, further study regarding the dietary intake of glucoraphanin-rich vegetables (or SFN supplements) in depressed patients with neuropathic pain is necessary to study their prophylactic effects.

CONCLUSION

The current study suggests that decreased Keap1-Nrf2 signaling in mPFC, hippocampus, and muscle is associated with individual differences of the anhedonia-like phenotype in rats with neuropathic pain, whereas in the spinal cords of SNI rats, it is associated with neuropathic pain. Therefore, it is likely that Nrf2 activators, including SFN, are a potential therapeutic target for comorbid pain and anhedonia in patients with neuropathic pain.

AUTHOR CONTRIBUTIONS

CY, KH, and AL designed the study. SL, CY, XF, GZ, and NH performed the behavioral tests. SL, XF, and JG performed western blot. SL, CY, and KH drafted the manuscript. JG and HX revised the manuscript. All the authors approved the manuscript and submission.

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REFERENCES

Al-Sawaf, O., Fragoulis, A., Rosen, C., Kan, Y. W., Sonmez, T. T., Pufe, T., et al. (2014). Nrf2 protects against TWEAK-mediated skeletal muscle wasting. *Sci. Rep.* 4:3625. doi: 10.1038/srep03625

Austin, P. J., Berglund, A. M., Siu, S., Fiore, N. T., Gerke-Duncan, M. B., Ollerenshaw, S. L., et al. (2015). Evidence for a distinct neuro-immune signature in rats that develop behavioural disability after nerve injury. *J. Neuroinflammation* 12:96. doi: 10.1186/s12974-015-0318-4

Austin, P. J., and Moalem-Taylor, G. (2010). The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. *J. Neuroimmunol.* 229, 26–50. doi: 10.1016/j.jneuroim.2010.08.013

Bair, M. J., Robinson, R. L., Katon, W., and Kroenke, K. (2003). Depression and pain comorbidity: a literature review. *Arch. Intern. Med.* 163, 2433–2445. doi: 10.1001/archinte.163.20.2433

Bhaskaran, N., Srivastava, J. K., Shukla, S., and Gupta, S. (2013). Chamomile confers protection against hydrogen peroxide-induced toxicity through activation of Nrf2-mediated defense response. *Phytother. Res.* 27, 118–125. doi: 10.1002/ptr.4701

Cuadrado, A., Manda, G., Hassan, A., Alcaraz, M. J., Barbas, C., Daiber, A., et al. (2018). Transcription factor NRF2 as a therapeutic target for chronic diseases: a systems medicine approach. *Pharmacol. Rev.* 70, 348–383. doi: 10.1124/pr.117.014753

Dinkova-Kostova, A. T., Fahey, J. W., Kostov, R. V., and Kensler, T. W. (2017). KEAP1 and Done? Targeting the NRF2 pathway with sulforaphane. *Trends Food Sci. Technol.* 69, 257–269. doi: 10.1016/j.tifs.2017.02.002

Efentakis, P., Rizakou, A., Christodoulou, E., Chatzianastasiou, A., López, M. G., León, R., et al. (2017). Saffron (*Crocus sativus*) intake provides nutritional preconditioning against myocardial ischemia-reperfusion injury in wild type and Poe(-/-) mice: involvement of Nrf2 activation. *Nutr. Metab. Cardiovasc. Dis.* 27, 919–929. doi: 10.1016/j.numecd.2017.08.005

Fahey, J. W., Wade, K. L., Wehage, S. L., Holtzclaw, W. D., Liu, H., Talalay, P., et al. (2017). Stabilized sulforaphane for clinical use: phytochemical delivery efficiency. *Mol. Nutr. Food Res.* 61, 1600766. doi: 10.1002/mnfr.201600766

Fahey, J. W., Zhang, Y., and Talalay, P. (1997). Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* 94, 10367–10372. doi: 10.1073/pnas.94.19.10367

Fang, X., Yang, C., Li, S., Zhan, G., Zhang, J., Huang, N., et al. (2018). Brain-derived neurotrophic factor-TrkB signaling in the medial prefrontal cortex plays a role in the anhedonia-like phenotype after spared nerve injury. *Eur. Arch. Psychiatry Clin. Neurosci.* doi: 10.1007/s00406-018-0909-z [Epub ahead of print].

Gui, W. S., Wei, X., Mai, C. L., Murugan, M., Wu, L. J., Xin, W. J., et al. (2016). Interleukin-1beta overproduction is a common cause for neuropathic pain, memory deficit, and depression following peripheral nerve injury in rodents. *Mol. Pain* 12:1744806916646784. doi: 10.1177/1744806916646784

Gustorff, B., Dorner, T., Likar, R., Grisold, W., Lawrence, K., Schwarz, F., et al. (2008). Prevalence of self-reported neuropathic pain and impact on quality of life: a prospective representative survey. *Acta Anaesthesiol. Scand.* 52, 132–136. doi: 10.1111/j.1399-6576.2007.01486.x

Kim, D., You, B., Jo, E. K., Han, S. K., Simon, M. I., and Lee, S. J. (2010). NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14851–14856. doi: 10.1073/pnas.1009926107

Kobayashi, E., Suzuki, T., and Yamamoto, M. (2013). Roles nrf2 plays in myeloid cells and related disorders. *Oxid. Med. Cell. Longev.* 2013:529219. doi: 10.1155/2013/529219

Ma, Q. (2013). Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Matsuura, A., Ishima, T., Fujita, Y., Iwayama, Y., Hasegawa, S., Kawahara-Miki, R., et al. (2018). Dietary glucoraphanin prevents the onset of psychosis in the adult offspring after maternal immune activation. *Sci. Rep.* 8:2158. doi: 10.1038/s41598-018-20538-3

Menghini, L., Ferrante, C., Leporini, L., Recinella, L., Chiavaroli, A., Leone, S., et al. (2016). An hydroalcoholic chamomile extract modulates inflammatory and immune response in HT29 cells and isolated rat colon. *Phytother. Res.* 30, 1513–1518. doi: 10.1002/ptr.5655

Menghini, L., Leporini, L., Vecchiotti, G., Locatelli, M., Carradori, S., Ferrante, C., et al. (2018). *Crocus sativus* L. stigmas and byproducts: qualitative fingerprint, antioxidant potentials and enzyme inhibitory activities. *Food Res. Int.* 109, 91–98. doi: 10.1016/j.foodres.2018.04.028

Monassi, C. R., Bandler, R., and Keay, K. A. (2003). A subpopulation of rats show social and sleep-waking changes typical of chronic neuropathic pain following peripheral nerve injury. *Eur. J. Neurosci.* 17, 1907–1920. doi: 10.1046/j.1460-9568.2003.02627.x

O'Connell, M. A., and Hayes, J. D. (2015). The keap1/Nrf2 pathway in health and disease: from the bench to the clinic. *Biochem. Soc. Trans.* 43, 687–689. doi: 10.1042/BST20150069

Radat, F., Margot-Duclot, A., and Attal, N. (2013). Psychiatric co-morbidities in patients with chronic peripheral neuropathic pain: a multicentre cohort study. *Eur. J. Pain* 17, 1547–1557. doi: 10.1002/j.1532-2149.2013.00334.x

Redondo, A., Chamorro, P., Riego, G., Leanez, S., and Pol, O. (2017). Treatment with sulforaphane produces antinociception and improves morphine effects during inflammatory pain in mice. *J. Pharmacol. Exp. Ther.* 363, 293–302. doi: 10.1124/jpet.117.244376

Rolfe, D. F., and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* 77, 731–758. doi: 10.1152/physrev.1997.77.3.731

Sehgal, N., Colson, J., and Smith, H. S. (2013). Chronic pain treatment with opioid analgesics: benefits versus harms of long-term therapy. *Expert Rev. Neurother.* 13, 1201–1220. doi: 10.1586/14737175.2013.846517

Shah, S., and Mehta, V. (2012). Controversies and advances in non-steroidal anti-inflammatory drug (NSAID) analgesia in chronic pain management. *Postgrad. Med. J.* 88, 73–78. doi: 10.1136/postgradmedj-2011-130291

Shelar, S. B., Narasimhan, M., Shanmugam, G., Litovsky, S. H., Gounder, S. S., Karan, G., et al. (2016). Disruption of nuclear factor (erythroid-derived-2)-like 2 antioxidant signaling: a mechanism for impaired activation of stem cells and delayed regeneration of skeletal muscle. *FASEB J.* 30, 1865–1879. doi: 10.1096/fj.201500153

Shirai, Y., Fujita, Y., and Hashimoto, K. (2012). Effects of the antioxidant sulforaphane on hyperlocomotion and prepulse inhibition deficits in mice after phencyclidine administration. *Clin. Psychopharmacol. Neurosci.* 10, 94–98. doi: 10.9758/cpn.2012.10.2.94

Shirai, Y., Fujita, Y., Hashimoto, R., Ohi, K., Yamamori, H., Yasuda, Y., et al. (2015). Dietary intake of sulforaphane-rich broccoli sprout extracts during juvenile and adolescence can prevent phencyclidine-induced cognitive deficits at adulthood. *PLoS One* 10:e0127244. doi: 10.1371/journal.pone.0127244

Suzuki, T., Motohashi, H., and Yamamoto, M. (2013). Toward clinical application of the keap1–Nrf2 pathway. *Trends Pharmacol. Sci.* 34, 340–346. doi: 10.1016/j.tips.2013.04.005

Suzuki, T., and Yamamoto, M. (2015). Molecular basis of the keap1-Nrf2 system. *Free Radic. Biol. Med.* 88, 93–100. doi: 10.1016/j.freeradbiomed.2015.06.006

Vanegas, H., Vazquez, E., and Tortorici, V. (2010). NSAIDs, opioids, cannabinoids and the control of pain by the central nervous system. *Pharmaceuticals* 3, 1335–1347. doi: 10.3390/ph3051335

Volkow, N. D., and Collins, F. S. (2017). The role of science in addressing the opioid crisis. *N. Engl. J. Med.* 377, 391–394. doi: 10.1056/NEJMsr1706626

Wardyn, J. D., Ponsford, A. H., and Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochem. Soc. Trans.* 43, 621–626. doi: 10.1042/BST20150014

White, P. F. (2005). The changing role of non-opioid analgesic techniques in the management of postoperative pain. *Anesth. Analg.* 101(5 Suppl.), S5–S22. doi: 10.1213/01.ANE.0000177099.28914.A7

Xie, X., Pascual, C., Lieu, C., Oh, S., Wang, J., Zou, B., et al. (2017). Analgesic microneedle patch for neuropathic pain therapy. *ACS Nano* 11, 395–406. doi: 10.1021/acsnano.6b06104

Yamamoto, M., Kensler, T. W., and Motohashi, H. (2018). The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* 98, 1169–1203. doi: 10.1152/physrev.00023.2017

Yao, W., Zhang, J. C., Ishima, T., Dong, C., Yang, C., Ren, Q., et al. (2016a). Role of keap1-Nrf2 signaling in depression and dietary intake of glucoraphanin confers stress resilience in mice. *Sci. Rep.* 6, 30659. doi: 10.1038/srep30659

Yao, W., Zhang, J. C., Ishima, T., Ren, Q., Yang, C., Dong, C., et al. (2016b). Antidepressant effects of TBE-31 and MCE-1, the novel Nrf2 activators, in an inflammation model of depression. *Eur. J. Pharmacol.* 793, 21–27. doi: 10.1016/j.ejphar.2016.10.037

Zhang, J. C., Yao, W., Dong, C., Han, M., Shirayama, Y., and Hashimoto, K. (2018). Keap1-Nrf2 signaling pathway confers resilience versus susceptibility to inescapable electric stress. *Eur. Arch. Psychiatry Clin. Neurosci.* doi: 10.1007/s00406-017-0848-0 [Epub ahead of print].

Zhang, J. C., Yao, W., Dong, C., Yang, C., Ren, Q., Ma, M., et al. (2017). Prophylactic effects of sulforaphane on depression-like behavior and dendritic changes in mice after inflammation. *J. Nutr. Biochem.* 39, 134–144. doi: 10.1016/j.jnutbio.2016.10.004

Zhang, Y., Talalay, P., Cho, C. G., and Posner, G. H. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U.S.A.* 89, 2399–2403. doi: 10.1073/pnas.89.6.2399

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Activation of Nrf2/HO-1 Pathway by Nardochinoid C Inhibits Inflammation and Oxidative Stress in Lipopolysaccharide-Stimulated Macrophages

Jin-Fang Luo[1,2], Xiu-Yu Shen[3], Chon Kit Lio[1,2], Yi Dai[3], Chun-Song Cheng[1,2], Jian-Xin Liu[4], Yun-Da Yao[1,2], Yang Yu[3], Ying Xie[1,2], Pei Luo[1,2], Xin-Sheng Yao[3], Zhong-Qiu Liu[5] and Hua Zhou[1,2,5]**

[1] Faculty of Chinese Medicine, Macau University of Science and Technology, Macau, China, [2] State Key Laboratory of Quality Research in Chinese Medicines, Macau University of Science and Technology, Macau, China, [3] Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou, China, [4] College of Pharmacy, Hunan University of Chinese Medicine, Changsha, China, [5] Joint Laboratory for Translational Cancer Research of Chinese Medicine of the Ministry of Education of the People's Republic of China, Guangzhou University of Chinese Medicine, Guangzhou, China

The roots and rhizomes of *Nardostachys chinensis* have neuroprotection and cardiovascular protection effects. However, the specific mechanism of *N. chinensis* is not yet clear. Nardochinoid C (DC) is a new compound with new skeleton isolated from *N. chinensis* and this study for the first time explored the anti-inflammatory and anti-oxidant effect of DC. The results showed that DC significantly reduced the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in lipopolysaccharide (LPS)-activated RAW264.7 cells. The expression of pro-inflammatory proteins including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were also obviously inhibited by DC in LPS-activated RAW264.7 cells. Besides, the production of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were also remarkably inhibited by DC in LPS-activated RAW264.7 cells. DC also suppressed inflammation indicators including COX-2, PGE_2, TNF-α, and IL-6 in LPS-stimulated THP-1 macrophages. Furthermore, DC inhibited the macrophage M1 phenotype and the production of reactive oxygen species (ROS) in LPS-activated RAW264.7 cells. Mechanism studies showed that DC mainly activated nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, increased the level of anti-oxidant protein heme oxygenase-1 (HO-1) and thus produced the anti-inflammatory and anti-oxidant effects, which were abolished by Nrf2 siRNA and HO-1 inhibitor. These findings suggested that DC could be a new Nrf2 activator for the treatment and prevention of diseases related to inflammation and oxidative stress.

Keywords: ***Nardostachys chinensis*, Nardochinoid C, Nrf2, HO-1, ROS**

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***Correspondence:**
Hua Zhou
hzhou@must.edu.mo
Zhong-Qiu Liu
liuzq@gzucm.edu.cn

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INTRODUCTION

Multiple inflammatory diseases, including RA (McInnes and Schett, 2011), AD (Akiyama et al., 2000), seriously endanger human health. It is well accepted that inflammation is linked with oxidative stress. Oxidative stress refers to elevated intracellular levels of ROS that is considered to be the most potent inflammatory mediators (Shin et al., 2008). Antioxidants play a significant role in reducing inflammation (Waxman, 1996). The activation of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway could inhibit the progression of inflammation (Xu et al., 2003). Nrf2-mediated antioxidant gene expression can reduce the macrophage M1 phenotype and ROS production (Kobayashi et al., 2016). Since Nrf2 pathway plays a critical role in inflammation, Nrf2 activators has become a potential therapeutic strategy for numerous disorders (Crunkhorn, 2012), such as inflammatory disorders (Kim et al., 2010), cardiovascular diseases (Li et al., 2009), neurodegenerative diseases (Joshi and Johnson, 2012), cancer (Sporn and Liby, 2012), type 2 diabetes (Chartoumpekis and Kensler, 2013), chronic kidney disease (Ruiz et al., 2013) and multiple sclerosis (Gold et al., 2012). However, there are only very little Nrf2 activators in clinics. Tecfidera (dimethyl fumarate), a potent Nrf2 activator, has been approved for the treatment of multiple sclerosis (Gold et al., 2012), but long-term use of this drug can cause resistance and other side effects (Deeks, 2014). So, the discovery of new and safer Nrf2 activator for clinical use has become an important task in drug discovery.

In recent years, the research showed that natural components extracted from plant have anti-inflammatory and antioxidant effects (Shen et al., 2014). The roots and rhizomes of *Nardostachys chinensis* have been used for blood disorders, herpes and infection (Arora, 1965; Pakrashi and Chatarji, 1994), the extracts of *N. chinensis* were also used for the treatment of epilepsy and hysteria (Bagchi et al., 1991). Above all, *N. chinensis* have neuroprotection and cardiovascular protection properties. However, the action mechanism of *N. chinensis* remains unclear. There are some studies reporting that the compounds isolated from *N. chinensis* suppressed LPS-induced activation of RAW264.7 cells (Hwang et al., 2012; Shin et al., 2015). The activation of Nrf2-mediated antioxidant pathway has the neuroprotective effect (Catino et al., 2016) and antioxidant could promote anti-inflammatory effect (Li et al., 2008).

Until now, the antioxidant activity of the compounds extracted from *N. chinensis* in macrophages remain unknown. Therefore, the anti-inflammatory activity and the antioxidant effect of Nardochinoid C (DC) (**Figure 1A**), a new compound with new skeleton isolated from *N. chinensis* was studied for the first time in this research.

Macrophages play a key role in the innate immune response. It serves as the first line of defense in the body against invading pathogens and promotes cell protection and repair processes (Linde et al., 2007). Activated macrophage produces a variety of pro-inflammatory mediators, such as interleukin -6 (IL-6), tumor necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2), and nitric oxide (NO) (Noguchi et al., 2003; Kang et al., 2006; Szekanecz and Koch, 2007), which can promote the development of inflammatory (Coussens and Werb, 2002).

Therefore, two inflammatory cell models, LPS-stimulated RAW264.7 macrophage and LPS-stimulated THP-1 macrophage, were chosen to examine the anti-inflammatory activity of DC in this study.

We found that: (1) DC had significant anti-inflammatory activity both in LPS-induced RAW264.7 cells model and LPS-induced THP-1 cells model. (2) DC produced anti-inflammatory effect mainly through activating Nrf2/HO-1 pathway, rather than inhibiting NF-κB and MAPK pathways in LPS-stimulated RAW264.7 cell. (3) DC activated Nrf2 antioxidant pathways to reduce ROS production in LPS-stimulated RAW264.7 cell. (4) DC produced anti-inflammatory effect mainly through increasing the expression and the activity of HO-1 antioxidant protein.

These findings suggest that DC could be a new potential Nrf2 activator for the treatment and prevention of diseases related to inflammation and oxidative stress.

MATERIALS AND METHODS

Materials

DC (HPLC purity > 98%) was obtained from the Institute of Traditional Chinese Medicine and Natural Products, Jinan University. LPS, SFN, DEX, ZnPP, PMA, hemin, bilirubin, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and antibody to α-Tubulin were obtained from Sigma (St. Louis, MO, United States). LPS was first dissolved in PBS and then diluted with the medium to get the final working concentration. All the other test compounds (DEX, SFN, DC, ZnPP, and hemin) were first dissolved in DMSO and then diluted with the medium or potassium phosphate buffer to reach the final working concentration, respectively. The final concentration of DMSO was less than 0.1%. Antibodies to iNOS, COX-2, p-IKKα/β, p-p65, IKKα/β, p65, p-JNK, p-ERK, p-p38, JNK, ERK, p38, Nrf2, and Keap1 were from Cell Signaling Technology (Boston, MA, United States). Antibodies to p62, HO-1 and NQO-1 were from Abcam (Abcam, Cambridge, United Kingdom). Griess reagent from Promega (Promega, United States). ELISA kit for PGE_2 were from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, United States), ELISA kits for IL-6 and TNF-α were from eBioscience (eBioscience, Inc., United States). siRNA for Nrf2 (sc-37049), non-specific siRNA (sc-37007) and antibody to β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA,

Abbreviations: AD, Alzheimer's disease; BSA, bovine serum albumin; COX-2, cyclooxygenase-2; DC, nardochinoid C; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DEX, dexamethasone; HO-1, heme oxygenase-1; IL-6, interleukin-6; iNOS, nitric oxide synthase; Keap1, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; Maf, musculoaponeurotic fibrosarcoma; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; NO, nitric oxide; NQO1, NAD(P)H quinoneoxidoreductase 1; Nrf2, nuclear factor-E2-related factor 2; PGE_2, prostaglandin E_2; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; ROS, reactive oxygen species; SFN, sulforaphane; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; ZnPP, zinc protoporphyrin IX.

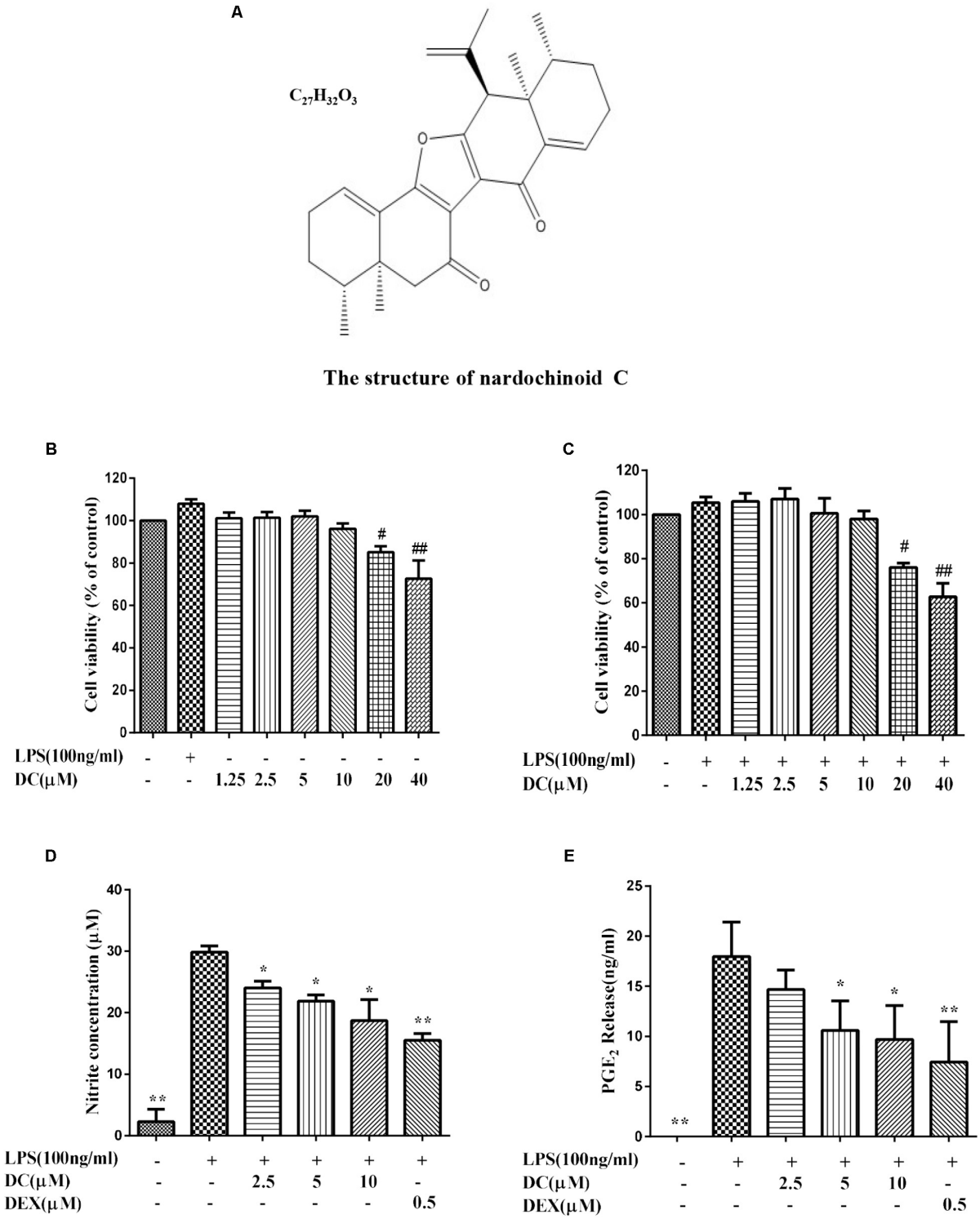

FIGURE 1 | The effect of Nardochinoid C (DC) on the release of NO and PGE_2 in LPS-stimulated RAW264.7 cells. **(A)** The chemical structure of DC. **(B)** Cytotoxicity of DC on LPS-unstimulated RAW264.7 cells. **(C)** Cytotoxicity of DC on LPS-stimulated RAW264.7 cells. The cells were treated with DC at various concentrations (1.25–40 μM) for 1 h, and then stimulated with or without LPS for 18 h, the cell viability was analyzed with MTT method. Effects of DC on the productions of NO **(D)** and PGE_2 **(E)** in LPS-stimulated RAW264.7 cells. The cells were incubated with indicated concentrations of DC or DEX for 1 h, and then stimulated with LPS for 18 h. The concentration of NO (expressed as nitrite) and PGE_2 in the culture medium were quantified by ELISA kits. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. LPS-unstimulated cells **(B,C)** or $^{*}p < 0.05$, $^{**}p < 0.01$, vs. LPS-stimulated cells **(D,E)**.

United States). Lipofectamine 2000 (Lipo2000) was obtained from Invitrogen (Carlsbad, CA, United States). The secondary antibodies for Western blot were from Li-COR Biotechnology (Lincoln, NE, United States).

Cell Culture

The RAW264.7 cells were from American Type Culture Collection (ATCC, Manassas, VA, United States). The cells were retained in Dulbecco's modified Eagle's medium (DMEM), which contained L-glutamine (2 mM), penicillin G (100 U/ml), 10% heat-inactivated FBS and streptomycin (100 mg/ml). The cells were incubated in a cell incubator remained at 37°C and a relative humidity of about 95% with 5% CO_2. Conventional cell subculture by trypsinizing cells for 1–2 min. The cells before the 30th generation were taken for experimental research.

THP-1 human monocytic leukemia cells were from ATCC. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. THP-1 cells were treated with 50 nM PMA (Sigma-Aldrich, St. Louis, MO, United States) for 48 h to induce differentiation of the cells into macrophages. Following differentiation, non-attached cells were removed by aspiration. The adherent macrophages were then washed three times with RPMI 1640 medium and maintained in a 37°C humidified incubator containing 5% CO_2. The cells before the 30th generation were used for the experiments.

Cytotoxicity Assay

Cell viability was determined by MTT assay. Briefly, RAW264.7 cells were seeded in a 96-well tissue culture plate at a density of 1.4×10^4 cells/well and incubated for 24 h, and then exposed to DC at various concentrations (1.25, 2.5, 5, 10, and 20 μM) for 18 h with or without LPS (100 ng/mL). THP-1 derived macrophages were seeded in a 96-well tissue culture plate at a density of 5×10^4 cells/well and incubated with various concentrations (1.25, 2.5, 5, 10, and 20 μM) of DC for 24 h with or without LPS (1 μg/mL). Then, each well was added 10 μL of MTT solution (5 g/L) respectively, incubated at 37°C for 4 h, and then added 100 μL of 10% SDS-HCl solution. At last, the optical density of each well was determined at 570 nm (the reference wavelength was 650 nm).

Measurement of NO, PGE_2, TNF-α, and IL-6

RAW264.7 cells were seeded in a 24 well plate at a density of 8×10^4 cells/well and incubated for 24 h. The cells were then pretreated with DC for 1 h, and next exposed to LPS (100 ng/mL) for 18 h. THP-1 derived macrophages (1×10^6 cells/well) were seeded in 6 well plates and incubated with various concentrations (2.5, 5, and 10 μM) of DC or DEX (0.5 μM) for 1 h, finally, exposed to LPS (1 μg/mL) for 24 h.

The cell supernatant of each well was collected, respectively, for detecting the concentration of PGE_2, TNF-α, and IL-6 with the ELISA kits according to the manufacturer's instructions. Detection of NO content is based on Griess Reagent System (Promega, United States) according to the kit instructions.

Real-Time PCR Analysis

RAW264.7 cells were seeded at a density of 8×10^4 cells/well, and then incubated for 24 h. The cells were pretreated with various concentrations (2.5, 5, and 10 μM) of DC for 1 h, and then exposed to LPS (100 ng/mL) for 18 h. THP-1 derived macrophages (1×10^6 cells/well) were seeded in 6 well plates and incubated with various concentration (2.5, 5, and 10 μM) of DC or DEX (0.5 μM) for 1 h, finally, exposed to LPS (1 μg/mL) for 24 h.

Total RNA was extracted from the cells by using the RNeasy Mini Kit (Qiagen, Germany). One microgram of total RNA was synthesized into cDNA by using the cDNA kit from Roche (Roche, Mannheim, Germany). Target RNA levels were determined by using ViiATM 7 real-time PCR.

PCR reaction system included 1 μl cDNA, 10 μl SYBR Green PCR Master Mix (Roche, Mannheim, Germany), 2 μl primers and 7 μl PCR-grade water. The reactions were performed with a denaturation step at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative mRNA expression levels were normalized to that of the internal control, using the $2^{-\Delta\Delta Ct}$ cycle threshold method.

The gene relative expression for target gene were first normalized with internal reference gene (β-actin or GAPDH) and then the relative expression level of target gene for each test group were normalized with control group value. This explain why the value in the control group was 1. Therefore, the value for the normal control group was always 1 unit for each independent experiment and there is no SEM for the control group. The primers used in this study were listed in **Tables 1**, **2**, respectively.

Protein Preparation and Western Blot Analysis

RAW264.7 cells (8×10^4 cells/well) were seeded in 24 well plates. After being incubated for 24 h, the cells were treated with various concentrations (2.5, 5, and 10 μM) of DC, DEX (0.5 μM) or SFN

TABLE 1 | The primers for real time PCR of RAW264.7 cells.

Target gene	Primer sequences
β-actin_F	5′-CGGTTCCGATGCCCTGAGGCTCTT-3′
β-actin_R	5′-CGTCACACTTCATGATGGAATTGA-3′
iNOS_F	5′-CAGCACAGGAAATGTTTCAGC-3′
iNOS_R	5′-TAGCCAGCGTACCGGATGA-3′
COX-2_F	5′-TTTGGTCTGGTGCCTGGTC-3′
COX-2_R	5′-CTGCTGGTTTGGAATAGTTGCTC-3′
TNF-α_F	5′-TATGGCTCAGGGTCCAACTC-3′
TNF-α_R	5′-CTCCCTTTGCAGAACTCAGG-3′
IL-6_F	5′-GGTGACAACCACGGCCTTCCC-3′
IL-6_R	5′-AAGCCTCCGACTTGTGAAGTGGT-3′
Nrf2_F	5′-AGCAGGACATGGAGCAAGTT-3′
Nrf2_R	5′-TTCTTTTTCCAGCGAGGAGA-3′
HO-1_F	5′-CCCACCAAGTTCAAACAGCTC-3′
HO-1_R	5′-AGGAAGGCGGTCTTAGCCTC-3′
NQO1_F	5′-TTCTGTGGCTTCCAGGTCTT-3′
NQO1_R	5′-AGGCTGCTTGGAGCAAAATA-3′

(10 μM) for 1 h, then exposed to LPS (100 ng/mL) for 6 h or 18 h. THP-1 derived macrophages (1×10^6 cells/well) were seeded in 6 well plates and incubated with various concentration (2.5, 5, and 10 μM) of DC or DEX (0.5 μM) for 1 h, finally, exposed to LPS (1 μg/mL) for 24 h.

The cells were lysed using RIPA lysis buffer and the protein concentration was detected, respectively, by using the Bradford Assay Reagent (Bio-Rad, Philadelphia, PA, United States). The nuclear and cytoplasmic protein fractions were extracted by using NE-PER nuclear and cytoplasmic extraction kit (Thermo, Pierce, United States). Equal amounts of proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane, the membrane was blocked by using 5% BSA solution, then incubated with the corresponding primary antibody for overnight at 4°C. Then, the membranes were incubated with the secondary antibodies for 1 h at 24°C. The band of antigen-antibody complexes were scanned by using the Odyssey CLx Imager (Li-COR, United States). Western blot data were analyzed by using ImageJ software. The image densities of specific bands for target protein were first normalized with the density of loading control reference protein band (β-actin or α-tubulin). Then, the relative expression level of target protein for each test group were normalized with the control group value. This explain why the value in the control group was 1. Therefore, the value for the normal control group was always 1 unit for each independent experiment and there is no SEM for the control group.

Immunofluorescence Analysis

For immunofluorescence analysis, RAW264.7 cells were seeded on glass coverslip in a six-well plate at a density of 2×10^5 cells/well, and then incubated overnight. After being treated with 10 μM DC or SFN for 6 h, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and subsequently permeabilized with 0.1% Triton X-100 for 30 min and blocked with 5% BSA for 30 min. The cells were then incubated with Nrf2 antibody for overnight, next incubated with the secondary antibody (Alexa Fluor 488-conjugated secondary antibody) for 1 h. At last, the cells were stained with DAPI for 5 min. The fluorescence images were captured by using a Leica TCS SP8 Confocal Laser Scanning Microscope System (Leica, Wetzlar, Germany).

Flow Cytometric Analysis

Reactive oxygen species induction and macrophage M1 subtype were detected by flow cytometry. The cells (3×10^5 cells/well) were cultured in a six-well plate, incubated for 18 h and then treated with 10 μM DC for 1 h, followed by stimulation with LPS (100 ng/mL) for 6 h. The cells were collected and stained for F/480 and CD11c to detect macrophage M1 subtype according to the manufacturer's directions (BioLegend, San Diego, CA, United States) or stained with the fluorescent probe DCFH-DA to determine ROS level according to the manufacturer's directions (Invitrogen, Carlsbad, CA, United States).

TABLE 2 | The primers for real time PCR of THP-1 cells.

Target gene	Primer sequences
GAPDH_F	5′- ACCAGCCTCAAGATCATCAGCA-3′
GAPDH_R	5′- TGCTAAGCAGTTGGTGGTGC-3′
TNF-α_F	5′- GCCCAGGCAGTCAGATCATC-3′
TNF-α_R	5′- CGGTTCAGCCACTGGAGCT-3′
IL-6_F	5′- GTGTTGCCTGCTGCCTTC-3′
IL-6_R	5′- AGTGCCTCTTTGCTGCTTTC-3′

Transfection Assay

Nrf2 siRNA and non-specific siRNA (NS siRNA) were transfected into RAW264.7 cells by using lipofectamine 2000 reagent according to the manufacturer's directions. In brief, the cells were seeded in a 24-well culture plate and incubated with the NS siRNA or Nrf2 siRNA at 300 nM for 24–48 h in serum-free OPTI-MEM media (Invitrogen, United States). After incubation, the transfected cells were pretreated with indicated concentration of DC for 1 h and stimulated with or without LPS (100 ng/ml) for 18 h. The cells were prepared and the expressions of Nrf2 and HO-1 were analyzed by real-time PCR and Western blot analysis.

Measurement of Heme Oxygenase Activity

Heme oxygenase activity was determined by the production of bilirubin. The production of bilirubin from hemin was determined upon addition of rat-liver cytosol as the source of biliverdin reductase (Srisook and Cha, 2004) with some modifications. Briefly, after the incubation, RAW264.7 macrophages were washed with PBS. Harvested cells were sonicated and centrifuged (18,000 × *g*, 10 min, 4°C). The supernatant (400 μl) was re-suspended in ice-cold potassium phosphate buffer (100 mM), then was added to a NADPH-generating system in 200 μl of reaction mixture containing 2 mg rat liver cytosol, 20 μM hemin, 1 mM NADPH, 2 mM glucose-6-phosphate, and 0.2 unit glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer, pH 7.4, at 37°C for 1 h. Bilirubin was then extracted with 1 ml chloroform and measured by the absorbance difference between 464 and 530 nm (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO-1 activity was expressed as nanomoles of bilirubin formed per mg of cell protein per hour.

Statistical Analysis

The results were expressed as mean ± SEM and represented three independent experiments. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests or unpaired *t*-test in GraphPad Prism7. $P < 0.05$ were considered statistically significant.

RESULTS

Nardochinoid C Reduced the Production of NO and PGE_2 in LPS-Stimulated Macrophages

The MTT results showed that DC at 1.25–10 μM concentrations was non-toxic to RAW264.7 cells with or without LPS stimulation

(**Figures 1B,C**). Based on these results, 2.5–10 μM DC were selected to study the anti-inflammatory activity. When the cells were exposed to LPS for 18 h, the production of NO in the cell supernatant was significantly increased ($P < 0.01$). DC obviously reduced the NO production induced by LPS in a concentration-dependent manner ($P < 0.05$, **Figure 1D**). Pretreatment with DC for 1 h also obviously and concentration-dependently reduced the production of PGE_2 induced by LPS in LPS-stimulated RAW264.7 cells ($P < 0.05$, **Figure 1E**). DEX is a classic anti-inflammatory drug that significantly inhibits the release of inflammatory indicators (Kim et al., 2014), therefore, it was selected as a positive control to evaluate the anti-inflammatory activity of DC in this study. As shown in **Figures 1D,E**, the levels of NO and PGE_2 were also significantly reduced by DEX ($P < 0.01$).

Nardochinoid C Suppressed the Expression of iNOS, COX-2, TNF-α, and IL-6 in LPS-Stimulated RAW264.7 Macrophages

In the inflammatory response, NO and PGE_2 are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively (Williams et al., 1999; Sampey et al., 2005). **Figures 2A,B** showed that iNOS and COX-2 proteins were increased in LPS treatment group, while DC pre-treatment obviously and concentration-dependently decreased the elevated expression levels of iNOS and COX-2 proteins, and DEX also inhibited the expressions of iNOS and COX-2 proteins ($P < 0.05 \sim 0.01$, **Figures 2A,B**).

As shown in **Figures 2C,D**, LPS stimulation increased the mRNA levels of iNOS and COX-2, DC pre-treatment obviously and concentration-dependently decreased the elevated mRNA levels of iNOS and COX-2, and DEX also showed inhibitory effects similar to DC ($P < 0.05 \sim 0.01$).

When inflammation occurs, activated macrophages acts as the major effector cells that secrete large amounts of inflammatory mediators (e.g., TNF-α and IL-6) to promote the development and progression of inflammation (Fujiwara and Kobayashi, 2005). Therefore, these related inflammatory mediators, including TNF-α and IL-6 were detected in this study. As shown in **Figures 2E,F**, LPS stimulation increased the protein levels of TNF-α and IL-6, while the increased protein levels of TNF-α and IL-6 were both significantly inhibited by DC in a concentration-dependent manner and by DEX ($P < 0.05 \sim 0.01$). LPS stimulation also increased the mRNA levels of TNF-α and IL-6 (**Figures 2G,H**), while these increases were significantly inhibited by DC concentration dependently and by DEX ($P < 0.05 \sim 0.01$, **Figures 2G,H**).

Nardochinoid C Suppressed the Levels of COX-2, PGE_2, TNF-α, and IL-6 in LPS-Stimulated THP-1 Macrophages

The MTT results showed that DC at 1.25–10 μM concentrations was non-toxic to THP-1 cells with or without LPS stimulation (**Figures 3A,B**). Based on these results, 2.5–10 μM DC were selected to study the anti-inflammatory activity in LPS-stimulated THP-1 macrophages.

Figure 3C showed that COX-2 proteins were increased in LPS treatment group, while DC pre-treatment obviously and concentration-dependently decreased the elevated expression levels of COX-2 proteins ($P < 0.05 \sim 0.01$, **Figure 3C**).

When the cells were exposed to LPS for 24 h, the production of PGE_2 in the cell supernatant was significantly increased ($P < 0.01$). DC obviously reduced the PGE_2 production induced by LPS in a concentration-dependent manner ($P < 0.05$, **Figure 3D**). As shown in **Figures 3C,D**, the levels of COX-2 and PGE_2 were also significantly reduced by DEX ($P < 0.01$).

As shown in **Figures 3E,F**, LPS stimulation also increased the mRNA levels of TNF-α and IL-6 in THP-1 cells model, while these increases were significantly inhibited by DC concentration dependently and DEX ($P < 0.05 \sim 0.01$, **Figures 3E,F**).

Nardochinoid C Failed to Inhibit the Activation of NF-κB and MAPK Pathway in LPS-Stimulated RAW264.7 Macrophages

Nuclear factor-κB (NF-κB) pathway is activated in inflammatory process and then promotes the expression of inflammatory mediators in different cells, including macrophages (DiDonato et al., 2012). LPS activates NF-κB pathway to induce the production of inflammatory cytokines (Ghosh and Hayden, 2008). MAPK pathway also plays an critical role in inflammatory response (Liu et al., 2007). The activation of NF-κB and MAPK signaling pathways are both involved in the development of inflammation (Liu et al., 2016). Therefore, the inhibition of NF-κB and MAPK signaling pathways are considered as the useful ways to regulate inflammatory reaction. In the non-inflammatory condition, NF-κB and IκBα are present in the cytoplasm as complex (Sahu et al., 2014). The activation of NF-κB resulted in the phosphorylation of IKKα/β, p65, and then leading to the transcription of inflammatory genes and the expression of inflammatory proteins (Ghosh et al., 1998).

As **Figure 4A** shows, LPS stimulation increased the phosphorylation of IKKα/β and p65. However, DC failed to inhibit the phosphorylation of these proteins (**Figure 4A**). LPS also increased the nuclear translocation of NF-κB p65 protein, but the pretreatment of DC failed to inhibit the nuclear translocation of NF-κB p65 induced by LPS (**Figure 4B**). MAPKs activation involves in regulating inflammation process (Kaminska, 2005). The activation of MAPK pathway resulted in the phosphorylation of p38, JNK and ERK (Rao, 2001), which may promote pro-inflammatory cytokines production (Sun et al., 2015). The results showed that LPS increased the protein levels of p-JNK, p-p38 and p-ERK (**Figure 4C**), but DC pretreatment didn't inhibit the increased levels of p-JNK, p-p38 and p-ERK proteins induced by LPS (**Figure 4C**).

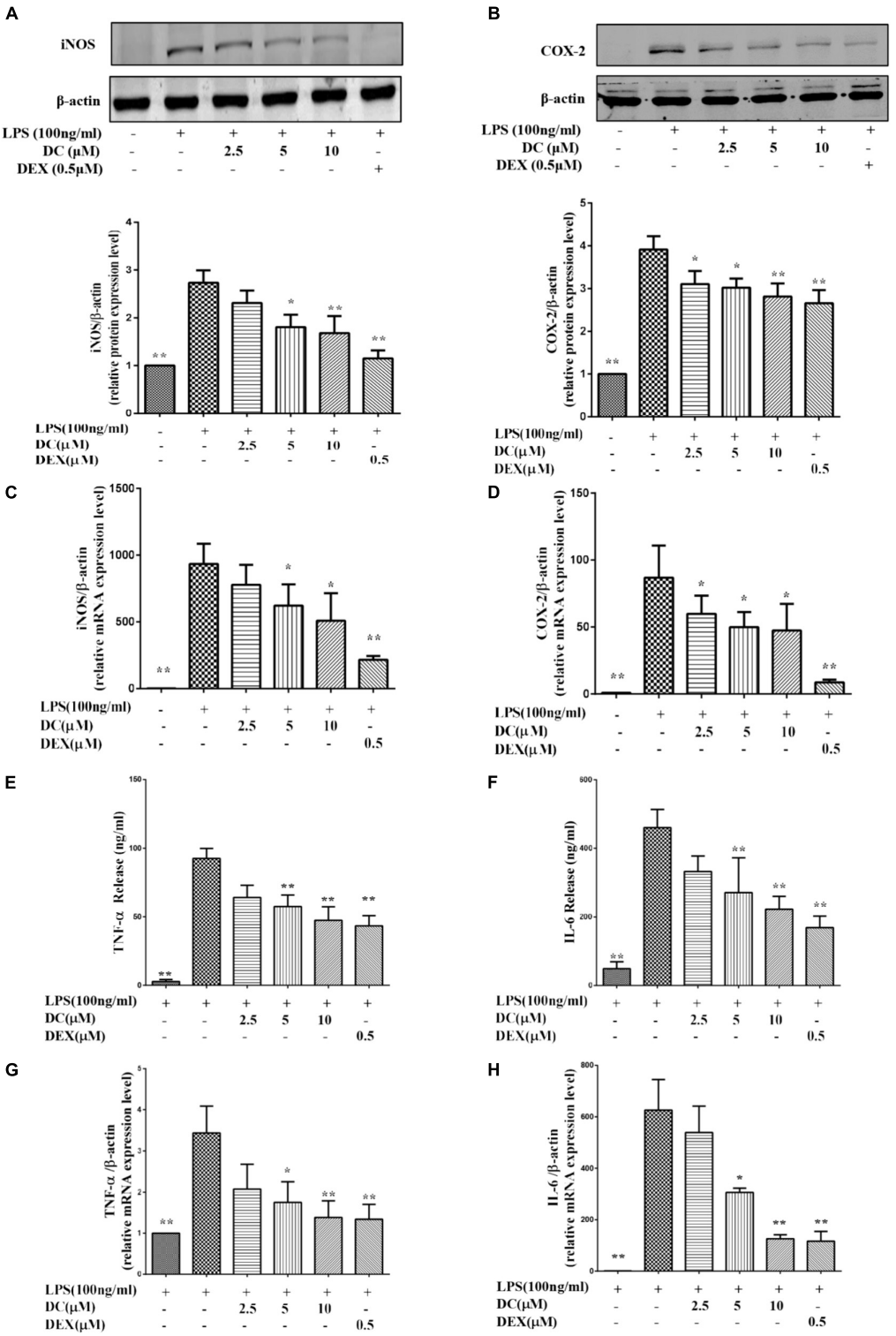

FIGURE 2 | Effects of DC on the expressions of iNOS, COX-2, TNF-α, and IL-6 in LPS-stimulated RAW264.7 cells. The cells were plated in 24-well plates and incubated for 24 h, next the cells were pretreated with indicated concentrations of DC for 1 h and stimulated with LPS for 18 h. The total proteins of the cells were prepared and the expressions of iNOS **(A)** and COX-2 **(B)** were analyzed by Western blot. Total mRNA was prepared, the mRNA expressions of iNOS **(C)** and COX-2 **(D)** were analyzed by real time PCR. The protein expression levels of TNF-α **(E)** and IL-6 **(F)** in cell supernatant were analyzed by ELISA. The mRNA expression of TNF-α **(G)** and IL-6 **(H)** were analyzed by real time PCR. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). *$p < 0.05$, **$p < 0.01$, vs. LPS-stimulated cells.

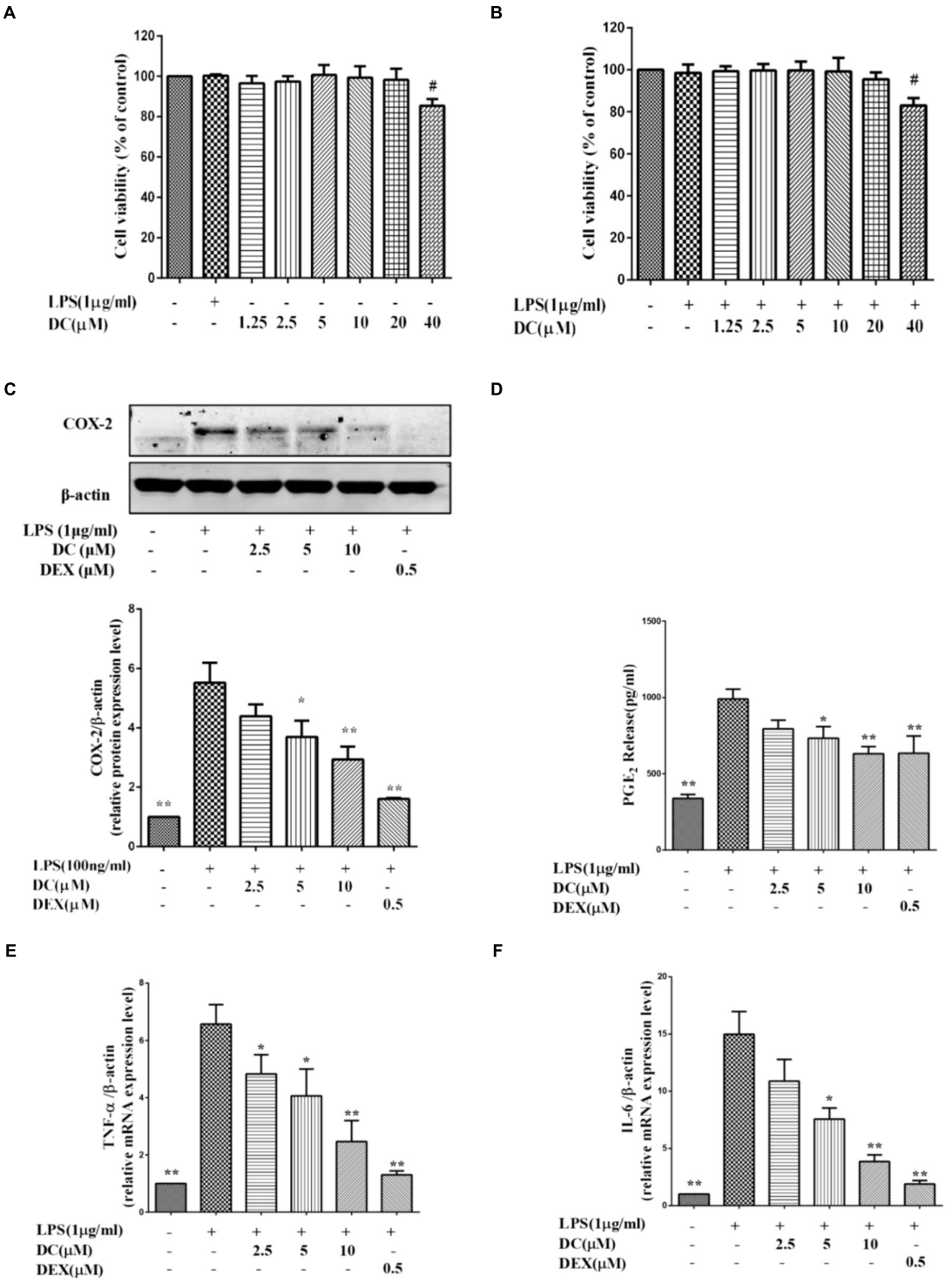

FIGURE 3 | Effects of DC on the levels of COX-2, PGE_2, TNF-α, and IL-6 in LPS-stimulated THP-1 cells. **(A)** Cytotoxicity of DC on LPS-unstimulated THP-1 cells. **(B)** Cytotoxicity of DC on LPS-stimulated THP-1 cells. **(C)** Effects of DC on the expression of COX-2 in LPS-stimulated THP-1 cells. **(D)** Effects of DC on the productions of PGE_2 in LPS-stimulated THP-1 cells. The mRNA expression of TNF-α **(E)** and IL-6 **(F)** were analyzed by real time PCR. THP-1 cells (1×10^6 cells/well) were seeded in six well plates. After being incubated for 24 h, the cells were treated with 50 nM PMA for 48 h, then incubated with various concentration (2.5, 5, and 10 μM) of DC or DEX (0.5 μM) for 1 h, finally, exposed to LPS (1 μg/mL) for 24 h. The cell supernatant, total proteins and mRNA of the cells were prepared as described before for Western blot and real time PCR. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. LPS-unstimulated cells **(A,B)** or $^{*}p < 0.05$, $^{**}p < 0.01$, vs. LPS-stimulated cells **(C–F)**.

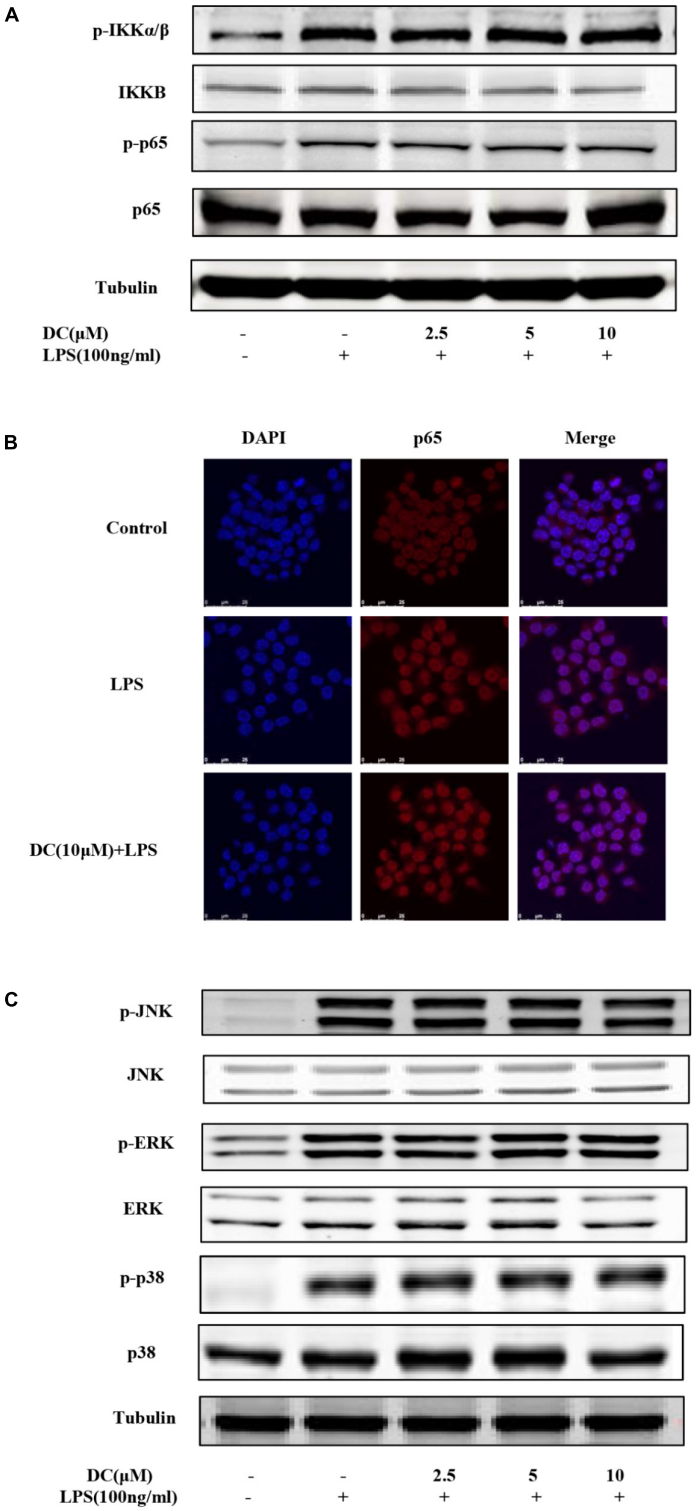

FIGURE 4 | The effect of DC on the activation of NF-κB and MAPK pathways in LPS-stimulated RAW264.7 cells. The cells were plated in 24-well plates and incubated for 24 h, next the cells were pretreated with indicated concentration of DC for 1 h and stimulated with LPS for 15 min. The total proteins of the cells were prepared and the expression of phosphorylation level of IKKα/β and p65 **(A)** were detected by Western blot. The cells were treated with DC (10 μM) for 1 h and stimulated with LPS for 15 min. The subcellular localization of p65 was detected with immunofluorescence assay **(B)** and the images were acquired using the Leica DM2500 fluorescent microscopy. The total proteins of the cells were prepared and the expressions of phosphorylated JNK, ERK and p38 **(C)** were detected by Western blot.

Nardochinoid C Attenuated LPS-Induced M1 Phenotype and ROS Generation and Also Increased the Levels of HO-1 and NQO1 in LPS-Stimulated RAW264.7 Macrophages

Previous reports showed that Nrf2-mediated antioxidant gene expression reduced the M1 phenotype and ROS production and contributed to anti-inflammation (Kobayashi et al., 2016). Macrophages could polarize to M1 phenotype under an inflammatory environment (Jaguin et al., 2013). As shown in **Figure 5A**, LPS induced an increase of macrophages in M1 phenotype and DC pretreatment at 10 μM suppressed this increase ($P < 0.05$). ROS participates in inflammation (Yang et al., 2015), the activation of Nrf2 could decrease the levels of ROS (Surh, 2003). As shown in **Figure 5B**, intracellular ROS was increased by LPS stimulation and DC pretreatment at 10 μM also suppressed the ROS generation induced by LPS ($P < 0.05$).

Nrf2 dimerizes with small Maf proteins in the nucleus, and then binds *cis*-oxidation reaction elements, i.e., HO-1 and NQO1, to activate their transcription (Buchman, 2001; Hotamisligil, 2006). Antioxidant protein HO-1 has anti-oxidative and anti-inflammatory effects (Linton and Fazio, 2003; McInnes and Schett, 2011). Many studies have shown that defective expression of HO-1 in humans is accompanied by an increase in the inflammatory state (Pae and Chung, 2009). Nrf2 also could increase the transcription of its target genes, including HO-1 and NQO1 (Hu et al., 2017). In order to understand whether DC could exert its anti-inflammatory effect through activating Nrf2 pathway, the downstream proteins (HO-1 and NQO1) of Nrf2 pathway were investigated in this study. As shown in **Figures 5C–F**, compared with LPS-stimulated RAW264.7 cell, DC concentration-dependently increased the levels of NQO1 and HO-1 proteins ($P < 0.05 \sim 0.01$, **Figures 5C,D**) and the expressions of NQO1 and HO-1 mRNA ($P < 0.05$, **Figures 5E,F**). However, DEX have no obvious influence on the protein and mRNA levels of NQO1 and HO-1, compared with LPS group (**Figures 5C–F**).

Nardochinoid C Promoted the Nucleus Translocation of Nrf2 Protein and Activated Nrf2 Pathway by Inhibiting Keap1 in LPS-Unstimulated RAW264.7 Macrophages

Above studies have showed that DC increased the protein levels of HO-1 and NQO1 in LPS-stimulated RAW264.7 cells. Next, the effects of DC on Nrf2 pathway in LPS-unstimulated RAW264.7 cells were also investigated. SFN, a potent Nrf2 activator (Fahey et al., 2002), was selected as a positive control. As shown in **Figures 6A,B**, pretreatment of DC at 10 μM significantly promoted Nrf2 protein entering into the nucleus in RAW264.7 cells, this is similar to SFN ($P < 0.01$). DC or SFN pretreatment both increased the level of NQO1 and HO-1 proteins ($P < 0.05 \sim 0.01$, **Figures 6C,D**) and the expressions of NQO1 and HO-1 mRNA ($P < 0.05$, **Figures 6E,F**) in unstimulated RAW264.7 cell. Keap1 is a negative regulator of the transcription factor Nrf2 (Satoh et al., 2006). Recently, p62 was thought to be an upstream protein of Nrf2 (Komatsu et al., 2010). **Figures 6G,H** show that DC decreased Keap1 protein expression in a concentration-dependent manner but didn't promote p62 protein expression in LPS-unstimulated RAW264.7 macrophages.

Nrf2 siRNA and HO-1 Inhibitor Significantly Abolished the Effect of Nardochinoid C

To further demonstrate the contribution of Nrf2 signaling pathway to the anti-inflammatory effect of DC, Nrf2 gene knockdown model was established by using Nrf2 siRNA transfection in RAW264.7 cells. The results showed that the protein and mRNA expressions of Nrf2 were significantly suppressed by using specific Nrf2 siRNA (**Figures 7A,B**). The increases in Nrf2 and HO-1 at protein levels caused by DC pretreatment at 10 μM were significantly suppressed by Nrf2 siRNA (**Figures 7C–F**). The suppressive effect of DC on NO production was also abolished by Nrf2 siRNA (**Figure 7G**). These results indicated that the expression of antioxidant proteins HO-1 mediated the anti-inflammatory effect of DC.

In this study, we found that DC decreased the expression of iNOS (**Figure 8A**) and the release of NO (**Figure 8C**). ZnPP, the inhibitor of HO-1 activity (Kim et al., 2008), significantly reversed the effect of DC on HO-1 protein activity (**Figure 8D**) rather than HO-1 expression (**Figure 8B**) to reverse the effect of DC on the expression of iNOS (**Figure 8A**) and the release of NO (**Figure 8B**).

DISCUSSION

When inflammation occurs, activated macrophages release a large number of different inflammatory mediators (NO, PGE_2, TNF-α, and IL-6) and regulatory enzymes (iNOS and COX-2) (Linton and Fazio, 2003; Chawla et al., 2011; McInnes and Schett, 2011; Balkwill and Mantovani, 2012). In this research, LPS stimulation elevated the levels of these inflammatory mediators (NO, PGE_2, TNF-α, and IL-6) and regulatory enzymes (iNOS and COX-2), but the increases of these inflammatory mediators and regulatory enzymes were significantly inhibited by DC (**Figures 1**, **2**), implying that DC has obvious anti-inflammatory activity in LPS-stimulated RAW264.7 macrophage cells model.

THP-1 cell line was used to estimate modulation of monocyte and macrophage activities (Chanput et al., 2014). In this study, we also use LPS-stimulated THP-1 macrophages to study the anti-inflammatory activity of DC. Our results showed that LPS stimulation elevated the levels of inflammatory protein (COX-2), inflammatory mediators (PGE_2) and inflammatory genes (TNF-α and IL-6), but these increases were significantly inhibited by DC (**Figures 3D,E**), implying that DC also has obvious anti-inflammatory activity in LPS-stimulated THP-1 macrophages, these effects are similar to these in LPS-stimulated RAW264.7 macrophages.

In LPS-stimulated THP-1 cell model, we did not detect the high expressed iNOS gene (data not shown), which is similar to

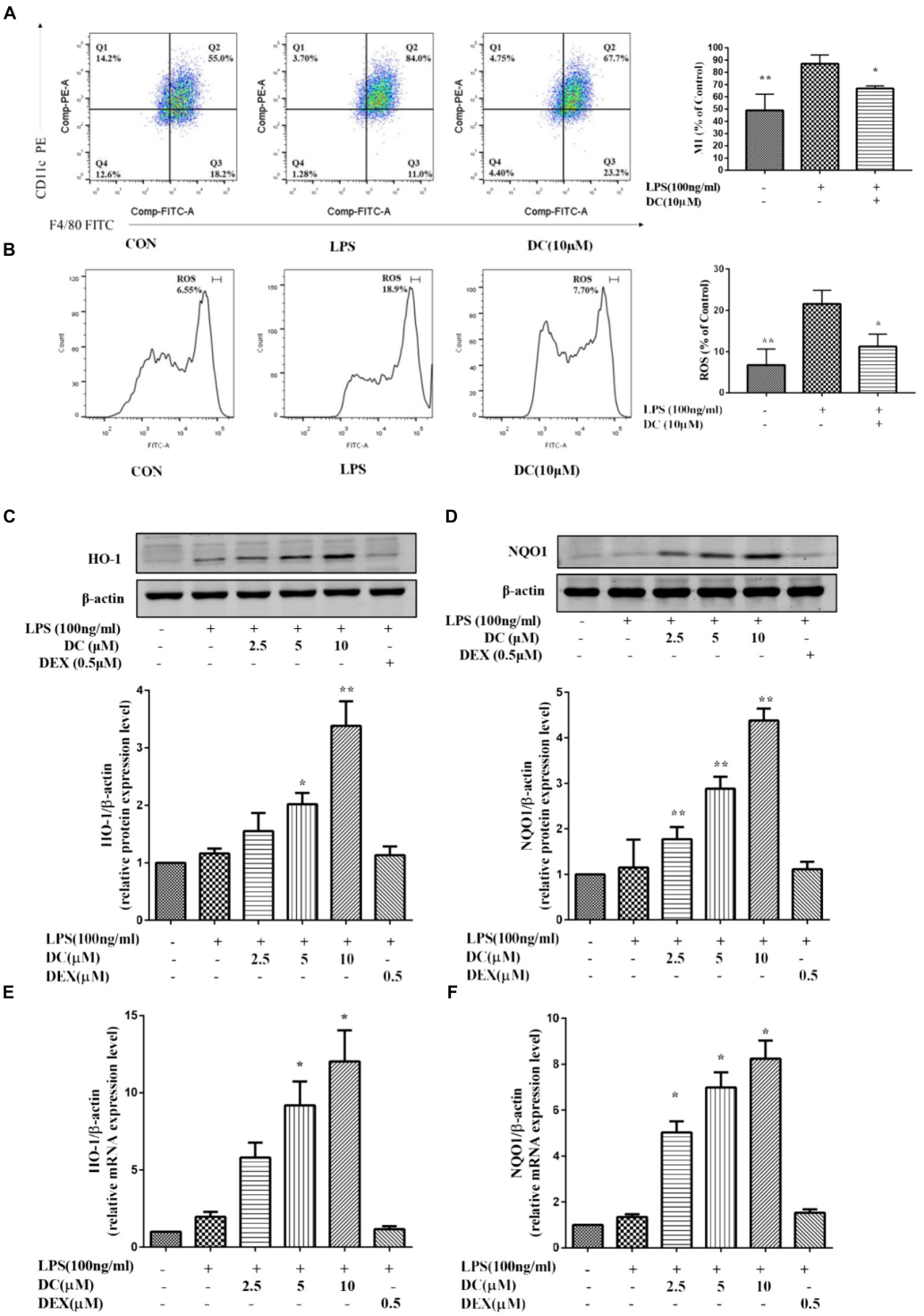

FIGURE 5 | The effect of DC on the expressions of M1, ROS, HO-1, and NQO1 in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with indicated concentrations of DC for 1 h, then cells were incubated for 6 h in the absence of LPS, then collected all the cells and cellular staining for F/480 and CD11c, the M1 markers **(A)** according to the manufacturer's directions. The intracellular level of ROS **(B)** was determined using the fluorescent probe DCFH-DA. The cells were plated in 24-well plates and incubated for 24 h, next the cells were pretreated with indicated concentrations of DC for 1 h and stimulated with LPS for 18 h. The total proteins of the cells were prepared and the expression of HO-1 **(C)** and NQO1 **(D)** were analyzed by Western blot. The mRNA expressions of HO-1 **(E)** and NQO1 **(F)** were analyzed by real time PCR. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). *$p < 0.05$, **$p < 0.01$, vs. LPS-stimulated cells.

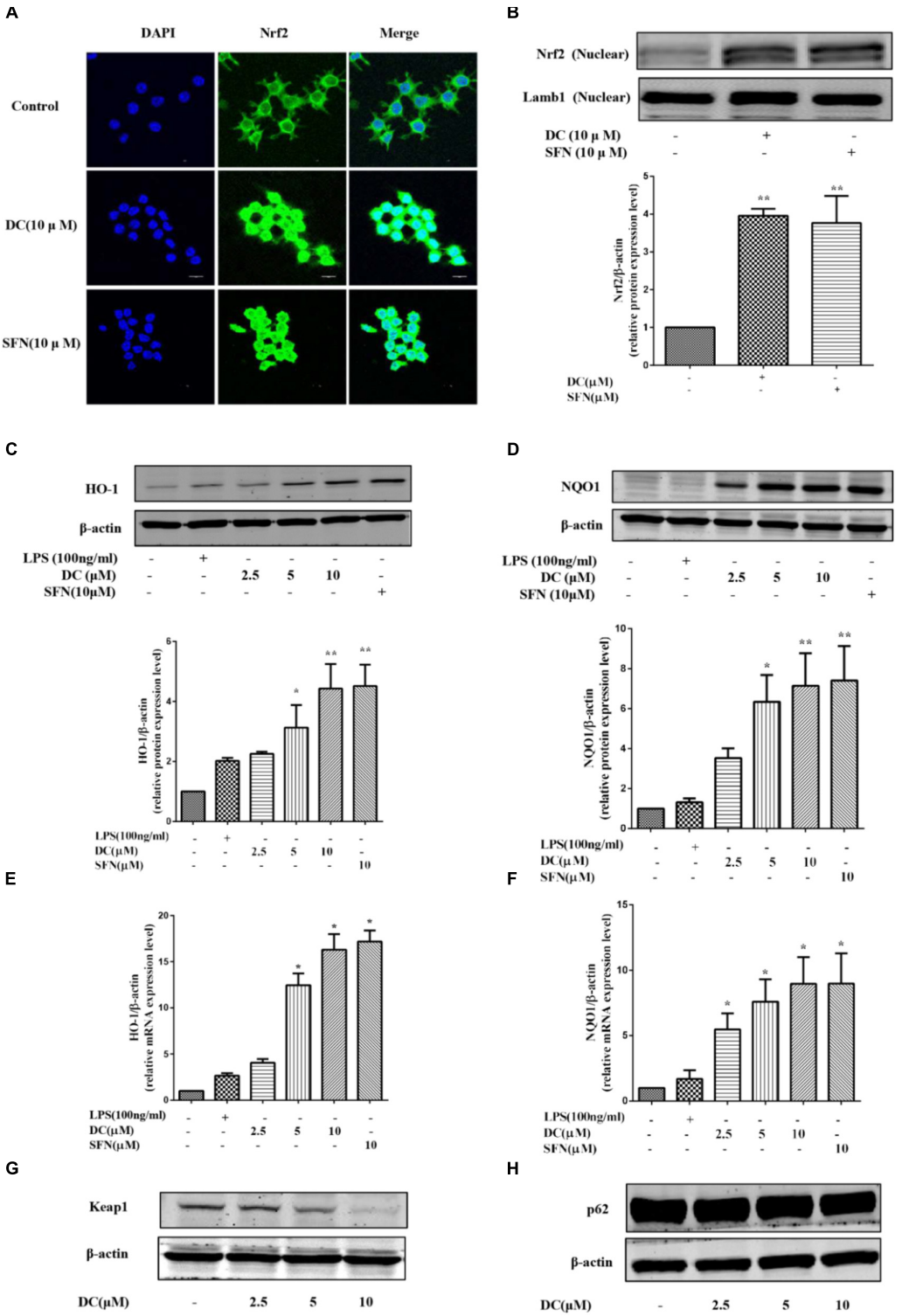

FIGURE 6 | The effect of DC on the nuclear translocation of Nrf2 and the expressions of HO-1, NQO1, Keap1, and p62 in LPS-unstimulated RAW264.7 cells. The cells were treated with SFN or DC (10 μM) for 6 h. The subcellular localization of Nrf2 was detected with immunofluorescence assay **(A)** and the images were acquired using the Leica DM2500 fluorescent microscopy. The nuclear protein of the cells was prepared and the expression of Nrf2 in nuclear protein **(B)** of the cells were analyzed by Western blot. The cells were plated in 24-well plates and incubated for 24 h, next the cells were pretreated with indicated concentrations of DC for 1 h and stimulated with or without LPS for 18 h. The total proteins of the cells were prepared and the expressions of HO-1 **(C)**, NQO1 **(D)**, Keap1 **(G)**, and p62 **(H)** were analyzed by Western blot. The mRNA levels of HO-1 **(E)** and NQO1 **(F)** were analyzed by real time PCR. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). *$p < 0.05$, **$p < 0.01$, vs. LPS-unstimulated cells.

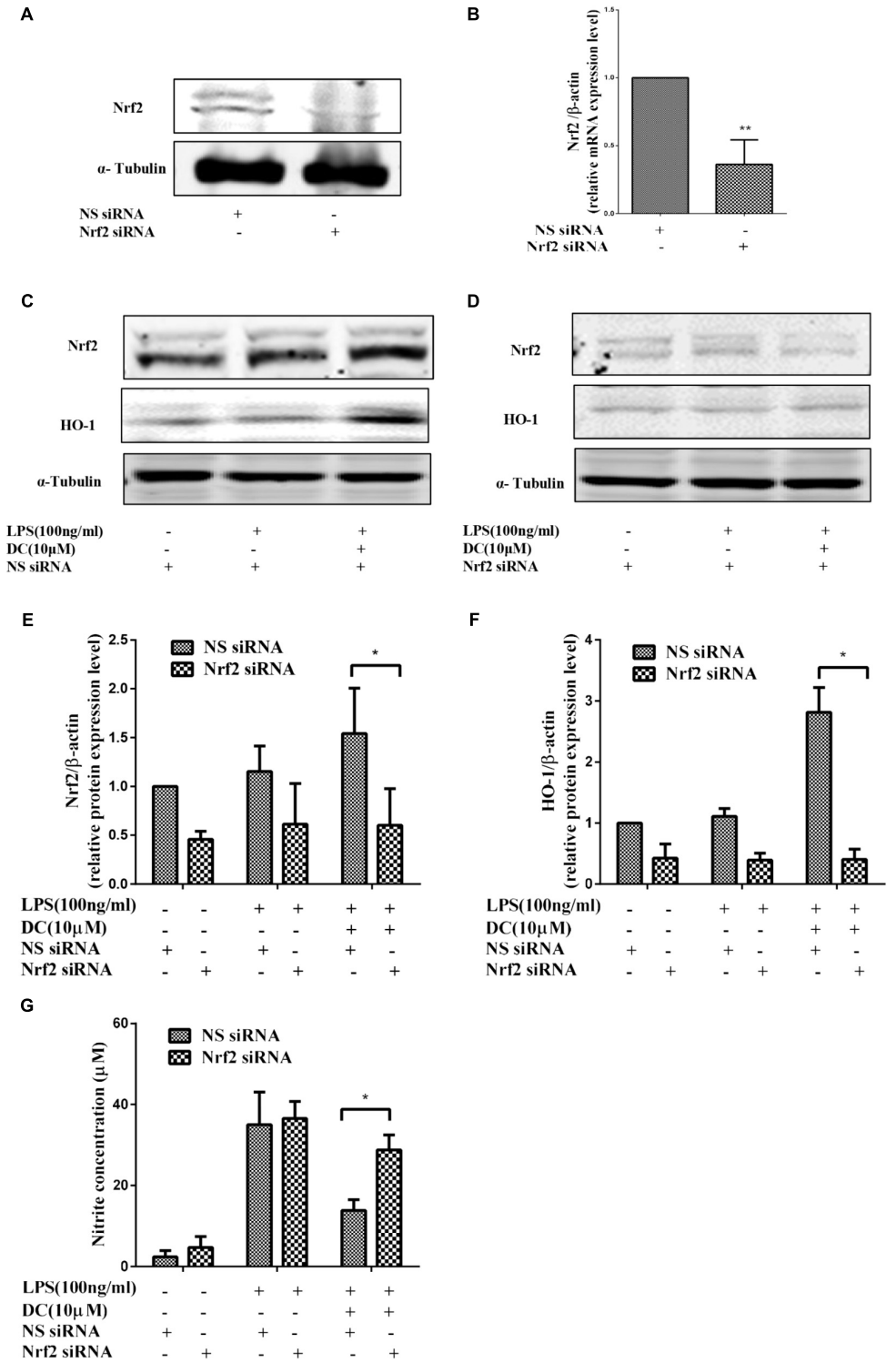

FIGURE 7 | The effect of Nrf2 siRNA and HO-1 inhibitor on the anti-inflammatory effect of DC. For transfection, the cells were seeded in 24-well culture plates and incubated with the control siRNA or Nrf2 siRNA at 300 nM for 24–48 h in serum-free OPTI-MEM media. The total proteins of Nrf2 **(A)** were analyzed by Western blot. The mRNA level of Nrf2 **(B)** were analyzed by real time PCR. Statistical analysis was carried out by using unpaired *t*-test in GraphPad Prism7 ($P < 0.05$, unpaired *t*-test). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. NS siRNA treated cells **(B)**. The transfected cells were also pretreated with indicated concentration of DC for 1 h and stimulated with or without LPS for 18 h. The total proteins of the cells were prepared and the expressions of Nrf2 **(C,E)** and HO-1 **(D,F)** were analyzed by Western blot. The concentration of NO **(G)** in the culture medium were quantified. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. DC, LPS, and NS siRNA treated cells **(E–G)**.

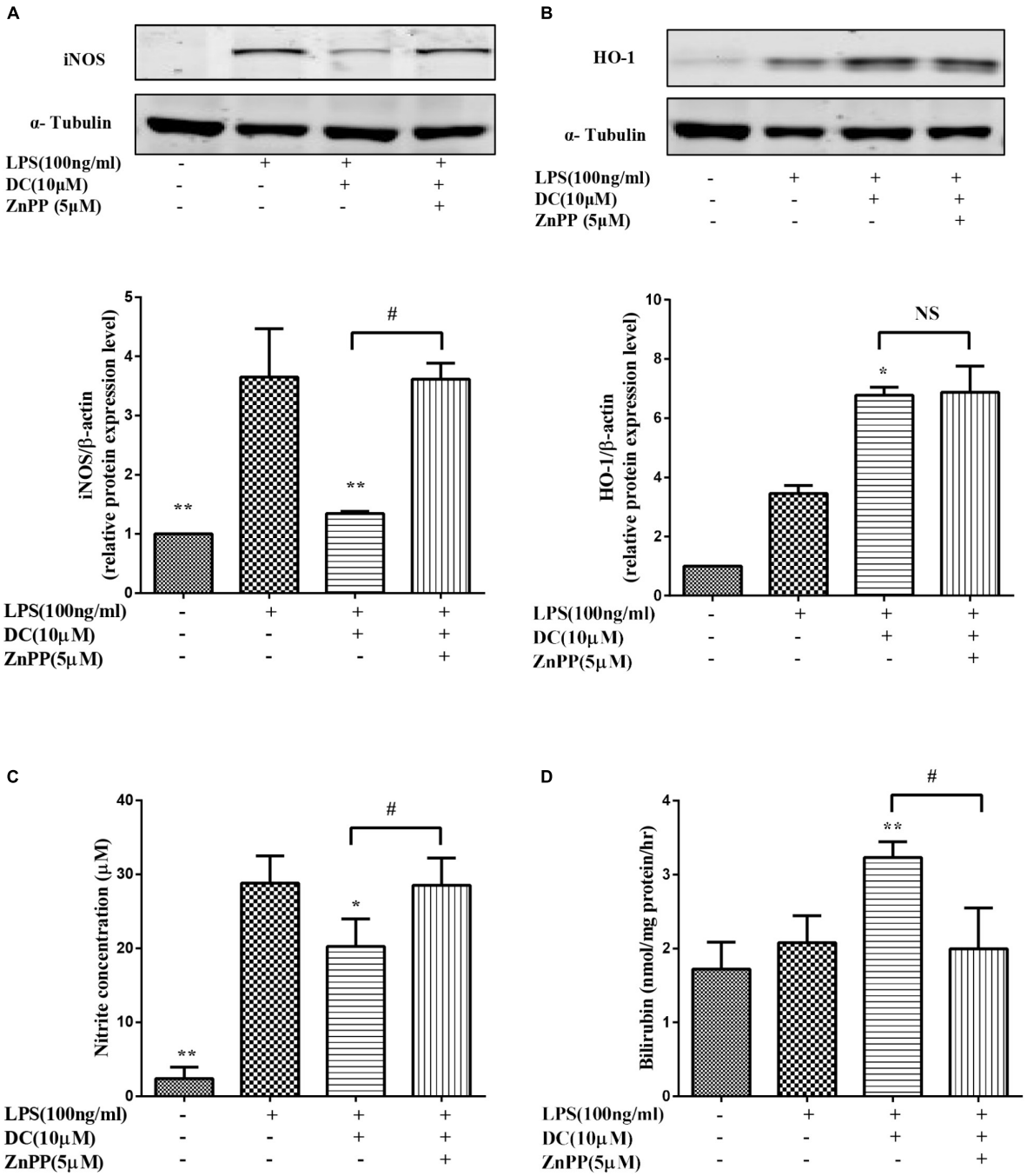

FIGURE 8 | The effect of HO-1 inhibitor on the anti-inflammatory effect of DC. The cells were pretreated with indicated concentration of ZnPP for 1 h, and then stimulated with DC for 1 h, at last, treated with LPS for 18 h. The total proteins of iNOS **(A)**, and HO-1 **(B)** were analyzed by Western blot. The concentration of NO **(C)** in the culture medium were quantified. The levels of Bilirubin **(D)** in cell microsome fraction were detected. Statistical analysis was carried out by using one-way ANOVA with Tukey's multiple comparison tests in GraphPad Prism7 ($P < 0.05$, ANOVA). Results are expressed as mean ± SEM of three independent experiments ($N = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. LPS treated cells, $^{\#}p < 0.05$ vs. ZnPP treated cells.

the paper reported that LPS could not up-regulated iNOS gene in THP-1 macrophages (Chanput et al., 2010). There is no doubt that iNOS is an important inflammatory index, it is expressed in response to a variety of inflammatory stimuli and generates NO in macrophage during the inflammatory process (Laskin and Pendino, 1995), the inhibition of iNOS was able to reduce the production of ROS (Egea et al., 2012). As described earlier, ROS play an important role in oxidative stress and inflammatory responses. LPS-stimulated THP-1 macrophages without the high expressed iNOS gene, so in the next study, we selected the

commonly used inflammatory model, LPS-stimulated RAW264.7 macrophages which expresses high levels of iNOS and ROS for in-depth study on the mechanisms underlying the anti-inflammatory effect of DC.

There were reports showing that the inhibition of LPS-induced NO, PGE_2, iNOS, COX-2, TNF-α, and IL-6 through the inactivation of NF-κB and MAPK pathway in RAW264.7 cells (Kim et al., 2007; Liu et al., 2016). Other previous studies have introduced that the extracts of *N. chinensis* inhibited the production of inflammatory mediators through the inhibition of p38 MAPK pathway instead of the inhibition of NF-κB pathway. In order to study the anti-inflammatory mechanism of DC, the effects of DC on the activation of NF-κB and MAPK pathways in LPS-stimulated RAW264.7 cells were investigated, but the results suggested that DC didn't inhibit the activation of NF-κB and MAPK pathways (**Figures 4A–C**), so DC may not act on NF-κB and MAPK pathways to exert its anti-inflammatory effects. This is common to other terpenoid- derived compounds with known anti-inflammatory effects (Ferrante et al., 2017; Locatelli et al., 2017).

It has been reported that the activation of Nrf2 anti-oxidant pathway prevents LPS-induced transcriptional upregulation of pro-inflammatory cytokines, including IL-6. Since the current results showed that DC inhibited IL-6 obviously (**Figure 2F**), so it was hypothesized that DC may activate Nrf2 pathway to exert its anti-inflammatory effect. As described before, Nrf2 pathway mediated antioxidant gene expression reduced the M1 phenotype and ROS production (Kobayashi et al., 2016). In order to verify the hypothesis of this study, the M1 phenotype and ROS production in LPS-stimulated RAW264.7 cells were examined, the results showed that the elevated M1 phenotype and ROS production induced by LPS were significantly inhibited by DC (**Figures 5A,B**), suggesting that DC is likely to exert anti-inflammatory and anti-oxidant effects by activating the Nrf2 anti-oxidant pathway.

The Nrf2-dependent anti-oxidant genes HO-1 and NQO-1 could block TNF-α and IL-6 inflammatory mediators. In Nrf2-knockout mice, the anti-inflammatory effect disappeared (Thimmulappa et al., 2006). Nrf2-knockout mice showed increased mRNA and protein levels of COX-2, iNOS, IL-6, and TNF-α (Rojo et al., 2010). Furthermore, the activation of Nrf2 leads to its nuclear translocation, resulting in the decrease of COX-2 and iNOS (Ho et al., 2007). In this study, it was found that DC inhibited the expressions of inflammatory proteins (COX-2 and iNOS, **Figures 2A,B**) and inflammatory cytokines (TNF-α and IL-6, **Figures 2E–H**) accompanied by the increase of antioxidant proteins HO-1 and NQO1 (**Figures 5C–F**). All of these results further indicated that DC may activate Nrf2 pathway to exert its anti-inflammatory effects.

In oxidative stress and inflammation condition, enhancement of HO-1 expression plays an important role in cell protection (Ryter et al., 2006; Chung et al., 2008). HO-1 can be rapidly induced by various oxidative-inducing agents, also including LPS (Chung et al., 2008). The current results also showed that LPS increased the level of HO-1 slightly, but compared with LPS group, DC further increased the level of HO-1 protein dramatically (**Figures 5C,E**). The high expression of HO-1 can inhibit LPS-induced NO production (Lin et al., 2003). In this study, DC reduced NO production while increasing HO-1 expression (**Figures 1D**, **5C,E**), the current results are consistent with that the high expression of HO-1 can inhibit LPS-induced NO production (Lin et al., 2003).

Sulforaphane is an known and potent Nrf2 activator and capable of preventing toxicity of organic chemicals (Fahey et al., 2002) and has antioxidant protection effects (Gao et al., 2001). In the current study, DEX had strong anti-inflammatory activity, but didn't show any effect on the activation of Nrf2 pathway, implying that DEX produced its anti-inflammatory effect through a Nrf2 independent way. Therefore, SFN was chosen as a positive drug to evaluate the antioxidant activity and underlying mechanisms of DC related to Nrf2 pathway. The current research showed that both DC and SFN increased the levels of HO-1 and NQO1 in LPS-unstimulated RAW264.7 cell (**Figures 6C–F**). Since DC increased the downstream antioxidant proteins (HO-1 and NQO1) of Nrf2 pathway, it was indicated that DC could also activate Nrf2 and make Nrf2 protein entering into the nucleus. In this research, we did find that DC increased the nuclear translocation of Nrf2 (**Figures 6A,B**), further supported that DC was able to activate Nrf2 pathway to promote the increase of antioxidant protein to reduce ROS production.

Keap1 is a negative regulator of the transcription factor Nrf2, which mediated the inactivation of Nrf2 and thus enhanced Nrf2 translocation into the nucleus (Satoh et al., 2006; Lv et al., 2018). It has been reported that protein p62, which is on the upstream of Nrf2 pathway, can inactivate Keap1 and activate Nrf2 (Komatsu et al., 2010). The current research found that DC inhibited the expression of Keap1 (**Figure 6G**) but didn't increase p62 expression (**Figure 6H**), suggesting that DC induced Nrf2 activation probably through inhibiting Keap1 expression instead of upregulating p62 protein.

To further confirm the pivotal role of Nrf2 pathway in the anti-inflammatory effect of DC, Nrf2 siRNA was employed to down regulate the protein and gene expressions. The results showed that the mRNA and protein level of Nrf2 were significantly suppressed by using specific Nrf2 siRNA (**Figures 7A,B**). The protein expressions of Nrf2 and HO-1 were decreased by Nrf2 siRNA (**Figures 7C–F**). The suppressive effect of DC on NO production induced by LPS was also abolished by Nrf2 siRNA (**Figure 7G**), these results suggested the anti-inflammatory effect of DC is mediated by the activation of Nrf2/HO-1 antioxidant pathway.

It has been reported in the literature (Srisook et al., 2006) that increasing the activity of HO-1 can reduce the production of iNOS and NO. Bilirubin, the metabolic products of HO-1, also exerts anti-inflammatory effect (Araujo et al., 2012). In order to further confirm the important role of HO-1 activity in mediating the anti-inflammatory effect of DC. ZnPP, a HO-1 activity inhibitor (Freitas et al., 2006), was used in the research.

In this study, we found that DC increased the expression (**Figure 8B**) and the activity of HO-1 protein (**Figure 8D**). At the same time, DC decreased the expression of iNOS (**Figure 8A**) and the release of NO (**Figure 8C**). ZnPP significantly reversed the effect of DC on HO-1 protein activity (**Figure 8D**) rather than HO-1 expression (**Figure 8B**) to block the effect of DC

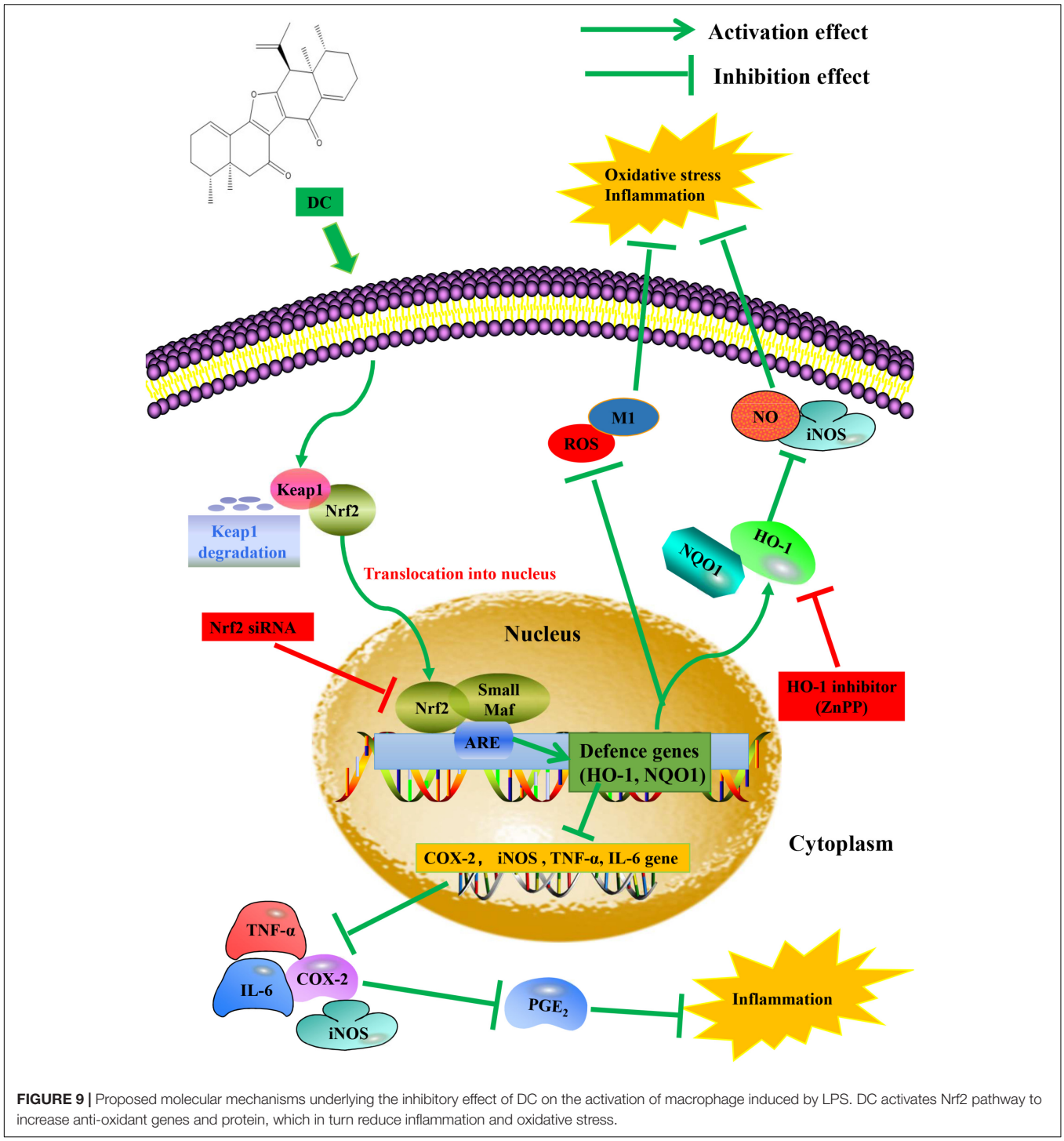

FIGURE 9 | Proposed molecular mechanisms underlying the inhibitory effect of DC on the activation of macrophage induced by LPS. DC activates Nrf2 pathway to increase anti-oxidant genes and protein, which in turn reduce inflammation and oxidative stress.

on the expression of iNOS (**Figure 8A**) and the release of NO (**Figure 8C**), these results further confirmed that the anti-inflammatory effect of DC was mediated by the increase of HO-1 activity. In short, DC activates Nrf2/HO-1 pathway to increase anti-oxidant proteins, which in turn reduce inflammation and oxidative stress to contribute to its anti-inflammatory and antioxidant effects (**Figure 9**).

Nrf2 activator can be used as potential therapies for numerous disorders (Crunkhorn, 2012), but there are very few Nrf2 activators applicable currently in clinics. In addition, the current available Nrf2 activator, dimethyl fumarate, has showed drug resistance and side effects (Deeks, 2014). Therefore, there is significant need to develop new and safer Nrf2 activator for clinical use. In this research, DC exerted anti-inflammatory

effects mainly through the activation of Nrf2 pathway. This implies that DC may be a new Nrf2 activator and has the potential to be developed as new drug. Nrf2 pathway plays an important role in oxidative stress response and metabolism, Nrf2 activation can significantly inhibit the onset of diabetes and prevent diabetes (Uruno et al., 2013). Since DC also activates Nrf2 pathway in the absence of LPS, it indicated that DC could not only treat inflammation but also prevent many other diseases related to oxidative stress response and metabolism, giving broader application situations to this compound.

CONCLUSION

Taken together, we discovered a novel Nrf2 activator, DC, which acts on Nrf2/HO-1 pathway to exert its anti-inflammatory effects against LPS-induced inflammation. Besides, the unique anti-inflammatory mechanism of DC may provide a new therapeutic window for the prevention and treatment of oxidative damage and inflammation-related diseases. Although other inflammation-related cell experiments, animal models and clinical trials are certainly needed to do in future depth research, this study now still could help providing a potential treatment mechanism of *N. chinensis* and also provide a novel natural compound with new skeleton for the treatment of the diseases related to inflammation and oxidative stress.

AUTHOR CONTRIBUTIONS

J-FL performed the experiments, analyzed the data, and wrote the paper. X-YS and YD provided the compound. CKL performed the experiments. C-SC, J-XL, and Y-DY analyzed the data. YY, YX, PL, and X-SY critically read the manuscript. Z-QL and HZ conceived the research idea and revise the manuscript. All authors contributed to final approval of the article.

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REFERENCES

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., et al. (2000). Inflammation and Alzheimer's disease. *Neurobiol. Aging* 21, 383–421. doi: 10.1016/S0197-4580(00)00124-X

Araujo, J. A., Zhang, M., and Yin, F. (2012). Heme oxygenase-1, oxidation, inflammation, and atherosclerosis. *Front. Pharmacol.* 3:119. doi: 10.3389/fphar.2012.00119

Arora, R. (1965). *Nardostachys jatamansi–a Chemical, Pharmacological and Clinical Appraisal, Monograph Special Series.* New Delhi: Indian Council of Medical Research, 51, 1–117.

Bagchi, A., Oshima, Y., and Hikino, H. (1991). Neolignans and lignans of *Nardostachys jatamansi* roots1. *Planta Med.* 57, 96–97. doi: 10.1055/s-2006-960037

Balkwill, F. R., and Mantovani, A. (2012). Cancer-related inflammation: common themes and therapeutic opportunities. *Semin. Cancer Biol.* 22, 33–40. doi: 10.1016/j.semcancer.2011.12.005

Buchman, A. L. (2001). Side effects of corticosteroid therapy. *J. Clin. Gastroenterol.* 33, 289–294. doi: 10.1097/00004836-200110000-00006

Catino, S., Paciello, F., Miceli, F., Rolesi, R., Troiani, D., Calabrese, V., et al. (2016). Ferulic acid regulates the Nrf2/heme oxygenase-1 system and counteracts trimethyltin-induced neuronal damage in the human neuroblastoma cell line SH-SY5Y. *Front. Pharmacol.* 6:305. doi: 10.3389/fphar.2015.00305

Chanput, W., Mes, J., Vreeburg, R. A., Savelkoul, H. F., and Wichers, H. J. (2010). Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. *Food Funct.* 1, 254–261. doi: 10.1039/c0fo00113a

Chanput, W., Mes, J. J., and Wichers, H. J. (2014). THP-1 cell line: an in vitro cell model for immune modulation approach. *Int. Immunopharmacol.* 23, 37–45. doi: 10.1016/j.intimp.2014.08.002

Chartoumpekis, D. V., and Kensler, T. W. (2013). New player on an old field; the keap1/Nrf2 pathway as a target for treatment of type 2 diabetes and metabolic syndrome. *Curr. Diabetes Rev.* 9, 137–145. doi: 10.2174/1573399811309020005

Chawla, A., Nguyen, K. D., and Goh, Y. S. (2011). Macrophage-mediated inflammation in metabolic disease. *Nat. Rev. Immunol.* 11, 738–749. doi: 10.1038/nri3071

Chung, S. W., Liu, X., Macias, A. A., Baron, R. M., and Perrella, M. A. (2008). Heme oxygenase-1–derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J. Clin. Invest.* 118, 239–247. doi: 10.1172/JCI32730

Coussens, L. M., and Werb, Z. (2002). Inflammation and cancer. *Nature* 420, 860–867. doi: 10.1038/nature01322

Crunkhorn, S. (2012). Deal watch: abbott boosts investment in NRF2 activators for reducing oxidative stress. *Nat. Rev. Drug Discov.* 11, 96–96. doi: 10.1038/nrd3655

Deeks, E. D. (2014). Nivolumab: a review of its use in patients with malignant melanoma. *Drugs* 74, 1233–1239. doi: 10.1007/s40265-014-0234-4

DiDonato, J. A., Mercurio, F., and Karin, M. (2012). NF-kappa B and the link between inflammation and cancer. *Immunol. Rev.* 246, 379–400. doi: 10.1111/j.1600-065X.2012.01099.x

Egea, J., Martin-de-Saavedra, M., Parada, E., Romero, A., Del Barrio, L., Rosa, A., et al. (2012). Galantamine elicits neuroprotection by inhibiting iNOS, NADPH oxidase and ROS in hippocampal slices stressed with anoxia/reoxygenation. *Neuropharmacology* 62, 1082–1090. doi: 10.1016/j.neuropharm.2011.10.022

Fahey, J. W., Haristoy, X., Dolan, P. M., Kensler, T. W., Scholtus, I., Stephenson, K. K., et al. (2002). Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo [a] pyrene-induced stomach tumors. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7610–7615. doi: 10.1073/pnas.112203099

Ferrante, C., Recinella, L., Locatelli, M., Guglielmi, P., Secci, D., Leporini, L., et al. (2017). Protective effects induced by microwave-assisted aqueous *Harpagophytum* extract on rat cortex synaptosomes challenged with amyloid β-peptide. *Phytother. Res.* 31, 1257–1264. doi: 10.1002/ptr.5850

Freitas, A., Alves-Filho, J., Secco, D., Neto, A., Ferreira, S., Barja-Fidalgo, C., et al. (2006). Heme oxygenase/carbon monoxide-biliverdin pathway down regulates neutrophil rolling, adhesion and migration in acute inflammation. *Br. J. Pharmacol.* 149, 345–354. doi: 10.1038/sj.bjp.0706882

Fujiwara, N., and Kobayashi, K. (2005). Macrophages in inflammation. *Curr. Drug Targets Inflamm. Allergy* 4, 281–286. doi: 10.2174/1568010054022024

Gao, X., Dinkova-Kostova, A. T., and Talalay, P. (2001). Powerful and prolonged protection of human retinal pigment epithelial cells, keratinocytes, and mouse leukemia cells against oxidative damage: the indirect antioxidant effects of sulforaphane. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15221–15226. doi: 10.1073/pnas.261572998

Ghosh, S., and Hayden, M. S. (2008). New regulators of NF-κB in inflammation. *Nat. Rev. Immunol.* 8, 837–848. doi: 10.1038/nri2423

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-kappa B and rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16, 225–260. doi: 10.1146/annurev.immunol.16.1.225

Gold, R., Kappos, L., Arnold, D. L., Bar-Or, A., Giovannoni, G., Selmaj, K., et al. (2012). Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *N. Engl. J. Med.* 367, 1098–1107. doi: 10.1056/NEJMoa1114287

Ho, F.-M., Kang, H.-C., Lee, S.-T., Chao, Y., Chen, Y.-C., Huang, L.-J., et al. (2007). The anti-inflammatory actions of LCY-2-CHO, a carbazole analogue, in vascular smooth muscle cells. *Biochem. Pharmacol.* 74, 298–308. doi: 10.1016/j.bcp.2007.04.008

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature* 444, 860–867. doi: 10.1038/nature05485

Hu, Y.-R., Ma, H., Zou, Z.-Y., He, K., Xiao, Y.-B., Wang, Y., et al. (2017). Activation of Akt and JNK/Nrf2/NQO1 pathway contributes to the protective effect of coptisine against AAPH-induced oxidative stress. *Biomed. Pharmacother.* 85, 313–322. doi: 10.1016/j.biopha.2016.11.031

Hwang, J. S., Lee, S. A., Hong, S. S., Han, X. H., Lee, C., Lee, D., et al. (2012). Inhibitory constituents of *Nardostachys chinensis* on nitric oxide production in RAW 264.7 macrophages. *Bioorg. Med. Chem. Lett.* 22, 706–708. doi: 10.1016/j.bmcl.2011.10.043

Jaguin, M., Houlbert, N., Fardel, O., and Lecureur, V. (2013). Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell Immunol.* 281, 51–61. doi: 10.1016/j.cellimm.2013.01.010

Joshi, G., and Johnson, J. A. (2012). The Nrf2-ARE pathway: a valuable therapeutic target for the treatment of neurodegenerative diseases. *Recent Pat. CNS Drug Discov.* 7, 218–229. doi: 10.2174/157488912803252023

Kaminska, B. (2005). MAPK signalling pathways as molecular targets for anti-inflammatory therapy - from molecular mechanisms to therapeutic benefits. *Biochim. Biophys. Acta Proteins Proteomics* 1754, 253–262. doi: 10.1016/j.bbapap.2005.08.017

Kang, Y.-J., Wingerd, B. A., Arakawa, T., and Smith, W. L. (2006). Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. *J. Immunol.* 177, 8111–8122. doi: 10.4049/jimmunol.177.11.8111

Kim, J., Cha, Y.-N., and Surh, Y.-J. (2010). A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat. Res.* 690, 12–23. doi: 10.1016/j.mrfmmm.2009.09.007

Kim, J.-B., Han, A.-R., Park, E.-Y., Kim, J.-Y., Cho, W., Lee, J., et al. (2007). Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-κB inactivation in RAW 264.7 macrophage cells. *Biol. Pharm. Bull.* 30, 2345–2351. doi: 10.1248/bpb.30.2345

Kim, K. M., Pae, H.-O., Zhung, M., Ha, H.-Y., Ha, Y. A., Chai, K.-Y., et al. (2008). Involvement of anti-inflammatory heme oxygenase-1 in the inhibitory effect of curcumin on the expression of pro-inflammatory inducible nitric oxide synthase in RAW264. 7 macrophages. *Biomed. Pharmacother.* 62, 630–636. doi: 10.1016/j.biopha.2008.01.008

Kim, M.-J., Kim, S.-J., Kim, S. S., Lee, N. H., and Hyun, C.-G. (2014). *Hypochaeris radicata* attenuates LPS-induced inflammation by suppressing p38, ERK, and JNK phosphorylation in RAW 264.7 macrophages. *EXCLI J.* 13, 123–136.

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., et al. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat. Commun.* 7:11624. doi: 10.1038/ncomms11624

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., et al. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of keap1. *Nat. Cell Biol.* 12, 213–223. doi: 10.1038/ncb2021

Laskin, D. L., and Pendino, K. J. (1995). Macrophages and inflammatory mediators in tissue injury. *Annu. Rev. Pharmacol. Toxicol.* 35, 655–677. doi: 10.1146/annurev.pa.35.040195.003255

Li, J., Ichikawa, T., Janicki, J. S., and Cui, T. (2009). Targeting the Nrf2 pathway against cardiovascular disease. *Expert Opin. Ther. Targets* 13, 785–794. doi: 10.1517/14728220903025762

Li, W., Khor, T. O., Xu, C., Shen, G., Jeong, W.-S., Yu, S., et al. (2008). Activation of Nrf2-antioxidant signaling attenuates NFκB-inflammatory response and elicits apoptosis. *Biochem. Pharmacol.* 76, 1485–1489. doi: 10.1016/j.bcp.2008.07.017

Lin, H.-Y., Juan, S.-H., Shen, S.-C., Hsu, F.-L., and Chen, Y.-C. (2003). Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264. 7 macrophages involves heme oxygenase-1. *Biochem. Pharmacol.* 66, 1821–1832. doi: 10.1016/S0006-2952(03)00422-2

Linde, A., Mosier, D., Blecha, F., and Melgarejo, T. (2007). Innate immunity and inflammation - New frontiers in comparative cardiovascular pathology. *Cardiovasc. Res.* 73, 26–36. doi: 10.1016/j.cardiores.2006.08.009

Linton, M. F., and Fazio, S. (2003). Macrophages, inflammation, and atherosclerosis. *Int. J. Obes. Relat. Metab. Disord.* 27(Suppl. 3), S35–S40. doi: 10.1038/sj.ijo.0802498

Liu, J., Tang, J., Zuo, Y., Yu, Y., Luo, P., Yao, X., et al. (2016). Stauntoside B inhibits macrophage activation by inhibiting NF-κB and ERK MAPK signalling. *Pharmacol. Res.* 111, 303–315. doi: 10.1016/j.phrs.2016.06.022

Liu, Y. S., Shepherd, E. G., and Nelin, L. D. (2007). MAPK phosphatases - regulating the immune response. *Nat. Rev. Immunol.* 7, 202–212. doi: 10.1038/nri2035

Locatelli, M., Ferrante, C., Carradori, S., Secci, D., Leporini, L., Chiavaroli, A., et al. (2017). Optimization of aqueous extraction and biological activity of *Harpagophytum procumbens* root on ex vivo rat colon inflammatory model. *Phytother. Res.* 31, 937–944. doi: 10.1002/ptr.5821

Lv, H., Xiao, Q., Zhou, J., Feng, H., Liu, G., and Ci, X. (2018). Licochalcone A upregulates Nrf2 antioxidant pathway and thereby alleviates acetaminophen-induced Hepatotoxicity. *Front. Pharmacol.* 9:147. doi: 10.3389/fphar.2018.00147

McInnes, I. B., and Schett, G. (2011). The pathogenesis of rheumatoid arthritis. *N. Engl. J. Med.* 365, 2205–2219. doi: 10.1056/NEJMra1004965

Noguchi, S., Nakatsuka, M., Konishi, H., Kamada, Y., Chekir, C., and Kudo, T. (2003). Nafamostat mesilate suppresses NF-κB activation and no overproduction in LPS-treated macrophages. *Int. Immunopharmacol.* 3, 1335–1344. doi: 10.1016/S1567-5769(03)00146-2

Pae, H.-O., and Chung, H.-T. (2009). Heme oxygenase-1: its therapeutic roles in inflammatory diseases. *Immune Netw.* 9, 12–19. doi: 10.4110/in.2009.9.1.12

Pakrashi, C. A., and Chatarji, A. (1994). *The Treatise on Indian Medicinal Plants*, Vol. 3, New Delhi: Publication and Information Directorate.

Rao, K. M. K. (2001). MAP kinase activation in macrophages. *J. Leukoc. Biol.* 69, 3–10.

Rojo, A. I., Innamorato, N. G., Martín-Moreno, A. M., De Ceballos, M. L., Yamamoto, M., and Cuadrado, A. (2010). Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. *Glia* 58, 588–598. doi: 10.1002/glia.20947

Ruiz, S., Pergola, P. E., Zager, R. A., and Vaziri, N. D. (2013). Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. *Kidney Int.* 83, 1029–1041. doi: 10.1038/ki.2012.439

Ryter, S. W., Alam, J., and Choi, A. M. (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol. Rev.* 86, 583–650. doi: 10.1152/physrev.00011.2005

Sahu, B. D., Kalvala, A. K., Koneru, M., Kumar, J. M., Kuncha, M., Rachamalla, S. S., et al. (2014). Ameliorative effect of fisetin on cisplatin-induced nephrotoxicity in rats via modulation of NF-κB activation and antioxidant defence. *PLoS One* 9:e105070. doi: 10.1371/journal.pone.0105070

Sampey, A. V., Monrad, S., and Crofford, L. J. (2005). Microsomal prostaglandin E synthase-1: the inducible synthase for prostaglandin E 2. *Arthritis Res. Ther.* 7, 114–117. doi: 10.1186/ar1748

Satoh, T., Okamoto, S.-I., Cui, J., Watanabe, Y., Furuta, K., Suzuki, M., et al. (2006). Activation of the Keap1/Nrf2 pathway for neuroprotection by electrophillic phase II inducers. *Proc. Natl. Acad. Sci. U.S.A.* 103, 768–773. doi: 10.1073/pnas.0505723102

Shen, T., Chen, X.-M., Harder, B., Long, M., Wang, X.-N., Lou, H.-X., et al. (2014). Plant extracts of the family Lauraceae: a potential resource for chemopreventive agents that activate the nuclear factor-erythroid 2-related factor 2/antioxidant response element pathway. *Planta Med.* 80, 426–434. doi: 10.1055/s-0034-1368197

Shin, E. M., Zhou, H. Y., Guo, L. Y., Kim, J. A., Lee, S. H., Merfort, I., et al. (2008). Anti-inflammatory effects of glycyrol isolated from *Glycyrrhiza uralensis* in LPS-stimulated RAW264. 7 macrophages. *Int. Immunopharmacol.* 8, 1524–1532. doi: 10.1016/j.intimp.2008.06.008

Shin, J. Y., Bae, G.-S., Choi, S.-B., Jo, I.-J., Kim, D.-G., Lee, D.-S., et al. (2015). Anti-inflammatory effect of desoxo-narchinol-A isolated from *Nardostachys jatamansi* against lipopolysaccharide. *Int. Immunopharmacol.* 29, 730–738. doi: 10.1016/j.intimp.2015.09.002

Sporn, M. B., and Liby, K. T. (2012). NRF2 and cancer: the good, the bad and the importance of context. *Nat. Rev. Cancer* 12, 564–571. doi: 10.1038/nrc3278

Srisook, K., and Cha, Y.-N. (2004). Biphasic induction of heme oxygenase-1 expression in macrophages stimulated with lipopolysaccharide. *Biochem. Pharmacol.* 68, 1709–1720. doi: 10.1016/j.bcp.2004.07.001

Srisook, K., Han, S.-S., Choi, H.-S., Li, M.-H., Ueda, H., Kim, C., et al. (2006). CO from enhanced HO activity or from CORM-2 inhibits both O2- and NO production and downregulates HO-1 expression in LPS-stimulated macrophages. *Biochem. Pharmacol.* 71, 307–318. doi: 10.1016/j.bcp.2005.10.042

Sun, L. D., Wang, F., Dai, F., Wang, Y. H., Lin, D., and Zhou, B. (2015). Development and mechanism investigation of a new piperlongumine derivative as a potent anti-inflammatory agent. *Biochem. Pharmacol.* 95, 156–169. doi: 10.1016/j.bcp.2015.03.014

Surh, Y. J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* 3, 768–780. doi: 10.1038/nrc1189

Szekanecz, Z., and Koch, A. E. (2007). Macrophages and their products in rheumatoid arthritis. *Curr. Opin. Rheumatol.* 19, 289–295. doi: 10.1097/BOR.0b013e32805e87ae

Thimmulappa, R. K., Scollick, C., Traore, K., Yates, M., Trush, M. A., Liby, K. T., et al. (2006). Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolide. *Biochem. Biophys. Res. Commun.* 351, 883–889. doi: 10.1016/j.bbrc.2006.10.102

Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., et al. (2013). The Keap1-Nrf2 system prevents onset of diabetes mellitus. *Mol. Cell. Biol.* 33, 2996–3010. doi: 10.1128/MCB.00225-13

Waxman, K. (1996). Shock: ischemia, reperfusion, and inflammation. *New Horiz.* 4, 153–160.

Williams, C. S., Mann, M., and DuBois, R. N. (1999). The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* 18, 7908–7916. doi: 10.1038/sj.onc.1203286

Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., et al. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* 112, 1821–1830. doi: 10.1172/JCI200319451

Yang, H. L., Lin, S. W., Lee, C. C., Lin, K. Y., Liao, C. H., Yang, T. Y., et al. (2015). Induction of Nrf2-mediated genes by *Antrodia salmonea* inhibits ROS generation and inflammatory effects in lipopolysaccharide-stimulated RAW264.7 macrophages. *Food Funct.* 6, 230–241. doi: 10.1039/c4fo00869c

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frontiers in Pharmacology

REVIEW
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Essential Role of Keap1-Nrf2 Signaling in Mood Disorders: Overview and Future Perspective

*Kenji Hashimoto**

Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba, Japan

Depression is one of the most common mood disorders with a high rate of relapse. Accumulating evidence suggests that the transcription factor Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1)-Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) system plays a key role in inflammation which is involved in depression. Preclinical studies demonstrated that the protein expressions of Keap1 and Nrf2 in the prefrontal cortex (PFC), CA3 and dentate gyrus (DG) of hippocampus in mice with depression-like phenotype were lower than control mice. In the learned helplessness paradigm, the protein levels of Keap1 and Nrf2 in the PFC and DG of hippocampus from rats with depression-like phenotype were also lower than control and resilient rats. Furthermore, rodents with depression-like phenotype have higher levels of pro-inflammatory cytokines. Interestingly, *Nrf2* knock-out (KO) mice exhibit depression-like phenotype, and higher serum levels of pro-inflammatory cytokines compared with wild-type mice. Furthermore, *Nrf2* KO mice have lower expression of brain-derived neurotrophic factor (BDNF) in the PFC, and CA3 and DG of hippocampus compared to wild-type mice. 7,8-Dihydroxyflavone, a TrkB agonist, showed antidepressant effects in *Nrf2* KO mice, by stimulating BDNF-TrkB in the PFC, CA3, and DG. Pretreatment with sulforaphane, a naturally occurring Nrf2 activator, prevented depression-like phenotype in mice after inflammation, or chronic social defeat stress. Interestingly, dietary intake of 0.1% glucoraphanin (a precursor of sulforaphane) containing food during juvenile and adolescent stages of mice could prevent depression-like phenotype in adulthood after chronic social defeat stress. Moreover, the protein expressions of Keap1 and Nrf2 in the parietal cortex from major depressive disorder and bipolar disorder were lower than controls. These findings suggest that Keap1-Nrf2 system plays a key role in the stress resilience which is involved in the pathophysiology of mood disorders. It is, therefore, possible that dietary intake of cruciferous vegetables including glucoraphanin (or SFN) may prevent or minimize relapse from remission, induced by stress and/or inflammation in depressed patients. In the review, the author would like to discuss the role of Keap1-Nrf2 system in mood disorders.

Keywords: brain-derived neurotrophic factor, glucoraphanin, keap1, Nrf2, nutrition, stress resilience, sulforaphane, TrkB

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Edited by:
Javier Egea,
Instituto de Investigación Sanitaria del Hospital Universitario de La Princesa, Spain

Reviewed by:
Giustino Orlando,
Università degli Studi G. d'Annunzio Chieti e Pescara, Italy
Claudio Ferrante,
Università degli Studi G. d'Annunzio Chieti e Pescara, Italy

***Correspondence:**
Kenji Hashimoto
hashimoto@faculty.chiba-u.jp

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INTRODUCTION

Depression, one of the most common psychiatric disorders in the world, is a mood disorder with a high rate of relapse. The World Health Organization (WHO) estimates that more than 320 million individuals of all ages suffer from depression, highlighting this disease as a major contributor to the global burden of disease (World Health Organization [WHO], 2017). Although the precise mechanisms underlying the pathophysiology of depression are currently unknown, accumulating evidence implicate inflammatory processes in the pathophysiology of depression (Dantzer et al., 2008; Hashimoto, 2009; Miller et al., 2009; Raison et al., 2010; Hashimoto, 2015; Mechawar and Savitz, 2016; Miller and Raison, 2016; Zhang et al., 2016; Miller et al., 2017). Meta-analysis demonstrated higher levels of pro-inflammatory cytokines in the blood of drug-free or medicated depressed patients compared to healthy controls (Dowlati et al., 2010; Young et al., 2014; Haapakoski et al., 2015; Köhler et al., 2018). Studies demonstrated elevated gene expression of pro-inflammatory cytokines in the postmortem brain samples from patients with a history of depression (Dean et al., 2010; Shelton et al., 2011). Collectively, it is likely that inflammation plays a key role in the pathophysiology of depression.

Over the past decade, there has been increasing interest in the potential benefits of early intervention for mood disorders. Several lines of evidence suggest that nutrition has a high impact on the development of depression (Lin et al., 2010; Murakami and Sasaki, 2010; Bazinet and Layé, 2014; Mello et al., 2014; El-Behadli et al., 2015; Opie et al., 2015; Lin et al., 2017; Hsu et al., 2018; Wang et al., 2018). Recent meta-analyses demonstrated that high intake of fruit, vegetables, fish, and whole grains are associated with a lower risk of depression (Lai et al., 2014; Liu et al., 2016; Saghafian et al., 2018).

In the review, the author would like to discuss the role of Keap1 [Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1)]-Nrf2 [Nuclear factor (erythroid 2-derived)-like 2] system in the pathophysiology of depression since Keap1-Nrf2 system plays a key role in inflammation. Furthermore, we also refer to the clinical significance of natural Nrf2 activator sulforaphane (SFN) as nutritional intervention for mood disorders.

Keap1-Nrf2 SYSTEM

Nrf2 is the transcription factor with a key role in cellular defense against oxidative stress. It binds to the antioxidant response elements (ARE) located in the promoter region of genes encoding many phase II detoxifying or antioxidant enzymes and related stress-responsive proteins (Kobayashi et al., 2013; Ma, 2013; Suzuki et al., 2013a; Suzuki and Yamamoto, 2015; Yamamoto et al., 2018). Under normal conditions, Nrf2 is repressed by Keap1, which is an adaptor protein for the degradation of Nrf2 (Suzuki et al., 2013a; Suzuki and Yamamoto, 2015). During oxidative stress, Nrf2 is de-repressed and activates the transcription of protective genes (Suzuki et al., 2013a; Suzuki and Yamamoto, 2015). Importantly, the Keap1-Nrf2 system plays a role in inflammation-associated pathogenesis (Kobayashi et al., 2013; Suzuki et al., 2013a; O'Connell and Hayes, 2015; Suzuki and Yamamoto, 2015; Wardyn et al., 2015; Yamamoto et al., 2018). In cancer cells, Nrf2 activation is beneficial and deleterious for the cancer-bearing host, depending on the time (initiation, promotion, and metastasis) and place (cancer cells or microenvironment) (Yamamoto et al., 2018).

Nrf2 ACTIVATORS

Based on the role of Nrf2 in the prevention of a wide variety of pathological conditions, great efforts have been made to isolate from natural sources or develop potent and specific Nrf2 activators (Yamamoto et al., 2018). The potent anti-inflammatory and naturally occurring compound sulforaphane (SFN: 1-isothiocyanato-4-methylsulfinylbutane) is an organosulfur compound derived from a glucosinolate precursor glucoraphanin (a glucosinolate, or β-thioglucoside-*N*-hydroxysulfate) (**Figure 1**) found in cruciferous vegetables, such

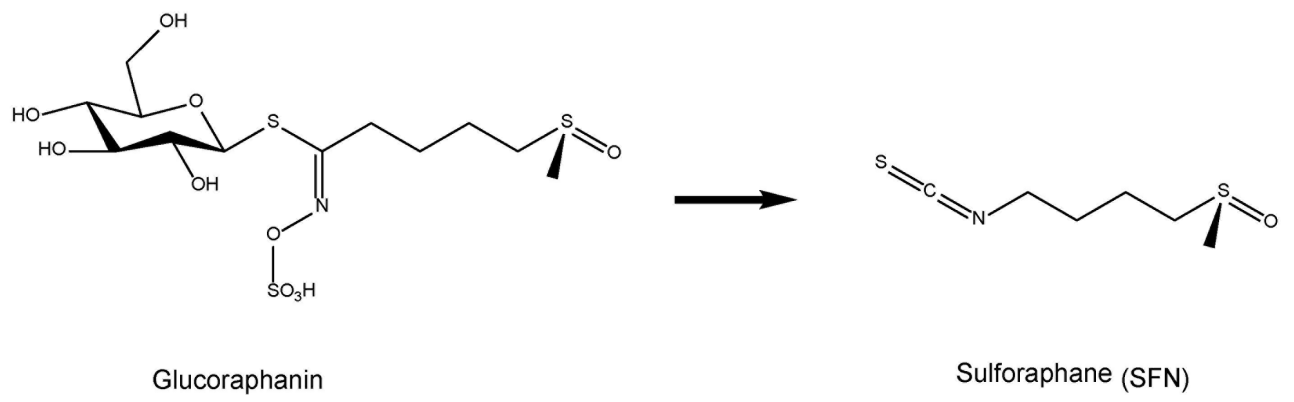

FIGURE 1 | Chemical structure of sulphoraphane (SFN) and its precursor glucoraphanin. Cruciferous vegetables contain glucoraphanin, a glucosinolate derivative of sulforaphane (SFN).

as broccoli sprout (Zhang et al., 1992; Fahey et al., 1997; Kwak and Kensler, 2010; Kensler et al., 2013; Fahey et al., 2015). It is well known that glucoraphanin can be converted to SFN by the endogenous enzyme, myrosinase (Fahey et al., 2015). Beneficial effect by SFN is thought to be mediated via activation of the Nrf2 pathway with subsequent up-regulation of phase II detoxification enzymes and antioxidant proteins, through ARE (Suzuki et al., 2013a; Suzuki and Yamamoto, 2015).

TBE-31 [(±)-(4bS,8aR,10aS)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydrophenanthrene-2,6-dicarbonitrile] and MCE-1 [(±)-3-ethynyl-3-methyl-6-oxocyclohexa-1,4-dienecarbonitrile] are the novel Nrf2 activators (Honda et al., 2007; Dinkova-Kostova et al., 2010; Honda et al., 2011; Kostov et al., 2015; **Figure 2**). Dimethyl fumarate (**Figure 2**) is a new oral drug for the treatment of multiple sclerosis, and has neuroprotective effects via Nrf2-dependent antioxidant response (Al-Jaderi and Maghazachi, 2016; Mills et al., 2018). Bardoxolone methyl, the C-28 methyl ester of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) known as CDDO-Me (**Figure 2**), is one of the derivatives of synthetic triterpenoids. Bardoxolone methyl has been used for the treatment of cancer (including leukemia and solid tumors), chronic kidney disease, and other diseases (Wang et al., 2014). Clinical trial of bardoxolone methyl is undergoing for diabetic nephropathy in Japan (Yamamoto et al., 2018), although its development was paused in the United States due to the occurrence of cardiac complications in patients with end-stage renal disease (Pergola et al., 2011; de Zeeuw et al., 2013).

EFFECTS OF Nrf2 ACTIVATORS ON NEURITE OUTGROWTH

The neuronal plasticity, including neurite outgrowth and neuroprotection, plays crucial role in the beneficial effect of therapeutic drugs in cellular level (Lu and Dwyer, 2005; Williams and Dwyer, 2009). Yao et al. (2016b) reported that SFN increased the number of cell with neurite outgrowth in PC12 cells. Furthermore, the potentiating effects of SFN on NGF-induced neurite outgrowth were blocked by treatment with *Nrf2* siRNA,

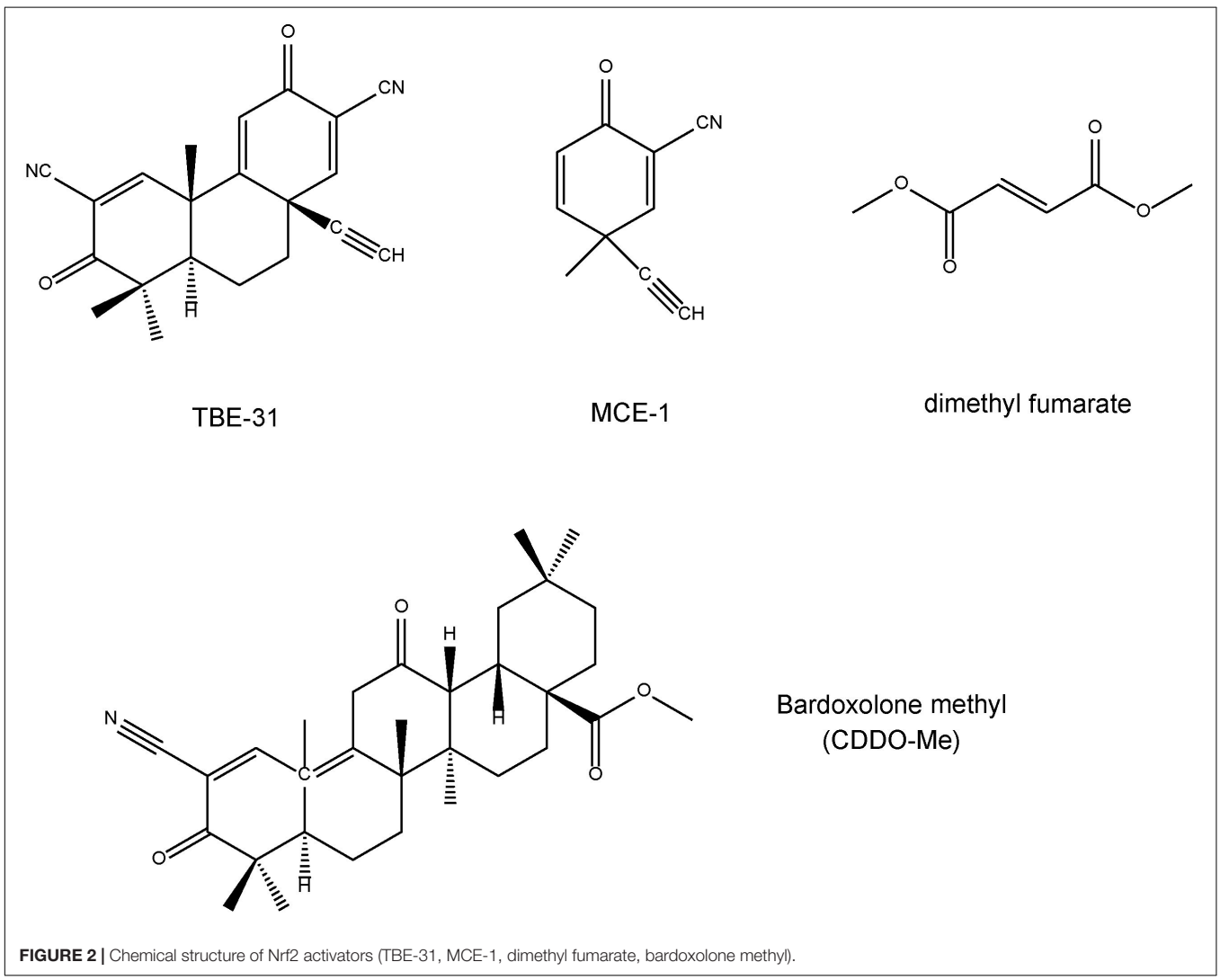

FIGURE 2 | Chemical structure of Nrf2 activators (TBE-31, MCE-1, dimethyl fumarate, bardoxolone methyl).

but not the negative control (Yao et al., 2016b), suggesting that SFN can potentiate NGF-induced neurite outgrowth via activation of Nrf2.

Yao et al. (2016a) reported that TBE-31 and MCE-1 also potentiated NGF-induced neurite outgrowth in PC12 cells. The *Nrf2* siRNA blocked the potentiating effects of TBE-31 and MCE-1 on neurite outgrowth in PC12 cells. Astragaloside IV is also reported to attenuate lead-induced inhibition of neurite outgrowth through activation of Akt-dependent Nrf2 pathway (Yu et al., 2017). Collectively, it is likely that Nrf2 activators can promote neurite outgrowth through Nrf2 activation (Yang et al., 2015c; Yao et al., 2016a,b).

ALTERATIONS IN Keap1-Nrf2 SIGNALING IN RODENTS WITH DEPRESSION-LIKE PHENOTYPE

Chronic social defeat stress (CSDS) model has been used widely as an animal model of depression (Krishnan and Nestler, 2008; Nestler and Hyman, 2010; Golden et al., 2011). Susceptible mice with depression-like phenotype after CSDS have higher blood levels of pro-inflammatory cytokines [e.g., tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and IL-1β] (Zhang et al., 2017a).

Western blot analysis showed that protein levels of Keap1 and Nrf2 in the CA3, DG, and PFC from mice with depression-like phenotype were significantly lower than those of control mice (Yao et al., 2016b). In contrast, protein levels of Keap1 and Nrf2 in the CA1 and NAc were not different compared to control (Yao et al., 2016b). These findings suggest that lower levels of Keap1 and Nrf2 in the CA3, DG, and PFC may be involved in depression-like phenotypes after CSDS.

Learned helplessness (LH) model has been also used as an animal model of depression (Krishnan and Nestler, 2008). In the LH paradigm, approximately 20–40% rats are resilient to inescapable stress (Yang et al., 2015a,b; Yang et al., 2016). LH (susceptible) rats have higher blood levels of IL-6 than control and resilient rats (Yang et al., 2015a), suggesting that peripheral inflammation may contribute to resilience versus susceptibility to stress. Protein levels of Keap1 and Nrf2 in the PFC and DG of hippocampus from LH (susceptible) rats were lower than control and non-LH (resilient) rats (Zhang et al., 2018). These results suggest that Keap1-Nrf2 signaling may contribute to stress resilience which is involved in the pathophysiology of major psychiatric disorders (Zhang et al., 2018).

ALTERATIONS IN Keap1-Nrf2 SIGNALING IN SAMPLES FROM PATIENTS WITH MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD) patients (n = 30) exhibited higher levels of Nrf2 and its regulator Keap1, as well as NF-κB in the cytoplasm of peripheral blood mononuclear cells compared to healthy controls (n = 35), suggesting that depression may be characterized by up-regulation of the transcription factor Keap1-Nrf2 (Lukic et al., 2014). Using genome-wide transcriptional profiling and promoter-based bioinformatic strategies, Mellon et al. (2016) measured leukocyte transcription factor (TF) activity in leukocytes from un-medicated MDD subjects (n = 20) versus age- and sex-matched healthy controls (n = 20). In leukocytes from un-medicated MDD subjects, the bioinformatic analysis showed an increased transcriptional activity of cAMP response element-binding/activating TF (CREB/ATF) and increased activity of TFs associated with Nrf2. Antidepressant therapy for 8 weeks was associated with significant reductions in depressive symptoms and reduced activity of Nrf2, but not in CREB/ATF activity. By contrast, other transcriptional regulation pathways, including nuclear factor kappa-B cells (NF-κB), early growth response proteins 1-4 (EGR1-4), the glucocorticoid receptor, and interferon-responsive TFs, showed either no difference as a function of disease or treatment. These results suggest that Nrf2 signaling may contribute to MDD by activating immune cell transcriptome dynamics that ultimately may influence motivational and affective processes via circulating mediators (Mellon et al., 2016).

Postmortem tissue from patients with psychiatric disorders is an underutilized substance that may be used to translate genetic and/or preclinical studies (Hashimoto et al., 2007; McCullumsmith et al., 2013; Mechawar and Savitz, 2016; Yang et al., 2017). A study using postmortem brain samples showed decreased expressions of Keap1 and Nrf2 in the parietal cortex from patients with MDD and bipolar disorder compared to control group (Zhang et al., 2018). A recent study showed the reduced (-21%) expression of Nrf2 in the dorsolateral prefrontal cortex from MDD patients (Martín-Hernández et al., 2018). These results suggest that decreased Keap1-Nrf2 signaling plays a key role in the pathophysiology of mood disorders such as MDD and bipolar disorder (Zhang et al., 2018).

SINGLE NUCLEOTIDE POLYMORPHISMS IN THE *NRF2* PROMOTER GENE IN HUMANS

The NRF2 activity in humans is regulated through protein stabilization, primarily by KEAP1, but is also regulated at the transcriptional level (Yamamoto et al., 2018). In humans, a *NRF2* promotor single nucleotide polymorphism (SNP: rs6721961) located 617 bp by upstream from the transcription start site lowers the level of *NRF2* transcription (Yamamoto et al., 2006; Yamamoto et al., 2018; **Figure 3**). Luciferase assays showed that polymorphism at position -617 (C to A) affect basal levels of NRF2, thereby resulting in attenuation of ARE-mediated gene transcription (Marzec et al., 2007; **Figure 3**). Interestingly, subjects who possess this SNP are more susceptible to acute lung injury and related diseases (Marzec et al., 2007), and this SNP is also found to correlate with the incidence of non-small cell lung cancer (Suzuki et al., 2013b). In addition, ethnic difference of this SNP is also reported (Marzec et al., 2007). Therefore, it is

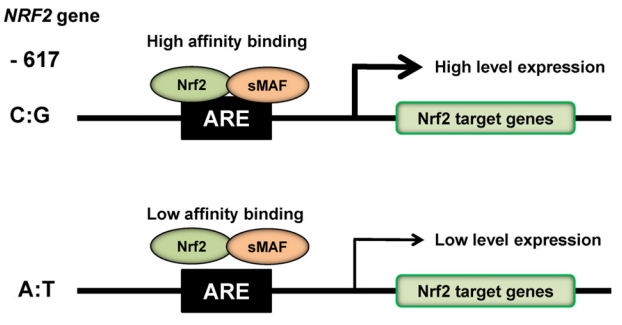

FIGURE 3 | SNP (rs6721961) in the promotor region of *NRF2* gene. The SNP (rs6721961) in the promotor of *NRF2* gene alters the transcription level of *NRF2* gene, resulting in alterations in the expression of *NRF2* target genes. The NRF2/sMAF (small MAF) protein complex regulates the oxidative stress response by occupying *cis*-acting enhancers containing an antioxidant response element (ARE). A slight modification of Yamamoto et al. (2018).

of interest to study whether this SNP can affect susceptibility to mood disorders.

DEPRESSION-LIKE PHENOTYPES IN *Nrf2* KO MICE

Serum levels of TNF-α, IL-6, IL-10, and IL-1β in the *Nrf2* KO mice were significantly higher than those of wild-type (WT) mice, suggesting that *Nrf2* KO mice have inflammation (Yao et al., 2016b). In the tail-suspension test (TST) and forced swimming test (FST), the immobility times of TST and FST in *Nrf2* KO mice were higher than those of WT mice. In the 1% sucrose preference test (SPT), the sucrose preference of *Nrf2* KO mice was lower than that of WT mice, suggesting that *Nrf2* KO mice have anhedonia. Furthermore, brain-derived neurotrophic factor (BDNF) and its receptor TrkB signaling in the CA3, DG and PFC of *Nrf2* KO mice were lower than those of WT mice. Moreover, protein levels of AMPA receptor 1 (GluA1) and postsynaptic density protein 95 (PSD-95) in the CA3, DG, and PFC of KO mice were lower than those of WT mice. Interestingly, 7,8-dihydroxyflavone (a TrkB agonist) produced antidepressant effects in *Nrf2* KO mice, by stimulating TrkB in the PFC, CA3, and DG (Yao et al., 2016b). Furthermore, the anti-inflammatory drug rofecoxib reversed depression-like behaviors in *Nrf2* KO mice (Martín-de-Saavedra et al., 2013). In addition, chronic treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine increased BDNF in cortex and hippocampus of corticosterone-treated *Nrf2* KO mice (Mendez-David et al., 2015), suggesting that Nrf2 signaling contributes to fluoxetine-induced neuroprotection. These all findings suggest that Nrf2 plays a key role in the depression-like phenotypes in rodents through potent anti-inflammatory action. Collectively, it is likely that *Nrf2* KO mice show depression-like phenotypes through inflammation, decreased BDNF-TrkB signaling and synaptogenesis (**Figure 5**; Yao et al., 2016b).

In contrast, Bouvier et al. (2017) reported that *Nrf2* KO mice did not display a depression-like phenotype although the KO mice were characterized by oxidative stress and by anatomical alterations in hippocampal CA3 pyramidal cells. However, when exposed to 3 weeks of chronic mild stress, *Nrf2* KO mice developed depression-like phenotypes which were prevented by pretreatment with antioxidant (Bouvier et al., 2017). This study also suggests the role of Nrf2-dependent persistent oxidative stress in stress-induced vulnerability to depression.

ANTIDEPRESSANT EFFECTS OF Nrf2 ACTIVATORS IN THE RODENT MODELS OF DEPRESSION

When lipopolysaccharide (LPS), the bacterial endotoxin, is administered to rodents, depression-like behaviors are observed 24 h after inflammation (Dantzer et al., 2008; O'Connor et al., 2009; Zhang et al., 2014; Zhang et al., 2016). Pretreatment with antidepressants, such as SSRIs and serotonin and norepinephrine reuptake inhibitors (SNRIs), can prevent depression-like behavior and alterations in serum pro-inflammatory cytokines, such as TNF-α, induced by LPS administration (Ohgi et al., 2013; Yao et al., 2015; Dong et al., 2016). These all findings suggest that inflammation is associated with depression, and that anti-inflammatory drugs could ameliorate depressive symptoms in patients with depression.

Pretreatment with SFN significantly blocked an increase in the serum TNF-α level after a single administration of LPS (Zhang et al., 2017b). Furthermore, SFN significantly potentiated increased serum levels of IL-10 after LPS administration. SFN attenuated an increase of the immobility time of TST and FST after LPS administration. In addition, SFN recovered to control levels for LPS-induced alterations in the proteins such as BDNF, PSD-95 and GluA1, and dendritic spine density in the brain regions (Zhang et al., 2017b). Furthermore, TBE-31 or MCE-1 attenuated an increase in serum levels of TNF-α after LPS administration. Administration of TBE-31 or MCE-1 attenuated an increase in the immobility time of TST and FST after LPS administration (Yao et al., 2016a).

Pretreatment with SFN attenuated the decreased social avoidance time and sucrose preference in CSDS model. Furthermore, SFN could attenuate the decreased levels of Nrf2 and Keap1 proteins in the PFC and hippocampus of mice with depression-like phenotype (Yao et al., 2016b). Li et al. (2018) reported that decreased Keap1-Nrf2 signaling in the PFC, hippocampus and skeletal muscle may contribute to anhedonia susceptibility after spared nerve injury (SNI), and that SFN exerts beneficial effects in SNI rats by normalization of decreased Keap1-Nrf2 signaling. These results suggest that Keap1-Nrf2 signaling plays a role in depression, and that SFN is prophylactic compound which can stimulate Keap1-Nrf2 signaling pathway (Yao et al., 2016b; Zhang et al., 2017b; Li et al., 2018). Taken all together, it is likely that the Nrf2 activators such as SFN, TBE-31, and MCE-1 might be potential prophylactic or therapeutic drugs for inflammation (or stress)-related depression (Yao et al., 2016a,b; Zhang et al., 2016, 2017b; Li et al., 2018).

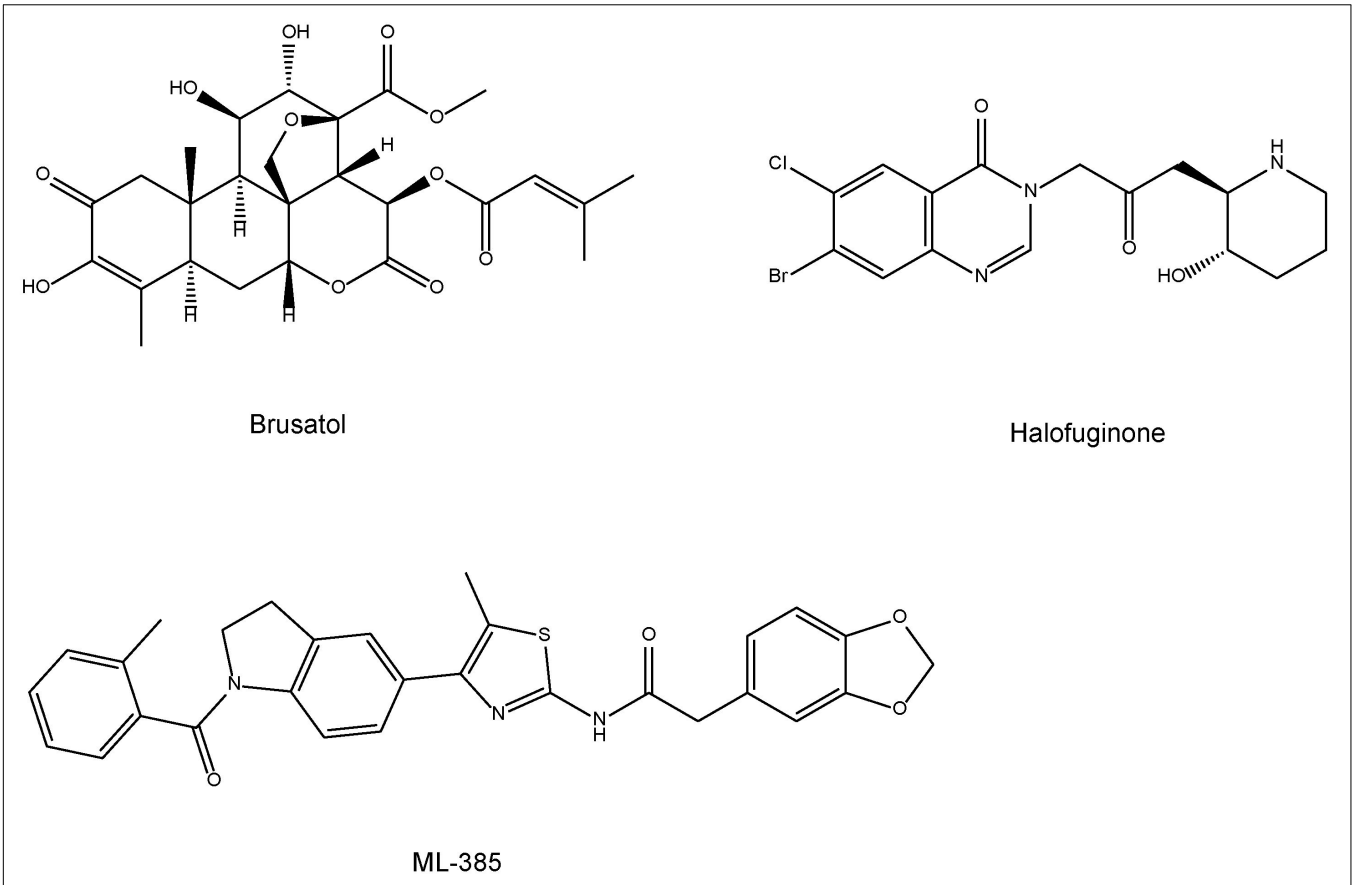

FIGURE 4 | Chemical structure of Nrf2 inhibitors (brustal, halofuginone, ML-385).

EFFECTS OF DIETARY INTAKE OF SFN PRECURSOR IN THE CSDS MODEL OF DEPRESSION

SFN is produced in the body from its precursor glucoraphanin which is involved in cruciferous vegetables. Previously, we demonstrated that dietary intake of 0.1% glucoraphanin –rich food during juvenile and adolescence prevented phencyclidine-induced cognitive deficits and loss of parvalbumin (PV)-positive cells in the PFC at adulthood (Shirai et al., 2015). Furthermore, we also reported that dietary intake of 0.1% glucoraphanin –rich food during juvenile and adolescence prevented the onset of psychosis in the adult offspring after maternal immune activation (Matsuura et al., 2018). These findings suggest that dietary-intake of glucoraphanin-rich vegetables in high-risk psychosis subjects might prevent the transition to psychosis in young adulthood (Hashimoto, 2014; Shirai et al., 2015; Matsuura et al., 2018).

Interestingly, dietary intake of 0.1% glucoraphanin containing food during juvenile and adolescent stages could prevent the depression-like phenotype in adulthood after CSDS (Yao et al., 2016b). Thus, the dietary intake of 0.1% glucoraphanin containing food during juvenile and adolescent periods could confer stress resilience in adulthood.

CLINICAL STUDY OF SFN IN PATIENTS WITH HEALTHY SUBJECTS, AND NEURODEVELOPMENTAL DISORDERS

Sedlak et al. (2018) reported that SFN increased blood glutathione (GSH) levels in healthy human subjects following 7 days of daily oral administration. Furthermore, a significant positive correlation between blood and thalamic GSH post- and pre-SFN treatment ratios was observed, in addition to a consistent increase in brain GSH levels in response to treatment. This study suggests the value of exploring relationships between peripheral GSH and clinical/neuropsychological measures, as well as the influences SFN has on functional measures that are altered in neuropsychiatric disorders.

A randomized, double-blinded, placebo-controlled study showed that SFN-rich broccoli sprout extract could improve social interaction, abnormal behavior and verbal communication in young male subjects with autism spectrum disorder (Singh et al., 2014; Lynch et al., 2017). In addition, a pilot study showed that supplementation with glucoraphanin-rich broccoli sprout extract for 8 weeks was effective in treating cognitive impairment in medicated patients with schizophrenia (Shiina et al., 2015). Collectively, it is likely that SFN would be potential therapeutic compound for neurodevelopmental disorders.

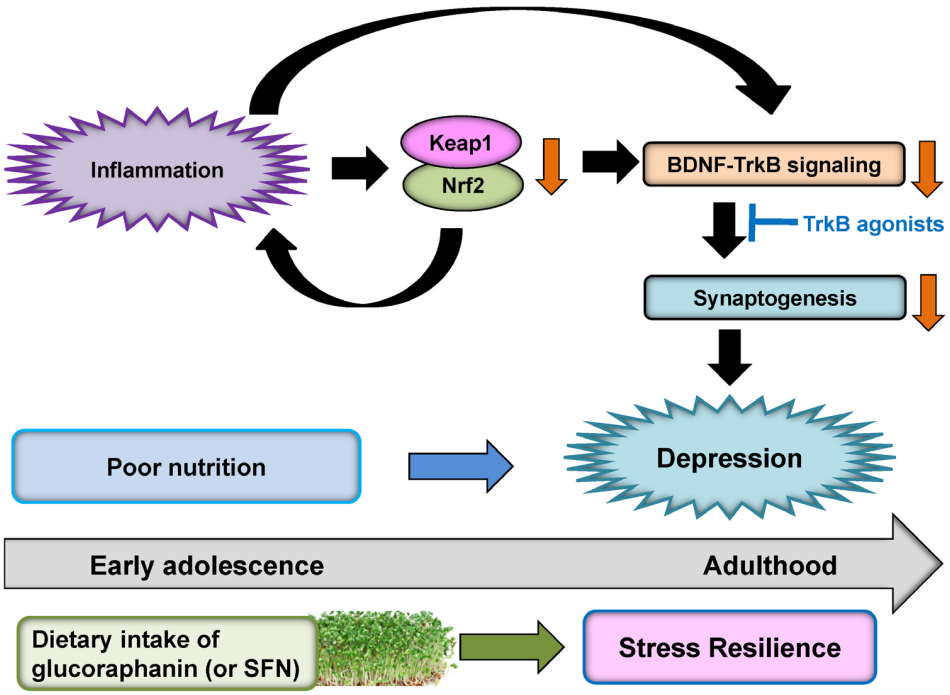

FIGURE 5 | Proposed hypothesis of role of Keap1-Nrf2 system in depression. Inflammation causes decreases of Keap1 and Nrf2 expression in the prefrontal cortex and hippocampus. Subsequently, inflammation-induced decreases in Keap1 and Nrf2 proteins can cause the decreased BDNF-TrkB signaling and synaptogenesis, resulting in depression-like phenotype. TrkB agonists might have antidepressant actions. Dietary intake of glucoraphanin (or SFN) in cruciferous vegetables during early adolescence may confer to stress resilience at adulthood whereas poor nutrition may play a role in the onset of depression by stress or inflammation.

ROLE OF Nrf2 IN THE MECHANISMS OF ANTIDEPRESSANT ACTION FOR OTHER POTENTIAL COMPOUNDS

Cilostazol is used in the treatment of the symptoms of intermittent claudication in patients with peripheral vascular disease. In the chronic restraint stress (CRS) model, cilostazol prevented depressive-like behaviors (Abuelezz and Hendawy, 2018). Furthermore, cilostazol modulated the Nrf2 protein and heme oxygenase-1 and NAD(P)H: quinone oxidoreductase-1 gene expression in the hippocampus of CRS rats. These findings suggest that cilostazol has the prophylactic antidepressant effect by preventing oxidative stress by stimulation of redox defense mechanisms mediated through the Nrf2 pathway (Abuelezz and Hendawy, 2018).

Dl-3-n-Butylphthalide (NBP), a small molecule compound extracted from the seeds of *Apium graveolens*, was approved by the State Food and Drug Administration of China for treating ischemic stroke (Abdoulaye and Yi, 2016). NBP attenuated the depression-like behaviors and increased expression of pro-inflammatory cytokines (e.g., IL-1β and IL-6) in rats. In addition with the anti-inflammation action, NBP reduced LPS-induced oxidative stress reactions in the hippocampus and enhanced Nrf2-targeted signals (Yang et al., 2018).

A randomized, double-blind, placebo-controlled trial showed that NBP showed greater effects than placebo on Alzheimer's disease assessment scale-cognitive subscale (ADAS-cog) and clinician's interview-based impression of change plus caregiver input (CIBIC-plus). NBP-related adverse events were uncommon and primarily consisted of mild gastrointestinal symptoms (Jia et al., 2016). Therefore, it is of interest to examine whether NBP can improve depressive symptoms in depressed patients.

Nrf2 INHIBITORS

Compared with Nrf2 activators, the development of Nrf2 inhibitors is in its infancy (Yamamoto et al., 2018). For example, cancers with persistent activation of Nrf2 exhibit high dependency on Nrf2 function for drug resistance and cell proliferation (Yamamoto et al., 2018). The plant-based product brusatol (**Figure 4**) decreases the protein levels of Nrf2 and sensitizes cancer cells to chemotherapy and radiotherapy (Ren et al., 2011). Another Nrf2 inhibitor halofuginone (**Figure 4**) is a synthetic halogenated derivative of febrifugine, a natural quinazolinone alkaloid which can be found in the Chinese herb *Dichroa febrifuga*. Halofuginone exerts a chemosensitizing effect on cancer cells exhibiting constitutive Nrf2 stabilization (Tsuchida et al., 2017). In addition, Singh et al. (2016) demonstrated that ML385 [*N*-[4-[2,3-dihydro-1-(2-methylbenzoyl)-1H-indol-5-yl]-5-methyl-2-thiazolyl]-1,3-benzodioxole-5-acetamide] (**Figure 4**) is a novel and specific Nrf2 inhibitor. Therefore, it is of interest to study whether these Nrf2 inhibitors can affect depression-like phenotypes in rodents.

CONCLUSION REMARKS AND FUTURE PERSPECTIVE

Rodents with depression-like phenotype have higher blood levels of pro-inflammatory cytokines, suggesting that inflammation plays a role in depression-like phenotype in rodents. Furthermore, rodents with depression-like phenotype have lower expression of Keap1 and Nrf2 in the PFC and hippocampus (Yao et al., 2016b; Zhang et al., 2017b). Interestingly, we found decreased expression of Keap1 and Nrf2 in the parietal cortex from patients with MDD and bipolar disorder (Zhang et al., 2018). Given the essential role of BDNF-TrkB signaling in depression (Nestler et al., 2002; Hashimoto et al., 2004; Duman and Monteggia, 2006; Hashimoto, 2010; Zhang et al., 2016), inflammation (or stress)-induced reduction of Keap1-Nrf2 system may contribute to decreased BDNF-TrkB signaling and synaptogenesis, resulting in depression-like phenotypes (**Figure 5**). It is noteworthy that TrkB agonist 7,8-DHF has antidepressant effects in *Nrf2* KO mice (Yao et al., 2016b), LPS-treated mice (Zhang et al., 2014) and CSDS susceptible mice (Zhang et al., 2015), suggesting a possible link between Keap1-Nrf2 system and BDNF-TrkB signaling (Mendez-David et al., 2015; Yao et al., 2016b; Zhang et al., 2017b; Li et al., 2018; **Figure 5**).

Nutritional status during early adolescence stage might have a great impact on the onset and severity of psychiatric diseases in adulthood (Paus et al., 2008; O'Connor and Cryan, 2014). Over the past decade, there has been increasing interest in the potential benefits of early intervention for psychiatric disorders such as depression (Paus et al., 2008; O'Connor and Cryan, 2014; Sarris et al., 2015; Correll et al., 2018).

Preclinical findings suggest that dietary intake of glucoraphanin during juvenile and adolescence can protect against depression-like behaviors after CSDS or LPS administration (Yao et al., 2016b; Zhang et al., 2017b), indicating prophylactic effects of glucoraphanin for depression. Thus, dietary intake of glucoraphanin (or SFN) during juvenile and adolescence might confer stress resilience at adulthood (**Figure 5**). Therefore, it is possible that dietary intake of glucoraphanin (or SFN) during childhood and adolescence stages could prevent the onset of depression in humans during adulthood. Since patients with mood disorder have high relapse rate, dietary intake of glucoraphanin (or SFN) may prevent or minimize relapse from remission, induced by inflammation and/or stress in depressed patients.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REFERENCES

Abdoulaye, I. A., and Yi, J. G. (2016). A review of recent advances in neuroprotective potential of 3-N-butylphthalide and its derivatives. *Biomed. Res. Int.* 2016:5012341. doi: 10.1155/2016/5012341

Abuelezz, S. A., and Hendawy, N. (2018). Insights into the potential antidepressant mechanisms of cilostazol in chronically restraint rats: impact on the Nrf2 pathway. *Behav. Pharmacol.* 29, 28–40. doi: 10.1097/FBP.0000000000000335

Al-Jaderi, Z., and Maghazachi, A. A. (2016). Utilization of dimethyl fumarate and related molecules for treatment of multiple sclerosis, cancer, and other diseases. *Front. Immunol.* 7:278. doi: 10.3389/fimmu.2016.00278

Bazinet, R. P., and Layé, S. (2014). Polyunsaturated fatty acids and their metabolites in brain function and disease. *Nat. Rev. Neurosci.* 15, 771–785. doi: 10.1038/nrn3820

Bouvier, E., Brouillard, F., Molet, J., Claverie, D., Cabungcal, J. H., Cresto, N., et al. (2017). Nrf2-dependent persistent oxidative stress results in stress-induced vulnerability to depression. *Mol. Psychiatry* 22, 1701–1713. doi: 10.1038/mp.2016.144

Correll, C. U., Galling, B., Pawar, A., Krivko, A., Bonetto, C., Ruggeri, M., et al. (2018). Comparison of early intervention services vs treatment as usual for early-phase psychosis: a systematic review, meta-analysis, and meta-regression. *JAMA Psychiatry* 75, 555–565. doi: 10.1001/jamapsychiatry.2018.0623

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., and Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* 9, 46–57. doi: 10.1038/nrn2297

de Zeeuw, D., Akizawa, T., Audhya, P., Bakris, G. L., Chin, M., Christ-Schmidt, H., et al. (2013). Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. *N. Eng. J. Med.* 369, 2492–2503. doi: 10.1056/NEJMoa1306033

Dean, B., Tawadros, N., Scarr, E., and Gibbons, A. S. (2010). Regionally-specific changes in levels of tumour necrosis factor in the dorsolateral prefrontal cortex obtained postmortem from subjects with major depressive disorder. *J. Affect. Disord.* 120, 245–248. doi: 10.1016/j.jad.2009.04.027

Dinkova-Kostova, A. T., Talalay, P., Sharkey, J., Zhang, Y., Holtzclaw, W. D., Wang, X. J., et al. (2010). An exceptionally potent inducer of cytoprotective enzymes: elucidation of the structural features that determine inducer potency and reactivity with Keap1. *J. Biol. Chem.* 285, 33747–33755. doi: 10.1074/jbc.M110.163485

Dong, C., Zhang, J. C., Yao, W., Ren, Q., Yang, C., Ma, M., et al. (2016). Effects of escitalopram, R-citalopram, and reboxetine on serum levels of tumor necrosis factor-α, interleukin-10, and depression-like behavior in mice after lipopolysaccharide administration. *Pharmacol. Biochem. Behav.* 144, 7–12. doi: 10.1016/j.pbb.2016.02.005

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., et al. (2010). A meta-analysis of cytokines in major depression. *Biol. Psychiatry* 67, 446–457. doi: 10.1016/j.biopsych.2009.09.033

Duman, R. S., and Monteggia, L. M. (2006). A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry* 59, 1116–1127. doi: 10.1016/j.biopsych.2006.02.013

El-Behadli, A. F., Sharp, C., Hughes, S. O., Obasi, E. M., and Nicklas, T. A. (2015). Maternal depression, stress and feeding styles: towards a framework for theory and research in child obesity. *Br. J. Nutr.* 113, S55–S71. doi: 10.1017/S000711451400333X

Fahey, J. W., Holtzclaw, W. D., Wehage, S. L., Wade, K. L., Stephenson, K. K., and Talalay, P. (2015). Sulforaphane bioavailability from glucoraphanin-rich broccoli: control by active endogenous myrosinase. *PLoS One* 10:e0140963. doi: 10.1371/journal.pone.0140963

Fahey, J. W., Zhang, Y., and Talalay, P. (1997). Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. U.S.A.* 94, 10367–10372. doi: 10.1073/pnas.94.19.10367

Golden, S. A., Covington, H. E., Berton, O., and Russo, S. J. (2011). A standardized protocol for repeated social defeat stress in mice. *Nat. Protoc.* 6, 1183–1191. doi: 10.1038/nprot.2011.361

Haapakoski, R., Mathieu, J., Ebmeier, K. P., Alenius, H., and Kivimäki, M. (2015). Cumulative meta-analysis of interleukins 6 and 1β, tumour necrosis factor α and C-reactive protein in patients with major depressive disorder. *Brain Behav. Immun.* 49, 206–215. doi: 10.1016/j.bbi.2015.06.001

Hashimoto, K. (2009). Emerging role of glutamate in the pathophysiology of major depressive disorder. *Brain Res. Rev.* 61, 1056–1123. doi: 10.1016/j.brainresrev.2009.05.005

Hashimoto, K. (2010). Brain-derived neurotrophic factor as a biomarker for mood disorders: an historical overview and future directions. *Psychiatry Clin. Neurosci.* 64, 341–357. doi: 10.1111/j.1440-1819.2010.02113.x

Hashimoto, K. (2014). Targeting of NMDA receptors in new treatments for schizophrenia. *Expert Opin. Ther. Targets* 18, 1049–1063. doi: 10.1517/14728222.2014.934225

Hashimoto, K. (2015). Inflammatory biomarkers as differential predictors of antidepressant response. *Int. J. Mol. Sci.* 16, 7796–7801. doi: 10.3390/ijms16047796

Hashimoto, K., Sawa, A., and Iyo, M. (2007). Increased levels of glutamate in brains from patients with mood disorders. *Biol. Psychiatry* 62, 1310–1316. doi: 10.1016/j.biopsych.2007.03.017

Hashimoto, K., Shimizu, E., and Iyo, M. (2004). Critical role of brain-derived neurotrophic factor in mood disorders. *Brain Res. Rev.* 45, 104–114. doi: 10.1016/j.brainresrev.2004.02.003

Honda, T., Sundararajan, C., Yoshizawa, H., Su, X., Honda, Y., Liby, K. T., et al. (2007). Novel tricyclic compounds having acetylene groups at C-8a and cyano enones in rings A and C: highly potent anti-inflammatory and cytoprotective agents. *J. Med. Chem.* 50, 1731–1734. doi: 10.1021/jm070141c

Honda, T., Yoshizawa, H., Sundararajan, C., David, E., Lajoie, M. J., Favaloro, F. G. Jr., et al. (2011). Tricyclic compounds containing nonenolizable cyano enones: a novel class of highly potent anti-inflammatory and cytoprotective agents. *J. Med. Chem.* 54, 1762–1778. doi: 10.1021/jm101445p

Hsu, M. C., Tung, C. Y., and Chen, H. E. (2018). Omega-3 polyunsaturated fatty acid supplementation in prevention and treatment of maternal depression: putative mechanism and recommendation. *J. Affect. Disord.* 238, 47–61. doi: 10.1016/j.jad.2018.05.018

Jia, J., Wei, C., Liang, J., Zhou, A., Zuo, X., Song, H., et al. (2016). The effects of DL-3-n-butylphthalide in patients with vascular cognitive impairment without dementia caused by subcortical ischemic small vessel disease: a multicentre, randomized, double-blind, placebo-controlled trial. *Alzheimers Dement.* 12, 89–99. doi: 10.1016/j.jalz.2015.04.010

Kensler, T. W., Egner, P. A., Agyeman, A. S., Visvanathan, K., Groopman, J. D., Chen, J. G., et al. (2013). Keap1-Nrf2 signaling: a target for cancer prevention by sulforaphane. *Top Curr. Chem.* 329, 163–177. doi: 10.1007/128_2012_339

Kobayashi, E., Suzuki, T., and Yamamoto, M. (2013). Roles Nrf2 plays in myeloid cells and related disorders. *Oxid. Med. Cell. Longev.* 2013:529219. doi: 10.1155/2013/529219

Köhler, C. A., Freitas, T. H., Stubbs, B., Maes, M., Solmi, M., Veronese, N., et al. (2018). Peripheral alterations in cytokine and chemokine levels after antidepressant drug treatment for major depressive disorder: systematic review and meta-analysis. *Mol. Neurobiol.* 55, 4195–4206. doi: 10.1007/s12035-017-0632-1

Kostov, R. V., Knatko, E. V., McLaughlin, L. A., Henderson, C. J., Zheng, S., Huang, J. T., et al. (2015). Pharmacokinetics and pharmacodynamics of orally administered acetylenic tricyclic bis(cyanoenone), a highly potent Nrf2 activator with a reversible covalent mode of action. *Biochem. Biophys. Res. Commun.* 465, 402–407. doi: 10.1016/j.bbrc.2015.08.016

Krishnan, V., and Nestler, E. J. (2008). The molecular neurobiology of depression. *Nature* 455, 894–902. doi: 10.1038/nature07455

Kwak, M. K., and Kensler, T. W. (2010). Targeting NRF2 signaling for cancer chemoprevention. *Toxicol. Appl. Pharmacol.* 244, 66–76. doi: 10.1016/j.taap.2009.08.028

Lai, J. S., Hiles, S., Bisquera, A., Hure, A. J., McEvoy, M., and Attia, J. (2014). A systematic review and meta-analysis of dietary patterns and depression in community-dwelling adults. *Am. J. Clin. Nutr.* 99, 181–197. doi: 10.3945/ajcn.113.069880

Li, S., Yang, C., Fang, X., Zhan, G., Huang, N., Gao, J., et al. (2018). Role of Keap1-Nrf2 signaling in anhedonia symptoms in a rat model of chronic neuropathic pain: improvement with sulforaphane. *Front. Pharmacol.* 9:887. doi: 10.3389/fphar.2018.00887

Lin, P. Y., Chang, C. H., Chong, M. F., Chen, H., and Su, K. P. (2017). Polyunsaturated fatty acids in perinatal depression: a systematic review and meta-analysis. *Biol. Psychiatry* 82, 560–569. doi: 10.1016/j.biopsych.2017.02.1182

Lin, P. Y., Huang, S. Y., and Su, K. P. (2010). A meta-analytic review of polyunsaturated fatty acid compositions in patients with depression. *Biol. Psychiatry* 68, 140–147. doi: 10.1016/j.biopsych.2010.03.018

Liu, X., Yan, Y., Li, F., and Zhang, D. (2016). Fruit and vegetable consumption and the risk of depression: a meta-analysis. *Nutrition* 32, 296–302. doi: 10.1016/j.nut.2015.09.009

Lu, X. H., and Dwyer, D. S. (2005). Second-generation antipsychotic drugs, olanzapine, quetiapine, and clozapine enhance neurite outgrowth in PC12 cells via PI3K/AKT, ERK, and pertussis toxin-sensitive pathways. *J. Mol. Neurosci.* 27, 43–64. doi: 10.1385/JMN:27:1:043

Lukic, I., Mitic, M., Djordjevic, J., Tatalovic, N., Bozovic, N., Soldatovic, I., et al. (2014). Lymphocyte levels of redox-sensitive transcription factors and antioxidative enzymes as indicators of pro-oxidative state in depressive patients. *Neuropsychology* 70, 1–9. doi: 10.1159/000362841

Lynch, R., Diggins, E. L., Connors, S. L., Zimmerman, A. W., Singh, K., Liu, H., et al. (2017). Sulforaphane from broccoli reduces symptoms of autism: a follow-up case series from a randomized double-blind study. *Glob. Adv. Health Med.* 6:21644957X17735826. doi: 10.1177/2164957X17735826

Ma, Q. (2013). Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Martín-de-Saavedra, M. D., Budni, J., Cunha, M. P., Gómez-Rangel, V., Lorrio, S., Del Barrio, L., et al. (2013). Nrf2 participates in depressive disorders through an anti-inflammatory mechanism. *Psychoneuroendocrinology* 38, 2010–2022. doi: 10.1016/j.psyneuen.2013.03.020

Martín-Hernández, D., Caso, J. R., Javier Meana, J., Callado, L. F., Madrigal, J. L. M., García-Bueno, B., et al. (2018). Intracellular inflammatory and antioxidant pathways in postmortem frontal cortex of subjects with major depression: effect of antidepressants. *J. Neuroinflammation* 15:251. doi: 10.1186/s12974-018-1294-2

Marzec, J. M., Christie, J. D., Reddy, S. P., Jedlicka, A. E., Vuong, H., Lanken, P. N., et al. (2007). Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *FASEB J.* 21, 2237–2246. doi: 10.1096/fj.06-7759com

Matsuura, A., Ishima, T., Fujita, Y., Iwayama, Y., Hasegawa, S., Kawahara-Miki, R., et al. (2018). Dietary glucoraphanin prevents the onset of psychosis in the adult offspring after maternal immune activation. *Sci. Rep.* 8:2158. doi: 10.1038/s41598-018-20538-3

McCullumsmith, R. E., Hammond, J. H., Shan, D., and Meador-Woodruff, J. H. (2013). Postmortem brain: an underutilized substrate for studying severe mental illness. *Neuropsychopharmacology* 39, 65–87. doi: 10.1038/npp.2013.239

Mechawar, N., and Savitz, J. (2016). Neuropathology of mood disorders: do we see the stigmata of inflammation? *Transl. Psychiatry* 6:e946. doi: 10.1038/tp.2016.212

Mello, A. H., Gassenferth, A., Souza, L. R., Fortunato, J. J., and Rezin, G. T. (2014). ω-3 and major depression: a review. *Acta Neuropsychiatr.* 26, 178–185. doi: 10.1017/neu.2013.52

Mellon, S. H., Wolkowitz, O. M., Schonemann, M. D., Epel, E. S., Rosser, R., Burke, H. B., et al. (2016). Alterations in leukocyte transcriptional control pathway activity associated with major depressive disorder and antidepressant treatment. *Transl. Psychiatry* 6:e821. doi: 10.1038/tp.2016.79

Mendez-David, I., Tritschler, L., Ali, Z. E., Damiens, M. H., Pallardy, M., David, D. J., et al. (2015). Nrf2-signaling and BDNF: a new target for the antidepressant-like activity of chronic fluoxetine treatment in a mouse model

of anxiety/depression. *Neurosci. Lett.* 597, 121–126. doi: 10.1016/j.neulet.2015.04.036

Miller, A. H., Haroon, E., and Felger, J. C. (2017). Therapeutic implications of brain-immune interactions: treatment in translation. *Neuropsychopharmacology* 42, 334–359. doi: 10.1038/npp.2016.167

Miller, A. H., Maletic, V., and Raison, C. L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol. Psychiatry* 65, 732–741. doi: 10.1016/j.biopsych.2008.11.029

Miller, A. H., and Raison, C. L. (2016). The role of inflammation in depression: from evolutionary imperative to modern treatment target. *Nat. Rev. Immunol.* 16, 22–34. doi: 10.1038/nri.2015.5

Mills, E. A., Ogrodnik, M. A., Plave, A., and Mao-Draayer, Y. (2018). Emerging understanding of the mechanism of action for dimethyl fumarate in the treatment of multiple sclerosis. *Front. Neurol.* 9:5. doi: 10.3389/fneur.2018.00005

Murakami, K., and Sasaki, S. (2010). Dietary intake and depressive symptoms: a systemic review of observational studies. *Mol. Nutr. Food. Res.* 54, 471–488. doi: 10.1002/mnfr.200900157

Nestler, E. J., Barrot, M., DiLeone, R. J., Eisch, A. J., Gold, S. J., and Monteggia, L. M. (2002). Neurobiology of depression. *Neuron* 34, 13–25. doi: 10.1016/S0896-6273(02)00653-0

Nestler, E. J., and Hyman, S. E. (2010). Animal models of neuropsychiatric disorders. *Nat. Rev. Neurosci.* 13, 1161–1169. doi: 10.1038/nn.2647

O'Connell, M. A., and Hayes, J. D. (2015). The Keap1/Nrf2 pathway in health and disease: from the bench to the clinic. *Biochem. Soc. Trans.* 43, 687–689. doi: 10.1042/BST20150069

O'Connor, J. C., Lawson, M. A., André, C., Moreau, M., Lestage, J., Castanon, N., et al. (2009). Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. *Mol. Psychiatry* 14, 511–522. doi: 10.1038/sj.mp.4002148

O'Connor, R. M., and Cryan, J. F. (2014). Adolescent brain vulnerability and psychopathology through the generations: role of diet and dopamine. *Biol. Psychiatry* 75, 4–6. doi: 10.1016/j.biopsych.2013.10.022

Ohgi, Y., Futamura, T., Kikuchi, T., and Hashimoto, K. (2013). Effects of antidepressants on alternations in serum cytokines and depressive-like behavior in mice after lipopolysaccharide administration. *Pharmacol. Biochem. Behav.* 103, 853–859. doi: 10.1016/j.pbb.2012.12.003

Opie, A., O'Neil, R. S., Itsiopoulos, C., and Jacka, F. N. (2015). The impact of whole-of-diet interventions on depression and anxiety: a systematic review of randomised controlled trials. *Public Health Nutr.* 18, 2074–2093. doi: 10.1017/S1368980014002614

Paus, T., Keshavan, M., and Giedd, J. N. (2008). Why do many psychiatric disorders emerge during adolescence? *Nat. Rev. Neurosci.* 9, 947–957. doi: 10.1038/nrn2513

Pergola, P. E., Raskin, P., Toto, R. D., Meyer, C. J., Huff, J. W., Grossman, E. B., et al. (2011). Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N. Eng. J. Med.* 365, 327–336. doi: 10.1056/NEJMoa1105351

Raison, C. L., Lowry, C. A., and Rook, G. A. (2010). Inflammation, sanitation, and consternation: loss of contact with coevolved, tolerogenic microorganisms and the pathophysiology and treatment of major depression. *Arch. Gen. Psychiatry* 67, 1211–1224. doi: 10.1001/archgenpsychiatry.2010.161

Ren, D., Villeneuve, N. F., Jiang, T., Wu, T., Lau, A., Toppin, H. A., et al. (2011). Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1433–1438. doi: 10.1073/pnas.1014275108

Saghafian, F., Malmir, H., Saneei, P., Milajerdi, A., Larijani, B., and Esmaillzadeh, A. (2018). Fruit and vegetable consumption and risk of depression: accumulative evidence from an updated systematic review and meta-analysis of epidemiological studies. *Br. J. Nutr.* 119, 1087–1101. doi: 10.1017/S0007114518000697

Sarris, J., Logan, A. C., Akbaraly, T. N., Amminger, G. P., Balanzá-Martínez, V., Freeman, M. P., et al. (2015). Nutritional medicine as mainstream in psychiatry. *Lancet Psychiatry* 2, 271–274. doi: 10.1016/S2215-0366(14)00051-0

Sedlak, T. W., Nucifora, L. G., Koga, M., Shaffer, L. S., Higgs, C., Tanaka, T., et al. (2018). Sulforaphane augments glutathione and influences brain metabolites in human subjects: a clinical pilot study. *Mol. Neuropsychiatry* 3, 314–222. doi: 10.1159/000487639

Shelton, R. C., Claiborne, J., Sidoryk-Wegrzynowicz, M., Reddy, R., Aschner, M., Lewis, D. A., et al. (2011). Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. *Mol. Psychiatry* 16, 751–762. doi: 10.1038/mp.2010.52

Shiina, A., Kanahara, N., Sasaki, H., Oda, Y., Hashimoto, T., Hasegawa, T., et al. (2015). An open study of sulforaphane-rich broccoli sprout extract in patients with schizophrenia. *Clin. Psychopharmacol. Neurosci.* 13, 62–67. doi: 10.9758/cpn.2015.13.1.62

Shirai, Y., Fujita, Y., Hashimoto, R., Ohi, K., Yamamori, H., Yasuda, Y., et al. (2015). Dietary intake of sulforaphane-rich broccoli sprout extracts during juvenile and adolescence can prevent phencyclidine-induced cognitive deficits at adulthood. *PLoS One* 10:e0127244. doi: 10.1371/journal.pone.0127244

Singh, A., Venkannagari, S., Oh, K. H., Zhang, Y. Q., Rohde, J. M., Liu, L., et al. (2016). Small molecule inhibitor of NRF2 selectively intervenes therapeutic resistance in KEAP1-deficient NSCLC tumors. *ACS Chem. Biol.* 11, 3214–3225. doi: 10.1021/acschembio.6b00651

Singh, K., Connors, S. L., Macklin, E. A., Smith, K. D., Fahey, J. W., Talalay, P., et al. (2014). Sulforaphane treatment of autism spectrum disorder (ASD). *Proc. Natl. Acad. Sci. U.S.A.* 111, 15550–15555. doi: 10.1073/pnas.1416940111

Suzuki, T., Motohashi, H., and Yamamoto, M. (2013a). Toward clinical application of the Keap1-Nrf2 pathway. *Trends Pharmacol. Sci.* 34, 340–346. doi: 10.1016/j.tips.2013.04.005

Suzuki, T., Shibata, T., Takaya, K., Shiraishi, K., Kohno, T., Kunitoh, H., et al. (2013b). Regulatory nexus of synthesis and degradation deciphers cellular Nrf2 expression levels. *Mol. Cell. Biol.* 33, 2402–2412. doi: 10.1128/MCB.00065-13

Suzuki, T., and Yamamoto, M. (2015). Molecular basis of the Keap1-Nrf2 system. *Free Radic. Biol. Med.* 88, 93–100. doi: 10.1016/j.freeradbiomed.2015.06.006

Tsuchida, K., Tsujita, T., Hayashi, M., Ojima, A., Keleku-Lukwete, N., Katsuoka, F., et al. (2017). Halofuginone enhances the chemo-sensitivity of cancer cells by suppressing NRF2 accumulation. *Free Radic. Biol. Med.* 103, 236–247. doi: 10.1016/j.freeradbiomed.2016.12.041

Wang, X., Zhao, F., Wang, X., Niu, Y., Niu, L., and Wang, C. (2018). Recent advances in nutrition for the treatment of depressive disorder. *Curr. Pharm. Des.* doi: 10.2174/1381612824666180803113106 [Epub ahead of print].

Wang, Y. Y., Yang, Y. X., Zhe, H., He, Z. X., and Zhou, S. F. (2014). Bardoxolone methyl (CDDO-Me) as a therapeutic agent: an update on its pharmacokinetic and pharmacodynamic properties. *Drug Des. Devel. Ther.* 8, 2075–2088. doi: 10.2147/DDDT.S68872

Wardyn, J. D., Ponsford, A. H., and Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochem. Soc. Trans.* 43, 621–626. doi: 10.1042/BST20150014

Williams, B., and Dwyer, D. S. (2009). Structure-based discovery of low molecular weight compounds that stimulate neurite outgrowth and substitute for nerve growth factor. *J. Neurochem.* 110, 1876–1884. doi: 10.1111/j.1471-4159.2009.06291.x

World Health Organization [WHO] (2017). *Depression*. Available at: http://www.who.int/en/news-room/fact-sheets/detail/depression

Yamamoto, M., Kensler, T. W., and Motohashi, H. (2018). The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* 98, 1169–1203. doi: 10.1152/physrev.00023.2017

Yamamoto, T., Kyo, M., Kamiya, T., Tanaka, T., Engel, J. D., Motohashi, H., et al. (2006). Predictive base substitution rules that determine the binding and transcriptional specificity of Maf recognition elements. *Genes Cells* 11, 575–591. doi: 10.1111/j.1365-2443.2006.00965.x

Yang, B., Ren, Q., Zhang, J. C., Chen, Q. X., and Hashimoto, K. (2017). Altered expression of BDNF, BDNF pro-peptide and their precursor proBDNF in brain and liver tissues from psychiatric disorders: rethinking the brain-liver axis. *Transl. Psychiatry* 7:e1128. doi: 10.1038/tp.2017.95

Yang, B., Yang, C., Ren, Q., Zhang, J. C., Chen, Q. X., Shirayama, Y., et al. (2016). Regional differences in the expression of brain-derived neurotrophic factor (BDNF) pro-peptide, proBDNF and preproBDNF in the brain confer stress resilience. *Eur. Arch. Psychiatry Clin. Neurosci.* 266, 765–769. doi: 10.1007/s00406-016-0693-6

Yang, C., Cheng, Y., Zhao, J., and Rong, J. (2015c). Releasing Nrf2 to promote neurite outgrowth. *Neural. Regen. Res.* 10, 1934–1935. doi: 10.4103/1673-5374.169618

Yang, C., Shirayama, Y., Zhang, J. C., Ren, Q., and Hashimoto, K. (2015a). Peripheral interleukin-6 promotes resilience versus susceptibility to

inescapable stress. *Acta Neuropsychiatr.* 27, 312–316. doi: 10.1017/neu.2015.36

Yang, C., Shirayama, Y., Zhang, J. C., Ren, Q., and Hashimoto, K. (2015b). Regional differences in brain-derived neurotrophic factor levels and dendritic spine density confer resilience to inescapable stress. *Int. J. Neuropsychopharmacol.* 18:yu121. doi: 10.1093/ijnp/pyu121

Yang, M., Dang, R., Xu, P., Guo, Y., Han, W., Liao, D., et al. (2018). Dl-3-n-Butylphthalide improves lipopolysaccharide-induced depressive-like behavior in rats: involvement of Nrf2 and NF-κB pathways. *Psychopharmacology* 235, 2573–2585. doi: 10.1007/s00213-018-4949-x

Yao, W., Zhang, J. C., Dong, C., Zhuang, C., Hirota, S., Inanaga, K., et al. (2015). Effects of amycenone on serum levels of tumor necrosis factor-α, interleukin-10, and depression-like behavior in mice after lipopolysaccharide administration. *Pharmacol. Biochem. Behav.* 136, 7–12. doi: 10.1016/j.pbb.2015.06.012

Yao, W., Zhang, J. W., Ishima, T., Dong, C., Yang, C., Ren, Q., et al. (2016a). Antidepressant effects of TBE-31 and MCE-1, the novel Nrf2 activators, in an inflammation model of depression. *Eur. J. Pharmacol.* 793, 21–27. doi: 10.1016/j.ejphar.2016.10.037

Yao, W., Zhang, J. W., Ishima, T., Dong, C., Yang, C., Ren, Q., et al. (2016b). Role of Keap1-Nrf2 signaling in depression and dietary intake of glucoraphanin confers stress resilience in mice. *Sci. Rep.* 6:30659. doi: 10.1038/srep30659

Young, J. J., Bruno, D., and Pomara, N. (2014). A review of the relationship between pro-inflammatory cytokines and major depressive disorder. *J. Affect. Disord.* 169, 15–20. doi: 10.1016/j.jad.2014.07.032

Yu, C., Pan, S., Dong, M., and Niu, Y. (2017). Astragaloside IV attenuates lead acetate-induced inhibition of neurite outgrowth through activation of Akt-dependent Nrf2 pathway in vitro. *Biochem. Biophys. Acta* 1863, 1195–1203. doi: 10.1016/j.bbadis.2017.03.006

Zhang, J. C., Wu, J., Fujita, Y., Yao, W., Ren, Q., Yang, C., et al. (2014). Antidepressant effects of TrkB ligands on depression-like behavior and dendritic changes in mice after inflammation. *Int. J. Neuropsychopharmacol.* 18:yu077. doi: 10.1093/ijnp/pyu077

Zhang, J. C., Yao, W., Dong, C., Han, M., Shirayama, Y., and Hashimoto, K. (2018). Keap1-Nrf2 signaling pathway confers resilience versus susceptibility to inescapable electric stress. *Eur. Arch. Psychiatry Clin. Neurosci.* doi: 10.1007/s00406-017-0848-0 [Epub ahead of print].

Zhang, J. C., Yao, W., Dong, C., Yang, C., Ren, Q., Ma, M., et al. (2017a). Blockade of interleukin-6 receptor in the periphery promotes rapid and sustained antidepressant actions: a possible role of gut-microbiota-brain axis. *Transl. Psychiatry* 7:e1138. doi: 10.1038/tp.2017.112

Zhang, J. C., Yao, W., Dong, C., Yang, C., Ren, Q., Ma, M., et al. (2015). Comparison of ketamine, 7,8-dihydroxyflavone, and ANA-12 antidepressant effects in the social defeat stress model of depression. *Psychopharmacology* 232, 4325–4335. doi: 10.1007/s00213-015-4062-3

Zhang, J. C., Yao, W., Dong, C., Yang, C., Ren, Q., Ma, M., et al. (2017b). Prophylactic effects of sulforaphane on depression-like behavior and dendritic changes in mice after inflammation. *J. Nutr. Biochem.* 39, 134–144. doi: 10.1016/j.jnutbio.2016.10.004

Zhang, J. C., Yao, W., and Hashimoto, K. (2016). Brain-derived neurotrophic factor (BDNF) – TrkB signaling in inflammation-related depression and potential therapeutic targets. *Curr. Neuropharmacol.* 14, 721–731. doi: 10.2174/1570159X14666160119094646

Zhang, Y., Talalay, P., Cho, C. G., and Posner, G. H. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U.S.A.* 89, 2399–2403. doi: 10.1073/pnas.89.6.2399

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frontiers in Pharmacology

MINI REVIEW
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Oxidative Stress in Age-Related Macular Degeneration: Nrf2 as Therapeutic Target

*Ilaria Bellezza**

Department of Experimental Medicine, University of Perugia, Perugia, Italy

Age-related macular degeneration is one of the leading causes of vision loss in the elderly. Genetics, environmental insults, and age-related issues are risk factors for the development of the disease. All these risk factors are linked to the induction of oxidative stress. In young subjects retinal pigment epithelial cells mitigate reactive oxygen generation by the elimination of dysfunctional mitochondria, via mitophagy, and by increasing antioxidant defenses via Nrf2 activation. The high amount of UV light absorbed by the retina, together with cigarette smoking, cooperate with the aging process to increase the amount of reactive oxygen species generated by retinal pigment epithelium where oxidative stress arises. Moreover, in the elderly both the mitophagic process and Nrf2 activation are impaired thus causing retinal cell death. This review will focus on the impact of oxidative stress on the pathogenesis of age-related macular degeneration and analyze the natural and synthetic Nrf2-activating compounds that have been tested as potential therapeutic agents for the disease.

Keywords: oxidative stress, light-induced photooxidative damage, cigarette smoke, aging, Nrf2 activators

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Soon Yew Tang, University of Pennsylvania, United States

***Correspondence:**
Ilaria Bellezza
ilaria.bellezza@unipg.it

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INTRODUCTION

The retina is a multilayered sensory structure that lines the inner surface of the back of the globe of the eye. The macula is a well-defined region of the retina with an approximate size of 0.6 mm devoted to the "high definition vision." It can be subdivided into two zones: a central zone, the fovea, and a peripheral zone, the parafovea. The fovea contains a high percentage of cones, the photoreceptor cells devoted to the photopic vision, i.e., vision under well lit conditions; the parafovea is rich in rods, the photoreceptor cells devoted to night vision. The interplay between the macula's cells guarantees central vision acuity which is indispensable for the most common daily activities (Datta et al., 2017).

The external layer of the retina is the retinal pigment epithelium (RPE) composed of highly specialized, polarized epithelial cells whose apical side is in contact with photoreceptor outer segments and basal side is in contact with Burch's membrane, the internal layer of the choroid. RPE is important for the health of phototeceptor cells. Indeed, RPE cells phagocytose photoreceptor outer segments daily to guarantee their optimal functionality. Moreover, RPE cells transport metabolic waste through Burch's membrane to the choroid to be eliminated (Kevany and Palczewski, 2010).

Age related macular degeneration (AMD), the principal cause of blindness in western countries (Congdon et al., 2004), is caused by the loss of RPE cells and photoreceptors in large zones of the macula. AMD is characterized by the presence of extracellular depositions, known as

drusen accumulating between Burch's membrane and the RPE. Advanced glycation end-products (AGEs) and carboxyethylpyrrole adducts (produced by the oxidative modification of fatty acids in photoreceptor tips) have been found in drusen isolated from AMD samples. The presence of these molecules, related to oxidative damage (Farboud et al., 1999; Crabb et al., 2002), underpins the concept that oxidative stress plays a major role in AMD pathogenesis and progression (Beatty et al., 2000). It is worth noting that AGEs can be recognized by receptor for advanced glycation endproducts (RAGE), a transmembrane receptor that exerts pro-inflammatory functions through nuclear factor-κB (NF-κB) signaling (Lin, 2006), thus implicating inflammation as another pathogenic causes of AMD.

Based on these premises, this review describes how oxidative stress contributes to macular degeneration and the effects of pharmacologically induced antioxidant defenses.

OXIDATIVE STRESS AND AMD

During cellular metabolism living organisms produce reactive oxygen species (ROS) from molecular oxygen. The major site of ROS production is the mitochondrial electron transport chain where some electrons leak from the transport process and spontaneously react with molecular oxygen, producing superoxide anion. Other enzymes, such as NADPH oxidase and xanthine oxidase contribute to ROS generation (Birben et al., 2012).

Reactive oxygen species levels are strictly regulated to maintain cellular homeostasis. Oxidative stress (OS) refers to a condition in which ROS levels accumulate to the extent that cellular macromolecules are damaged and apoptosis ensues (Birben et al., 2012). On the other hand, reductive stress is the name given to the condition when ROS levels are too low, such that the normal functions of the cell are affected. In homeostatic conditions, ROS are maintained at levels that support the normal cell functioning and guarantee redox signaling (Bellezza et al., 2018). The correct levels of ROS are underwritten by the antioxidant system, which is compromised of enzymes and non-enzymatic molecules. Non-enzymatic antioxidants include low-molecular-weight compounds, i.e., vitamins C and E, β-carotene, and glutathione. The majority of the enzymatic antioxidant defenses, i.e., SODs, catalase, and the enzymes responsible for glutathione metabolism, are regulated at transcriptional levels by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Bellezza et al., 2010) (see below).

The main risk factors for the development of AMD are aging, ethnicity, genetics and environmental insults, including cigarette smoking, high fat diet and light-induced photooxidative reactions (Organisciak and Vaughan, 2010; Datta et al., 2017). Aging, cigarette smoking and photo-oxidative reactions share the capacity to increase in ROS generation and promote OS.

Light-Induced Oxidative Stress

Light is an electromagnetic radiation which can be translated into a visual information by complex interactions between the eye and the brain. Only a portion of the electromagnetic spectrum interacts with the eye and includes wavelengths from ultraviolet (100–400 nm) to infrared (above 760 nm) (Ivanov et al., 2018).

As long ago as 1966 Noell and co-workers theorized light damage hypotheses, including the occurrence of light-induced oxidative reactions. In particular, ultraviolet and blue light are considered responsible for the retinal damage associated with AMD (Noell et al., 1966; Chalam et al., 2011). It is predicted that increasing exposure to video displays and the use of light-emitting diodes (LEDs) as light sources will increase the contribution of blue light-induced phototoxicity to human retinal diseases (Jaadane et al., 2015). Photo-oxidative damage occurs when light interacts with an endogenous chromophore in the ocular tissue including visual pigments, proteins, flavoproteins and the naturally occurring pigment granules of melanin and lipofuscin in the RPE (Ivanov et al., 2018). The absorption of light by chromophores causes their excitation to a triplet state that, being a highly reactive, rapidly interact with other molecules, including molecular oxygen thus leading to generation of ROS (Chalam et al., 2011; Ivanov et al., 2018). Therefore, OS has been identified as one of the major players in light-induced cellular stress.

Exposure to ultraviolet radiation induces degeneration of RPE mitochondria, known to increase ROS generation, accompanied by a reduction in ATP generation. Since one of the major function of RPE is the phagocytosis of photoreceptor outer segments after photoactivation, a decrease in ATP generation might be responsible for a reduction in RPE phagocytic ability that culminates in RPE hyperpigmentation, a risk factor for AMD (Chalam et al., 2011). Moreover, ultraviolet radiation drives the upregulation of pro-inflammatory molecules through NF-κB activation, a condition that may accelerate drusen formation (Chalam et al., 2011).

Oxidative Stress and Aging

The aging process is defined as the sum of deteriorative alterations that decreases both the fitness of the organism and the ability to maintain homeostasis (Finch and Ruvkun, 2001). The "free radical theory" of aging, proposed in 1956 by Harman (Harman, 1956), states that the accumulation of free radicals during lifespan leads to the accrual of oxidative damage to various classes of macromolecules that, in the end, is responsible for the decline in the physiological fitness of the organism. This was followed by the "oxidative stress theory" of aging stating that the aging process is driven by an imbalance between pro-oxidant species and antioxidant defenses (Sies and Cadenas, 1985). It is important to note, however, that ROS are not only hazardous molecules causing OS, but have a fundamental role in cellular signaling, namely redox signaling, that ensure correct cellular functions. The concept of reductive stress, defined as a condition of sustained increase in cellular reducing equivalents, associated with excessive Nrf2 activation, has recently come to the attention of the scientific community (Bellezza et al., 2018). For these reasons the "redox stress hypothesis" of aging has recently been proposed and states that aging-associated functional declines is primarily driven by a progressive disruption of the redox-regulated signaling mechanisms (Sohal and Orr, 2012).

An increase in oxidative modifications of macromolecules and a concomitant decrease in the antioxidant defenses are associated to the aging process (excellently reviewed in Sohal and Orr, 2012; Jacob et al., 2013; Ewald, 2018). The leakage of electrons from the electron transport chain might increase with age, explaining the age-related increase in ROS generation (Cadenas and Davies, 2000; Sohal and Orr, 2012). In addition, a diminished antioxidant capacity and an impaired adaptive induction of antioxidants has been observed during the aging process (Zhang et al., 2015).

Retinal pigment epithelium, having a high metabolic activity, possesses an elevated number of mitochondria (Datta et al., 2017) to generate enough ATP to accomplish all its physiological functions. Therefore, the age-related mitochondrial malfunctioning can increase OS in the RPE thus leading to AMD (Golestaneh et al., 2016).

Aging is also associated with a chronic low-grade inflammation, known as inflammageing, a condition characterized by elevated levels of inflammatory markers that provides high susceptibility to morbidity, including AMD (Zhuang and Lyga, 2014).

Also a role for cellular senescence of the RPE in the etiology of AMD has been proposed (Kozlowski, 2012). Cell senescence, i.e., the state of permanent cellular division arrest, has been involved in aging and in age-related diseases. Senescent cells has been found in eye diseases such as cataracts, and glaucoma (Naylor et al., 2013). Mitochondrial ROS have a causative role in cellular senescence and exposure to pro-oxidants induce the senescence process in proliferating human RPE *in vitro* (Blasiak et al., 2017).

The eye contains a circadian system and aging affects the circadian rhythm of the retina (Baba and Tosini, 2018). As an example, melatonin through melatonin receptors, regulates the daily rhythm of photoreceptor phagocytosis and melatonine receptors knock-out mice showed lipofuscin accumulation in the RPE (Laurent et al., 2017). These results suggest that alterations in the circadian rhythm can be involved in AMD pathogenesis, but more research in this area is warranted. Circadian clock regulates the expression of half of the mammalian protein which, in turn, are involved in drug transport/metabolism or are drug targets themselves (Ruben et al., 2018), thus circadian rhythm can be considered as target for AMD therapy.

CIGARETTE SMOKING AND OXIDATIVE STRESS

Cigarette smoke is a strong oxidant composed of approximately 4700 chemical components including ROS, epoxides, peroxides, nitric oxide, peroxynitrite (Rahman and MacNee, 1996). Although cigarette smoking is one of the principal non-genetic factors associated with AMD pathogenesis, a direct damage of the RPE cells by cigarette smoke has been demonstrated only in 2008 by Fujihara and co-workers (Fujihara et al., 2008; Cano et al., 2010). Mice exposed to 6 months of cigarette smoke in a chamber that produces emphysema with evidence of oxidative damage, also develop RPE apoptosis and basal drusen-like deposits. Moreover, cigarette smoke extract mediates autophagy-impairment in RPE cells and affect cell viability by inducing ROS generation (Govindaraju et al., 2017). Cigarette smoke extract and one of its components, 2-ethylpyridine, enhance mitochondrial fragmentation and dysfunction (Mansoor et al., 2014; Huang et al., 2015) suggesting a potential role for cigarette smoking in the reduced phagocytic capacity associated with AMD.

Moreover, exposure to cigarette smoke results in production and release of pro-inflammatory molecules by immune cells via the activation of the NF-κB pathway (Rom et al., 2013; Marinucci et al., 2018).

ANTIOXIDANT DEFENSES

Retinal pigment epithelium redox homeostasis relies on the activation of the transcription factor Nrf2. Under basal conditions, Nrf2 activity is maintained at low levels by the binding to its inhibitor Kelch ECH-associated protein 1 (Keap1), a Cul3-based E3 ligase that polyubiquitinates Nrf2 leading to its constitutive degradation by the proteasome. The low basal Nrf2 activity allows the maintenance of redox homeostasis. Under OS conditions, two redox-sensitive cysteine residues of Keap1 become oxidized resulting in the inhibition of its ubiquitin ligase activity. As a consequence, newly synthesized Nrf2 is not degraded, and translocates to the nucleus where it binds to the antioxidant and electrophilic responsive element (ARE/EpRE) sequences on the regulatory region of target genes leading to the induction of the antioxidant response (Bellezza et al., 2018). Besides Keap1, other two ubiquitin ligase complexes can regulate Nrf2 activation, i.e., F-box/WD repeat-containing protein 1A (βTRCP) and synoviolin (HRD1) (Tebay et al., 2015; Rojo de la Vega et al., 2018), whose possible involvement in AMD is worthy of investigation. It is also to note that expression of Nrf2 can be controlled by the molecular clock protein, BMAL1, (Early et al., 2018).

Due to the high amount of ROS produced in the retina, the RPE has adapted to life under OS conditions (Handa, 2012). The presence of several chromophores in the retina can provide protection against light induced damage by absorbing excess light energy. In the aging RPE, an accumulation of melanofuscine granules, containing both melanin and lipofuscin, has been observed, and this phenomenon correlates with AMD development (Chalam et al., 2011). Moreover, as discussed above, mitochondria are the main source of ROS in the RPE and their leakage increases with aging. To maintain the correct cell functions, the RPE removes damaged or malfunctioning mitochondria through the process of mitophagy, a mitochondrial-specific type of autophagy. It has been hypothesized that mitophagy impairment may play a role in AMD pathogenesis (Hyttinen et al., 2018). The outer mitochondrial membrane proteins B-cell leukemia/lymphoma 2 (BCL-2)/adenovirus E1B interacting protein 3 (BNIP3) and Nip-like protein X (NIX) cause an increase in ROS generation which, in turn, induces mitochondrial depolarization, autophagy and mitophagy (Vande Velde et al., 2000; Ding et al., 2010; Ney, 2015). However, a role for BNIP3 and NIX in AMD is still to be elucidated.

The accumulation of oxidatively damaged molecules found in AMD suggests that the antioxidant defense cannot cope with the increasing amount of ROS (Datta et al., 2017). Mounting evidence suggests that aging induces a decline in the antioxidant capacity via a reduction in Nrf2 signaling (Sachdeva et al., 2014; Zhang et al., 2015). Nrf2 activation has been linked to mitochondrial structural and functional integrity where its role is of particular importance under stressful conditions (Dinkova-Kostova and Abramov, 2015). Furthermore, the known cross-talk between Nrf2 and NF-κB implicates that a decline in Nrf2 signaling in exacerbated NF-κB activation further increasing inflammation (Bellezza et al., 2010, 2018; Wardyn et al., 2015).

Nrf2 ACTIVATING COMPOUNDS IN AMD THERAPY

Awareness of the importance of Nrf2 in retinal disease came from the finding that Nrf2-deficient mice develop ocular pathology similar to human AMD (Zhao et al., 2011).

Different types of stresses have been employed to induce OS in retinal epithelial cells such as UV exposure, hydrogen peroxide, or acrolein, a component of cigarette smoke. In these experimental settings several known or potential antioxidant compounds have been tested either *in vitro* or *in vivo* and a number of clinical trials have investigated the effects of antioxidants on AMD progression (Nakagami, 2016).

The first experimental evidence that Nrf2 activation can protect the RPE from photooxidative damage came in 2004 when Gao and Talalay demonstrated that sulforaphane, contained in broccoli and cabbages, protects human adult RPE cells from ultraviolet light-induced damage by increasing Nrf2-regulated glutathione levels and NAD(P)H:quinone oxidoreductase activity (Gao and Talalay, 2004). Sulforaphane protects mouse retina from ultraviolet light by upregulating the expression of thioredoxin, an antioxidant protein whose expression is driven by Nrf2. Moreover, sulforaphane exerted protection of ARPE-19 cell line exposed to 400 μM H_2O_2 by up-regulating the translation of thioredoxin and Nrf2 (Tanito et al., 2005) and has been suggested to promote regeneration of retinal cells (Dulull et al., 2018). Recently the protective effect of sulforaphane on RPE cells was correlated with preventing mitochondrial fission independently of Nrf2 activation (O'Mealey et al., 2017).

Curcumin, a natural compound found in *Curcuma longa*, protects ARPE-19 cells from up to 1mM H_2O_2 exposure by inducing the Nrf2 driven gene hemeoxygenase 1 (HO-1) (Mandal et al., 2009) and a curcumin analog, 1, 5-bis (2-trifluoromethylphenyl)-1, 4-pentadien-3-one, exerted protection against acrolein-induced oxidative damage by inducing Nrf2 (Li et al., 2013). Moreover, curcumin fed rats (0.2% for 2 weeks) were protected from light-induced retinal degeneration by down-regulating inflammatory genes via NF-κB inhibition (Mandal et al., 2009).

The carotenoids zeaxanthin and lutein preserve photoreceptors against light damage by mitigating OS

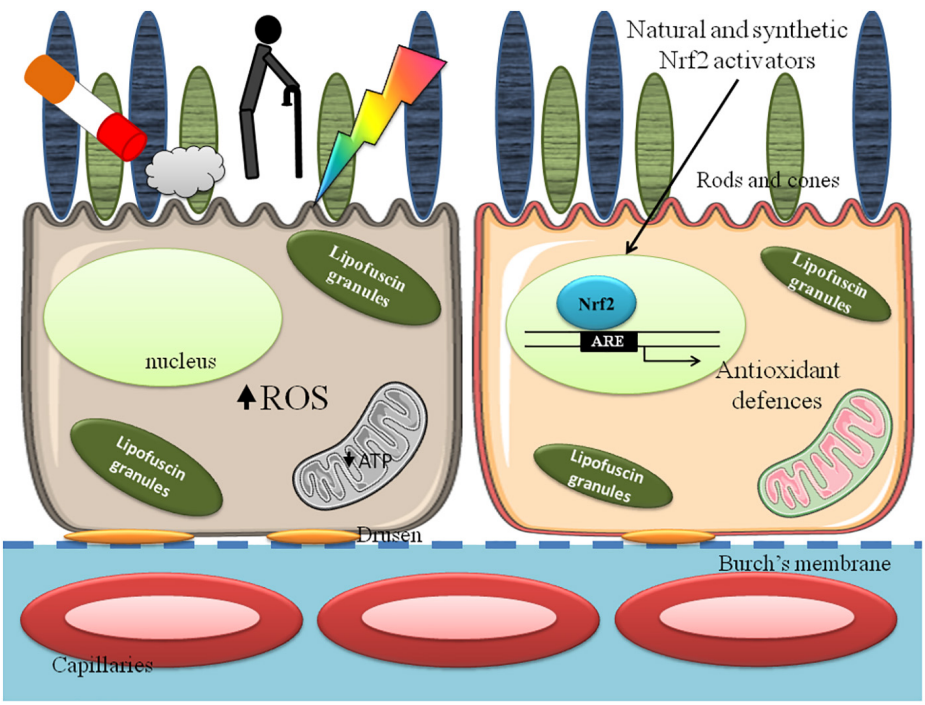

FIGURE 1 | Retinal pigment epithelium (RPE) in age related macular degeneration (AMD). Cigarette smoke, aging and light absorption increase reactive oxygen species (ROS) formation and decrease mitochondrial function, lowering ATP synthesis and affect RPE cell functions **(Left)**. The exposure to Nrf2 activating compounds increases antioxidant defenses and ameliorate mitochondrial and cellular functions **(Right)**.

(Yu et al., 2018). It has been shown that lutein activates Nrf2 in ARPE-19 cells (Frede et al., 2017) and that mesozeaxanthin protects against chronic and cumulative eye damage by reducing OS (Orhan et al., 2016). A prospective, randomized controlled study with 114 early AMD patients demonstrated that 25 g of Goji berries supplementation per day for 90d improves macular pigment optical density by increasing serum zaxantin levels (Li et al., 2018) and, as excellently reviewed by Buscemi and co-workers high lutein intake, either through diet or as nutritional supplement, has beneficial effects on AMD (Buscemi et al., 2018). Lutein and Zeaxantin can also reduce NF-κB activation in the retina (Tuzcu et al., 2017).

Carnosic acid from *Rosmarinus officinalis* and *Salvia officinalis*, salvianolic acids from Radix *Salvia miltiorrhiza,* mangostin from *Garcinia mangostana,* taxifolin a flavonol from conifers, were all effective against OS damages in retinal cells via activation of Nrf2 (Rezaie et al., 2012; Zhang et al., 2014; Liu et al., 2016b; Xie et al., 2017).

Also the Mediterranean diet, characterized by high consumption of plant foods, olive oil as primary fat source, and moderate consumption of wine (Willett et al., 1995) can be regarded as an Nrf2 activator (Martucci et al., 2017). A prospective cohort study of the Rotterdam Study I (RS-I) and the Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires (Alienor) Study showed that higher adherence to the Mediterranean diet is associated with a reduced risk of advanced AMD (Merle et al., 2018). Furthermore, a nested case–control study within the Coimbra Eye Study demonstrated that high adherence to Mediterranean diet confers protection against the development of AMD (Raimundo et al., 2018).

The beneficial effects of Nrf2 activation on retinal cells has led to the synthesis of Nrf2 activators such as RS9 (Nakagami et al., 2015). RS9 decreases light-induced retinal cell death *in vivo* and *in vitro* (Inoue et al., 2017). Very recently it has been reported that RS9 protects RPE cells from sodium iodate-induced OS and adult zebrafish retina from light-induced damage by increasing Nrf2-dependent HO-1 expression (Saito et al., 2018). RTA 408, a synthetic triterpenoid able to activate Nrf2, protects cultured RPE cells from OS (Liu et al., 2016a). Investigation of other molecules that exert cytoprotection by activating Nrf2 and potentially by inhibiting NF-κB (Bellezza et al., 2014; Grottelli et al., 2016) are warranted to ascertain vision preservation in early AMD patients.

CONCLUSION

Oxidative stress is an important contributor of AMD, and Nrf2 activation exerts protective effects that can be enhanced by pharmacologic meaning (**Figure 1**).

An increase of the antioxidant defenses can provide novel and effective therapeutic strategies for this disease. However, it will be important to apply Nrf2 activators mindful of the concept of redox homeostasis, since there is a fine line between beneficial and potentially damaging effects of Nrf2 activation. However, adverse systemic effects during the treatment of ocular diseases might be minimized by local pharmacological intervention such as intravitreal injections or by the use of eye drops.

AUTHOR CONTRIBUTIONS

IB conceived the work, analyzed bibliogaphical data, and wrote the manuscript.

REFERENCES

Baba, K., and Tosini, G. (2018). Aging alters circadian rhythms in the mouse eye. *J. Biol. Rhythms* 33, 441–445. doi: 10.1177/0748730418783648

Beatty, S., Koh, H., Phil, M., Henson, D., and Boulton, M. (2000). The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv. Ophthalmol.* 45, 115–134. doi: 10.1016/S0039-6257(00)00140-5

Bellezza, I., Giambanco, I., Minelli, A., and Donato, R. (2018). Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim. Biophys. Acta* 1865, 721–733. doi: 10.1016/j.bbamcr.2018.02.010

Bellezza, I., Grottelli, S., Mierla, A. L., Cacciatore, I., Fornasari, E., Roscini, L., et al. (2014). Neuroinflammation and endoplasmic reticulum stress are coregulated by cyclo(His-Pro) to prevent LPS neurotoxicity. *Int. J. Biochem. Cell Biol.* 51, 159–169. doi: 10.1016/j.biocel.2014.03.023

Bellezza, I., Mierla, A. L., and Minelli, A. (2010). Nrf2 and NF-κB and their concerted modulation in cancer pathogenesis and progression. *Cancers* 2, 483–497. doi: 10.3390/cancers2020483

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., and Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organ. J.* 5, 9–19.

Blasiak, J., Piechota, M., Pawlowska, E., Szatkowska, M., Sikora, E., and Kaarniranta, K. (2017). Cellular senescence in age-related macular degeneration: can autophagy and DNA damage response play a role? *Oxid Med. Cell. Longev.* 2017:5293258. doi: 10.1155/2017/5293258

Buscemi, S., Corleo, D., Di Pace, F., Petroni, M. L., Satriano, A., and Marchesini, G. (2018). The effect of lutein on eye and extra-eye health. *Nutrients* 10:E1321. doi: 10.3390/nu10091321

Cadenas, E., and Davies, K. J. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* 29, 222–230. doi: 10.1016/S0891-5849(00)00317-8

Cano, M., Thimmalappula, R., Fujihara, M., Nagai, N., Sporn, M., Wang, A. L., et al. (2010). Cigarette smoking, oxidative stress, the anti-oxidant response through Nrf2 signaling, and age-related macular degeneration. *Vis. Res.* 50, 652–664. doi: 10.1016/j.visres.2009.08.018

Chalam, K. V., Khetpal, V., Rusovici, R., and Balaiya, S. (2011). A review: role of ultraviolet radiation in age-related macular degeneration. *Eye Contact Lens* 37, 225–232. doi: 10.1097/ICL.0b013e31821fbd3e

Congdon, N., O'Colmain, B., Klaver, C. C., Klein, R., Munoz, B., Friedman, D. S., et al. (2004). Causes and prevalence of visual impairment among adults in the United States. *Arch. Ophthalmol.* 122:477–485. doi: 10.1001/archopht.122.4.477

Crabb, J. W., Miyagi, M., Gu, X., Shadrach, K., West, K. A., Sakaguchi, H., et al. (2002). Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14682–14687. doi: 10.1073/pnas.222551899

Datta, S., Cano, M., Ebrahimi, K., Wang, L., and Handa, J. T. (2017). The impact of oxidative stress and inflammation on RPE degeneration in non-neovascular AMD. *Prog. Retin. Eye Res.* 60, 201–218. doi: 10.1016/j.preteyeres.2017.03.002

Ding, W. X., Ni, H. M., Li, M., Liao, Y., Chen, X., Stolz, D. B., et al. (2010). Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated

mitochondrial priming. *J. Biol. Chem.* 285, 27879–27890. doi: 10.1074/jbc.M110.119537

Dinkova-Kostova, A. T., and Abramov, A. Y. (2015). The emerging role of Nrf2 in mitochondrial function. *Free Radic. Biol. Med.* 88(Pt B), 179–188. doi: 10.1016/j.freeradbiomed.2015.04.036

Dulull, N. K., Dias, D. A., Thrimawithana, T. R., and Kwa, F. A. A. (2018). L-Sulforaphane confers protection against oxidative stress in an in vitro model of age-related macular degeneration. *Curr. Mol. Pharmacol.* 11, 237–253. doi: 10.2174/1874467211666180125163009

Early, J. O., Menon, D., Wyse, C. A., Cervantes-Silva, M. P., Zaslona, Z., Carroll, R. G., et al. (2018). Circadian clock protein BMAL1 regulates IL-1β in macrophages via NRF2. *Proc. Natl. Acad. Sci. U.S.A.* 115, E8460–E8468. doi: 10.1073/pnas.1800431115

Ewald, C. Y. (2018). Redox signaling of NADPH oxidases regulates oxidative stress responses, immunity and aging. *Antioxidants* 7:E130. doi: 10.3390/antiox7100130

Farboud, B., Aotaki-Keen, A., Miyata, T., Hjelmeland, L. M., and Handa, J. T. (1999). Development of a polyclonal antibody with broad epitope specificity for advanced glycation endproducts and localization of these epitopes in Bruch's membrane of the aging eye. *Mol. Vis.* 5:11.

Finch, C. E., and Ruvkun, G. (2001). The genetics of aging. *Annu. Rev. Genomics Hum. Genet.* 2, 435–462. doi: 10.1146/annurev.genom.2.1.435

Frede, K., Ebert, F., Kipp, A. P., Schwerdtle, T., and Baldermann, S. (2017). Lutein activates the transcription factor NRF2 in human retinal pigment epithelial cells. *J. Agric. Food Chem.* 65, 5944–5952. doi: 10.1021/acs.jafc.7b01929

Fujihara, M., Nagai, N., Sussan, T. E., Biswal, S., and Handa, J. T. (2008). Chronic cigarette smoke causes oxidative damage and apoptosis to retinal pigmented epithelial cells in mice. *PLoS One* 3:e3119. doi: 10.1371/journal.pone.0003119

Gao, X., and Talalay, P. (2004). Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10446–10451. doi: 10.1073/pnas.0403886101

Golestaneh, N., Chu, Y., Cheng, S. K., Cao, H., Poliakov, E., and Berinstein, D. M. (2016). Repressed SIRT1/PGC-1α pathway and mitochondrial disintegration in iPSC-derived RPE disease model of age-related macular degeneration. *J Transl. Med.* 14:344. doi: 10.1186/s12967-016-1101-8

Govindaraju, V. K., Bodas, M., and Vij, N. (2017). Cigarette smoke induced autophagy-impairment regulates AMD pathogenesis mechanisms in ARPE-19 cells. *PLoS One* 12:e0182420. doi: 10.1371/journal.pone.0182420

Grottelli, S., Ferrari, I., Pietrini, G., Peirce, M. J., Minelli, A., and Bellezza, I. (2016). The role of cyclo(His-Pro) in neurodegeneration. *Int. J. Mol. Sci.* 17:E1332. doi: 10.3390/ijms17081332

Handa, J. T. (2012). How does the macula protect itself from oxidative stress? *Mol. Aspects Med.* 33, 418–435. doi: 10.1016/j.mam.2012.03.006

Harman, D. (1956). Aging:A theory based on free radical and radiation chemistry. *J. Gerontol.* 11, 298–300. doi: 10.1093/geronj/11.3.298

Huang, C., Wang, J. J., Ma, J. H., Jin, C., Yu, Q., and Zhang, S. X. (2015). Activation of the UPR protects against cigarette smoke-induced RPE apoptosis through up-regulation of Nrf2. *J. Biol. Chem.* 290, 5367–5380. doi: 10.1074/jbc.M114.603738

Hyttinen, J. M. T., Viiri, J., Kaarniranta, K., and Błasiak, J. (2018). Mitochondrial quality control in AMD: does mitophagy play a pivotal role? *Cell Mol. Life Sci.* 75, 2991–3008. doi: 10.1007/s00018-018-2843-7

Inoue, Y., Shimazawa, M., Noda, Y., Nagano, R., Otsuka, T., Kuse, Y., et al. (2017). RS9, a novel Nrf2 activator, attenuates light-induced death of cells of photoreceptor cells and Müller glia cells. *J. Neurochem.* 141, 750–765. doi: 10.1111/jnc.14029

Ivanov, I. V., Mappes, T., Schaupp, P., Lappe, C., and Wahl, S. (2018). Ultraviolet radiation oxidative stress affects eye health. *J Biophotonics.* 11:e201700377. doi: 10.1002/jbio.201700377

Jaadane, I., Boulenguez, P., Chahory, S., Carré, S., Savoldelli, M., Jonet, L., et al. (2015). Retinal damage induced by commercial light emitting diodes (LEDs). *Free Radic. Biol. Med.* 84, 373–384. doi: 10.1016/j.freeradbiomed.2015.03.034

Jacob, K. D., Noren Hooten, N., Trzeciak, A. R., and Evans, M. K. (2013). Markers of oxidant stress that are clinically relevant in aging and age-related disease. *Mech. Ageing Dev.* 134, 139–157. doi: 10.1016/j.mad.2013.02.008

Kevany, B. M., and Palczewski, K. (2010). Phagocytosis of retinal rod and cone photoreceptors. *Physiology* 25, 8–15. doi: 10.1152/physiol.00038.2009

Kozlowski, M. R. (2012). RPE cell senescence: a key contributor to age-related macular degeneration. *Med. Hypotheses* 78, 505–510. doi: 10.1016/j.mehy.2012.01.018

Laurent, V., Sengupta, A., Sánchez-Bretaño, A., Hicks, D., and Tosini, G. (2017). Melatonin signaling affects the timing in the daily rhythm of phagocytic activity by the retinal pigment epithelium. *Exp. Eye Res.* 165, 90–95. doi: 10.1016/j.exer.2017.09.007

Li, S., Liu, N., Lin, L., Sun, E. D., Li, J. D., and Li, P. K. (2018). Macular pigment and serum zeaxanthin levels with Goji berry supplement in early age-related macular degeneration. *In.t J. Ophthalmol.* 11, 970–975.

Li, Y., Zou, X., Cao, K., Xu, J., Yue, T., Dai, F., et al. (2013). Curcumin analog 1, 5-bis (2-trifluoromethylphenyl)-1, 4-pentadien-3-one exhibits enhanced ability on Nrf2 activation and protection against acrolein-induced ARPE-19 cell toxicity. *Toxicol. Appl. Pharmacol.* 272, 726–735. doi: 10.1016/j.taap.2013.07.029

Lin, L. (2006). RAGE on the Toll Road? *Cell Mol. Immunol.* 3, 351–358.

Liu, X., Ward, K., Xavier, C., Jann, J., Clark, A. F., Pang, I. H., et al. (2016a). The novel triterpenoid RTA 408 protects human retinal pigment epithelial cells against H2O2-induced cell injury via NF-E2-related factor 2 (Nrf2) activation. *Redox. Biol.* 8, 98–109. doi: 10.1016/j.redox.2015.12.005

Liu, X., Xavier, C., Jann, J., and Wu, H. (2016b). Salvianolic Acid B (Sal B) Protects Retinal Pigment Epithelial Cells from Oxidative Stress-Induced Cell Death by Activating Glutaredoxin 1 (Grx1). *Int J Mol Sci* 17, E1835. doi: 10.3390/ijms17111835

Mandal, M. N., Patlolla, J. M., Zheng, L., Agbaga, M. P., Tran, J. T., Wicker, L., et al. (2009). Curcumin protects retinal cells from light-and oxidant stress-induced cell death. *Free Radic. Biol. Med.* 1, 672–679. doi: 10.1016/j.freeradbiomed.2008.12.006

Mansoor, S., Gupta, N., Falatoonzadeh, P., Kuppermann, B. D., and Kenney, M. C. (2014). 2-ethylpyridine, a cigarette smoke component, causes mitochondrial damage in human retinal pigment epithelial cells in vitro. *Indian J. Ophthalmol.* 62, 16–22. doi: 10.4103/0301-4738.126168

Marinucci, L., Balloni, S., Fettucciari, K., Bodo, M., Talesa, V. N., and Antognelli, C. (2018). Nicotine induces apoptosis in human osteoblasts via a novel mechanism driven by H2O2and entailing Glyoxalase 1-dependent MG-H1 accumulation leading to TG2-mediated NF-kB desensitization: implication for smokers-related osteoporosis. *Free Radic. Biol. Med.* 117, 6–17.

Martucci, M., Ostan, R., Biondi, F., Bellavista, E., Fabbri, C., Bertarelli, C., et al. (2017). Mediterranean diet and inflammaging within the hormesis paradigm. *Nutr. Rev.* 75, 442–455. doi: 10.1093/nutrit/nux013

Merle, B. M. J., Colijn, J. M., Cougnard-Grégoire, A., de Koning-Backus, A. P. M., Delyfer, M. N., Kiefte-de Jong, J. C., et al. (2018). Mediterranean diet and incidence of advanced age-related macular degeneration: the EYE-RISK consortium. *Ophthalmology* pii: S0161-6420(18)30721-8. doi: 10.1016/j.ophtha.2018.08.006 [Epub ahead of print].

Nakagami, Y. (2016). Nrf2 Is an attractive therapeutic target for retinal diseases. *Oxid. Med. Cell. Longev.* 2016:7469326. doi: 10.1155/2016/7469326

Nakagami, Y., Masuda, K., Hatano, E., Inoue, T., Matsuyama, T., Iizuka, M., et al. (2015). Novel Nrf2 activators from microbial transformation products inhibit blood-retinal barrier permeability in rabbits. *Br. J. Pharmacol.* 172, 1237–1249. doi: 10.1111/bph.12999

Naylor, R. M., Baker, D. J., and van Deursen, J. M. (2013). Senescent cells: a novel therapeutic target for aging and age-related diseases. *Clin. Pharmacol. Ther.* 93, 105–116. doi: 10.1038/clpt.2012.193

Ney, P. A. (2015). Mitochondrial autophagy: origins, significance, and role of BNIP3 and NIX. *Biochim. Biophys. Acta* 1853, 2775–2783. doi: 10.1016/j.bbamcr.2015.02.022

Noell, W. K., Walker, V. S., Kang, B. S., and Berman, S. (1966). Retinal damage by light in rats. *Invest. Ophthalmol.* 5, 450–473.

O'Mealey, G. B., Berry, W. L., and Plafker, S. M. (2017). Sulforaphane is a Nrf2-independent inhibitor of mitochondrial fission. *Redox. Biol.* 11, 103–110. doi: 10.1016/j.redox.2016.11.007

Organisciak, D. T., and Vaughan, D. K. (2010). Retinal light damage: mechanisms and protection. *Prog. Retin Eye Res.* 29, 113–134. doi: 10.1016/j.preteyeres.2009.11.004

Orhan, C., Akdemir, F., Tuzcu, M., Sahin, N., Yilmaz, I., Deshpande, J., et al. (2016). Mesozeaxanthin protects retina from oxidative stress in a rat model. *J. Ocul. Pharmacol. Ther.* 32, 631–637. doi: 10.1089/jop.2015.0154

Rahman, I., and MacNee, W. (1996). Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 21, 669–681. doi: 10.1016/0891-5849(96)00155-4

Raimundo, M., Mira, F., Cachulo, M. D. L., Barreto, P., Ribeiro, L., Farinha, C., et al. (2018). Adherence to a mediterranean diet, lifestyle and age-related macular degeneration: the coimbra eye study - report 3. *Acta Ophthalmol.* doi: 10.1111/aos.13775 [Epub ahead of print].

Rezaie, T., McKercher, S. R., Kosaka, K., Seki, M., Wheeler, L., Viswanath, V., et al. (2012). Protective effect of carnosic acid, a pro-electrophilic compound, in models of oxidative stress and light-induced retinal degeneration. *Invest. Ophthalmol. Vis. Sci.* 53, 7847–7854. doi: 10.1167/iovs.12-10793

Rojo de la Vega, M., Chapman, E., and Zhang, D. D. (2018). NRF2 and the hallmarks of cancer. *Cancer Cell* 34, 21–43. doi: 10.1016/j.ccell.2018.03.022

Rom, O., Avezov, K., Aizenbud, D., and Reznick, A. Z. (2013). Cigarette smoking and inflammation revisited. *Respir. Physiol. Neurobiol.* 187, 5–10. doi: 10.1016/j.resp.2013.01.013

Ruben, M. D., Wu, G., Smith, D. F., Schmidt, R. E., Francey, L. J., Lee, Y. Y., et al. (2018). A database of tissue-specific rhythmically expressed human genes has potential applications in circadian medicine. *Sci. Transl. Med.* 10:eaat8806.

Sachdeva, M. M., Cano, M., and Handa, J. T. (2014). Nrf2 signaling is impaired in the aging RPE given an oxidative insult. *Exp. Eye Res.* 119, 111–114. doi: 10.1016/j.exer.2013.10.024

Saito, Y., Kuse, Y., Inoue, Y., Nakamura, S., Hara, H., and Shimazawa, M. (2018). Transient acceleration of autophagic degradation by pharmacological Nrf2 activation is important for retinal pigment epithelium cell survival. *Redox. Biol.* 19, 354–363. doi: 10.1016/j.redox.2018.09.004

Sies, H., and Cadenas, E. (1985). Oxidative stress: damage to intact cells and organs. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 311, 617–631. doi: 10.1098/rstb.1985.0168

Sohal, R. S., and Orr, W. C. (2012). The redox stress hypothesis of aging. *Free Radic. Biol. Med.* 52, 539–555. doi: 10.1016/j.freeradbiomed.2011.10.445

Tanito, M., Masutani, H., Kim, Y. C., Nishikawa, M., Ohira, A., and Yodoi, J. (2005). Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice. *Invest. Ophthalmol. Vis. Sci.* 46, 979–987. doi: 10.1167/iovs.04-1120

Tebay, L. E., Robertson, H., Durant, S. T., Vitale, S. R., Penning, T. M., Dinkova-Kostova, A. T., et al. (2015). Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radic. Biol. Med.* 88(Pt B), 108–146. doi: 10.1016/j.freeradbiomed.2015.06.021

Tuzcu, M., Orhan, C., Muz, O. E., Sahin, N., Juturu, V., and Sahin, K. (2017). Lutein and zeaxanthin isomers modulates lipid metabolism and the inflammatory state of retina in obesity-induced high-fat diet rodent model. *BMC Ophthalmol.* 17:129. doi: 10.1186/s12886-017-0524-1

Vande Velde, C., Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., et al. (2000). BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol. Cell. Biol.* 20, 5454–5468. doi: 10.1128/MCB.20.15.5454-5468.2000

Wardyn, J. D., Ponsford, A. H., and Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochem. Soc. Trans.* 43, 621–626. doi: 10.1042/BST20150014

Willett, W. C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E., et al. (1995). Mediterranean diet pyramid: a cultural model for healthy eating. *Am. J. Clin. Nutr.* 61, 1402S–1406S. doi: 10.1093/ajcn/61.6.1402S

Xie, X., Feng, J., Kang, Z., Zhang, S., Zhang, L., Zhang, Y., et al. (2017). Taxifolin protects RPE cells against oxidative stress-induced apoptosis. *Mol. Vis.* 23, 520–528.

Yu, M., Yan, W., and Beight, C. (2018). Lutein and zeaxanthin isomers protect against light-induced retinopathy via decreasing oxidative and endoplasmic reticulum stress in BALB/cJ Mice. *Nutrients* 10:E842. doi: 10.3390/nu10070842

Zhang, H., Davies, K. J. A., and Forman, H. J. (2015). Oxidative stress response and Nrf2 signaling in aging. *Free Radic. Biol. Med.* 88(Pt B), 314–336. doi: 10.1016/j.freeradbiomed.2015.05.036

Zhang, H., Liu, Y. Y., Jiang, Q., Li, K. R., Zhao, Y. X., Cao, C., et al. (2014). Salvianolic acid A protects RPE cells against oxidative stress through activation of Nrf2/HO-1 signaling. *Free Radic. Biol. Med.* 69, 219–228. doi: 10.1016/j.freeradbiomed.2014.01.025

Zhao, Z., Chen, Y., Wang, J., Sternberg, P., Freeman, M. L., Grossniklaus, H. E., et al. (2011). Age-related retinopathy in NRF2-deficient mice. *PLoS One* 6:e19456. doi: 10.1371/journal.pone.0019456

Zhuang, Y., and Lyga, J. (2014). Inflammaging in skin and other tissues - the roles of complement system and macrophage. *Inflamm. Allergy Drug Targets.* 13, 153–161. doi: 10.2174/1871528113666140522112003

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REVIEW
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The Role of Natural Products in Targeting Cardiovascular Diseases via Nrf2 Pathway: Novel Molecular Mechanisms and Therapeutic Approaches

Bee Kee Ooi[1], Kok-Gan Chan[2,3], Bey Hing Goh[4,5,6,7] and Wei Hsum Yap[1]**

[1] School of Biosciences, Taylor's University, Subang Jaya, Malaysia, [2] Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, [3] International Genome Centre, Jiangsu University, Zhenjiang, China, [4] Biofunctional Molecule Exploratory Research Group, School of Pharmacy, Monash University Malaysia, Bandar Sunway, Malaysia, [5] Novel Bacteria and Drug Discovery Research Group, School of Pharmacy, Monash University Malaysia, Bandar Sunway, Malaysia, [6] Asian Centre for Evidence Synthesis in Population, Implementation and Clinical Outcomes, Health and Well-Being Cluster, Global Asia in the 21st Century Platform, Monash University Malaysia, Bandar Sunway, Malaysia, [7] Center of Health Outcomes Research and Therapeutic Safety, School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand

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***Correspondence:**
Kok-Gan Chan
kokgan@um.edu.my
Wei Hsum Yap
weihsum.yap@taylors.edu.my

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Cardiovascular diseases (CVDs) are closely linked to cellular oxidative stress and inflammation. This may be resulted from the imbalance generation of reactive oxygen species and its role in promoting inflammation, thereby contributing to endothelial dysfunction and cardiovascular complications. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that plays a significant role in regulating expression of antioxidant and cytoprotective enzymes in response to oxidative stress. Natural products have emerged as a potential source of bioactive compounds which have shown to protect against atherogenesis development by activating Nrf2 signaling. This review aims to provide a comprehensive summary of the published data on the function, regulation and activation of Nrf2 as well as the molecular mechanisms of natural products in regulating Nrf2 signaling. The beneficial effects of using natural bioactive compounds as a promising therapeutic approach for the prevention and treatment of CVDs are reviewed.

Keywords: nuclear factor erythroid 2-related factor 2 (Nrf2), cardiovascular diseases (CVDs), natural products, oxidative stress, nuclear factor-κB (NF-κB)

INTRODUCTION

Cardiovascular disease (CVD) is a major health complication which accounts for 15.2 million deaths worldwide in 2016 (World Health Organization, 2018). Atherosclerosis, as characterized by the formation of plaques with bulks of modified low density lipoprotein (LDL), immune cells, smooth muscle cells and cellular debris in the arterial intima, is the primary cause of CVD. The molecular mechanisms underlying CVD have been extensively investigated over the past decades. It has been demonstrated that the involvement of oxidative stress and inflammation are associated with the pathogenesis of CVD. Oxidative stress which results from excessive generation of reactive oxygen species/reactive nitrogen species (ROS/RNS) can trigger inflammation,

which contribute to LDL oxidation, endothelial dysfunction, atherosclerotic plaque formation, plaque rupture, vascular remodeling, and atherothrombosis (Pashkow, 2011; Hajjar and Gotto, 2013; Hussain et al., 2016). In response to increased ROS/RNS levels under oxidative stress condition, the cells will induce the expression of antioxidant proteins and phase II detoxification enzymes such as heme oxygenase 1 (HO-1), aldo-keto reductase (AKR), peroxiredoxin 1 (PRX), γ-glutamyl cysteine ligase (γ-GCL) glutamate-cysteine ligase modifier subunit (GCLM), superoxide dismutase (SOD), NADPH quinine oxidoreductase 1 (NQO1) and others (Menegon et al., 2016; Jeddi et al., 2017). Transcriptional regulation of these enzymes is mainly controlled by nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that plays as a central role in intracellular redox homeostasis. In addition, Nrf2 also protects against macrophage foam cells formation by regulating expression of scavenger receptors, ATP-binding cassette (ABC) transporters, and multidrug resistance-associated proteins (MRPs) (Jeddi et al., 2017; Ooi et al., 2017). For instance, deficiency of Nrf2 in the bone marrow has been shown to aggravate atherosclerosis in LDL receptor-null ($LDLR^{-/-}$) mice (Collins et al., 2012; Ruotsalainen et al., 2013). These evidences support the notion that Nrf2 protects against atherosclerosis. Although some studies showed that Nrf2 exhibits pro-atherogenic effects, its molecular mechanisms remain unclear (Sussan et al., 2008; Barajas et al., 2011; Freigang et al., 2011; Harada et al., 2012; Ruotsalainen et al., 2018).

Natural products offer unique structural and chemical diversity that serve as a source of novel drug leads and therapeutic agents. Natural products have been shown to alleviate oxidative stress-induced diseases such as CVD, neurodegenerative diseases, cancer and metabolic disorders by regulating the Nrf2/antioxidant responsive element (ARE) pathway (Waltenberger et al., 2016; Basak et al., 2017; Matzinger et al., 2017). Natural products derived from olive oil (hydroxytyrosol) and red wine (resveratrol) have been demonstrated to inhibit ROS production. Meanwhile, both bioactive compounds have also been reported to enhance Nrf2 nuclear translocation and decrease miRNA-146a expression, a pro-inflammatory marker (Bigagli et al., 2017). Recent studies revealed that sulforaphane, an isothiocyanate derived from cruciferous vegetables, protects against CVD due to its antioxidant and anti-inflammatory effects mediated through the Nrf2 signaling pathway (Bai et al., 2015). These evidences suggested that natural products may serve as a promising therapeutic approach for the prevention and treatment of CVD associated with oxidative stress. This review will discuss on the current knowledge on the molecular mechanisms of cardioprotective bioactive compounds targeting the Nrf2/ARE signaling pathway (**Figure 1**).

REGULATION OF Nrf2 SIGNALING PATHWAY

Structural Features of Nrf2

Human Nrf2 (*NFE2L2*) protein comprises of 605 amino acid residues with molecular weight of 67.7 kDa (Cho, 2013). The Nrf2 gene consists of seven functional domains, also known as the Nrf2-ECH homology (Neh) domains (Namani et al., 2014; Canning et al., 2015). The position of each functional domain of Nrf2 is illustrated in **Figure 2A**. Neh 1 domain comprises of highly conserved basic region-leucine zipper (CNC-bZIP) region that dimerizes with small musculoaponeurotic fibrosarcoma (Maf) proteins and binds to ARE, a *cis*-acting enhancer sequence found in the promoter region of many genes encoding antioxidant and phase II detoxification enzymes or proteins. Neh 2 domain acts as a negative regulatory domain as it contains the two degrons, known as high-affinity ETGE motif and the lower-affinity DLG motif. These motifs specifically interact with Kelch-like ECH-associated protein 1 (Keap1) which mediate ubiquitination and degradation of Nrf2. The carboxy-terminal of Neh 3 domain is a transactivation domain that recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD 6) and drives ARE- gene expression. Both Neh 4 and Neh 5 also function as transactivation domains which are involved in the interaction with cAMP response element-binding protein (CREB)-binding protein (CBP) and receptor-associated coactivator 3 (RAC 3). Meanwhile, Neh 6 domain negatively regulates Nrf2 stability via glycogen synthase kinase-3 (GSK-3)/β-transducin repeat-containing protein (β-TrCP)-mediated degradation. It contains two highly conserved redox-independent degrons known as DSGIS and DSAPGS motifs. DSAPGS motif interacts with β-TrCP, which serves as a substrate receptor for the S-phase kinase-associated protein 1- Cullin 1- RING box protein-1/regulator of cullins-1 (Skp1–Cul1–Rbx1/Roc1) ubiquitin ligase complex. This results in ubiquitination and degradation of Nrf2 via the Keap1-independent pathway. Besides, suppression of Nrf2/ARE signaling pathway may be mediated via interacting with Neh 7 domain and the DNA-binding domain of retinoid X receptor α (RXRα).

Regulation of Nrf2 Activity

Keap1-Dependent Regulation of Nrf2 Activity

Keap1 is a cysteine rich adaptor protein for cullin (Cul3)-containing E3 ubiquitin ligase complex which mediates Nrf2 ubiquitination and degradation by 26S proteasomes. It consists of five sub-sections (**Figure 2B**), namely the N-terminal region, BTB dimerization domain (Broad-Complex, Tramtrack, and Bric-a-Brac), cysteine-rich intervening (IVR) domain, six Kelch/double glycine repeats (DRG) domain, and C-terminal region (Namani et al., 2014; Basak et al., 2017). Under normal homeostatic condition, Nrf2 has a short half-life of approximately 20 min. They are maintained at low level and constantly targeted for proteasomal degradation (Kobayashi and Yamamoto, 2006). The Keap1-dependent Nrf2 regulatory pathway supports the notion that exposure to ROS or Nrf2 inducers such as epigallocatechin-3-gallate (EGCG), sulforaphane, dimethyl fumarate (DMF), and *tert*-butylhydroquinone (tBHQ) will result in conformational changes in Keap1 cysteine residues, which interferes the interaction between Kelch domain and DLG motif where the ETGE motif still bound to Nrf2. Consequently, Keap1 fails to align with the E2 ubiquitin-conjugating enzyme and thus Nrf2 are no longer targeted for ubiquitination and degradation.

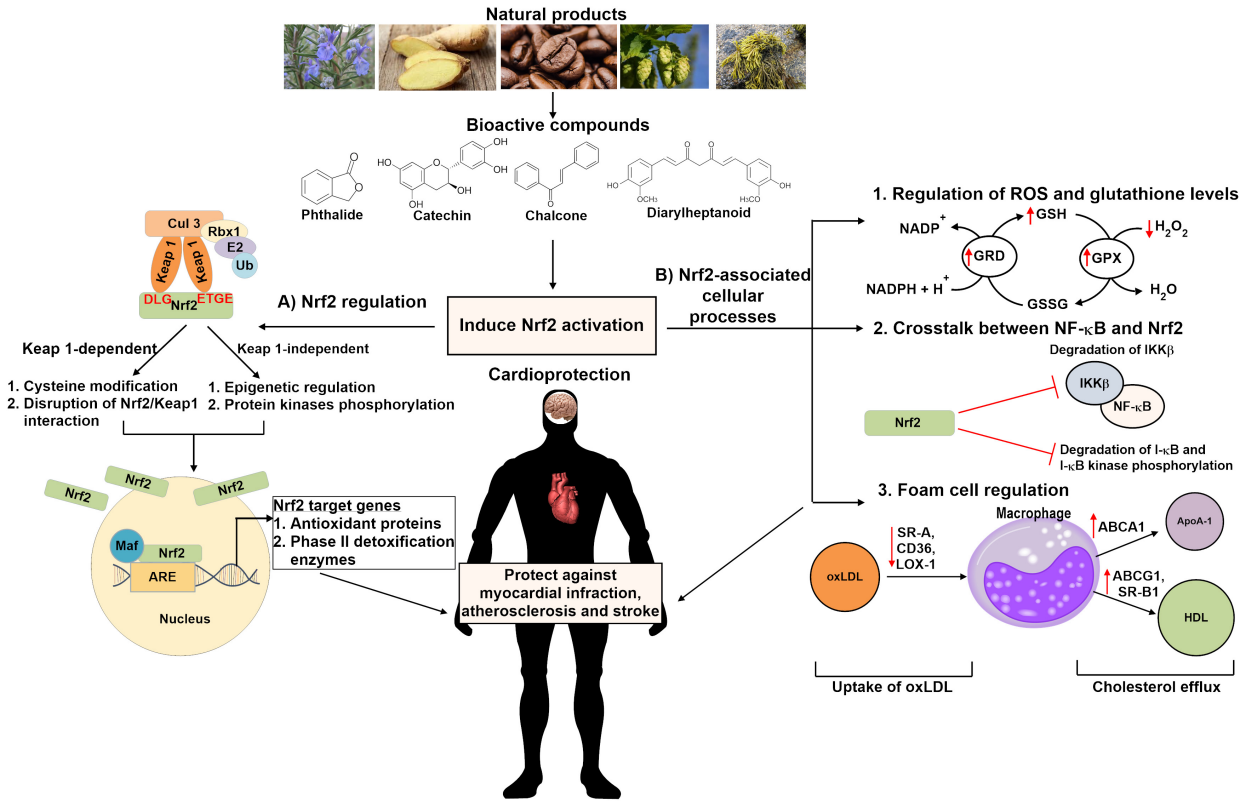

FIGURE 1 | Schematic overview of the cardioprotective mechanisms of bioactive compounds derived from natural products in activating Nrf2 signaling pathway. Natural compounds may induce Nrf2 activation via the (A) Keap1-dependent or Keap1-independent pathway which involves cysteine modification, disruption of Nrf2/Keap1 interaction, epigenetic regulation and/or protein kinases phosphorylation. Nrf2 activation might also be induced through (B) Nrf2-associated cellular processes, including regulation of ROS and glutathione levels, inhibiting NF-κB inflammatory signaling pathway as well as controlling oxLDL uptake and cholesterol efflux in foam cells regulation. These bioactive compounds protect against cardiovascular diseases such as stroke, atherosclerosis and myocardial infraction. Nrf2 indicates nuclear factor erythroid 2-related factor 2; Keap 1, Kelch-like ECH-associated protein 1; Ub, ubiquitin; Maf, musculoaponeurotic fibrosarcoma; ARE, antioxidant response element; ROS, reactive oxygen species; GRD, glutathione reductase; GPX, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; NF-κB, nuclear factor kappa-B kinase; IKK-β, inhibitor of nuclear factor kappa-B kinase subunit beta; oxLDL, oxidized low density lipoprotein; SR-A, scavenger receptor class A; CD36, scavenger receptor class B; LOX-1, lectin-type oxidized LDL receptor 1; ABC, ATP-binding cassette transporter; SR-B1, scavenger receptor class B type 1; ApoA-1, apolipoprotein A-1; HDL, high density lipoprotein.

The accumulation of free cytosolic Nrf2 is translocated into the nucleus where it dimerizes with Maf protein and binds to ARE sequences, resulting in the expression of downstream target genes (**Figure 3**) (Basak et al., 2017; Matzinger et al., 2017; Ooi et al., 2017).

Keap1-Independent Regulation of Nrf2 Activity

Apart from Keap1-dependent mechanism, emerging bodies of evidences revealed that Nrf2 can also be regulated through a number of mechanisms independent of Keap1. These mechanisms include transcriptional and epigenetic regulation, autophagy and other signaling pathways (**Figure 3**).

Transcriptional regulation of Nrf2

Binding of Aryl hydrocarbon receptor-aryl hydrocarbon receptor nuclear translocator (AhR-ARNT) to xenobiotic response element (XRE) sequences is known to regulate Nrf2 activation. AhR is a member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors that functions as xenobiotic chemical sensor in eukaryotes (Furue et al., 2017; Nebert, 2017). The inactive form of AhR is stabilized in the cytoplasm in a complex form with heat shock protein 90 (HSP90), X-associated protein 2 (XAP2), and HSP90 co-chaperone p23 (Mimura and Fujii-Kuriyama, 2003; Quintana, 2013; Furue et al., 2017). Upon exposure to polycyclic aromatic hydrocarbon, AhR ligand complex translocate into the nucleus where it dissociates from HSP90 complex and dimerizes with ARNT and binds to XRE sequences at the promoter region and upregulates the expression of phase I and II metabolic enzymes, cytochrome P450 family members, NQO1, Ya subunit of glutathione *S*-transferase (GST), δ-aminolevulinic acid synthase, UDP-glucuronosyltransferase and others (Beischlag et al., 2008; Furue et al., 2017). There are studies reporting the cross-talk between AhR and Nrf2 signaling pathway (Miao et al., 2005; Korashy and El-Kadi, 2006; Tsuji et al., 2012; Dietrich, 2016). It was shown that Nrf2 promoter contains three XRE-like elements (XREL) located at position −712 (XREL1), +755 (XREL2) and +850 (XREL3). The activity of 2, 3, 7,

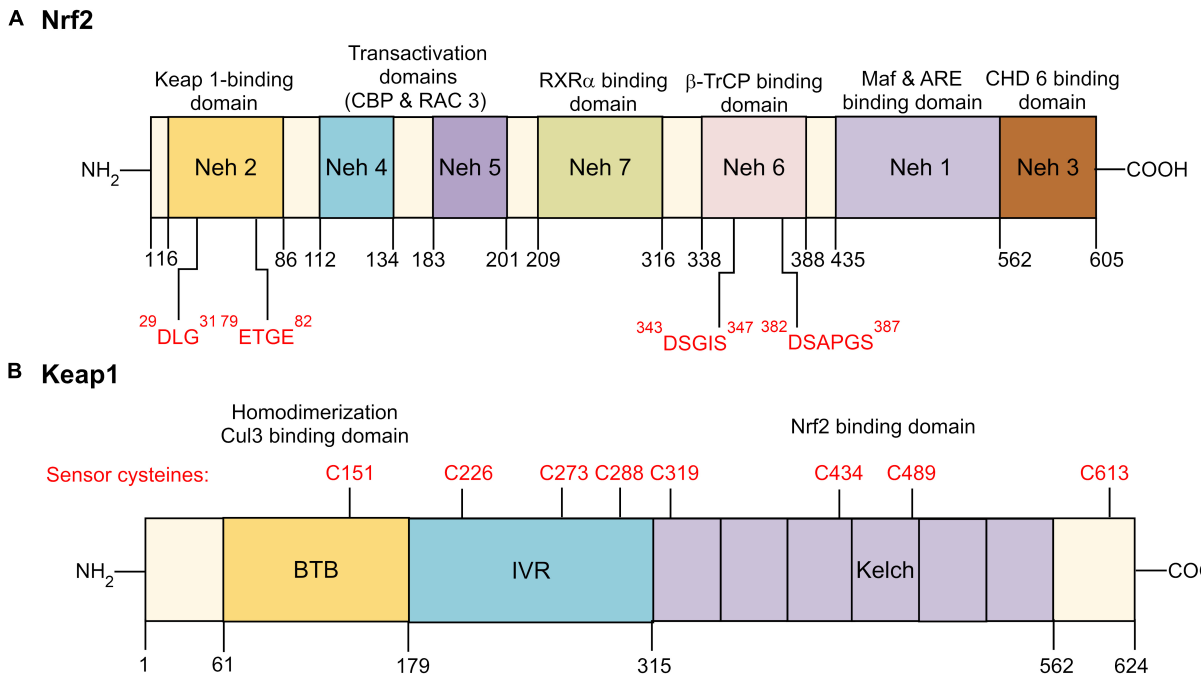

FIGURE 2 | Domain structure of Nrf2 and Keap1. **(A)** Functional Nrf2-ECH homology (Neh) domains: Neh 1 is the binding site for small Maf proteins and ARE. Neh 2 serves as the binding site for Keap1 by interacting with low-affinity DLG and the high affinity ETGE motifs. Neh 3-5 are transactivation domains for Nrf2. Neh 6 is a serine-rich domain that negatively controls the Nrf2 stability by β-TrCP interacts with DSGIS and DSAPGS motifs. Neh 7 interacts with RXRα, a nuclear receptor responsible for suppression of Nrf2/ARE signaling pathway. **(B)** Functional Keap1 domains: N-terminal region, BTB dimerization domain, cysteine-rich IVR domain, six Kelch/DRG domain, and C-terminal region. BTB is responsible for Keap1 homodimerization and association with cullin (Cul3)-containing E3 ubiquitin ligase complex. IVR consists of reactive cysteine residues, including C226, C273 and C288. DRG domain is responsible for Nrf2 binding to DLG and ETGE motifs. Nrf2 indicates nuclear factor erythroid 2-related factor 2; Keap 1, Kelch-like ECH-associated protein 1; RXRα, retinoid X receptor α; β-TrCP, β-transducin repeat-containing protein; Maf, musculoaponeurotic fibrosarcoma; ARE, antioxidant response element; CHD 6, chromo-ATPase/helicase DNA-binding protein 6; BTB, Broad-Complex, Tramtrack, and Bric-a-Brac; IVR, intervening region; DRG, double glycine repeats.

8-tetrachlorodibenzo-p-dioxin (TCDD)-induced Nrf2 mRNA was abolished in transient AhR-silenced cell line tao (Miao et al., 2005). Besides, activation of AhR nuclear translocation by ketoconazole (KCZ) has shown to upregulate cytochrome P450 family 1 Subfamily A Member 1 (CYP1A1) expression. Meanwhile, it also induced the Nrf2 nuclear translocation, resulting in upregulation of NQO1 expression (Tsuji et al., 2012). These evidences suggest that activation of Nrf2 can be regulated by AhR-ARNT pathway.

Epigenetic regulation of Nrf2

Epigenetics modifications including DNA methylation, histone modification, and microRNAs (miRNAs) expressions are involved in Nrf2 regulation (Guo et al., 2015). For instance, the expression of Nrf2 and its downstream gene NQO1 were lower in transgenic adenocarcinoma of mouse prostate (TRAMP) C1 cells (Yu et al., 2010; Zhang et al., 2013). Treatment with DNA methyltransferases (DNMTs) inhibitor 5-aza-2′-deoxycytidine (5-Aza) and histone deacetylase (HDAC) inhibitor trichostatin A (TSA) has shown to restore the epigenetically silenced Nrf2 gene and increase NQO1 expression, which helps to prevent prostate cancer progression in TRAMP mice and protects against Alzheimer's development in a mouse neuroblastoma N2a cellular model (Yu et al., 2010; Cao et al., 2016). In addition, TSA also increased Nrf2-regulated HO-1, NQO1, glutamate-cysteine ligase catalytic (GCLC) in neuron cultures and brain tissue by promoting Nrf2 dissociation from Keap1 and nuclear Nrf2 translocation (Wang et al., 2012).

MiRNAs are short, single-stranded, small non-coding RNA molecules of approximately 18–25 nucleotides long. It also has been implicated in regulation of Nrf2 at the post-transcriptional level (Lujambio and Lowe, 2012). Recent study showed that increased miR-200a expression leads to Keap1 degradation and Nrf2 protein stabilization, thereby protecting OB-6 osteoblastic cells from dexamethasone-induced oxidative stress and apoptosis (Zhao et al., 2017). A similar relationship was observed between miR-141 and Nrf2/Keap1 pathway in hepatocellular carcinoma cells. Increased levels of miR-141 expression in hepatocellular carcinoma cells such as HepG2, SMMC-7721, and HuH7 cell lines has shown to downregulate Keap1 expression via the Keap1 3′ untranslated region (3′ UTR), resulting in transcriptional activation of Nrf2-dependent HO-1 gene (Shi et al., 2015). Interestingly, an inverse correlation between miRNAs and Nrf2 has also been reported. Downregulation of miRNAs such as miR-93, miR-142-5p and miR-218 exhibited protective effects against cerebral ischemic injury and high glucose (HG)-induced apoptosis through upregulation of Nrf2/ARE signaling pathway (Wang et al., 2016, 2017; Liu et al., 2017).

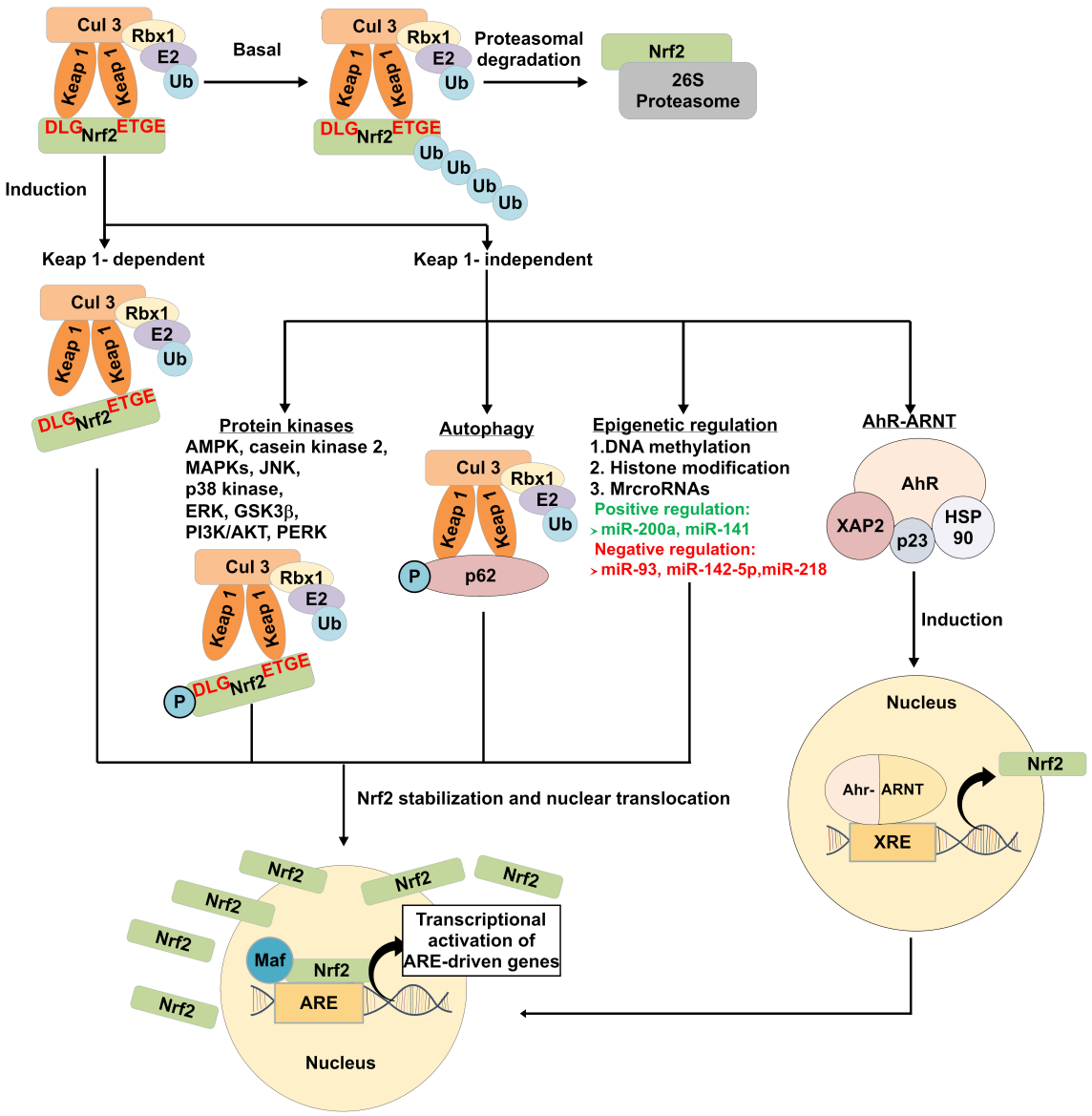

FIGURE 3 | Modulation of Keap1-dependent and Keap1-independent mediated Nrf2 signaling pathways. Under basal condition, Nrf2 is constantly targeted by Keap1 for proteasomal degradation. Nrf2 may be induced by Keap1-dependent and Keap1-independent pathways. Under the Keap1-dependent pathway, exposure to oxidative stress and Nrf2 activators induce conformational change in the cysteine residues of Keap1, which disrupts the interaction between Kelch domain and DLG motif bound to Nrf2. This results in the release of Nrf2 from Keap1. Phosphorylation of protein kinases and autophagy adaptor proteins, epigenetic regulation and AhR-ARNT binding to XRE are associated with the release of Nrf2 in Keap1-independent-mediated pathways. Stabilized free cytosolic Nrf2 translocate into the nucleus, binds to ARE and induce transcription of antioxidant and detoxifying enzymes and proteins. Ub indicates ubiquitin; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase cascades; JNK, c-Jun N-terminal kinase; ERK, extracellular-signal-regulated kinase; GSK3β, glycogen synthase kinase-3β; PI3K/AKT, phosphatidylinositol-3-kinase; PERK, pancreatic endoplasmic reticulum kinase; p62, sequestosome-1; AhR-ARNT, aryl hydrocarbon receptor-aryl hydrocarbon receptor nuclear translocator; HSP90, heat shock protein 90; XAP2, X-associated protein 2; p23, HSP90 co-chaperone; ARE, antioxidant response element, and; XRE, xenobiotic response element.

Autophagy

Autophagy, a bulk-lysosomal degradation process that is responsible for the clearance of aggresomes and abnormal organelles, can enhance cell survival under stress condition. The functional role of autophagy adaptor proteins, also known as sequestosome-1 (p62/SQSTM1) in regulating Nrf2 and its downstream target genes has been elucidated (Bryan et al., 2013; Kapuy et al., 2018). Under oxidative stress condition, p62 is phosphorylated, which increases its binding affinity to Keap1. The binding of p62 to Keap1 results in the dissociation of Nrf2 from Keap1, thereby promoting Nrf2 stabilization and subsequent activation of downstream target genes. This is indicated in previous research showing that knockdown of p62 significantly promoted the accumulation of Keap1,

thus enhanced Nrf2 degradation (Sun et al., 2016). In addition, depletion of SQSTM1 significantly doubled the half-life of Keap1 and lead to simultaneous decrease in Nrf2 protein and mRNA levels (Copple et al., 2010). Mammalian target of rapamycin complex 1 (mTORC1)-induced S351 phosphorylation in the Keap1-interacting region (KIR) motif of p62 markedly increase binding affinity of p62 for Keap1, thereby increasing the transcriptional activation of Nrf2 target genes (Ichimura et al., 2013). This suggests that p62 can compete with Nrf2 for binding to Keap1 via KIR motif, which has a sequence similar to the ETGE motif in Nrf2 (Jain et al., 2010). Apart from mTOR kinase-induced p62 phosphorylation, several other kinases such as class III phosphoinositide 3-kinase (PI3K) vacuolar protein sorting 34 (VPS34) (Jiang et al., 2017) and TGF-β-activated kinase 1 (TAK1) (Hashimoto et al., 2016) have also been demonstrated to phosphorylate p62 and facilitate the Keap1-p62 complex interaction, thus increasing Nrf2 expression levels. Overall, these studies revealed that p62/SQSTM1 creates a positive feedback loop for enhancing Nrf2 expression.

Other signaling pathways

Several protein kinases including AMP-activated protein kinase (AMPK), casein kinase 2, mitogen-activated protein kinase cascades (MAPKs): JUN-N-terminal kinase (JNK), p38 kinase, extracellular-signal-regulated kinase (ERK), GSK3β, phosphatidylinositol 3-kinase (PI3K/AKT) and pancreatic endoplasmic reticulum kinase (PERK) have been implicated in Nrf2/Keap1 interaction. Phosphorylation of the serine (Ser), threonine (Thr), and tyrosine (Tyr) residues could lead to enhanced Nrf2 stability, nuclear accumulation, and subsequent transactivation activity. Phosphorylation at Thr 172 and Ser 550 by AMPK (Zimmermann et al., 2015; Joo et al., 2016) and at Ser40 by casein kinase 2 (Apopa et al., 2008) could induce Nrf2 accumulation for ARE-driven gene transactivation. Furthermore, studies have shown that MAPKs signaling pathways have a role in the Nrf2 regulation. Recent research reported that Andrographolide, a labdane diterpenoid exerts a potential therapeutic effect against neuroinflammatory diseases through upregulation of Nrf2/HO-1 expression in astrocytes via p38 MAPK and ERK-dependent pathways (Wong et al., 2016). Similarly, it was shown that activation of p38 MAPK/Nrf2 pathway is required to induce the expression of HO-1 induction by fungal β-glucan-containing particles (β-GPs) (Ishida et al., 2018). Stimulation of p38 MAPK by anisomycin was found to phosphorylate Nrf2 protein, which promotes the interaction of Nrf2 with Keap1, thereby inhibiting nuclear translocation of Nrf2 (Keum et al., 2006). In addition, GSK3β has been reported to negatively regulate Nrf2 activity. Inhibition of GSK-3β can increase the nuclear accumulation of Nrf2 and antioxidant response in hepatocytes as well as rat with cerebral ischemia-reperfusion (Jiang Y. et al., 2015; Chen et al., 2016). Moreover, PI3K/AKT and PERK also have been reported positively regulate Nrf2 activation (Cullinan and Diehl, 2004; Zou et al., 2013).

Role of Nrf2 in Cardiovascular Diseases

A growing body of evidence showed that Nrf2 and its downstream target genes protect against CVD development, including oxidative stress-induced endothelial dysfunction and atherosclerosis. Endothelial dysfunction marks the early stages of atherosclerosis where oxidative stress enhances endothelium cell permeability, LDL oxidation, monocyte adherence, platelet activation, vascular inflammation, as well as proliferation and infiltration of vascular smooth muscle cells (VSMCs) from media to arterial intima (Hadi et al., 2005; Grover-Páez and Zavalza-Gómez, 2009). Transplantation of Nrf2-deficient bone marrow cells in $LDLR^{-/-}$ mice model showed a reduction in the expression levels of antioxidant enzymes [NAD(P)H dehydrogenase, NQO1, catalase and GPX1], increased macrophage migration, production of pro-inflammatory cytokines, and atherosclerotic lesions (Collins et al., 2012). Meanwhile, silencing of Nrf2 in U937 monocytic cells led to an elevation of pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), and endoplasmic reticulum (ER) stress markers expression (Song et al., 2015). Further evidence showed that overexpression of Nrf2 in VSMCs of rabbit model showed increased expression of antioxidant enzymes (HO-1 and NQO1) and inhibition of VSMCs proliferation and vascular inflammation (Levonen et al., 2007). In addition, Nrf2 deficiency in macrophage promoted pro-inflammatory cytokines production (MCP-1, IL-6, and TNF-α) and enhanced oxidized low density lipoprotein (oxLDL) uptake, leading to foam cell formation (Ruotsalainen et al., 2013).

Interestingly, some studies reported that Nrf2-mediates pro-atherogenic effects (Sussan et al., 2008; Barajas et al., 2011; Freigang et al., 2011; Harada et al., 2012; Ruotsalainen et al., 2018). Nrf2 deficiency in $ApoE^{-/-}$ mice developed smaller atherosclerotic plaques by reducing CD36 expression, a type of scavenger receptor which is responsible for taking up modified LDLs (Sussan et al., 2008). Moreover, a reduction in pro-inflammatory cytokine IL-1-mediated vascular inflammation was observed in Nrf2-deficient $ApoE^{-/-}$ mice (Freigang et al., 2011). Recent studies have shown that deficiency of Nrf2 in $LDLR^{-/-}$ and $LDLR^{-/-}$ mice expressing apoB-100 only ($LDLR^{-/-}ApoB^{100/100}$) reduced atherosclerotic lesion development. However, Nrf2 deficiency in aged $LDLR^{-/-}ApoB^{100/100}$ mice exhibit enhanced plaque inflammation and calcification (Ruotsalainen et al., 2018). These evidences suggest that Nrf2 activation plays a dual role in CVD.

PROTECTIVE EFFECTS OF NATURAL PRODUCTS AGAINST CARDIOVASCULAR DISEASES

Natural products derived from plants, marine organisms and animals have been a reliable source of new structural leads for treatment of various diseases. Most bioactive compounds are produced as secondary metabolites, which can be classified as phenolics, flavonoids, chalcones, terpenoids, carotenoids, anthocyanins, quinones, and others. These bioactive compounds possess a wide range of biological activities including anti-tumor, anti-inflammatory, anti-carcinogenic, anti-viral, anti-microbial, anti-diarrheal, anti-oxidant, and

other activities (Manivasagan et al., 2014; Hamed et al., 2015; Wang et al., 2018). Bioactive compounds have been shown to reduce atherosclerosis formation and risk of developing CVD (Rangel-Huerta et al., 2015; Dal and Sigrist, 2016). Recently, the PREvencion con DIeta MEDiterannea (PREDIMED) trial reported that dietary polyphenols intake such as extra-virgin olive oil and nuts were associated with improved CVD risk factors and decreased inflammatory biomarkers levels in high CVD risk participants. It was shown that polyphenol intake decreased blood pressure (BP), increased plasma high density lipoprotein (HDL) and decreased the inflammatory biomarkers of CVD, including vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), IL-6, TNF-α as well as MCP-1 (Medina-Remón et al., 2017). Similarly, Health, Alcohol and Psychosocial factors In Eastern Europe (HAPIEE) study also reported that dietary polyphenols (phenolic acids and stilbenes) intake were found to be inversely correlated to metabolic syndrome (MetS) which is closely link to risk factors of CVD including glucose intolerance, dyslipidemia, high BP and abdominal obesity (Grosso et al., 2017). Apart from dietary supplement human trials, accumulating evidence from both *in vivo* and *in vitro* studies are also supporting the cardioprotective effects of natural products. For instance, $ApoE^{-/-}$ mice fed with high cholesterol diet supplemented with ellagic acid (EA) exerts an atheroprotective effect by improving the antioxidant capacity, attenuated hypochlorous acid (HOCl)-induced endothelial dysfunction and increased the expression of HO-1 and Nrf2 (Ding et al., 2014). Treatment with quercetin ameliorated the high fat diet-induced MetS such as abdominal obesity, cardiovascular remodeling and liver complications in rats by increasing the expression of Nrf2, HO-1, carnitine palmitoyltransferase 1 (CPT1) and decreasing NF-κB (Panchal et al., 2012). Furthermore, cucurmin, a natural diarylheptanoids can prevent copper sulfate-induced LDL peroxidation, which is the earliest stage of atherosclerotic plaque that contributes to CVD (Mahfouz et al., 2009). Maslinic acid, a natural triterpenoid has also been shown to protect VSMCs against oxidative stress through activation of Akt/Nrf2/HO-1 pathway (Qin et al., 2014). Treatment with Tanshindiol C, a quinone derivative, attenuated oxLDL-induced macrophage foam cell formation by upregulating antioxidant peroxiredoxin 1 (Prdx1) and ATP-binding cassette transporter A1 (ABCA1) via Nrf2/Sirtuin 1 (Sirt1) signaling pathway (Yang et al., 2018b).

MOLECULAR MECHANISMS OF CARDIOPROTECTIVE NATURAL PRODUCTS TARGETING THE Nrf2 SIGNALING PATHWAY

The cardioprotective role of natural products targeting Nrf2 signaling pathway has been widely investigated. There are multiple mechanisms that are involved in activating Nrf2, including interaction with cysteine residues on Keap1, disruption of Nrf2/Keap1 interaction, epigenetic modification and activation of protein kinases. The molecular mechanisms of natural products targeting Nrf2 signaling pathway will be discussed and summarized as schematically outlined in **Table 1**.

Interaction With Keap1 Cysteine Residues

Keap1 is a cysteine rich adaptor protein. Human Keap1 have a total of 27 cysteine residues which can be modified by oxidants and electrophiles. Among the cysteine residues in human Keap1, Cys 151, 273 and 288 are highly reactive and play an essential role for repression of Nrf2/ARE activation (Saito et al., 2016). Studies suggest that Keap1 cysteine residues modification by bioactive compounds derived from natural products are involved in activating Nrf2 antioxidant defense system. These bioactive compounds are reported to modify Keap1 cysteine residues via oxidation, alkylation or thiol disulfide interchange. A recent report investigating the effects of rutin, a flavonoid abundantly present in citrus fruit demonstrated that it protects against H_2O_2-induced oxidative stress in human umbilical vein endothelial cells (HUVECs). The study showed that rutin can target the Cys 151 of Keap1, and form an adduct with Keap1 which results in Nrf2 activation and upregulation of glutamate cysteine ligase, a glutathione biosynthesis rate-limiting enzyme which plays an important role in the endogenous antioxidant system (Sthijns et al., 2017). Honaucin A, an anti-inflammatory compound isolated from marine filamentous cyanobacterium *Leptolyngbya crossbyana* has been reported to induce alkylation of Keap1 cysteine thiols and thereby activating Nrf2/ARE pathway (Mascuch et al., 2018). Similarly, treatment with withaferin A, a steroidal lactone significantly increased expression of HO-1 in HUVECs and EA.hy926 endothelial cells by enhancing nuclear translocation of Nrf2. Analysis of the *in vitro* and *in silico* model suggested that withaferin A can interact with Cys 151, Cys 319, Cys 434, Cys 489, and Cys 613 (Heyninck et al., 2016). Apart from that, a number of bioactive compounds including xanthohumol, sulforaphane, falcarindiol, carnosic acid and (6)-shogaol have also been showed to interact with cysteine residues on Keap1, thereby stimulating its dissociation from Nrf2 and promoting Nrf2 nuclear accumulation which induces antioxidant proteins and phase II detoxification enzymes (Satoh et al., 2008; Ohnuma et al., 2010; Hu et al., 2011; Chen et al., 2014; Yao et al., 2015). The activation of Nrf2 and stimulation of downstream antioxidants and phase II detoxification enzymes expression suggest that these natural bioactive compounds represent a potential therapeutic source for prevention and treatment of CVDs.

Disruption of Nrf2/Keap1 Interaction

Apart from modification of Keap1 cysteine residues, studies have demonstrated that bioactive compounds from natural products can disrupt the Nrf2/Keap1 interaction, thereby promoting Nrf2 nuclear translocation. For instance, EGCG is a well-known Nrf2 activator that promotes the dissociation of Nrf2/Keap1 and activate ARE genes transcription, thereby inhibiting TNF-α-induced NF-κB activation in human THP-1 cells (Jiang et al., 2012). Similar study also found that ECGC protects mice model against diabetic nephropathy

TABLE 1 | Molecular mechanisms of bioactive compounds from natural products targeting Nrf2/Keap1 pathway.

Mode of action	Bioactive compounds	Classification	Sources	Model	Reference
Interaction with cysteine residues of Keap1	**Marine products**				
	Honaucin A	(S)-3-hydroxy-γ-butyrolactone and 4-chlorocrotonic acid connected via ester linkage	Cyanobacterium *Leptolyngbya crossbyana*	MCF7 breast cancer cell line	Mascuch et al., 2018
	Plants				
	Rutin	Flavonoid	Citrus fruits, black tea and buckwheat bran	HUVEC endothelial cells	Sthijns et al., 2017
	Withaferin A	Steroidal lactone	*Withania somnifera*	HUVEC endothelial cells, EA.hy926 endothelial cells and *in vitro* and *in silico* evaluations	Heyninck et al., 2016
	Xanthohumol	Chalcone	Hops (*Humulus lupulus*)	Rat adrenal PC12 pheochromocytoma cell line	Yao et al., 2015
	[6]-Shogaol	Phenylpropanoid	Ginger	HCT-116 colorectal carcinoma cell line	Chen et al., 2014
	Sulforaphane	Isothiocyanate	Broccoli	*In vitro* protein/chemical interaction (Keap1/sulforaphane)	Hu et al., 2011
	Falcarindiol	Polyacetylene	Parsley and carrots	HEK293 embryonic kidney cell line	Ohnuma et al., 2010
	Carnosic acid	Diterpene	Rosemary from *Rosmarinus officinalis*	Rat adrenal PC12h pheochromocytoma cell line and COS7 fibroblast-like cell line	Satoh et al., 2008
Disruption of Nrf2/Keap1 interaction	**Plants**				
	Khayandirobilide A	Andirobin-type limonoid	*Khaya senegalensis*	RAW 264.7 macrophage cell line and BV-2 microglia cells line	Zhou et al., 2018
	Epigallocatechin gallate	Catechin	Tea	THP-1 monocytic cell line and mice	Jiang et al., 2012; Sun et al., 2017
	Ethyl acetate extract	N/A	*Salvia miltiorrhiza*	Mouse mesangial cell (MMC) line SV40-MES-13 and mice	An et al., 2017
	Carexanes	Stilbenoid	*Carex distachya* Desf.	AGS gastric epithelial cell line	Buommino et al., 2017
	α-Linolenic acid	Polyunsaturated fatty acid	Canola, soybean, wild berries, perilla, and walnut	Rats	Yu et al., 2013
Epigenetic modulation	**Marine products**				
	Fucoxanthin	Carotenoid	Microalgae and seaweeds	HepG2 immortalized and human hepatoma cell line and JB6 P+ epidermal cells	Yang et al., 2018c

(Continued)

TABLE 1 | Continued

Mode of action	Bioactive compounds	Classification	Sources	Model	Reference
	Plants				
	Sulforaphane	Isothiocyanate	Broccoli	N2a neuroblastoma cell line	Zhao F. et al., 2018
	Corosolic acid	Pentacyclic triterpene acid	*Schisandra chinensis, Eriobotrya japonica, Lagerstroemia speciosa* L., *Orthosiphon stamineus* and *Weigela subsessilis*	Transgenic cell line of C57BL/6 mice (TRAMP-C1 cells)	Jie et al., 2018
	Dioscin	Steroid saponin	*Dioscorea nipponica* Makino	H9c2 embryonic cardiomyocyte cell line	Zhao L. et al., 2018
	Taxifolin	Flavanonol	*Pseudotsuga taxifolia, Taxus chinensis, Cedrus deodara* and *Pinus roxburghii*	HepG2 immortalized and human hepatoma cell line and JB6 P+ epidermal cells	Kuang et al., 2017
	Reserpine	Indole alkaloid	*Rauvolfia verticillata*	HepG2-C8 immortalized and human hepatoma cell line and JB6 P+ epidermal cells	Hong et al., 2016
	Quercetin	Flavonol	Red kidney bean, caper, radish, onion	Mice	Liu et al., 2015
	Z-Ligustilide	Phthalides	Radix Angelicae Sinensis	Transgenic cell line of C57BL/6 mice (TRAMP C1 cells)	Su et al., 2013
	Curcumin	Diarylheptanoid	Turmeric	Mice and rats	Khor et al., 2011; Muta et al., 2016
ERK phosphorylation	**Marine products**				
	Astaxanthin	Carotenoid	Red-colored aquatic organisms	HUVEC endothelial cells	Niu et al., 2018
	Plants				
	Methyleugenol	Phenylpropanoid	Clove, lemon grass, anise and laurel leaf oils	RAW 264.7 and J774A.1 macrophage cell lines	Zhou et al., 2017
	Dihydromyricetin	Flavanonol	Vine tea	HUVEC endothelial cells	Luo et al., 2017
	Sodium tanshinone IIA sulfonate	Water-soluble derivative of tanshinone IIA	*Salvia miltiorrhiza* Bunge (Danshen)	Rats	Wei et al., 2013
AMPK/GSK3β phosphorylation	**Plants**				
	Methyleugenol	Phenylpropanoid	Clove, lemon grass, anise and laurel leaf oils	RAW 264.7 and J774A.1 macrophage cell lines	Zhou et al., 2017
	Butin	Flavanone	*Dalbergia odorifera*	Mice and H9c2 embryonic cardiomyocyte cell line	Duan et al., 2017
	Betulin	Triterpene	Birch tree bark	RAW 264.7 macrophage cell line and mice	Ci et al., 2017

(Continued)

TABLE 1 | Continued

Mode of action	Bioactive compounds	Classification	Sources	Model	Reference
	Xanthohumol	Chalcone	Hops (*Humulus lupulus*)	Mice	Lv et al., 2017
p38 MAPK phosphorylation	**Plants**				
	Fisetin	Flavonol	Strawberries, persimmons and apples	Rat adrenal pheochromocytoma cells (PC12 cells)	Yen et al., 2017
PI3K/AKT phosphorylation	**Plants**				
	Dihydromyricetin	Flavanonol	Vine tea	HUVEC endothelial cells	Luo et al., 2017
	Paeonol and danshensu combination	Polyphenol	*Cortex Moutan* and *Radix Salvia miltiorrhiza*	Rats	Li et al., 2016
	Punicalagin	Phenolic	*Punica granatum* L.	Mouse macrophage cells (RAW 264.7 cells)	Xu et al., 2015
	3-Caffeoyl, 4-dihydrocaffeoyl quinic acid	Chlorogenic acid derivative	*Salicornia herbacea*	Hepa1c1c7 c hepatoma cell line	Hwang et al., 2009

through inhibiting the function of Keap1 by forming hydrogen bonds with specific residues such as Ser 508, Ser 555, Ser 602, Tyr 525, Tyr 572, Gln 530, and Arg 483 (Sun et al., 2017). Besides, α-linolenic acid (ALA) has been found to protect against DOX-induced cardiotoxicity by exerting anti-oxidative and anti-apoptosis properties in rat model. The underlying mechanism is associated with the enhancement of antioxidant defense system through Nrf2/Keap1 pathway by promoting the degradation of Keap1 and thus facilitating nuclear translocation of Nrf2, as well as activation of AKT/ERK pathway (Yu et al., 2013). Similarly, khayandirobilide A (KLA) also exhibits anti-inflammatory effect via elevated expression of HO-1 by inducing Keap1 autophagic degradation and thus facilitating Nrf2 nuclear translocation (Zhou et al., 2018).

Epigenetic Modification

Epigenetic mechanisms have been reported to be associated with the pathogenesis of CVD. Epigenomics study in atherosclerotic human aorta demonstrated a genome-wide increase in DNA methylation during the onset and progression of atherosclerosis (Zaina et al., 2014). Studies have demonstrated that DNA demethylation and histone (de)acetylation can trigger or increase Nrf2 expression. Natural compounds derived from plants (such as sulforaphane, corosolic acid, taxifolin, reserpine, quercetin, Z-ligustilide and curcumin) and marine constituents (fucoxanthin) have been shown to activate Nrf2 signaling through epigenetic regulation (Khor et al., 2011; Su et al., 2013; Liu et al., 2015; Hong et al., 2016; Muta et al., 2016; Kuang et al., 2017; Jie et al., 2018; Yang et al., 2018c; Zhao F. et al., 2018). Quercetin, a natural flavonoid, significantly inhibited nickel-induced inflammation in mouse liver by decreasing Nrf2 DNA methylation and inhibiting the p38 MAPK signaling pathway (Liu et al., 2015). In addition, some natural compounds are involved in regulating miRNAs expression, which in turn activate Nrf2 pathway. For instance, treatment with dioscin, a natural steroid saponin markedly decreased the expression level of miRNA-140-5p, and subsequently activates Nrf2 and silent information regulator factor 2-related enzyme 2 (Sirt2) in cardiac H9c2 cells, thereby upregulating downstream target genes such as HO-1, NQO1, GST, GCLM, and forkhead box O3 (FOXO3a) (Zhao L. et al., 2018). However, the mechanism underlying epigenetic pathway responsible for the cardioprotective effects of natural product remains to be elucidated.

Protein Kinases Modulation

Protein kinases such as ERK, AMPK, GSK3β, p38 MAPK, and PI3K/AKT can mediate Nrf2 phosphorylation which enhance Nrf2 stability, thereby promoting nuclear Nrf2 translocation and transactivation activity (Nguyen et al., 2003; Bryan et al., 2013). Several natural compounds, including sodium tanshinone IIA sulfonate (STS), dihydromyricetin (DMY), methyleugenol (MLG), and astaxanthin have been shown to phosphorylate ERK, and upregulate Nrf2 expression (Wei et al., 2013; Luo et al., 2017; Zhou et al., 2017; Niu et al., 2018). STS, a water-soluble derivative of tanshione IIA, was found to protect against isoproterenol (ISO)-induced myocardial infarction (MI) in rat model. Pre-treatment with STS has dramatically increased ERK phosphorylation, and subsequently enhanced the expressions of Nrf2 and HO-1 (Wei et al., 2013). DMY has also been shown to ameliorate oxLDL-induced oxidative injury in HUVECs through activation of Akt/ERK/Nrf2/HO-1 (Luo et al., 2017). Similarly, MLG

protects against t-BHP-triggered cytotoxicity and attenuated ROS generation by inducing antioxidant enzymes expression and ERK phosphorylation in RAW 264.7 and J774A.1 murine macrophage cell lines. Interestingly, MLG has also shown to phosphorylate AMPK and GSK3β, which leads to upregulation of Nrf2 and its downstream target genes (Zhou et al., 2017). Besides targeting ERK pathway, other cardioprotective natural products such as butin, xanthohumol, botulin, fisetin, paeonol and danshensu combination (PDSS), punicalagin and 3-caffeoyl, 4-dihydrocaffeoyl quinic acid can activate Nrf2 through phosphorylation AMPK, GSK3, p38 MAPK, and PI3K/AKT.

THE CARDIOPROTECTIVE MECHANISMS OF NATURAL PRODUCTS REGULATING Nrf2-ASSOCIATED CELLULAR PROCESSES IN CARDIOVASCULAR DISEASES

Apart from targeting the Nrf2/Keap1 interaction, some cardioprotective natural products were found to regulate Nrf2-associated cellular processes. The mechanisms of natural products in regulating Nrf2-associated cellular processes were summarized and tabulated in **Table 2**.

Regulation of ROS and Glutathione Levels

Glutathione (GSH) is a sulfhydryl group tripeptide composed of glutamate, cysteine and glycine. It plays an important role in cellular redox homeostasis. Several studies suggested that GSH can protect cells against oxidative stress due to its capability in interacting with antioxidant enzymes. Examples of GSH-linked defense enzymes include GSH peroxidase (GPX), GST, glutathione reductase (GRD), thioredoxins (Trx), glutaredoxin (GRX2), and PRX (Ribas et al., 2014; Ye et al., 2015). Glutathione is present mainly in reduced form and it is only oxidized into glutathione disulfide (GSSG) in the presence of oxidative stress. Thus, the ratio of GSH and GSSG within the cells is often used as the index for intracellular oxidative stress. Furthermore, clinical evidences have shown an association between GSH level and CVD. Low GSH level was observed in patients with the most severe cases of heart failure. Patients with cardiac diseases showed 21% depletion in blood GSH than healthy controls (Damy et al., 2009). Chronic depletion of myocardial GSH levels was reported in GCLM-deficient mice after transverse aortic constriction-induced pressure overload, and this condition leads to increased left ventricular dilation, myocardial fibrosis, and dysfunction (Watanabe et al., 2013).

Recent studies have reported the role of natural products at inducing glutathione-linked enzymes via Nrf2 signaling pathway. Azafrin, a natural carotenoid, have shown to induce Nrf2 downstream target genes such as HO-1, GCLC, GCLM, Trx1 and GST (Yang et al., 2018a). This is in consistent with another study showing that pre-treatment with triptolide, a diterpenoid epoxide protected Wistar rat from myocardial ischemia/reperfusion injuries by suppressing the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and inducing Nrf2-regulated antioxidant enzymes (SOD, GSH, GPx, and HO-1) (Yu et al., 2016). Besides, coffee constituent 5-*O*-caffeoylquinic acid (CGA) has also been shown to enhance Nrf2 nuclear translocation and increase the transcriptional expression of γ-GCL, HO-1 and GSTA1 (Boettler et al., 2011). Curcumin on the other hand has been found to attenuate hemin-induced ROS production and increase the ratio of GSH/GSSG in primary cultures of rat cerebellar granule neurons (CGNs). Furthermore, it also increased the cytoprotective enzymes such as HO-1, GR, GST, and SOD via inducing Nrf2 nuclear translocation (Gonzlez-Reyes et al., 2013).

Crosstalk With NF-κB Inflammatory Signaling Pathway

The transcription factor NF-κB plays a crucial role in regulating innate immunity and inflammatory responses, and it is also involved in the pathogenesis of atherosclerotic plaques formation (Maracle et al., 2018). Recent studies showed that there are potential crosstalk between NF-κB and Nrf2. Nrf2 deficient mice were found to have increased NF-κB activation, inflammatory cytokines TNF-α, IL-1β, and IL-6 production, and ICAM-1 expression in brain after traumatic brain injury compared to wild-type mice (Jin et al., 2008). Evidence suggests that Nrf2/Keap1 pathway can inactivate NF-κB activity through ubiquitin-mediated degradation of IKKβ (Lee et al., 2009). Besides, it has been reported that p65, a canonical NF-κB subunit antagonized the transcriptional activity of Nrf2 by depriving Nrf2 transcriptional co-activator CBP (Liu et al., 2008). These data suggest that there is a crosstalk between Nrf2 and NF-κB in regulating the transcription of its downstream target proteins.

Several natural compounds have been reported to protect against CVDs by targeting both Nrf2 and NF-κB signaling pathways. For instance, Ligustilide, a phthalide compound inhibited VCAM-1, ICAM-1 and E-selectin expression by suppressing the NF-κB activation as well as inducing Nrf2-mediated HO-1 expression in TNF-α-stimulated HUVECs (Choi et al., 2018). Similarly, bioactive compounds such as cyanidin-3-*O*-glucoside and isothiocyanates have been reported to counteract the pathogenesis of endothelial dysfunction, including upregulation of Nrf2-dependent antioxidant response elements (HO-1, GCLC and GCLM) and downregulation of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) via inhibition of NF-κB activation (Huang et al., 2013; Fratantonio et al., 2015). Furthermore, *Antrodia salmonea*, a medicinal fungal species exerts an anti-angiogenic and anti-atherogenic activity in human vascular endothelial cell line (EA.hy 926) and human leukemic monocyte lymphoma cell line (U937). It significantly suppressed TNF-α-induced matrix metalloproteinase-9 (MMP-9) and ICAM-1 expression via suppressing I-κB degradation and I-κB kinase phosphorylation, as well as upregulating the expression of

TABLE 2 | Molecular mechanisms of bioactive compounds from natural products targeting Nrf2-associated cellular processes.

Nrf2-associated cellular processes in CVD	Bioactive compounds	Classification	Sources	Mechanisms of action	Model	Reference
Regulation of ROS and glutathione levels	Curcumin	Diarylheptanoid	Turmeric	↑ Nrf2, ↑ HO-1, GSH, GRD, GST and SOD, ↑ GSH/GSSG ratio	Primary cultures of rats cerebellar granule neurons	Gonzlez-Reyes et al., 2013
	5-*O*-caffeoylquinic acid	Chlorogenic acid	Coffee	↑ Nrf2, ↑ γ-GCL, HO-1 and GSTA1	HT29 colon carcinoma cell line	Boettler et al., 2011
	Azafrin	Carotenoid	Dried root of *Centranthera grandiflora*	↑ Nrf2, ↑ mRNA expression levels of HO-1, NQO1, GCLC, GCLM, Trx1 and GST	HEK293 embryonic kidney and H9c2 embryonic cardiomyocyte cell lines	Yang et al., 2018a
	Triptolide	Diterpenoid epoxide	*Tripterygium wilfordii* Hook F	↑ Nrf2, ↓ TNF-α, IL-1β, IL-6 and MDA, ↑ HO-1, SOD, GSH and GPx	Rats	Yu et al., 2016
Crosstalk with NF-κB inflammatory signaling pathway	Ligustilide	Phthalide	*Cnidii Rhizoma* and *Angelicae Gigantis Radix*	↑ Nrf2, ↑ HO-1, ↑ intracellular NO synthesis, ↓ TNF-α-ROS, ↓ NF-κB, ↓ ICAM-1, VCAM-1 and E-selectin	HUVEC endothelial cells and HL-60 leukemia cells	Choi et al., 2018
	(-)-7(S)-hydroxymatairesinol	Lignan	Norway spruce (*Picea abies*)	↑ Nrf2, ↑ superoxide dismutase and HO-1, ↓ phosphorylation of ERK and Akt, ↓p65, ↓ NF-κB, ↓ TNF-α-induced VCAM-1, IL-6 and iNOS, ↓ ROS	Rat aortic endothelial cells (RAECs)	Yang et al., 2017
	Baicalein	Flavone	*Scutellaria baicalensis* and *Scutellaria lateriflora*	↑ Nrf2, ↑ HO-1, ↓ IκBα phosphorylation and p65, ↓ NF-κB, ↓ TBARS, iNOS and nitrites	Mice	Sahu et al., 2016
	Cyanidin-3-*O*-glucoside	Anthocyanins	Food plants rich in anthocyanins	↑ Nrf2, ↑ HO-1 and NQO-1, ↓ NF-κB, ↓ E-selectin and VCAM-1	HUVEC endothelial cells	Fratantonio et al., 2015
	Curcumin	Diarylheptanoid	Turmeric	↑ Nrf2, ↑ HO-1, GCLC, and NQO-1, ↓ NF-κB, ↓ TNF-α, IL-1β and IL-6, ↓ caspase-3, Bax and ↑ Bcl2, ↓ TGF-β	H9c2 embryonic cardiomyocyte cell line	Zeng et al., 2015
	Antrodia salmonea	Fungus	Rotten trunk of *Cunninghamia konishii*	↑ Nrf2, ↑ HO-1 and γ-GCLC, ↓ NF-κB, ↓ I-κB degradation and phosphorylation of IKKα, ↓ MMP-9 and ICAM-1	EA.hy926 endothelial cells and U937 leukemic monocyte lymphoma cell line	Yang et al., 2014
	Sulforaphane, benzyl isothiocyanate and phenethyl isocyanate	Isothiocyanates	Cruciferous vegetables	↑ Nrf2,↑ HO-1, GCLC and GCLM, ↓ ROS, ↓ NF-κB, ↓ ICAM-1, VCAM-1 and E-selectin	HUVEC endothelial cells and HL-60 leukemia cell line	Huang et al., 2013
Regulation of cholesterol uptake and efflux	Tanshinone IIA	Phenanthrenequinone	*Salvia miltiorrhiz*a Bunge (Danshen)	↑ Nrf2, ↑ HO-1, ↓ SR-A, ↑ ABCA1 and ABCG1	THP-1 monocytic cell line and Mice	Liu et al., 2014
	Tanshindiol C	Phenanthrenequinone	Root of *Salvia miltiorrhiz*a Bge.	↑ Nrf2 and Sirt1, ↑Prdx1, ↑ABCA1	Primary cultures of rats cerebellar granule neurons	Yang et al., 2018b
	Epigallocatechin-3-gallate	Catechin	Tea	↑ Nrf2, ↓TNF-α-induced NF-κB activation, ↑ABCA1	HT29 colon carcinoma cell line	Jiang et al., 2012

(Continued)

TABLE 2 | Continued

Nrf2-associated cellular processes in CVD	Bioactive compounds	Classification	Sources	Mechanisms of action	Model	Reference
	4-*O*-methylhonokiol	Phenolic	*Magnolia officinalis*	↑ Nrf2 and Akt2, ↓ CD36	HEK293 embryonic kidney and H9c2 embryonic cardiomyocyte cell lines	Zhang et al., 2015
	Oleanolic acid	Pentacyclic triterpenoid	*Fructus Ligustrum lucidum* and *Forsythiae fructus*	↑ Nrf2, ↑HO-1, ↓LOX-1 and NADPH oxidase subunits	Rats	Jiang Q. et al., 2015
	Salidroside	Tyrosol glucoside	*Rhodiola rosea*	↓ Phosphorylation of JNK, ERK, p38 MAPK,↑ Akt, ↑ Nrf2, ↓ LOX-1, ↑ABCA1	HUVEC endothelial cells and HL-60 leukemia cell line	Ni et al., 2017

An upward-pointing arrow (↑) indicates increase; a downward-pointing arrow (↓) indicates decrease.

HO-1 and γ-GCLC through Nrf2 signaling pathway (Yang et al., 2014).

Regulation of Cholesterol Uptake and Efflux (Foam Cell Formation)

Macrophage foam cell formation represents the early hallmarks of atherosclerosis lesion formation. Foam cells formation is closely associated with abnormal cholesterol metabolism that results from imbalanced cholesterol uptake and efflux. Macrophages may take up modified LDL via scavenger receptors or through the pinocytosis process. Scavenger receptors such as scavenger receptors class A (SR-A), scavenger receptor class B (CD36), and lectin-type oxidized LDL receptor (LOX-1) have been implicated in the pathogenesis of atherosclerosis (Kunjathoor et al., 2002; Schaeffer et al., 2009). Several studies suggested that SR-A and CD36 exert pro-atherogenic properties due to their ability to interact with modified LDL, thereby contributing to the foam cell formation. Silencing SR-A or CD36 alone in $LDLR^{-/-}$ ApoB100 mice was shown to profoundly protect against atherosclerosis (Mäkinen et al., 2010). Besides, studies have shown that cholesterol efflux transporters such as scavenger receptor class B type 1 (SR-B1), ABCA1 and ABCG1 promote efflux of free cholesterol to apolipoproteinA-1 (apoA-1) and HDL. Deficiency of either one or both efflux transporters (ABCA1 or ABCG1) have shown to enhance lipopolysaccharide (LPS)-induced inflammatory gene expression, reduce aortic endothelial NO synthase (eNOS), increase monocyte adhesion and infiltration into atherosclerotic plaque (Westerterp et al., 2016). Similarly, double-knockout ABCA1 and ABCG1 mice administered with a high cholesterol diet exhibited extensive infiltration of macrophage foam cells in the myocardium and spleen and have shown accelerated progression in atherosclerosis (Yvan-Charvet et al., 2007; Westerterp et al., 2013).

The role of Nrf2 in the transcriptional regulation of these scavenger receptors and cholesterol efflux transporters has been established. Many recent studies have reported the role of natural compounds in modulating cholesterol uptake and efflux receptors by targeting the Nrf2 signaling pathway. Oleanolic acid (OA), a natural pentacyclic triterpenoid, exerts anti-atherosclerotic effect in quail models and HUVECs where it was shown to inhibit oxLDL-induced LOX-1 and NADPH oxidase subunits expression while increasing the expression of Nrf2 and HO-1 (Jiang Q. et al., 2015). Furthermore, salidroside protects against foam cells formation by upregulating ABCA1 and downregulation of LOX-1 via activation of MAPK/Akt/Nrf2 pathways (Ni et al., 2017). This is consistent with another study which showed that Tanshinone IIA (Tan) inhibit atherosclerotic plaque formation in $ApoE^{-/-}$ mice and it is suggested that Tan regulates cholesterol metabolism by reducing the expression of SR-A, while further enhancing ABCA1 and ABCG1 expression in human THP-1 cells via activation of ERK/Nrf2/HO-1 pathway (Liu et al., 2014). Other plant constituents (as shown in **Table 2**) such as Tanshindiol C, epigallocatechin-3-gallate, and 4-*O*-methylhonokiol (MH) can regulate SR-A and CD36 receptors via Nrf2 activation and its downstream effects.

RECENT INSIGHTS OF THE CLINICAL INVESTIGATION OF Nrf2-ACTIVATORS

Nrf2 is a potential therapeutic target for the treatment of multiple sclerosis, diabetes mellitus, Parkinson's disease, cancer, and others diseases. Several Nrf2 activators were currently being tested in human clinical trials. For instance, bardoxolone methyl is an OA derivative tested in the Phase II/III clinical trials for the treatment of Alport syndrome, a genetic disorder characterized by glomerulonephritis, eye abnormalities, and hearing loss (Chin et al., 2018; Gross et al., 2018). Bardoxolone methyl activates Nrf2 by disrupting the Nrf2/Keap1 interaction and inhibits IKKβ kinase activity (Wang et al., 2014). It was also reported for its beneficial effect in Phase III clinical trial for type 2 diabetes and stage 4 chronic kidney disease patients, where it was shown to improve the estimated glomerular filtration rate (GFR) (Chin et al., 2018). It is suggested that bardoxolone methyl increases GFR by restoring endothelial dysfunction and reducing angiotensin II-induced glomerular mesangial cell contraction (Aminzadeh et al., 2013; Ding et al., 2013). In addition, Protandim®, a nutritional supplement containing five natural Nrf2 activators such as bacosides, silymarin, withanolides, ECGC and curcumin has been shown to reduce oxidative stress and increase antioxidant enzymes SOD and catalase expression (Nelson et al., 2006). Similarly, increased SOD expression was also observed after oral Protandim® supplementation in runners (Ueberschlag et al., 2016). Considering that many studies have reported the role of natural compounds in activating Nrf2 pathway, further investigations and validations in the clinical setting may help to accelerate its development as therapeutics protecting against CVD.

CONCLUSION

Transcription factor Nrf2 serves as the master regulator of cellular antioxidant defense system which has shown to protect against endothelial dysfunction, foam cells formation and atherosclerotic lesion development. Compelling evidences in this paper have demonstrated that a wide range of bioactive compounds derived from natural sources activate Nrf2/Keap1 signaling and protect against CVD development. These studies suggest that bioactive compounds may serve as new therapeutic strategies targeting CVD via Nrf2 pathway.

AUTHOR CONTRIBUTIONS

This writing was performed by BO. BG and K-GC provided vital guidance and insight to the work. WY did some literature review and amended the review. K-GC, BG, and WY contributed to the funding of the project. The project was conceptualized by WY and BG.

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REFERENCES

Aminzadeh, M. A., Reisman, S. A., Vaziri, N. D., Shelkovnikov, S., Farzaneh, S. H., Khazaeli, M., et al. (2013). The synthetic triterpenoid RTA dh404 (CDDO-dhTFEA) restores endothelial function impaired by reduced Nrf2 activity in chronic kidney disease. *Redox Biol.* 1, 527–531. doi: 10.1016/j.redox.2013.10.007

An, L., Zhou, M., Marikar, F. M. M. T., Hu, X. W., Miao, Q. Y., Li, P., et al. (2017). Salvia miltiorrhiza lipophilic fraction attenuates oxidative stress in diabetic nephropathy through activation of nuclear factor erythroid 2-related factor 2. *Am. J. Chin. Med.* 45, 1441–1457. doi: 10.1142/s0192415x17500781

Apopa, P. L., He, X., and Ma, Q. (2008). Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. *J. Biochem. Mol. Toxicol.* 22, 63–76. doi: 10.1002/jbt.20212

Bai, Y., Wang, X., Zhao, S., Ma, C., Cui, J., and Zheng, Y. (2015). Sulforaphane protects against cardiovascular disease via Nrf2 activation. *Oxid. Med. Cell. Longev.* 2015:407580. doi: 10.1155/2015/407580

Barajas, B., Che, N., Yin, F., Rowshanrad, A., Orozco, L. D., Gong, K. W., et al. (2011). NF-E2-related factor 2 promotes atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow antioxidant protection. *Arterioscler. Thromb. Vasc. Biol.* 31, 58–66. doi: 10.1161/ATVBAHA.110.210906

Basak, P., Sadhukhan, P., Sarkar, P., and Sil, P. C. (2017). Perspectives of the Nrf-2 signaling pathway in cancer progression and therapy. *Toxicol. Rep.* 4, 306–318. doi: 10.1016/j.toxrep.2017.06.002

Beischlag, T. V., Morales, J. L., Hollingshead, B. D., and Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. *Crit. Rev. Eukaryot. Gene Expr.* 18, 207–250.

Bigagli, E., Cinci, L., Paccosi, S., Parenti, A., D'Ambrosio, M., and Luceri, C. (2017). Nutritionally relevant concentrations of resveratrol and hydroxytyrosol mitigate oxidative burst of human granulocytes and monocytes and the production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 *macrophages*. *Int. Immunopharmacol.* 43, 147–155. doi: 10.1016/j.intimp.2016.12.012

Boettler, U., Sommerfeld, K., Volz, N., Pahlke, G., Teller, N., Somoza, V., et al. (2011). Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression. *J. Nutr. Biochem.* 22, 426–440. doi: 10.1016/j.jnutbio.2010.03.011

Bryan, H. K., Olayanju, A., Goldring, C. E., and Park, B. K. (2013). The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochem. Pharmacol.* 85, 705–717. doi: 10.1016/j.bcp.2012.11.016

Buommino, E., D'Abrosca, B., Donnarumma, G., Parisi, A., Scognamiglio, M., Fiorentino, A., et al. (2017). Evaluation of the antioxidant properties of carexanes in AGS cells transfected with the *Helicobacter* pylori's protein HspB. *Microb. Pathog.* 108, 71–77. doi: 10.1016/j.micpath.2017.05.007

Canning, P., Sorrell, F. J., and Bullock, A. N. (2015). Structural basis of Keap1 interactions with Nrf2. *Free Rad. Biol. Med.* 88(Pt B), 101–107. doi: 10.1016/j.freeradbiomed.2015.05.034

Cao, H., Wang, L., Chen, B., Zheng, P., He, Y., Ding, Y., et al. (2016). DNA demethylation upregulated Nrf2 expression in Alzheimer's disease cellular model. *Front. Aging Neurosci.* 7:244. doi: 10.3389/fnagi.2015.00244

Chen, H., Fu, J., Chen, H., Hu, Y., Soroka, D. N., Prigge, J. R., et al. (2014). Ginger compound [6]-shogaol and its cysteine-conjugated metabolite (M2) activate nrf2 in colon epithelial cells in vitro and in vivo. *Chem. Res. Toxicol.* 27, 1575–1585. doi: 10.1021/tx500211x

Chen, X., Liu, Y., Zhu, J., Lei, S., Dong, Y., Li, L., et al. (2016). GSK-3β downregulates Nrf2 in cultured cortical neurons and in a rat model of cerebral ischemia-reperfusion. *Sci. Rep.* 6:20196. doi: 10.1038/srep20196

Chin, M. P., Bakris, G. L., Block, G. A., Chertow, G. M., Goldsberry, A., Inker, L. A., et al. (2018). Bardoxolone methyl improves kidney function in patients with chronic kidney disease stage 4 and type 2 diabetes: post-Hoc analyses from bardoxolone methyl evaluation in patients with chronic kidney disease and type 2 diabetes study. *Am. J. Nephrol.* 47, 40–47. doi: 10.1159/000486398

Cho, H. (2013). Genomic structure and variation of nuclear factor (erythroid-derived 2)-like 2. *Oxid. Med. Cell. Longev.* 2013:24. doi: 10.1155/2013/286524

Choi, E. S., Yoon, J. J., Han, B. H., Jeong, D. H., Lee, Y. J., Kang, D. G., et al. (2018). Ligustilide attenuates vascular inflammation and activates Nrf2/HO-1 induction and, NO synthesis in HUVECs. *Phytomedicine* 38, 12–23. doi: 10.1016/j.phymed.2017.09.022

Ci, X., Zhou, J., Lv, H., Yu, Q., Peng, L., and Hua, S. (2017). Betulin exhibits anti-inflammatory activity in LPS-stimulated macrophages and endotoxin-shocked mice through an AMPK/AKT/Nrf2-dependent mechanism. *Cell Death Amp. Dis.* 8:e2798. doi: 10.1038/cddis.2017.39

Collins, A. R., Gupte, A. A., Ji, R., Ramirez, M. R., Minze, L. J., Liu, J. Z., et al. (2012). Myeloid deletion of nuclear factor erythroid 2-related factor 2 increases atherosclerosis and liver injury. *Arterioscler. Thromb. Vasc. Biol.* 32, 2839–2846. doi: 10.1161/ATVBAHA.112.300345

Copple, I. M., Lister, A., Obeng, A. D., Kitteringham, N. R., Jenkins, R. E., Layfield, R., et al. (2010). Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. *J. Biol. Chem.* 285, 16782–16788. doi: 10.1074/jbc.M109.096545

Cullinan, S. B., and Diehl, J. A. (2004). PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J. Biol. Chem.* 279, 20108–20117. doi: 10.1074/jbc.M314219200

Dal, S., and Sigrist, S. (2016). The protective effect of antioxidants consumption on diabetes and vascular complications. *Diseases* 4:24. doi: 10.3390/diseases4030024

Damy, T., Kirsch, M., Khouzami, L., Caramelle, P., Le Corvoisier, P., Roudot-Thoraval, F., et al. (2009). Glutathione deficiency in cardiac patients is related to the functional status and structural cardiac abnormalities. *PLoS One* 4:e4871. doi: 10.1371/journal.pone.0004871

Dietrich, C. (2016). Antioxidant functions of the aryl hydrocarbon receptor. *Stem Cells Int.* 2016:10. doi: 10.1155/2016/7943495

Ding, Y., Stidham, R., Bumeister, R., Trevino, I., Winters, A., Sprouse, M., et al. (2013). The synthetic triterpenoid, RTA405, increases glomerular filtration rate and reduces angiotensin II-induced contraction of glomerular mesangial cells. *Kidney Int.* 83, 845–854. doi: 10.1038/ki.2012.393

Ding, Y., Zhang, B., Zhou, K., Chen, M., Wang, M., Jia, Y., et al. (2014). Dietary ellagic acid improves oxidant-induced endothelial dysfunction and atherosclerosis: Role of Nrf2 activation. *Int. J. Cardiol.* 175, 508–514. doi: 10.1016/j.ijcard.2014.06.045

Duan, J., Guan, Y., Mu, F., Guo, C., Zhang, E., Yin, Y., et al. (2017). Protective effect of butin against ischemia/reperfusion-induced myocardial injury in diabetic mice: involvement of the AMPK/GSK-3β/Nrf2 signaling pathway. *Sci. Rep.* 7:41491. doi: 10.1038/srep41491

Fratantonio, D., Speciale, A., Ferrari, D., Cristani, M., Saija, A., and Cimino, F. (2015). Palmitate-induced endothelial dysfunction is attenuated by cyanidin-3-O-glucoside through modulation of Nrf2/Bach1 and NF-κB pathways. *Toxicol. Lett.* 239, 152–160. doi: 10.1016/j.toxlet.2015.09.020

Freigang, S., Ampenberger, F., Spohn, G., Heer, S., Shamshiev, A. T., Kisielow, J., et al. (2011). Nrf2 is essential for cholesterol crystal-induced inflammasome activation and exacerbation of atherosclerosis. *Eur. J. Immunol.* 41, 2040–2051. doi: 10.1002/eji.201041316

Furue, M., Uchi, H., Mitoma, C., Hashimoto-Hachiya, A., Chiba, T., Ito, T., et al. (2017). Antioxidants for healthy skin: the emerging role of aryl hydrocarbon receptors and nuclear factor-erythroid 2-related factor-2. *Nutrients* 9:223. doi: 10.3390/nu9030223

Gonzlez-Reyes, S., Guzmn-Beltrn, S., Medina-Campos, O. N., and Pedraza-Chaverri, J. (2013). Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. *Oxid. Med. Cell. Longev.* 2013:14. doi: 10.1155/2013/801418

Gross, O., Appel, G., Block, G., Chin, M., Goldsberry, A., Inker, L., et al. (2018). A phase 2/3 study of the efficacy and safety of bardoxolone methyl in patients with Alport syndrome. *Nephrol. Dialys. Trans.* 33(Suppl._1), i384–i385. doi: 10.1093/ndt/gfy104.SP121

Grosso, G., Stepaniak, U., Micek, A., Stefler, D., Bobak, M., and Pająk, A. (2017). Dietary polyphenols are inversely associated with metabolic syndrome in Polish adults of the HAPIEE study. *Eur. J. Nutr.* 56, 1409–1420. doi: 10.1007/s00394-016-1187-z

Grover-Páez, F., and Zavalza-Gómez, A. B. (2009). Endothelial dysfunction and cardiovascular risk factors. *Diabetes. Res. Clin. Pract* 84, 1–10. doi: 10.1016/j.diabres.2008.12.013

Guo, Y., Yu, S., Zhang, C., and Kong, A.-N. T. (2015). Epigenetic regulation of Keap1-Nrf2 signaling. *Free Radic. Biol. Med.* 88(Pt B), 337–349. doi: 10.1016/j.freeradbiomed.2015.06.013

Hadi, H. A. R., Carr, C. S., and Al Suwaidi, J. (2005). Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc. Health Risk Manag.* 1, 183–198.

Hajjar, D. P., and Gotto, A. M. (2013). Biological relevance of inflammation and oxidative stress in the pathogenesis of arterial diseases. *Am. J. Pathol.* 182, 1474–1481. doi: 10.1016/j.ajpath.2013.01.010

Hamed, I., Özogul, F., Özogul, Y., and Regenstein, J. M. (2015). Marine bioactive compounds and their health benefits: a review. *Compr. Rev. Food Sci. Food Saf.* 14, 446–465. doi: 10.1111/1541-4337.12136

Harada, N., Ito, K., Hosoya, T., Mimura, J., Maruyama, A., Noguchi, N., et al. (2012). Nrf2 in bone marrow-derived cells positively contributes to the advanced stage of atherosclerotic plaque formation. *Free Rad. Biol. Med.* 53, 2256–2262. doi: 10.1016/j.freeradbiomed.2012.10.001

Hashimoto, K., Simmons, A. N., Kajino-Sakamoto, R., Tsuji, Y., and Ninomiya-Tsuji, J. (2016). TAK1 regulates the Nrf2 antioxidant system through modulating p62/ SQSTM1. *Antioxid. Redox Signal.* 25, 953–964. doi: 10.1089/ars.2016.6663

Heyninck, K., Sabbe, L., Chirumamilla, C. S., Szarc, vel Szic, K., Vander Veken, P., et al. (2016). Withaferin A induces heme oxygenase (HO-1) expression in endothelial cells via activation of the Keap1/Nrf2 pathway. *Biochem. Pharmacol.* 109, 48–61. doi: 10.1016/j.bcp.2016.03.026

Hong, B., Su, Z., Zhang, C., Yang, Y., Guo, Y., Li, W., et al. (2016). Reserpine inhibit the JB6 P + cell transformation through epigenetic reactivation of Nrf2-mediated anti-oxidative stress pathway. *AAPS J.* 18, 659–669. doi: 10.1208/s12248-016-9901-6

Hu, C., Eggler, A. L., Mesecar, A. D., and van Breemen, R. B. (2011). Modification of Keap1 cysteine residues by sulforaphane. *Chem. Res. Toxicol.* 24, 515–521. doi: 10.1021/tx100389r

Huang, C. S., Lin, A. H., Liu, C. T., Tsai, C. W., Chang, I. S., Chen, H. W., et al. (2013). Isothiocyanates protect against oxidized LDL-induced endothelial dysfunction by upregulating Nrf2-dependent antioxidation and suppressing NFκB activation. *Mol. Nutr. Food Res.* 57, 1918–1930. doi: 10.1002/mnfr.201300063

Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M. C. B., and Rahu, N. (2016). Oxidative stress and inflammation: What polyphenols can do for us? *Oxid. Med. Cell. Longev.* 2016:7432797. doi: 10.1155/2016/7432797

Hwang, Y. P., Yun, H. J., Chun, H. K., Chung, Y. C., Kim, H. K., Jeong, M. H., et al. (2009). Protective mechanisms of 3-caffeoyl, 4-dihydrocaffeoyl quinic acid from *Salicornia herbacea* against tert-butyl hydroperoxide-induced oxidative damage. *Chem. Biol. Interact* 181, 366–376. doi: 10.1016/j.cbi.2009.07.017

Ichimura, Y., Waguri, S., Sou, Y.-S., Kageyama, S., Hasegawa, J., Ishimura, R., et al. (2013). Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Mol. Cell* 51, 618–631. doi: 10.1016/j.molcel.2013.08.003

Ishida, Y., Ohta, K., Naruse, T., Kato, H., Fukui, A., Shigeishi, H., et al. (2018). Candida albicans β-glucan-containing particles increase HO-1 expression in oral keratinocytes via a reactive oxygen species/p38 mitogen-activated protein kinase/Nrf2 pathway. *Infect. Immun.* 86, e575–e517. doi: 10.1128/iai.00575-17

Jain, A., Lamark, T., Sjøttem, E., Bowitz Larsen, K., Atesoh Awuh, J., Øvervatn, A., et al. (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.* 285, 22576–22591. doi: 10.1074/jbc.M110.118976

Jeddi, F., Soozangar, N., Sadeghi, M. R., Somi, M. H., and Samadi, N. (2017). Contradictory roles of Nrf2/Keap1 signaling pathway in cancer

prevention/promotion and chemoresistance. *DNA Repair.* 54, 13–21. doi: 10.1016/j.dnarep.2017.03.008

Jiang, J., Mo, Z., Yin, K., Zhao, G., Lv, Y., Ouyang, X., et al. (2012). Epigallocatechin-3-gallate prevents TNF-α-induced NF-κB activation thereby upregulating ABCA1 via the Nrf2/Keap1 pathway in macrophage foam cells. *Int. J. Mol. Med.* 29, 946–956. doi: 10.3892/ijmm.2012.924

Jiang, Q., Wang, D., Han, Y., Han, Z., Zhong, W., and Wang, C. (2015). Modulation of oxidized-LDL receptor-1 (LOX1) contributes to the antiatherosclerosis effect of oleanolic acid. *Int. J. Biochem. Cell Biol.* 69, 142–152. doi: 10.1016/j.biocel.2015.10.023

Jiang, Y., Bao, H., Ge, Y., Tang, W., Cheng, D., Luo, K., et al. (2015). Therapeutic targeting of GSK3β enhances the Nrf2 antioxidant response and confers hepatic cytoprotection in hepatitis C. *Gut* 64, 168–179. doi: 10.1136/gutjnl-2013-306043

Jiang, X., Bao, Y., Liu, H., Kou, X., Zhang, Z., Sun, F., et al. (2017). VPS34 stimulation of p62 phosphorylation for cancer progression. *Oncogene* 36:6850. doi: 10.1038/onc.2017.295

Jie, Y., Renyi, W., Wenji, L., Linbo, G., Yuqing, Y., Ping, L., et al. (2018). The triterpenoid corosolic acid blocks transformation and epigenetically reactivates Nrf2 in TRAMP-C1 prostate cells. *Mol. Carcinog.* 57, 512–521. doi: 10.1002/mc.22776

Jin, W., Wang, H., Yan, W., Xu, L., Wang, X., Zhao, X., et al. (2008). Disruption of Nrf2 enhances upregulation of nuclear factor-κB activity, proinflammatory cytokines, and intercellular adhesion molecule-1 in the brain after traumatic brain injury. *Mediat. Inflamm.* 2008:725174. doi: 10.1155/2008/725174

Joo, M. S., Kim, W. D., Lee, K. Y., Kim, J. H., Koo, J. H., and Kim, S. G. (2016). AMPK facilitates nuclear accumulation of nrf2 by phosphorylating at serine 550. *Mol. Cell. Biol.* 36, 1931–1942. doi: 10.1128/mcb.00118-16

Kapuy, O., Papp, D., Vellai, T., Bánhegyi, G., and Korcsmáros, T. (2018). Systems-level feedbacks of Nrf2 controlling autophagy upon oxidative stress response. *Antioxidants* 7:39. doi: 10.3390/antiox7030039

Keum, Y.-S., Yu, S., Chang, P. P.-J., Yuan, X., Kim, J.-H., Xu, C., et al. (2006). Mechanism of action of sulforaphane: Inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element–mediated heme oxygenase-1 in human hepatoma HepG2 cells. *Cancer Res.* 66, 8804–8813. doi: 10.1158/0008-5472.can-05-3513

Khor, T. O., Huang, Y., Wu, T.-Y., Shu, L., Lee, J., and Kong, A.-N. T. (2011). Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation. *Biochem. Pharmacol.* 82, 1073–1078. doi: 10.1016/j.bcp.2011.07.065

Kobayashi, M., and Yamamoto, M. (2006). Nrf2–Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* 46, 113–140. doi: 10.1016/j.advenzreg.2006.01.007

Korashy, H. M., and El-Kadi, A. O. S. (2006). The role of aryl hydrocarbon receptor in the pathogenesis of cardiovascular diseases. *Drug Metab. Rev.* 38, 411–450. doi: 10.1080/03602530600632063

Kuang, H., Tang, Z., Zhang, C., Wang, Z., Li, W., Yang, C., et al. (2017). Taxifolin activates the Nrf2 anti-oxidative stress pathway in mouse skin epidermal JB6 P + cells through epigenetic modifications. *Int. J. Mol. Sci.* 18:1546. doi: 10.3390/ijms18071546

Kunjathoor, V. V., Febbraio, M., Podrez, E. A., Moore, K. J., Andersson, L., Koehn, S., et al. (2002). Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J. Biol. Chem.* 277, 49982–49988. doi: 10.1074/jbc.M209649200

Lee, D.-F., Kuo, H.-P., Liu, M., Chou, C.-K., Xia, W., Du, Y., et al. (2009). Keap1 E3 ligase-mediated down-regulation of NF-κB signaling by targeting IKKβ. *Mol. Cell.* 36, 131–140. doi: 10.1016/j.molcel.2009.07.025

Levonen, A., Inkala, M., Heikura, T., Jauhiainen, S., Jyrkkänen, H., Kansanen, E., et al. (2007). Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth in vitro and reduces oxidative stress in rabbit aorta in vivo. *Arterioscler. Thromb. Vasc. Biol.* 27, 741–747. doi: 10.1161/01.ATV.0000258868.80079.4d

Li, H., Song, F., Duan, L.-R., Sheng, J.-J., Xie, Y.-H., Yang, Q., et al. (2016). Paeonol and danshensu combination attenuates apoptosis in myocardial infarcted rats by inhibiting oxidative stress: Roles of Nrf2/HO-1 and PI3K/Akt pathway. *Sci. Rep.* 6:23693. doi: 10.1038/srep23693

Liu, C. M., Ma, J. Q., Xie, W. R., Liu, S. S., Feng, Z. J., Zheng, G. H., et al. (2015). Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-κB pathway. *Food Chem. Toxicol.* 82, 19–26. doi: 10.1016/j.fct.2015.05.001

Liu, G. H., Qu, J., and Shen, X. (2008). NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochimica et Biophysica Acta Mol. Cell Res.* 1783, 713–727. doi: 10.1016/j.bbamcr.2008.01.002

Liu, X., Zhang, Y., Wu, H., Zhu, P., Mo, X., and Ying, J. (2017). Suppression of microRNA-128 attenuates high glucose-induced podocyte apoptosis through activation of Nrf2-ARE signaling pathway. *Int. J. Clin. Exp. Pathol.* 10, 922–931.

Liu, Z., Wang, J., Huang, E., Gao, S., Li, H., Lu, J., et al. (2014). Tanshinone IIA suppresses cholesterol accumulation in human macrophages: role of heme oxygenase-1. *J. Lipid Res.* 55, 201–213. doi: 10.1194/jlr.M040394

Lujambio, A., and Lowe, S. W. (2012). The microcosmos of cancer. *Nature* 482:347. doi: 10.1038/nature10888

Luo, Y., Lu, S., Dong, X., Xu, L., Sun, G., and Sun, X. (2017). Dihydromyricetin protects human umbilical vein endothelial cells from injury through ERK and Akt mediated Nrf2/HO-1 signaling pathway. *Apoptosis* 22, 1013–1024. doi: 10.1007/s10495-017-1381-3

Lv, H., Liu, Q., Wen, Z., Feng, H., Deng, X., and Ci, X. (2017). Xanthohumol ameliorates lipopolysaccharide (LPS)-induced acute lung injury via induction of AMPK/GSK3β-Nrf2 signal axis. *Redox Biol.* 12, 311–324. doi: 10.1016/j.redox.2017.03.001

Mahfouz, M. M., Zhou, S. Q., and Kummerow, F. A. (2009). Curcumin prevents the oxidation and lipid modification of LDL and its inhibition of prostacyclin generation by endothelial cells in culture. *Prostagland. Other Lipid Mediat.* 90, 13–20. doi: 10.1016/j.prostaglandins.2009.06.005

Mäkinen, P. I., Lappalainen, J. P., Heinonen, S. E., Leppänen, P., Lähteenvuo, M. T., Aarnio, J. V., et al. (2010). Silencing of either SR-A or CD36 reduces atherosclerosis in hyperlipidaemic mice and reveals reciprocal upregulation of these receptors. *Cardiovasc. Res.* 88, 530–538. doi: 10.1093/cvr/cvq235

Manivasagan, P., Venkatesan, J., Sivakumar, K., and Kim, S. K. (2014). Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiol. Res.* 169, 262–278. doi: 10.1016/j.micres.2013.07.014

Maracle, C. X., Agca, R., Helder, B., Meeuwsen, J. A. L., Niessen, H. W. M., Biessen, E. A. L., et al. (2018). Noncanonical NF-κB signaling in microvessels of atherosclerotic lesions is associated with inflammation, atheromatous plaque morphology and myocardial infarction. *Atherosclerosis* 270, 33–41. doi: 10.1016/j.atherosclerosis.2018.01.032

Mascuch, S. J., Boudreau, P. D., Carland, T. M., Pierce, N. T., Olson, J., Hensler, M. E., et al. (2018). Marine natural product Honaucin A attenuates inflammation by activating the Nrf2-ARE pathway. *J. Nat. Prod.* 81, 506–514. doi: 10.1021/acs.jnatprod.7b00734

Matzinger, M., Fischhuber, K., and Heiss, E. H. (2017). Activation of Nrf2 signaling by natural products-can it alleviate diabetes. *Biotechnol. Adv.* 36, 1738–1767. doi: 10.1016/j.biotechadv.2017.12.015

Medina-Remón, A., Casas, R., Tressserra-Rimbau, A., Ros, E., Martínez-González, M. A., Fitó, M., et al. (2017). Polyphenol intake from a Mediterranean diet decreases inflammatory biomarkers related to atherosclerosis: a substudy of the PREDIMED trial. *Br. J. Clin. Pharmacol.* 83, 114–128. doi: 10.1111/bcp.12986

Menegon, S., Columbano, A., and Giordano, S. (2016). The dual roles of Nrf2 in cancer. *Trends Mol. Med.* 22, 578–593. doi: 10.1016/j.molmed.2016.05.002

Miao, W., Hu, L., Scrivens, P. J., and Batist, G. (2005). Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. *J. Biol. Chem.* 280, 20340–20348. doi: 10.1074/jbc.M412081200

Mimura, J., and Fujii-Kuriyama, Y. (2003). Functional role of AhR in the expression of toxic effects by TCDD. *Biochimica et Biophysica Acta General Subjects* 1619, 263–268. doi: 10.1016/S0304-4165(02)00485-3

Muta, K., Obata, Y., Oka, S., Abe, S., Minami, K., Kitamura, M., et al. (2016). Curcumin ameliorates nephrosclerosis via suppression of histone acetylation independent of hypertension. *Nephrol. Dialys. Transplant.* 31, 1615–1623. doi: 10.1093/ndt/gfw036

Namani, A., Li, Y., Wang, X. J., and Tang, X. (2014). Modulation of Nrf2 signaling pathway by nuclear receptors: implications for cancer. *Biochimica et Biophysica Acta Mol. Cell Res.* 1843, 1875–1885. doi: 10.1016/j.bbamcr.2014.05.003

Nebert, D. W. (2017). Aryl hydrocarbon receptor (AHR): "Pioneer member" of the basic-helix/loop/helix per-Arnt-sim (bHLH/PAS) family of "sensors" of foreign and endogenous signals. *Prog. Lipid Res.* 67, 38–57. doi: 10.1016/j.plipres.2017.06.001

Nelson, S. K., Bose, S. K., Grunwald, G. K., Myhill, P., and McCord, J. M. (2006). The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy. *Free Rad. Biol. Med.* 40, 341–347. doi: 10.1016/j.freeradbiomed.2005.08.043

Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., and Pickett, C. B. (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element: degradation of Nrf2 by the 26 S proteasome. *J. Biol. Chem.* 278, 4536–4541. doi: 10.1074/jbc.M207293200

Ni, J., Li, Y., Li, W., and Guo, R. (2017). Salidroside protects against foam cell formation and apoptosis, possibly via the MAPK and AKT signaling pathways. *Lipids Health Dis.* 16:198. doi: 10.1186/s12944-017-0582-7

Niu, T., Xuan, R., Jiang, L., Wu, W., Zhen, Z., Song, Y., et al. (2018). Astaxanthin induces the Nrf2/HO-1 antioxidant pathway in human umbilical vein endothelial cells by generating trace amounts of ROS. *J. Agric. Food Chem.* 66, 1551–1559. doi: 10.1021/acs.jafc.7b05493

Ohnuma, T., Nakayama, S., Anan, E., Nishiyama, T., Ogura, K., and Hiratsuka, A. (2010). Activation of the Nrf2/ARE pathway via S-alkylation of cysteine 151 in the chemopreventive agent-sensor Keap1 protein by falcarindiol, a conjugated diacetylene compound. *Toxicol. Appl. Pharmacol.* 244, 27–36. doi: 10.1016/j.taap.2009.12.012

Ooi, B. K., Goh, B. H., and Yap, W. H. (2017). Oxidative stress in cardiovascular diseases: Involvement of Nrf2 antioxidant redox signaling in macrophage foam cells formation. *Int. J. Mol. Sci.* 18:2336. doi: 10.3390/ijms18112336

Panchal, S. K., Poudyal, H., and Brown, L. (2012). Quercetin ameliorates cardiovascular, hepatic, and metabolic changes in diet-induced metabolic syndrome in rats. *J. Nutr.* 142, 1026–1032. doi: 10.3945/jn.111.157263

Pashkow, F. J. (2011). Oxidative stress and inflammation in heart disease: do antioxidants have a role in treatment and/or prevention? *Int. J. Inflamm.* 2011:9. doi: 10.4061/2011/514623

Qin, X., Qiu, C., and Zhao, L. (2014). Maslinic acid protects vascular smooth muscle cells from oxidative stress through Akt/Nrf2/HO-1 pathway. *Mol. Cell. Biochem.* 390, 61–67. doi: 10.1007/s11010-013-1956-4

Quintana, F. J. (2013). The aryl hydrocarbon receptor: a molecular pathway for the environmental control of the immune response. *Immunology* 138, 183–189. doi: 10.1111/imm.12046

Rangel-Huerta, O., Pastor-Villaescusa, B., Aguilera, C., and Gil, A. (2015). A systematic review of the efficacy of bioactive compounds in cardiovascular disease: Phenolic compounds. *Nutrients* 7, 5177–5216. doi: 10.3390/nu7075177

Ribas, V., García-Ruiz, C., and Fernández-Checa, J. C. (2014). Glutathione and mitochondria. *Front. Pharmacol.* 5:151. doi: 10.3389/fphar.2014.00151

Ruotsalainen, A. K., Inkala, M., Partanen, M. E., Lappalainen, J. P., Kansanen, E., Mäkinen, P. I., et al. (2013). The absence of macrophage Nrf2 promotes early atherogenesis. *Cardiovasc. Res.* 98, 107–115. doi: 10.1093/cvr/cvt008

Ruotsalainen, A. K., Lappalainen, J. P., Heiskanen, E., Merentie, M., Sihvola, V., Näpänkangas, J., et al. (2018). Nuclear factor E2-related factor 2 deficiency impairs atherosclerotic lesion development but promotes features of plaque instability in hypercholesterolaemic mice. *Cardiovasc. Res.* 36:cvy143. doi: 10.1093/cvr/cvy143

Sahu, B. D., Kumar, J. M., Kuncha, M., Borkar, R. M., Srinivas, R., and Sistla, R. (2016). Baicalein alleviates doxorubicin-induced cardiotoxicity via suppression of myocardial oxidative stress and apoptosis in mice. *Life Sci.* 144, 8–18. doi: 10.1016/j.lfs.2015.11.018

Saito, R., Suzuki, T., Hiramoto, K., Asami, S., Naganuma, E., Suda, H., et al. (2016). Characterizations of three major cysteine sensors of Keap1 in stress response. *Mol. Cell. Biol.* 36, 271–284. doi: 10.1128/MCB.00868-15

Satoh, T., Kosaka, K., Itoh, K., Kobayashi, A., Yamamoto, M., Shimojo, Y., et al. (2008). Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. *J. Neurochem.* 104, 1116–1131. doi: 10.1111/j.1471-4159.2007.05039.x

Schaeffer, D. F., Riazy, M., Parhar, K. S., Chen, J. H., Duronio, V., Sawamura, T., et al. (2009). LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma. *J. Lipid Res.* 50, 1676–1684. doi: 10.1194/jlr.M900167-JLR200

Shi, L., Wu, L., Chen, Z., Yang, J., Chen, X., Yu, F., et al. (2015). MiR-141 activates Nrf2-dependent antioxidant pathway via down-regulating the expression of Keap1 conferring the resistance of hepatocellular carcinoma cells to 5-fluorouracil. *Cell Physiol. Biochem.* 35, 2333–2348. doi: 10.1159/000374036

Song, M. G., Ryoo, I. G., Choi, H. Y., Choi, B. H., Kim, S. T., Heo, T. H., et al. (2015). NRF2 signaling negatively regulates phorbol-12-myristate-13-acetate (PMA)-induced differentiation of human monocytic U937 cells into pro-inflammatory macrophages. *PLoS One* 10:e0134235. doi: 10.1371/journal.pone.0134235

Sthijns, M. M. J. P. E., Schiffers, P. M., Janssen, G. M., Lemmens, K. J. A., Ides, B., Vangrieken, P., et al. (2017). Rutin protects against H2O2-triggered impaired relaxation of placental arterioles and induces Nrf2-mediated adaptation in human umbilical vein endothelial cells exposed to oxidative stress. *Biochimica et Biophysica Acta General Sub.* 1861(5 Part A), 1177–1189. doi: 10.1016/j.bbagen.2017.03.004

Su, Z. Y., Khor, T. O., Shu, L., Lee, J. H., Saw, C. L. L., Wu, T. Y., et al. (2013). Epigenetic reactivation of Nrf2 in murine prostate cancer TRAMP C1 cells by natural phytochemicals Z-ligustilide and Radix angelica sinensis via promoter CpG demethylation. *Chem. Res. Toxicol.* 26, 477–485. doi: 10.1021/tx300524p

Sun, W., Liu, X., Zhang, H., Song, Y., Li, T., Liu, X., et al. (2017). Epigallocatechin gallate upregulates Nrf2 to prevent diabetic nephropathy via disabling Keap1. *Free Rad. Biol. Med.* 108, 840–857. doi: 10.1016/j.freeradbiomed.2017.04.365

Sun, X., Ou, Z., Chen, R., Niu, X., Chen, D., Kang, R., et al. (2016). Activation of the p62-Keap1-NRF2 pathway protects against ferroptosis in hepatocellular carcinoma cells. *Hepatology* 63, 173–184. doi: 10.1002/hep.28251

Sussan, T. E., Jun, J., Thimmulappa, R., Bedja, D., Antero, M., Gabrielson, K. L., et al. (2008). Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice. *PLoS One* 3:e3791. doi: 10.1371/journal.pone.0003791

Tsuji, G., Takahara, M., Uchi, H., Matsuda, T., Chiba, T., Takeuchi, S., et al. (2012). Identification of ketoconazole as an Ahr-Nrf2 activator in cultured human keratinocytes: the basis of its anti-inflammatory effect. *J. Investigat. Dermatol.* 132, 59–68. doi: 10.1038/jid.2011.194

Ueberschlag, S. L., Seay, J. R., Roberts, A. H., DeSpirito, P. C., Stith, J. M., Folz, R. J., et al. (2016). The effect of Protandim(®) supplementation on athletic performance and oxidative blood markers in runners. *PLoS One* 11:e0160559. doi: 10.1371/journal.pone.0160559

Waltenberger, B., Mocan, A., Šmejkal, K., Heiss, E., and Atanasov, A. (2016). Natural products to counteract the epidemic of cardiovascular and metabolic disorders. *Mol.* 21:807. doi: 10.3390/molecules21060807

Wang, B., Zhu, X., Kim, Y., Li, J., Huang, S., Saleem, S., et al. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Rad. Biol. Med.* 52, 928–936. doi: 10.1016/j.freeradbiomed.2011.12.006

Wang, N., Zhang, L., Lu, Y., Zhang, M., Zhang, Z., Wang, K., et al. (2017). Down-regulation of microRNA-142-5p attenuates oxygen-glucose deprivation and reoxygenation-induced neuron injury through up-regulating Nrf2/ARE signaling pathway. *Biomed. Pharmacother.* 89, 1187–1195. doi: 10.1016/j.biopha.2017.03.011

Wang, P., Liang, X., Lu, Y., Zhao, X., and Liang, J. (2016). MicroRNA-93 downregulation ameliorates cerebral ischemic injury through the Nrf2/HO-1 defense pathway. *Neurochem. Res.* 41, 2627–2635. doi: 10.1007/s11064-016-1975-0

Wang, T. Y., Li, Q., and Bi, K. S. (2018). Bioactive flavonoids in medicinal plants: structure, activity and biological fate. *Asian J. Pharmaceut. Sci.* 13, 12–23. doi: 10.1016/j.ajps.2017.08.004

Wang, Y.-Y., Yang, Y.-X., Zhe, H., He, Z.-X., and Zhou, S.-F. (2014). Bardoxolone methyl (CDDO-Me) as a therapeutic agent: An update on its pharmacokinetic and pharmacodynamic properties. *Drug Design Dev. Ther.* 8, 2075–2088. doi: 10.2147/DDDT.S68872

Watanabe, Y., Watanabe, K., Kobayashi, T., Saito, Y., Fujioka, D., Nakamura, T., et al. (2013). Chronic depletion of glutathione exacerbates ventricular remodelling and dysfunction in the pressure-overloaded heart. *Cardiovasc. Res.* 97, 282–292. doi: 10.1093/cvr/cvs333

Wei, B., You, M. G., Ling, J. J., Wei, L. L., Wang, K., Li, W. W., et al. (2013). Regulation of antioxidant system, lipids and fatty acid β-oxidation contributes to the cardioprotective effect of sodium tanshinone IIA sulphonate in isoproterenol-induced myocardial infarction in rats. *Atherosclerosis* 230, 148–156. doi: 10.1016/j.atherosclerosis.2013.07.005

Westerterp, M., Murphy, A. J., Wang, M., Pagler, T. A., Vengrenyuk, Y., Kappus, M. S., et al. (2013). Deficiency of ABCA1 and ABCG1 in macrophages increases inflammation and accelerates atherosclerosis in mice. *Circ. Res* 112, 1456–1465.

Westerterp, M., Tsuchiya, K., Tattersall, I. W., Fotakis, P., Bochem, A. E., Molusky, M. M., et al. (2016). Deficiency of ATP-binding cassette transporters A1 and G1 in endothelial cells accelerates atherosclerosis in mice. *Arterioscler. Thromb. Vasc. Biol.* 36, 1328–1337. doi: 10.1161/ATVBAHA.115.306670

Wong, S. Y., Tan, M. G. K., Wong, P. T. H., Herr, D. R., and Lai, M. K. P. (2016). Andrographolide induces Nrf2 and heme oxygenase 1 in astrocytes by activating p38 MAPK and ERK. *J. Neuroinflamm.* 13:251. doi: 10.1186/s12974-016-0723-3

World Health Organization (2018). *Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and By Region, 2000-2016* Available: http://www.who.int/healthinfo/global_burden_disease/estimates/en/ [accessed May 28, 2018].

Xu, X., Li, H., Hou, X., Li, D., He, S., Wan, C., et al. (2015). Punicalagin induces Nrf2/HO-1 expression via upregulation of PI3K/AKT pathway and inhibits LPS-induced oxidative stress in RAW264.7 macrophages. *Mediat. Inflamm.* 2015:380218. doi: 10.1155/2015/380218

Yang, D., Xiao, C. X., Su, Z. H., Huang, M. W., Qin, M., Wu, W. J., et al. (2017). (-)-7(S)-hydroxymataresinol protects against tumor necrosis factor-α-mediated inflammation response in endothelial cells by blocking the MAPK/NF-κB and activating Nrf2/HO-1. *Phytomedicine* 32, 15–23. doi: 10.1016/j.phymed.2017.04.005

Yang, H. L., Chang, H. C., Lin, S. W., Senthil Kumar, K. J., Liao, C. H., Wang, H. M., et al. (2014). Antrodia salmonea inhibits TNF-α-induced angiogenesis and atherogenesis in human endothelial cells through the down-regulation of NF-κB and up-regulation of Nrf2 signaling pathways. *J. Ethnopharmacol.* 151, 394–406. doi: 10.1016/j.jep.2013.10.052

Yang, S., Chou, G., and Li, Q. (2018a). Cardioprotective role of azafrin in against myocardial injury in rats via activation of the Nrf2-ARE pathway. *Phytomedicine* 123, 92–100. doi: 10.1016/j.phymed.2018.04.042

Yang, Y., Li, X., Peng, L., An, L., Sun, N., Hu, X., et al. (2018b). Tanshindiol C inhibits oxidized low-density lipoprotein induced macrophage foam cell formation via a peroxiredoxin 1 dependent pathway. *Biochimica et Biophysica Acta Mol. Basis Dis.* 1864, 882–890. doi: 10.1016/j.bbadis.2017.12.033

Yang, Y., Yang, I., Cao, M., Su, Z.-Y., Wu, R., Guo, Y., et al. (2018c). Fucoxanthin elicits epigenetic modifications, Nrf2 activation and blocking transformation in mouse skin JB6 P + cells. *AAPS J.* 20:32. doi: 10.1208/s12248-018-0197-6

Yao, J., Zhang, B., Ge, C., Peng, S., and Fang, J. (2015). Xanthohumol, a polyphenol chalcone present in hops, activating Nrf2 enzymes to confer protection against oxidative damage in PC12 cells. *J. Agric. Food Chem.* 63, 1521–1531. doi: 10.1021/jf505075n

Ye, Z. W., Zhang, J., Townsend, D. M., and Tew, K. D. (2015). Oxidative stress, redox regulation and diseases of cellular differentiation. *Biochim. Biophys. Acta* 1850, 1607–1621. doi: 10.1016/j.bbagen.2014.11.010

Yen, J., Wu, P., Chen, S., and Wu, M. (2017). Fisetin protects PC12 cells from tunicamycin-mediated cell death via reactive oxygen species scavenging and modulation of Nrf2-driven gene expression, SIRT1 and MAPK signaling in PC12 cells. *Int. J. Mol. Sci.* 18:852. doi: 10.3390/ijms18040852

Yu, H., Shi, L., Zhao, S., Sun, Y., Gao, Y., Sun, Y., et al. (2016). Triptolide attenuates myocardial ischemia/reperfusion injuries in rats by inducing the activation of Nrf2/HO-1 defense pathway. *Cardiovasc. Toxicol.* 16, 325–335. doi: 10.1007/s12012-015-9342-y

Yu, S., Khor, T. O., Cheung, K. L., Li, W., Wu, T. Y., Huang, Y., et al. (2010). Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. *PLoS One* 5:e8579. doi: 10.1371/journal.pone.0008579

Yu, X., Cui, L., Zhang, Z., Zhao, Q., and Li, S. (2013). α-Linolenic acid attenuates doxorubicin-induced cardiotoxicity in rats through suppression of oxidative stress and apoptosis. *Acta Biochimica et Biophysica Sinica* 45, 817–826. doi: 10.1093/abbs/gmt082

Yvan-Charvet, L., Ranalletta, M., Wang, N., Han, S., Terasaka, N., Li, R., et al. (2007). Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. *J. Clin. Invest.* 117, 3900–3908. doi: 10.1172/JCI33372

Zaina, S., Heyn, H., Carmona, F. J., Varol, N., Sayols, S., Condom, E., et al. (2014). DNA methylation map of human atherosclerosis. *Circulation* 7, 692–700. doi: 10.1161/circgenetics.113.000441

Zeng, C., Zhong, P., Zhao, Y., Kanchana, K., Zhang, Y., Khan, Z. A., et al. (2015). Curcumin protects hearts from FFA-induced injury by activating Nrf2 and inactivating NF-κB both in vitro and in vivo. *J. Mol. Cell Cardiol.* 79, 1–12. doi: 10.1016/j.yjmcc.2014.10.002

Zhang, C., Su, Z. Y., Khor, T. O., Shu, L., and Kong, A. N. T. (2013). Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. *Biochem. Pharmacol.* 85, 1398–1404. doi: 10.1016/j.bcp.2013.02.010

Zhang, Z., Chen, J., Zhou, S., Wang, S., Cai, X., Conklin, D. J., et al. (2015). Magnolia bioactive constituent 4-O-methylhonokiol prevents the impairment of cardiac insulin signaling and the cardiac pathogenesis in high-fat diet-induced obese mice. *Int. J. Biol. Sci.* 11, 879–891. doi: 10.7150/ijbs.12101

Zhao, F., Zhang, J., and Chang, N. (2018). Epigenetic modification of Nrf2 by sulforaphane increases the antioxidative and anti-inflammatory capacity in a cellular model of Alzheimer's disease. *Eur. J. Pharmacol* 824, 1–10. doi: 10.1016/j.ejphar.2018.01.046

Zhao, L., Tao, X., Qi, Y., Xu, L., Yin, L., and Peng, J. (2018). Protective effect of dioscin against doxorubicin-induced cardiotoxicity via adjusting microRNA-140-5p-mediated myocardial oxidative stress. *Redox Biol.* 16, 189–198. doi: 10.1016/j.redox.2018.02.026

Zhao, S., Mao, L., Wang, S.-G., Chen, F.-L., Ji, F., and Fei, H.-D. (2017). MicroRNA-200a activates Nrf2 signaling to protect osteoblasts from dexamethasone. *Oncotarget* 8, 104867–104876. doi: 10.18632/oncotarget.20452

Zhou, J., Ma, X., Cui, Y., Song, Y., Yao, L., Liu, Y., et al. (2017). Methyleugenol protects against t-BHP-triggered oxidative injury by induction of Nrf2 dependent on AMPK/GSK3β and ERK activation. *J. Pharmacol. Sci.* 135, 55–63. doi: 10.1016/j.jphs.2017.09.003

Zhou, M. M., Zhang, W. Y., Li, R. J., Guo, C., Wei, S. S., Tian, X. M., et al. (2018). Anti-inflammatory activity of Khayandirobilide A from Khaya senegalensis via NF-κB, AP-1 and p38 MAPK/Nrf2/HO-1 signaling pathways in lipopolysaccharide-stimulated RAW 264.7 and BV-2 cells. *Phytomedicine* 42, 152–163. doi: 10.1016/j.phymed.2018.03.016

Zimmermann, K., Baldinger, J., Mayerhofer, B., Atanasov, A. G., Dirsch, V. M., and Heiss, E. H. (2015). Activated AMPK boosts the Nrf2/HO-1 signaling axis—A role for the unfolded protein response. *Free Radic. Biol. Med.* 88, 417–426. doi: 10.1016/j.freeradbiomed.2015.03.030

Zou, W., Chen, C., Zhong, Y., An, J., Zhang, X., Yu, Y., et al. (2013). PI3K/Akt pathway mediates Nrf2/ARE activation in human L02 hepatocytes exposed to low-concentration HBCDs. *Environ. Sci. Technol.* 47, 12434–12440. doi: 10.1021/es401791s

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Sulforaphane Inhibited the Nociceptive Responses, Anxiety- and Depressive-Like Behaviors Associated With Neuropathic Pain and Improved the Anti-allodynic Effects of Morphine in Mice

*Pablo Ferreira-Chamorro[1,2], Alejandro Redondo[1,2], Gabriela Riego[1,2], Sergi Leánez[1,2] and Olga Pol[1,2]**

[1] Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau, Barcelona, Spain, [2] Grup de Neurofarmacologia Molecular, Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain

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***Correspondence:**
Olga Pol
opol@santpau.es

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Chronic neuropathic pain is associated with anxiety- and depressive-like disorders. Its treatment remains a serious clinical problem due to the lack of efficacy of the available therapeutic modalities. We investigated if the activation of the transcription factor Nrf2 could modulate the nociceptive and emotional disorders associated with persistent neuropathic pain and potentiated the analgesic activity of morphine. The possible mechanisms implicated in these effects have been also evaluated. Therefore, in C57BL/6 mice with neuropathic pain induced by the chronic constriction of the sciatic nerve (CCI), we assessed the antinociceptive, anxiolytic, and anti-depressant effects of the repeated intraperitoneal administration of a Nrf2 inducer, sulforaphane (SFN), and the effects of this treatment on the local antinociceptive actions of morphine. The protein levels of Nrf2, heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), CD11b/c (a microglial activator marker), mitogen-activated protein kinases (MAPK) and μ opioid receptors (MOR) in the spinal cord, prefrontal cortex and hippocampus from mice, at 28 days after CCI, were also evaluated. Our results showed that the repeated administration of SFN besides inhibiting nociceptive responses induced by sciatic nerve injury also diminished the anxiety- and depressive-like behaviors associated with persistent neuropathic pain. Moreover, SFN treatment normalized oxidative stress by inducing Nrf2/HO-1 signaling, reduced microglial activation and JNK, ERK1/2, p-38 phosphorylation induced by sciatic nerve injury in the spinal cord and/or hippocampus and prefrontal cortex. Interestingly, treatment with SFN also potentiated the antiallodynic effects of morphine in sciatic nerve-injured mice by regularizing the down regulation of MOR in the spinal cord and/or hippocampus. This study suggested that treatment with SFN might be an interesting approach for the management of persistent neuropathic pain and comorbidities associated as well as to improve the analgesic actions of morphine.

Keywords: analgesia, anxiety, depression, Nrf2 transcription factor, opioids, oxidative stress, chronic pain

INTRODUCTION

Chronic pain, especially neuropathic, significantly alters human living conditions. That is, patients with neuropathic pain experience emotional disorders such as anxiety and depression that produce a significant deterioration in their quality of life (Maletic and Raison, 2009; Attal et al., 2011). Therapies to treat neuropathic pain and associated comorbidities are limited with modest efficacy and significant side effects. Therefore, the investigation of new strategies to effectively relieve neuropathic pain and the emotional disorders associated is indispensable.

It is well known that the transcription factor Nrf2 coordinates the redox state in the cell and its activation serves as a barrier against the reactive species of oxygen through the activation of antioxidant and detoxifying enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase1 (NQO1), glutathione peroxidase (GPx1) enzymes, etc. . .(de Vries et al., 2008). The transcription factor Nrf2 also modulates inflammatory responses of the organism by inhibiting the synthesis of various proinflammatory mediators, such as the inducible nitric oxide synthase (NOS2), cyclooxygenase-2, interleukin (IL)-1α, IL-6, and mitogen-activated protein kinase (MAPK), among others (Kim et al., 2009; Davidson et al., 2013; Kobayashi et al., 2016). Furthermore, diverse studies revealed that the stimulation of Nrf2 reduced acute and inflammatory pain as well as neuropathic pain accompanying to diabetes (Negi et al., 2011; Di et al., 2016; McDonnell et al., 2017; Redondo et al., 2017), but the potential inhibitory role played by this transcription factor on the allodynia and hyperalgesia caused by nerve injury-induced persistent neuropathic pain has not been evaluated.

Numerous studies have demonstrated the anxiolytic and anti-depressant effects induced by Nrf2 in several stress paradigms (Wu et al., 2016) and in different animal models of depression (Yao et al., 2016). Moreover, the fact that mice with the Nrf2 gene silenced by pharmacological or genetic tools exhibited greater anxiety- and depressive-like behaviors sustained the anxiolytic and anti-depressant properties induced by Nrf2 activation in different experimental conditions (Muramatsu et al., 2013; Khalifeh et al., 2015). Nonetheless, the effects of the repetitive treatment with sulforaphane (SFN), a Nrf2 inducer (de Figueiredo et al., 2015), in the anxiety- and depressive-like behaviors associated with chronic pain have not yet studied. Our objective was to evaluate if the activation of Nrf2 besides reducing nociception might also inhibited the anxiety- and depressive-like behaviors associated with persistent neuropathic pain and normalized oxidative stress and microglial activation induced by sciatic nerve injury.

Sulforaphane is an isothiocyanate bioactive metabolite derived from glucoraphanin which is abundantly found in cruciferous vegetables (Dinkova-Kostova et al., 2017; Fahey et al., 2017). Several studies have demonstrated the potent anti-inflammatory, antioxidant, anti-cancer, antibiotic, as well as the protective effects of SFN for cognitive and memory impairments (Zhang et al., 1994; Guerrero-Beltran et al., 2012; Lee et al., 2014; Catanzaro et al., 2017; Pu et al., 2018). SFN has been also proven in humans demonstrating that this treatment improves the glucose levels in patients with type 2 diabetes (Axelsson et al., 2017), has beneficial clinical effects against autism disorders (Singh et al., 2014) and gastrointestinal diseases (Yanaka, 2018). Recent studies also showed the analgesic properties of SFN in several animal pain models and its capacity to potentiate the antinociceptive effects of opioids in animals with inflammatory pain or diabetic neuropathy (McDonnell et al., 2017; Redondo et al., 2017). Taking account the lower efficacy of opioids, particularly μ-opioid receptor (MOR) agonists in the management of neuropathic pain (Hervera et al., 2013; Zychowska et al., 2013; Popiolek-Barczyk and Mika, 2016), the investigation of new tools for increasing their analgesic efficacy is a priority in the current pain research. In this study we evaluated the possible potentiation of the analgesic actions of morphine triggered by SFN in animals with persistent neuropathic pain.

Then, in a neuropathic pain model induced by the chronic constriction of sciatic nerve (CCI) in mice, at 28 days after surgery, our aims were to assess the effects of treatment with SFN on: (1) the allodynia and hyperalgesia induced by CCI; (2) the anxiety- and depressive-like behaviors associated with persistent neuropathic pain; (3) the local antinociceptive effects of morphine during neuropathic pain and (4) the expression of Nrf2/HO-1/NQO1 signaling pathway, microglial activation, MAPK phosphorylation and MOR protein levels in the spinal cord, prefrontal cortex and hippocampus from animals with persistent neuropathic pain.

MATERIALS AND METHODS

Animals

The experiments were carried out with male C57BL/6J mice purchased from Envigo Laboratories (Barcelona, Spain). Mice weighing 21–25 g were housed under standard 12/12-h light/dark conditions in a room with a controlled temperature of 22°C and relative humidity of 66%. The animals had free access to food and drink and were used after 6 days of acclimatization to the environmental conditions mentioned. All experiments were conducted between 9:00 a.m. and 5:00 p.m., and executed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona. All efforts were made to diminish the suffering and amount of animals used in this study.

Induction of Neuropathic Pain

Neuropathic pain was induced by CCI (Hervera et al., 2012). Briefly, sciatic nerve ligation was performed under isoflurane anesthesia (3% induction, 2% maintenance). The biceps femoris and the gluteus superficialis were separated by blunt dissection, and the right sciatic nerve was exposed. The injury was produced by tying three ligatures around the sciatic nerve as described by Bennett and Xie (1988). The ligatures (4/0 silk) were tied loosely around the nerve with 1 mm spacing, until they elicited a brief twitch in the respective hindlimb, which prevented overtightening of the ligations, taking

care to preserve epineural circulation. In these experiments Sham-operated mice, whose surgery was exactly the same as described above without sciatic nerve ligament, were used as control animals.

Experimental Protocol

In a first set of experiments, we investigated the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects of the intraperitoneal daily administration of 10 mg/kg SFN in sciatic nerve-injured or Sham-operated mice from days 14 to 28 after surgery ($n = 6$ animals per group). The evaluation of the antinociceptive effect was carried out on days 14, 18, 21, 25, and 28 post-surgery, at 3 h after SFN or vehicle injection.

In other group of animals, we evaluated the effects of treatment with SFN, also administered at 10 mg/kg during 15 consecutive days (from days 14 to 28 post-surgery), on the anxiety- and depressive-like responses associated with persistent neuropathic pain, 28 days after surgery, using the EPM test and TST, respectively ($n = 8$ animals per group).

In other experiments, the evaluation of the effects produced by the intraperitoneal injection of 10 mg/kg of SFN combined with 50 μg of morphine, subplantarly administered, on the allodynia and hyperalgesia induced by CCI was carried out at 3 h after SFN administration ($n = 6$ animals per group). The doses of SFN and morphine were selected in accordance to other studies (Redondo et al., 2017; Wang and Wang, 2017).

Finally, at day 15 of treatment with SFN or vehicle (dimethylsulfoxide 1% in 0.9% saline solution), sciatic nerve-injured and Sham-operated mice were euthanized by cervical dislocation and the Nrf2, HO-1, NQO1, CD11b/c, MAPK, and MOR levels in the ipsilateral site of the spinal cord, prefrontal cortex and hippocampus were evaluated. In these experiments, Sham-operated mice treated with vehicle were used as controls ($n = 4$ samples per group).

The number of animals per group was determined from a pilot study taking into account a value of $\alpha = 0.05$ and $\beta = 0.20$ (power analysis of 0.80). In this work we used 60 animals in total.

Nociceptive Behavioral Tests

Mechanical Allodynia

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. Animals were placed in methacrylate cylinders (20 cm high × 9 cm diameter) with a wire grid bottom through which the von Frey filaments (North Coast Medical, Inc., San Jose, CA, United States), with a bending force in the range of 0.008–3.5 g, were applied by using a modified version of the up–down paradigm, described by Chaplan et al. (1994). The filament of 0.4 g was used first and the 3.0 g filament was used as a cut-off and the strength of the next filament was reduced or increased according to the response. The threshold of response was calculated from the sequence of filament strength used during the up-down procedure by using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that includes curve fitting of the data. Both ipsilateral and contralateral hind paws were tested. Animals were allowed to habituate for 1 h prior to testing to allow appropriate behavioral immobility.

Thermal Hyperalgesia

Thermal hyperalgesia was evaluated as proposed by Hargreaves et al. (1988). Latency of paw withdrawal in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Varese, Italy). In summary, mice were placed in methyl acrylate cylinders (20 cm high × 9 cm diameter) positioned on a glass surface. The heat source was positioned under the plantar surface of the hind paw and activated with a light beam intensity. A cut-off time of 12 s was used to avoid tissue damage in the absence of response. Mean paw withdrawal latencies (from both hind paws) were determined from the average of three separate trials, taken at 5 min intervals to avoid thermal sensitization and behavioral alterations. Mice were habituated to the environment for 1 h prior to the experiment so that the animals were quiet at the time of testing.

Thermal Allodynia

Thermal allodynia to cold stimulus was evaluated using the hot/cold plate analgesia meter (Ugo Basile) previously described by Bennett and Xie (1988). The number of elevations of each hind paw from mice exposed to the cold plate (4 ± 0.5°C) was recorded for 5 min.

Anxiety-Like Behavior

The anxiety-like behavior was measured by using the elevated plus maze (EPM) test such as described by Walf and Frye (2007). An apparatus with 4 arms of 5 cm wide and 35 cm long, two of which are open and two closed with walls of 15 cm high. The distance of the EPM to the ground is 45 cm. The animal was placed in the central square of maze facing one of the open arms and its behavior was recorded by a digital camera for 5 min. The number of entries in the open and closed arms, as well as the percentage of time spent in the open arms was calculated for each animal.

Depressive-Like Behavior

The evaluation of the depressive-like behavior was performed by using the tail suspension test (TST), in which the total duration of immobility of the animals was quantified according to the method described by Steru et al. (1985) with some modifications. Briefly, mice were individually suspended by the tail from a horizontal wooden bar (35 cm above the floor) using an adhesive tape (1 cm from the tip of the tail). The immobility time in seconds was recorded over a total period of 6 min.

All the behavioral experiments were performed by an experimenter blinded to the treatment applied.

Western Blot Analysis

Sham-operated and sciatic nerve-injured mice were killed at 28 days after surgery by cervical dislocation and tissues from the ipsilateral side lumbar section of the spinal cord, prefrontal cortex and hippocampus were extracted immediately after killing, frozen in liquid nitrogen and stored at −80°C. Samples from two to three animals were collected in an experimental sample to obtain enough protein levels for doing western blot analysis of Nrf2, HO-1, NQO1, CD11b/c, MAPK (JNK, ERK1/2, and P38) and MOR. Tissues were homogenized in ice-cold lysis

buffer (50 mM Tris·Base, 150 nM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM Na_3VO_4, 25 mM NaF, 0.5% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail). All reagents were purchased from Sigma (St. Louis, MO) excluding NP-40 which was acquired from Calbiochem (Darmstadt, Germany). The crude homogenate was solubilized for 1 h at 4° C, sonicated for 10 s and centrifuged at 4°C for 15 min at 700 g.

Then, 60 μg of total proteins were mixed with 4 × laemmli loading buffer and loaded onto 4% stacking/10% separation sodium dodecyl sulfate polyacrylamide gels. Proteins were electrophoretically transferred onto PVDF membrane for 120 min, blocked with PBS or TBST + 5% non-fat dry milk or BSA and successively incubated overnight at 4°C with rabbit anti Nrf2 (1:160, Abcam, Cambridge, United Kingdom), HO-1 (1:300, Abcam, Cambridge, United Kingdom), NQO1 (1:333, Sigma, St. Louis, MO, United States), CD11b/c (1:200, Novus Biologicals, Littleton, CO, United States), phospho JNK, total JNK, phospho ERK1/2, total ERK1/2, phospho P38 and total P38 (1:250, Cell Signaling Technology, Danvers, MA, United States), MOR (1:333, Merck, Billerica, MA, United States) or GAPDH antibody (1:5000, Merck, Billerica, MA, United States) which was used as a loading control. Proteins were detected by a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and visualized with chemiluminescent reagents (ECL kit; GE Healthcare) and by exposure to hyperfilm (GE Healthcare). The intensity of blots was quantified by densitometry.

Drugs

Sulforaphane was acquired from Merck Chemicals and Life Science S.A.U. (Madrid, Spain), dissolved in dimethylsulfoxide (1% in 0.9% saline solution) and intraperitoneally administered at 10 mg/kg, in a final volume of 10 ml/kg, 3 h before testing. Morphine hydrochloride was purchased from Alcaiber S.A. (Madrid, Spain), dissolved in 0.9% saline solution and administered via subplantar, in a final volume of 30 μl, at 30 min before conducting the behavioral tests. All drugs were prepared daily prior to its administration. For each group treated with a drug, the respective control group received the same volume of the corresponding vehicle.

Statistical Analysis

All data were expressed as mean ± SEM. Statistical analysis was carried out using the SPSS program (version 17 for Windows, IBM, Madrid, Spain). The effects of chronic treatment with SFN on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by CCI were evaluated using the three way analysis of variance (ANOVA) repeated measures followed by a one way ANOVA and the Student–Newman–Keuls test, when appropriate. The antinociceptive effects produced by the combination of SFN plus morphine were assessed by using a one way ANOVA followed by the Student–Newman–Keuls test.

The effects of chronic administration with SFN on the anxiety-like behavior identified by the number of open arm entries, the percentage of time of stay in them and the number of closed arm entries obtained in EPM as well as on the depressive-like behavior defined by the immobility time in TST, in Sham and animals with neuropathic pain, were evaluated by using a two way ANOVA followed by a one way ANOVA and the Student–Newman–Keuls test.

Antinociception in the von Frey filaments and plantar tests is expressed as the percentage of maximal possible effect, where the test latencies predrug (baseline) and postdrug administration are compared and calculated in accordance with the following equation:

$$\text{Maximal possible effect (\%)} = [(drug - baseline)/(\text{cut} - \text{off} - \text{baseline})] \times 100$$

In the cold plate, antinociception is expressed according to the following equation:

$$\text{Inhibition (\%)} = [(\text{number of paw elevations at baseline} - \text{number of paw elevations after drug})/\text{number of paw elevations at baseline})] \times 100.$$

Changes in the protein levels were analyzed using a one way ANOVA followed by the Student–Newman–Keuls. A value of $P < 0.05$ was considered significant.

RESULTS

Effects of SFN on the Mechanical Allodynia, Thermal Hyperalgesia and Thermal Allodynia Induced by Sciatic Nerve Injury in Mice

For mechanical allodynia, the three way ANOVA repeated measures indicated significant effects of surgery, treatment and time ($P < 0.022$), an interaction of treatment with time ($P < 0.007$), surgery with treatment ($P < 0.001$) and among surgery, treatment and time ($P < 0.006$). Indeed, nerve injury reduced the threshold of paw withdrawal to a mechanical stimulus from days 13 to 28 after surgery as compared to Sham-operated mice treated with vehicle ($P < 0.001$, one way ANOVA) (**Figure 1A**). This mechanical allodynia was progressively reduced from 1 to 12 days of SFN administration ($P < 0.001$; one way ANOVA vs. CCI-vehicle treated mice) and completely inhibited at 15 days of treatment. In contrast, SFN did not produce any effect in Sham-operated mice for the total duration of the experiment.

Sciatic nerve injury also significantly decreased threshold for evoking paw withdrawal to a thermal stimulus from days 13 to 28 after surgery ($P < 0.001$; one way ANOVA vs. Sham-operated mice) (**Figure 1B**). Three way ANOVA repeated measures showed significant actions of surgery, treatment and time ($P < 0.001$) and interactions among treatment

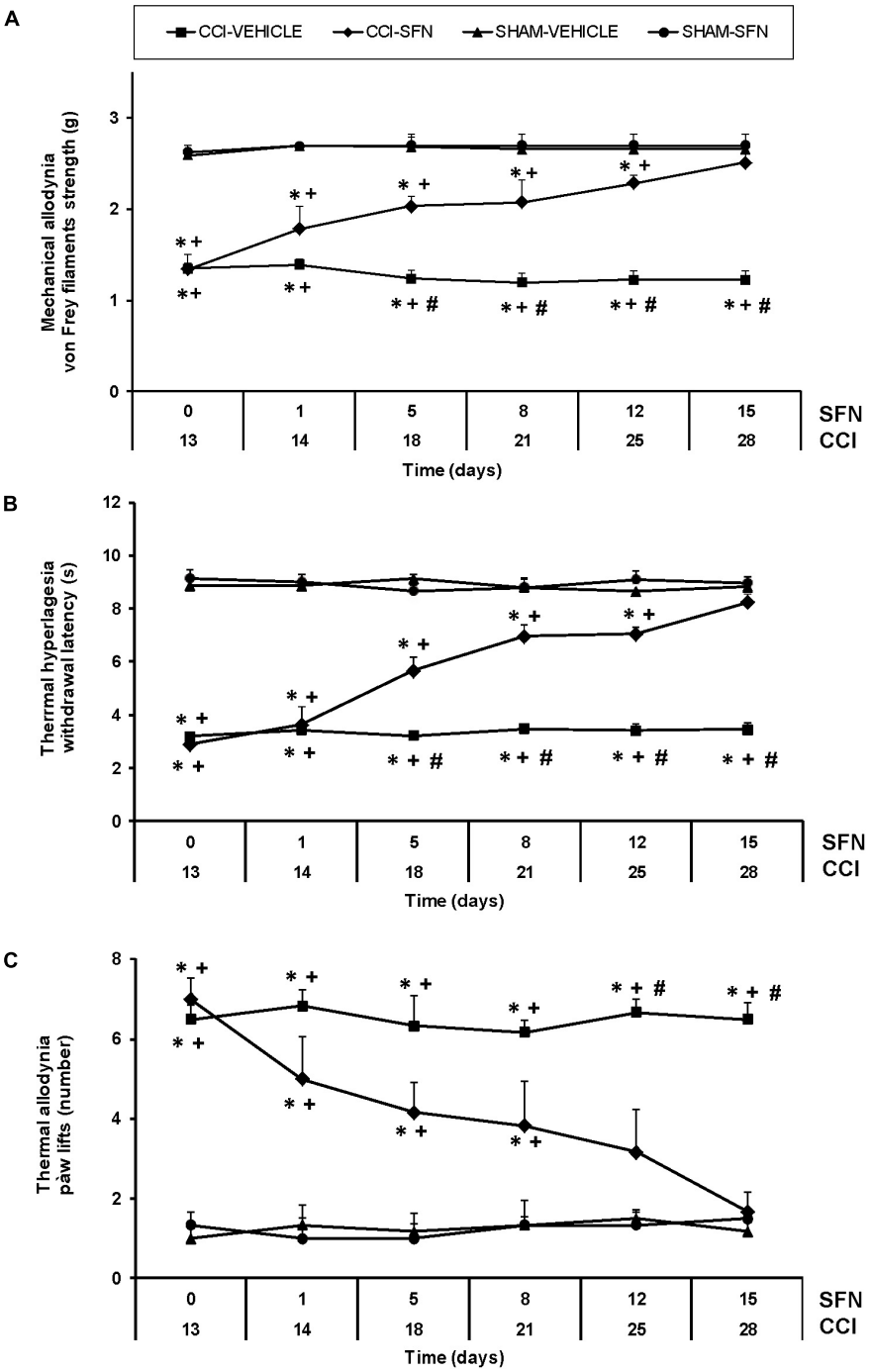

FIGURE 1 | The antinociceptive effects of SFN during neuropathic pain. Mechanical antiallodynic **(A)**, thermal antihyperalgesic **(B)**, and thermal antiallodynic **(C)** effects produced by the repeated administration of 10 mg/kg SFN or vehicle from days 14 to 28 after sciatic nerve-injury (CCI) or Sham-operation (SHAM). In all panels, for each day and treatment evaluated, * indicates significant differences vs. Sham-operated animals treated with vehicle, + vs. Sham-operated animals treated with SFN and # vs. sciatic nerve-injured animals treated with SFN ($P < 0.05$, one way ANOVA followed by Student–Newman–Keuls test). Results are shown as mean values ± SEM; $n = 6$ animals per experimental group.

and time ($P < 0.001$), surgery and treatment ($P < 0.001$), surgery and time ($P < 0.001$) and between the three factors ($P < 0.001$). Thermal hyperalgesia induced by CCI was progressively reduced from 1 to 12 days of SFN administration ($P < 0.001$; one way ANOVA vs. CCI-vehicle treated mice) and completely reversed after 15 days of treatment (**Figure 1B**). In Sham-operated mice, SFN did not cause any effect throughout the experiment.

Regarding thermal allodynia, three way ANOVA repeated measures also demonstrated relevant effects of surgery and time ($P < 0.011$) and interactions among treatment and time ($P < 0.05$) as well as between surgery and treatment ($P < 0.016$) (**Figure 1C**). That is, increased paw lifts number induced by cold thermal stimulation was observed in CCI-mice from days 13 to 28 after surgery ($P < 0.001$; one way ANOVA vs. Sham-operated mice). This thermal allodynia was progressively reduced from 1 to 12 days of SFN treatment ($P < 0.001$; one way ANOVA vs. CCI-vehicle treated mice), and totally reversed at 15 days of treatment. SFN did not produce any effect in Sham-operated mice during the course of the experiment. In all tests, the administration of SFN did not affect the contralateral paw of sciatic nerve-injured or Sham-operated mice (data not shown).

Effects of Chronic Administration With SFN on the Anxiety- and Depressive-Liked Behaviors Associated With Persistent Neuropathic Pain in Mice

Anxiety-like behavior was observed in sciatic nerve-injured animals at day 28 post-CCI. The two way ANOVA revealed a significant effect of the surgery ant treatment on the number of entries and on the percentage of time of animals spent in open arms ($P < 0.001$). That is, the EPM test showed a significant decrease in the number of entries into open arms (**Figure 2A**, $P < 0.05$, ANOVA) and a reduced percentage of the time spent in these arms in sciatic nerve-injured mice treated with vehicle (**Figure 2B**; one way ANOVA; as compared to Sham-operated mice treated with vehicle). No differences in the number of entries to the closed arms were observed between sciatic nerve-injured and Sham-operated mice treated with SFN or vehicle (**Figure 2C**). Chronic treatment with SFN inhibited this anxiety-like behavior by normalizing the decreased number of entries into open arms (**Figure 2A**; $P < 0.001$; ANOVA) and the reduced time spent in open arms (**Figure 2B**; $P < 0.01$, ANOVA) observed in CCI vehicle treated mice, without altering the number of entries to the closed arms (**Figure 3C**). In Sham-operated mice, SFN also had an anxiolytic effect as demonstrated by an increase in the number of entries (**Figure 2A**) and the time spent in open arms as compared to Sham-operated vehicle treated mice (**Figure 2B**; $P < 0.05$, ANOVA).

Regarding the TST test, significant effects of surgery ($P < 0.033$) and treatment ($P < 0.001$) have been also demonstrated by the two way ANOVA. Indeed, sciatic nerve injury induced a depressive-like behavior in the TST, proved

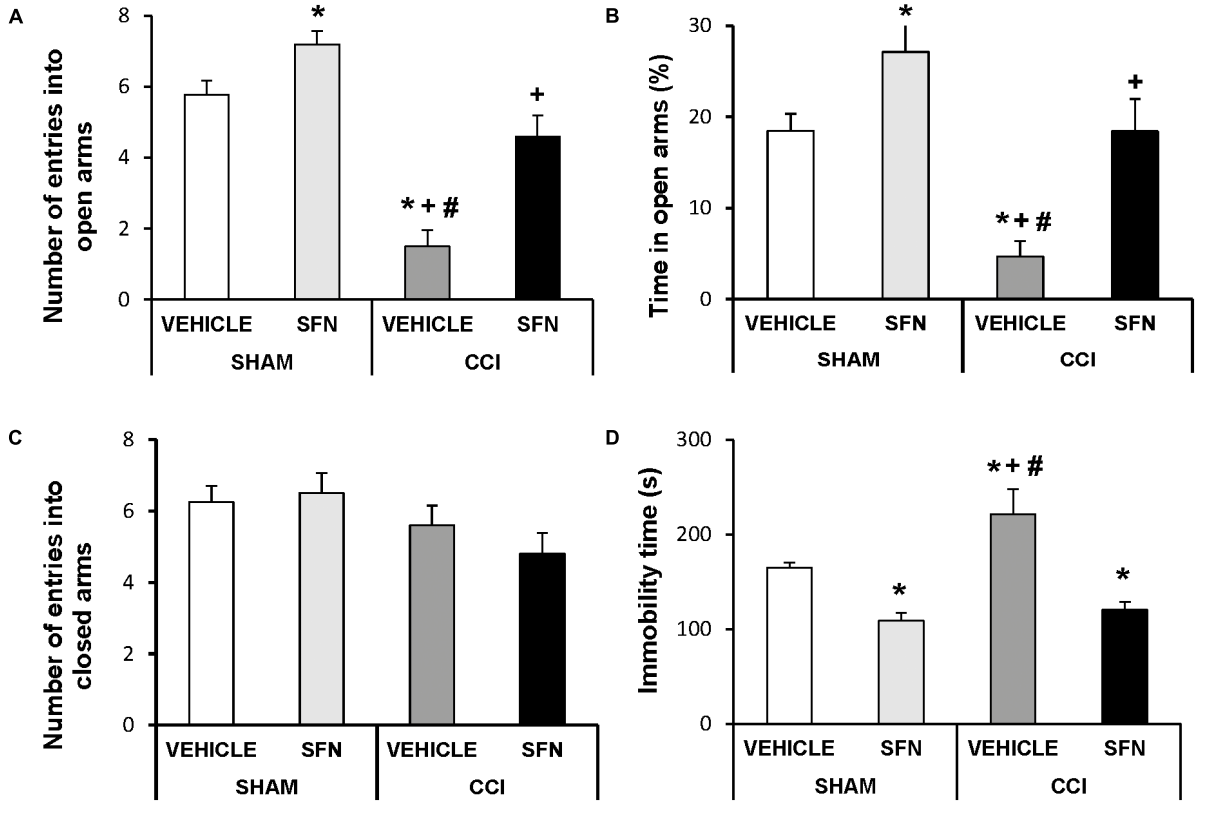

FIGURE 2 | The anxiolytic and anti-depressant effects induced by SFN in animals with neuropathic pain. Effects of the repetitive administration of 10 mg/kg SFN or vehicle from days 14 to 28 after sciatic nerve injury (CCI) or Sham-operation (SHAM) on the anxiety- (elevated plus maze, **A–C**) and depressive-liked (tail suspension test, **D**) behaviors associated with neuropathic pain. In all panels, * indicates significant differences vs. Sham-operated animals treated with vehicle, + vs. Sham-operated animals treated with SFN and # vs. sciatic nerve-injured animals treated with SFN ($P < 0.05$, one way ANOVA followed by Student–Newman–Keuls test). Results are shown as mean values ± SEM; $n = 8$ animals per experimental group.

by a significant increase in the immobility time ($P < 0.05$; one way ANOVA, as compared to Sham-operated mice treated with vehicle) (**Figure 2D**), which was significantly reduced by the administration of SFN. This treatment also decreased the immobility time in Sham-operated mice confirming its anti-depressant effects ($P < 0.05$; one way ANOVA, as compared to Sham-operated mice treated with vehicle).

Effects of SFN Combined With Morphine on the Nociception Induced by Sciatic Nerve Injury in Mice

The co-administration of SFN with morphine in animals with CCI-induced neuropathic pain resulted in a significant increase in the mechanical (**Figure 3A**; one way ANOVA vs. mice treated with vehicle, morphine or SFN alone) and thermal antiallodynic effects of morphine (**Figure 3C**; $P < 0.008$; one way ANOVA vs. mice treated with vehicle, morphine or SFN alone). In contrast, no significant changes in the antihyperalgesic effects of morphine were observed in SFN treated mice (**Figure 3B**).

Effects of SFN Treatment on the Expression of Nrf2, HO-1, NQO1, CD11b/c, MOR, MAPK in the Spinal Cord, Prefrontal Cortex and Hippocampus of Animals With Neuropathic Pain

Our results showed that treatment with SFN normalized the decreased expression of Nrf2 induced by sciatic nerve-injury in the spinal cord ($P < 0.001$; one way ANOVA, **Figure 4A**), prefrontal cortex ($P < 0.013$; one way ANOVA, **Figure 5A**) and hippocampus ($P < 0.030$; one way ANOVA, **Figure 6A**) as compared to their respective Sham-operated vehicle treated mice. SFN treatment was also able to normalize the decreased expression of HO-1 induced by CCI in the spinal cord ($P < 0.008$; one way ANOVA, **Figure 4B**), prefrontal cortex ($P < 0.032$; one way ANOVA, **Figure 5B**) and hippocampus ($P < 0.039$; one way ANOVA, **Figure 6B**). In contrast, the NQO1 levels were not significantly altered in the spinal cord (**Figure 4C**), prefrontal cortex (**Figure 5C**) or hippocampus (**Figure 6C**) from animals with neuropathic pain.

Nevertheless, SFN reduced the increased expression of CD11b/c induced by CCI in the spinal cord ($P < 0.028$; one way ANOVA, **Figure 4D**) and hippocampus ($P < 0.021$; one way ANOVA, **Figure 6D**). Finally, the decreased expression of MOR induced by nerve injury in the spinal cord ($P < 0.012$, one way ANOVA vs. Sham-operated vehicle treated mice; **Figure 4E**) and hippocampus ($P < 0.018$, one way ANOVA vs. Sham-operated vehicle treated mice; **Figure 6E**) was also normalized by SFN treatment. Non-changes in the expression of CD11b/c (**Figure 5D**) or MOR (**Figure 5E**) were observed in the prefrontal cortex of sciatic nerve-injured mice treated with vehicle or SFN. Examples of western blots of these proteins in the spinal cord (**Figure 4F**), prefrontal cortex (**Figure 5F**), and hippocampus (**Figure 6F**) were shown.

The effects of the repeated treatment with SFN on the expression of MAPK in the spinal cord from sciatic nerve-injured mice have been also evaluated. Results showed that the augmented protein levels of p-JNK (**Figure 7A**), p-ERK ½ (**Figure 7B**, and p-P38 (**Figure 7C**) induced by sciatic nerve injury ($P < 0.012$; one way ANOVA vs. Sham-operated vehicle treated mice) were all normalized by SFN treatment.

DISCUSSION

This study demonstrated that SFN besides inhibiting nociceptive responses induced by sciatic nerve injury also diminished the anxiety- and depressive-like behaviors associated with persistent neuropathic pain, at 28 days after surgery. Moreover, the normalization of oxidative stress, microglial activation and/or MAPK phosphorylation produced by SFN in the spinal cord, hippocampus and/or prefrontal cortex of sciatic nerve-injured mice might explain the analgesic, anxiolytic, and anti-depressant actions of this treatment. In addition, treatment with SFN also potentiated the antiallodynic effects of morphine by normalizing the down regulation of MOR in the spinal cord and/or hippocampus from sciatic nerve-injured mice.

Our results showed that the mechanical and thermal allodynia as well as the thermal hyperalgesia caused by sciatic nerve injury, at 28 days after induction, were inhibited by the repetitive administration of SFN from day 14 to 28 after sciatic nerve injury in mice. These results are supported by the antinociceptive effects produced by SFN in the early stages of neuropathic pain, from days 7 to 14 after surgery (Wang and Wang, 2017) as well as during inflammatory pain or diabetic neuropathy (McDonnell et al., 2017; Redondo et al., 2017), and additionally demonstrated the analgesic properties of this Nrf2 inductor in advance stages of neuropathic pain.

It is well known that Nrf2 transcription factor is a regulator of two important cytoprotective pathways: anti-oxidative and anti-inflammatory in the peripheral and central nervous system (Kobayashi et al., 2016). In order to study the possible mechanisms involved in the antinociceptive actions induced by SFN in animals with persistent neuropathic pain, we evaluated the effects of this treatment on the expression of Nrf2, the antioxidant enzymes (HO-1 and NQO1) as well as on the protein levels of CD11b/c (a microglial marker) in the spinal cord from animals with persistent neuropathic pain. In agreement with Riego et al. (2018), peripheral nerve injury provoked prevailing oxidative stress conditions in the spinal cord as demonstrated by the decreased expression of Nrf2 and HO-1 in this tissue. Interestingly, treatment with SFN reduced oxidative stress by normalizing the decreased expression of Nrf2 and HO-1. In accordance to these data, Riego et al. (2018) also demonstrated that treatment with an HO-1 inducer, cobalt protoporphyrin IX (CoPP), was able to compensate for the reduced synthesis of HO-1 induced by neuropathic pain in this tissue. Taking account that HO-1 is downstream of Nrf2 and its induction in the spinal cord exerts potent antinociceptive effects during chronic pain (Liu et al., 2016), it is suitable to propose that the antinociceptive effects induced by SFN during persistent neuropathic pain were principally associated with the activation of the Nrf2/HO-1 signaling pathway.

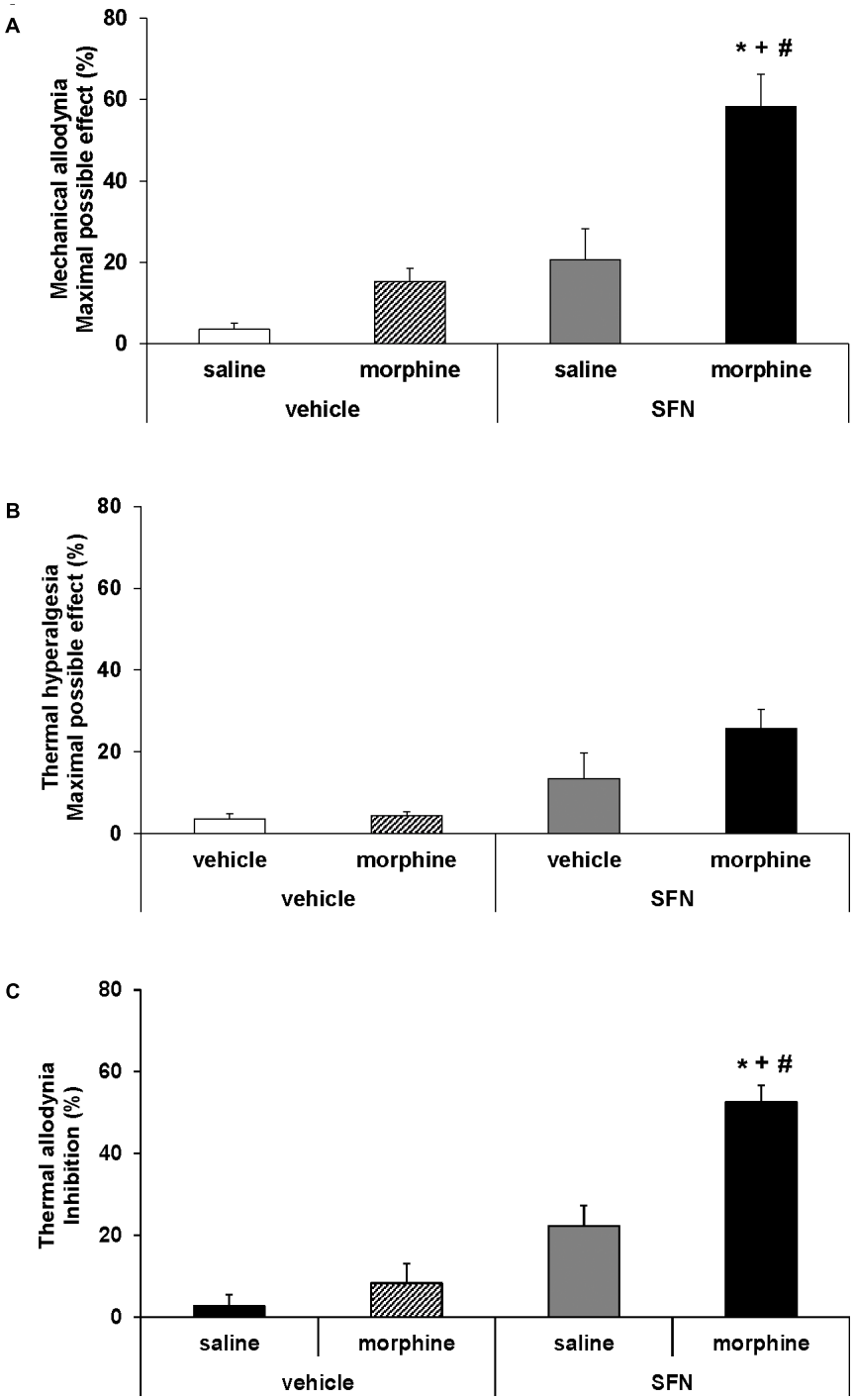

FIGURE 3 | Effects of SFN on the local antinociceptive effects of morphine during neuropathic pain. The mechanical antiallodynic **(A)**, thermal antihyperalgesic **(B)**, and thermal antiallodynic **(C)** effects produced by the acute administration of 10 mg/kg SFN or vehicle combined with a subanalgesic dose (50 μg) of morphine or saline administered on the ipsilateral paw of sciatic nerve-injured mice at 28 days after surgery were shown. In all panels,* indicates significant differences vs. vehicle plus saline treated mice, + vs. vehicle plus morphine treated mice and # vs. SFN plus saline treated mice ($P < 0.05$, one way ANOVA followed by Student–Newman–Keuls test). Results are shown as mean values ± SEM; $n = 6$ animals per experimental group.

On the contrary, the protein levels of NQO1 were not altered by either nerve injury or SFN treatment in animals with neuropathic pain. This is in contrast to that happens during inflammatory pain or diabetic neuropathy, where SFN increased the expression of NQO1 or re-established its down-regulation in the spinal cord or sciatic nerve, respectively

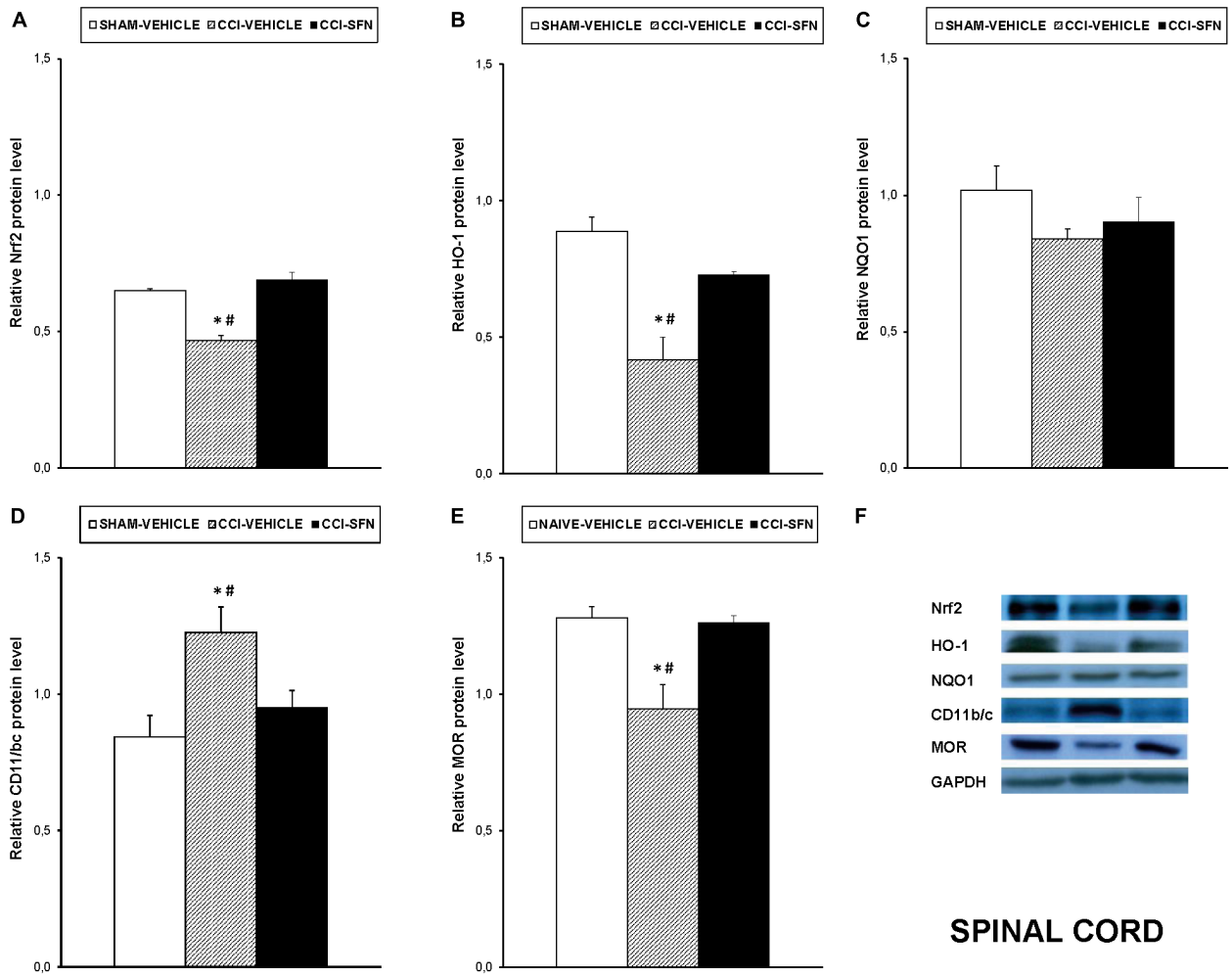

FIGURE 4 | Effects of SFN on the expression of Nrf2, HO-1, NQO1, CD11b/c, and MOR in the spinal cord from animals with neuropathic pain. Effects of repetitive treatment with 10 mg/kg SFN or vehicle from days 14 to 28 after sciatic nerve injury (CCI) on Nrf2 **(A)**, HO-1 **(B)**, NQO1 **(C)**, CD11b/c **(D)**, and MOR **(E)** protein levels in the ipsilateral site of the spinal cord from CCI-induced neuropathic pain in mice are represented. The protein levels from Sham-operated (SHAM) mice treated with vehicle has been also represented as controls. Examples of western blots for Nrf2 (75 kDa), HO-1 (32 kDa), NQO1 (28 kDa), CD11b/c (160 kDa), and MOR (50 kDa) proteins in which GAPDH (36 kDa) was used as a loading control are also shown **(F)**. In all panels, * indicates significant differences vs. Sham vehicle treated mice and # indicates significant differences vs. CCI plus SFN treated mice ($P < 0.05$, one-way ANOVA followed by Student–Newman–Keuls test). Data are expressed as mean values ± SEM; $n = 4$ samples per group.

(McDonnell et al., 2017; Redondo et al., 2017). Thus, suggesting that this enzyme is not implicated in the antinociceptive effects of SFN in sciatic nerve injury induced neuropathic pain.

It is well known that microglial activation promotes the consolidation and progression of neuropathic pain state by the up-regulation of several inflammatory mediators, such as the inducible nitric oxide synthase (NOS2), COX-2, interleukins, etc. (Popiolek-Barczyk and Mika, 2016). In this study, we demonstrated that spinal microglial activation induced by nerve injury, at 28 days after surgery, was inhibited by SFN indicating that, in addition to the antioxidative effects performed by this treatment, it also exerted anti-inflammatory effects in this tissue contributing all of them to the inhibition of neuropathic pain. In agreement with our results, other previous studies also demonstrated anti-inflammatory effects of SFN in chronic pain that were produced by inhibiting of NOS2 and COX-2 expression in sciatic nerves from nerve-injured mice or diabetic animals (Negi et al., 2011; Wang and Wang, 2017).

It is well recognized that MAPK, such as JNK, ERK ½ and p38, are involved in pain sensitization after nerve injury. Moreover, while their phosphorylation participated in the maintenance of pain hypersensitivity, its inhibition attenuated chronic pain (Ji et al., 2009; Edelmayer et al., 2014). The fact that SFN inhibited the phosphorylation of JNK, ERK ½ and p38 in the spinal cord of sciatic nerve-injured mice showed that the inhibition of MAPK might be also involved in the anti-allodynic and anti-hyperalgesic effects of this treatment during persistent neuropathic pain. In according to these data, this signaling pathway was also involved in the analgesic effects of SFN during inflammatory pain (Redondo et al., 2017). In summary, the modulation of persistent

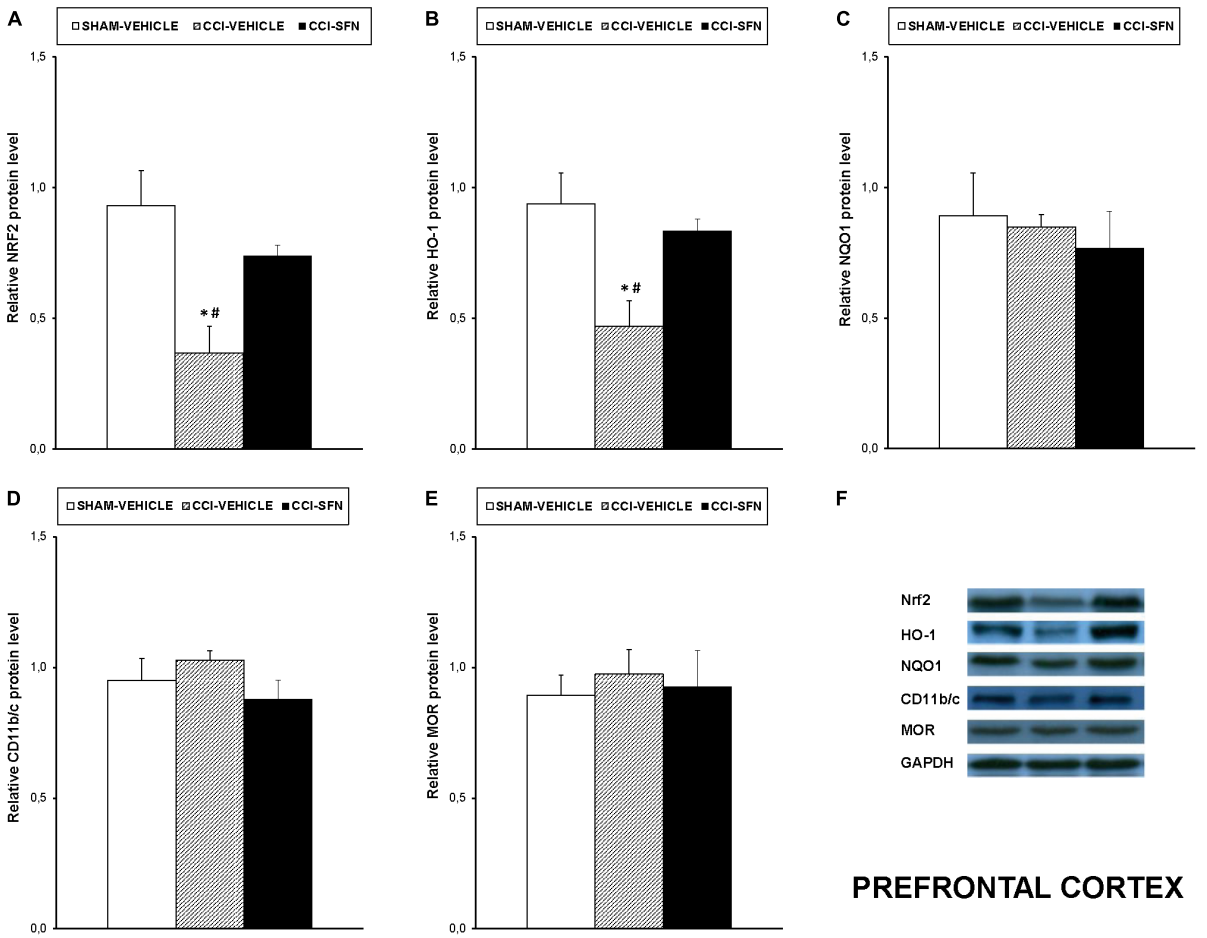

FIGURE 5 | Effects of SFN on the expression of Nrf2, HO-1, NQO1, CD11b/c, and MOR in the prefrontal cortex from animals with neuropathic pain. Effects of repetitive treatment with 10 mg/kg SFN or vehicle from days 14 to 28 after sciatic nerve injury (CCI) on Nrf2 **(A)**, HO-1 **(B)**, NQO1 **(C)**, CD11b/c **(D)**, and MOR **(E)** protein expression in the prefrontal cortex from CCI-induced neuropathic pain in mice are represented. The protein levels from Sham-operated (SHAM) mice treated with vehicle has been also represented as controls. Examples of western blots for Nrf2 (75 kDa), HO-1 (32 kDa), NQO1 (28 kDa), CD11b/c (160 kDa), and MOR (50 kDa) proteins in which GAPDH (36 kDa) was used as a loading control are also shown **(F)**. In all panels, * indicates significant differences vs. Sham vehicle treated mice and # indicates significant differences vs. CCI plus SFN treated mice ($P < 0.05$, one way ANOVA followed by Student–Newman–Keuls test). Data are expressed as mean values ± SEM; $n = 4$ samples per group.

sciatic nerve injury-induced neuropathic pain with the repeated administration of SFN might be explained by the activation of Nrf2/HO-1 signaling and the inhibition of microglial activation and MAPK phosphorylation induced by this treatment in the spinal cord.

In accordance with other preclinical pain models, our data verified that neuropathic pain induced by CCI, at 28 days after surgery, was associated with anxiety- and depressive-like behaviors (Jesse et al., 2010; Zhao et al., 2014; Alba-Delgado et al., 2016). That is, while anxiety-like response was demonstrated by the reduced number of entries and time spent into open arms in the EPM test, the depressive-like behavior was confirmed by the increased immobility time in the TST. These results are in agreement with the clinical data showing a relationship among chronic pain and emotional disorders (Micó et al., 2006).

More interesting is the finding that SFN treatment inhibited the anxiety-like behavior associated with persistent neuropathic pain, as demonstrated by the increased number of entries and the percentage of time spent in open arms in the EPM test observed in SFN treated mice. In addition, the fact that the number of entries into closed arms was not affected by SFN excluded the possibility that its anxiolytic effects might be result from alterations of mice locomotor activity. Our results supported the demonstrated anxiolytic effects produced by SFN in several stress models (Muramatsu et al., 2013; Wu et al., 2016) and further revealed, for first time, the anxiolytic action of this compound in the anxiety-like behavior accompanying to chronic neuropathic pain. Moreover, the anxiolytic effects induced by SFN in Sham-operated mice evaluated in the EPM test confirmed its anxiolytic effects measured in the open field test (Wu et al., 2016).

Our data further revealed, for the first time, the anti-depressant effects induced by the repeated administration of SFN in animals with depressive-like behavior associated

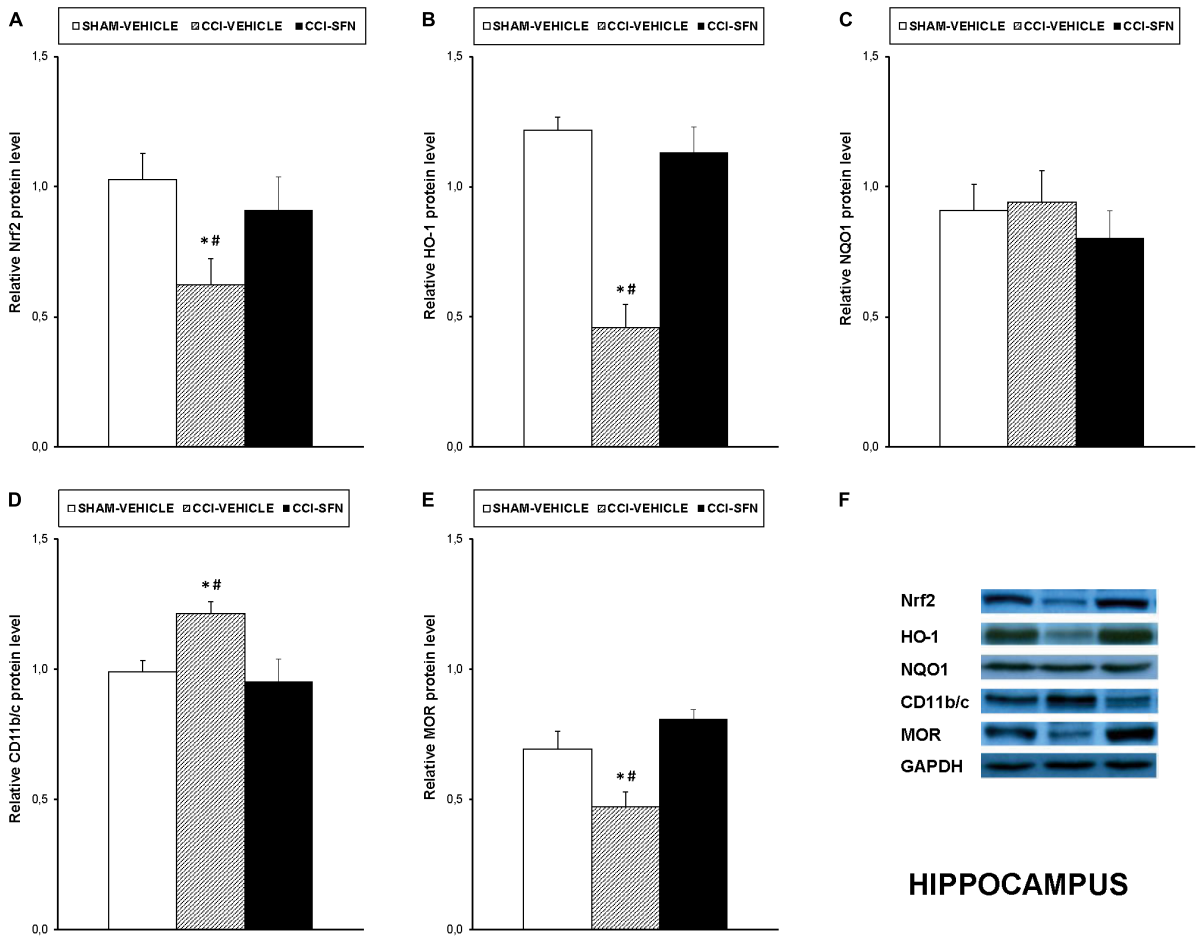

FIGURE 6 | Effects of SFN on the expression of Nrf2, HO-1, NQO1, CD11b/c, and MOR in the hippocampus from animals with neuropathic pain. Effects of repetitive treatment with 10 mg/kg SFN or vehicle from days 14 to 28 after sciatic nerve injury (CCI) on Nrf2 **(A)**, HO-1 **(B)**, NQO1 **(C)**, CD11b/c **(D)**, and MOR **(E)** protein expression in the hippocampus from CCI-induced neuropathic pain in mice are represented. The protein levels from Sham-operated (SHAM) mice treated with vehicle has been also represented as controls. Examples of western blots for Nrf2 (75 kDa), HO-1 (32 kDa), NQO1 (28 kDa), CD11b/c (160 kDa), and MOR (50 kDa) proteins in which GAPDH (36 kDa) was used as a loading control are also shown **(F)**. In all panels, * indicates significant differences vs. Sham vehicle treated mice and # indicates significant differences vs. CCI plus SFN treated mice ($P < 0.05$, one way ANOVA followed by Student–Newman–Keuls test). Data are expressed as mean values ± SEM; $n = 4$ samples per group.

with neuropathic pain, as demonstrated by the decreased immobility time in the TST. These data are in agreement with the anti-depressant activities induced by some Nrf2 activators in several animal models of depression as well as to the depressive phenotype manifested in Nrf2 knockout mice (Wu et al., 2016; Yao et al., 2016; Zhang et al., 2017). In addition, the anti-depressant effects induced by SFN in our Sham-operated mice assessed in the TST confirmed the anti-depressant capacity of this treatment previously demonstrated in the forced swimming test (Wu et al., 2016). It's important to mention that in contrast to the effects produced by several typical antidepressants, such as pregabalin that inhibited the anxiety-like behavior, but not the depressive-like response associated with neuropathic pain (La Porta et al., 2016), the repeated administration of SFN induced both anxiolytic and anti-depressant effects in animals with neuropathic pain. In brief, these results suggested the Nrf2 signaling pathway activation as a new therapeutic target for the treatment of anxiety and depression associated with chronic pain.

New theories of anxiety and depressive disorders revealed that beside inflammation, oxidative stress is also amply implicated in their development. Consequently, a significant decreased expression of the antioxidant enzymes activated by the Nrf2 signaling pathway in several anxiety- and depressive-like states has been demonstrated (Hashimoto, 2015; Khalifeh et al., 2015; Martín-Hernández et al., 2016). Thus, in order to evaluate the mechanisms implicated in the anxiolytic and anti-depressant properties of SFN in animals with chronic neuropathic pain, we evaluated its effects on the expression of Nrf2/HO-1/NQO1 signaling and CD11b/C in the prefrontal cortex and hippocampus of these animals. Our results showed a down-regulation of Nrf2 and HO-1 in prefrontal cortex and hippocampus, in addition to an up-regulation of CD11b/c in the hippocampus from animals with neuropathic pain, revealing

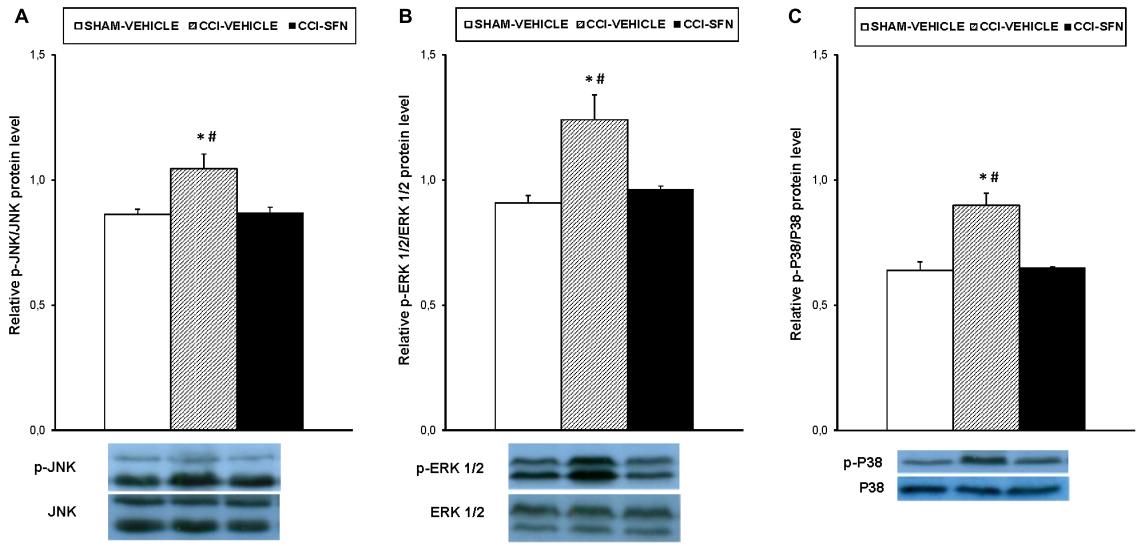

FIGURE 7 | Effect of SFN on the expression of MAPK in the spinal cord from animals with neuropathic pain. Effects of repetitive treatment with 10 mg/kg SFN or vehicle from days 14 to 28 after sciatic nerve injury (CCI) on the protein levels of p-JNK **(A)**, p-ERK ½ **(B)**, and p-P38 **(C)** in the spinal cord are represented. The protein levels from Sham-operated (SHAM) mice treated with vehicle has been also represented as controls. In all panels, * indicates significant differences vs. Sham-operated vehicle treated mice and # indicates significant differences vs. CCI-SFN treated mice ($P < 0.05$, one way ANOVA followed by Student–Newman–Keuls test). Each column represents the mean and vertical bars indicate the standard error of the mean (SEM); $n = 4$ samples per group. Representative examples of western blots for p-JNK/total JNK protein (46–54 kDa), p-ERK ½/total ERK ½ (42–44 kDa) and p-P38/total P38 (38 kDa) are also shown.

that both brain areas are involved in the affective disorders associated to chronic pain. Interestingly, the fact that treatment with SFN normalized the diminished expression of Nrf2 and HO-1 and avoided the activation of microglia induced by nerve injury in hippocampus and prefrontal cortex might explain the anxiolytic and anti-depressant effects of SFN in animals with neuropathic pain. The lack of changes in the expression of CD11b/c in prefrontal cortex confirmed that sciatic nerve injury only activates microglia in specific brain areas (Riego et al., 2018).

Finally, our data also revealed that SFN improved the mechanical and thermal antiallodynic effects produced by morphine during neuropathic pain. Considering that neuropathic pain is difficult to treat with the most potent analgesic compounds, such as MOR agonists (Obara et al., 2009; Hervera et al., 2013), the fact that SFN potentiated the antiallodynic effects of morphine represented a very interesting stratagem for the treatment of sciatic nerve injury induced neuropathic pain. These effects might be linked with the normalization of the decreased expression of MOR induced by SFN in the spinal cord and/or hippocampus from sciatic nerve-injured mice. These data are consistent with the up-regulation of MOR induced by the activation of Nrf2 in animals with peripheral inflammation (Redondo et al., 2017) as well as with the neutralization of the down regulation of these receptors induced by CoPP in sciatic nerve-injured mice or diabetic animals (Hervera et al., 2013; Castany et al., 2016; Jurga et al., 2017). These results suggested that the modulation of the expression of MOR by SFN treatment is probably mediated via Nrf2/HO-1 activation. Nevertheless and considering that the pharmacological inhibition of microglia also increased the analgesic effects of MOR agonists during neuropathic pain (Zychowska et al., 2013), we cannot discard that the inhibition of microglial activation made by SFN in our animals might be also implicated in the increased antiallodynic effects of morphine in SFN pretreated animals.

CONCLUSION

Our results revealed that treatment with SFN reduced allodynia and hyperalgesia induced by sciatic nerve injury and inhibited the anxiety and depressive-like behaviors associated with persistent neuropathic pain by normalizing oxidative stress, inhibiting microglial activation and MAPK phosphorylation in the specific areas evaluated. It is also interesting to note that treatment with SFN enhanced the antiallodynic activity of morphine by restoring the down regulation of MOR in the spinal cord and/or hippocampus from sciatic nerve-injured mice. In summary, this study showed that treatment with SFN might be an interesting approach for the management of persistent neuropathic pain and comorbidities associated, as well as to improve the analgesic actions of morphine in animals with neuropathic pain.

AUTHOR CONTRIBUTIONS

PF-C performed the behavioral tests. AR, GR, and SL performed the western blot assays. PF-C and OP performed the statistical analysis. PF-C and OP designed the study. OP wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REFERENCES

Alba-Delgado, C., Cebada-Aleu, A., Mico, J. A., and Berrocoso, E. (2016). Comorbid anxiety-like behavior and locus coeruleus impairment in diabetic peripheral neuropathy: a comparative study with the chronic constriction injury model. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 71, 45–56. doi: 10.1016/j.pnpbp.2016.06.007

Attal, N., Lanteri-Minet, M., Laurent, B., Fermanian, J., and Bouhassira, D. (2011). The specific disease burden of neuropathic pain: results of a French nationwide survey. *Pain* 152, 2836–2843. doi: 10.1016/j.pain.2011.09.014

Axelsson, A. S., Tubbs, E., Mecham, B., Chacko, S., Nenonen, H. A., Tang, Y., et al. (2017). Sulforaphane reduces hepatic glucose production and improves glucose control in patients with type 2 diabetes. *Sci. Transl. Med.* 9:394. doi: 10.1126/scitranslmed.aah4477

Bennett, G. J., and Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33, 87–107.

Castany, S., Carcolé, M., Leánez, S., and Pol, O. (2016). The induction of heme oxygenase 1 decreases painful diabetic neuropathy and enhances the antinociceptive effects of morphine in diabetic mice. *PLoS One* 11:e0146427. doi: 10.1371/journal.pone.0146427

Catanzaro, E., Calcabrini, C., Turrini, E., Sestili, P., and Fimognari, C. (2017). Nrf2: a potential therapeutic target for naturally occurring anticancer drugs? *Exp. Opin. Ther. Targets* 21, 781–793. doi: 10.1080/14728222.2017.1351549

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., and Yaksh, T. L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* 53, 55–63.

Davidson, R. K., Jupp, O., de Ferrars, R., Kay, C. D., Culley, K. L., Norton, R., et al. (2013). Sulforaphane represses matrix-degrading proteases and protects cartilage from destruction in vitro and in vivo. *Arthritis Rheum.* 65, 3130–3140. doi: 10.1002/art.38133

de Figueiredo, S. M., Binda, N. S., Nogueira-Machado, J. A., Vieira-Filho, S. A., and Caligiorne, R. B. (2015). The antioxidant properties of organosulfur compounds (sulforaphane). *Recent. Pat. Endocr. Metab. Immune. Drug Discov.* 9, 24–39.

de Vries, H. E., Witte, M., Hondius, D., Rozemuller, A. J., Drukarch, B., Hoozemans, J., et al. (2008). Nrf2-induced antioxidant protection: a promising target to counteract ROS-mediated damage in neurodegenerative disease? *Free Radic. Biol. Med.* 45, 1375–1383. doi: 10.1016/j.freeradbiomed.2008.09.001

Di, W., Shi, X., Lv, H., Liu, J., Zhang, H., Li, Z., et al. (2016). Activation of the nuclear factor E2-related factor 2/anitioxidant response element alleviates the nitroglycerin-induced hyperalgesia in rats. *J. Headache Pain* 17:99. doi: 10.1186/s10194-016-0694-x

Dinkova-Kostova, A. T., Fahey, J. W., Kostov, R. V., and Kensler, T. W. (2017). KEAP1 and done? targeting the NRF2 pathway with sulforaphane. *Trends Food Sci. Technol.* 69(Pt B), 257–269. doi: 10.1016/j.tifs.2017.02.002

Edelmayer, R. M., Brederson, J. D., Jarvis, M. F., and Bitner, R. S. (2014). Biochemical and pharmacological assessment of MAP-kinase signaling along pain pathways in experimental rodent models: a potential tool for the discovery of novel antinociceptive therapeutics. *Biochem. Pharmacol.* 87, 390–398. doi: 10.1016/j.bcp.2013.11.019

Fahey, J. W., Wade, K. L., Wehage, S. L., Holtzclaw, W. D., Liu, H., Talalay, P., et al. (2017). Stabilized sulforaphane for clinical use: phytochemical delivery efficiency. *Mol. Nutr. Food Res.* 61:1600766. doi: 10.1002/mnfr.201600766

Guerrero-Beltran, C. E., Calderon-Oliver, M., Pedraza-Chaverri, J., and Chirino, Y. I. (2012). Protective effect of sulforaphane against oxidative stress: recent advances. *Exp. Toxicol. Pathol.* 64, 503–508. doi: 10.1016/j.etp.2010.11.005

Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32, 77–88.

Hashimoto, K. (2015). Inflammatory biomarkers as differential predictors of antidepressant response. *Int. J. Mol. Sci.* 16, 7796–7801. doi: 10.3390/ijms16047796

Hervera, A., Leánez, S., Motterlini, R., and Pol, O. (2013). Treatment with carbon monoxide-releasing molecules and an HO-1 inducer enhances the effects and expression of μ-opioid receptors during neuropathic pain. *Anesthesiology* 118, 1180–1197. doi: 10.1097/ALN.0b013e318286d085

Hervera, A., Leánez, S., Negrete, R., Motterlini, R., and Pol, O. (2012). Carbon monoxide reduces neuropathic pain and spinal microglial activation by inhibiting nitric oxide synthesis in mice. *PLoS One* 7:e43693. doi: 10.1371/journal.pone.0043693

Jesse, C. R., Wilhelm, E. A., and Nogueira, C. W. (2010). Depression-like behavior and mechanical allodynia are reduced by bis selenide treatment in mice with chronic constriction injury: a comparison with fluoxetine, amitriptyline, and bupropion. *Psychopharmacology* 212, 513–522. doi: 10.1007/s00213-010-1977-6

Ji, R. R., Gereau, R. W., Malcangio, M., and Strichartz, G. R. (2009). MAP kinase and pain. *Brain Res. Rev.* 60, 135–148.

Jurga, A. M., Piotrowska, A., Makuch, W., Przewlocka, B., and Mika, J. (2017). Blockade of P2X4 receptors inhibits neuropathic pain-related behavior by preventing MMP-9 activation and, consequently, pronociceptive interleukin release in a rat model. *Front. Pharmacol.* 8:48. doi: 10.3389/fphar.2017.00048

Khalifeh, S., Oryan, S., Digaleh, H., Shaerzadeh, F., Khodagholi, F., Maghsoudi, N., et al. (2015). Involvement of Nrf2 in development of anxiety-like behavior by linking Bcl2 to oxidative phosphorylation: estimation in rat hippocampus, amygdala, and prefrontal cortex. *J. Mol. Neurosci.* 55, 492–499. doi: 10.1007/s12031-014-0370-z

Kim, H. A., Yeo, Y., Kim, W. U., and Kim, S. (2009). Phase 2 enzyme inducer sulphoraphane blocks matrix metalloproteinase production in articular chondrocytes. *Rheumatology* 48, 932–938. doi: 10.1093/rheumatology/kep132

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., et al. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat. Commun.* 7:11624. doi: 10.1038/ncomms11624

La Porta, C., Lara-Mayorga, I. M., Negrete, R., and Maldonado, R. (2016). Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice. *Eur. J. Pain* 20, 1454–1466. doi: 10.1002/ejp.868

Lee, S., Kim, J., Seo, S. G., Choi, B. R., Han, J. S., Lee, K. W., et al. (2014). Sulforaphane alleviates scopolamine-induced memory impairment in mice. *Pharmacol. Res.* 85, 23–32. doi: 10.1016/j.phrs.2014.05.003

Liu, X., Zhang, Z., Cheng, Z., Zhang, J., Xu, S., Liu, H., et al. (2016). Spinal heme oxygenase-1 (HO-1) exerts antinociceptive effects against neuropathic pain in a mouse model of L5 spinal nerve ligation. *Pain Med.* 17, 220–229.

Maletic, V., and Raison, C. L. (2009). Neurobiology of depression, fibromyalgia and neuropathic pain. *Front. Biosci.* 14:5291–5338.

Martín-Hernández, D., Bris, ÁG., MacDowell, K. S., García-Bueno, B., Madrigal, J. L., Leza, J. C., et al. (2016). Modulation of the antioxidant nuclear factor (erythroid 2-derived)-like 2 pathway by antidepressants in rats. *Neuropharmacology* 103, 79–91. doi: 10.1016/j.neuropharm.2015.11.029

McDonnell, C., Leánez, S., and Pol, O. (2017). The induction of the transcription factor Nrf2 enhances the antinociceptive effects of delta-opioid receptors in diabetic mice. *PLoS One* 12:e0180998. doi: 10.1371/journal.pone.0180998

Micó, J. A., Ardid, D., Berrocoso, E., and Eschalier, A. (2006). Antidepressants and pain. *Trends Pharmacol. Sci.* 27, 348–354.

Muramatsu, H., Katsuoka, F., Toide, K., Shimizu, Y., Furusako, S., and Yamamoto, M. (2013). Nrf2 deficiency leads to behavioral, neurochemical and transcriptional changes in mice. *Genes Cells* 18, 899–908. doi: 10.1111/gtc.12083

Negi, G., Kumar, A., and Sharma, S. S. (2011). Nrf2 and NF-κB modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes. *Curr. Neurovasc. Res.* 8, 294–304.

Obara, I., Parkitna, J. R., Korostynski, M., Makuch, W., Kaminska, D., Przewlocka, B., et al. (2009). Local peripheral opioid effects and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. *Pain* 141, 283–291. doi: 10.1016/j.pain.2008.12.006

Popiolek-Barczyk, K., and Mika, J. (2016). Targeting the microglial signaling pathways: new insights in the modulation of neuropathic pain. *Curr. Med. Chem.* 23, 2908–2928.

Pu, D., Zhao, Y., Chen, J., Sun, Y., Lv, A., Zhu, S., et al. (2018). Protective effects of sulforaphane on cognitive impairments and ad-like lesions in diabetic mice are associated with the upregulation of Nrf2 transcription activity. *Neuroscience* 381, 35–45. doi: 10.1016/j.neuroscience.2018.04.017

Redondo, A., Chamorro, P. A. F., Riego, G., Leánez, S., and Pol, O. (2017). Treatment with sulforaphane produces antinociception and improves morphine effects during inflammatory pain in mice. *J. Pharmacol. Exp. Ther.* 363, 293–302. doi: 10.1124/jpet.117.244376

Riego, G., Redondo, A., Leánez, S., and Pol, O. (2018). Mechanism implicated in the anti-allodynic and anti-hyperalgesic effects induced by the activation of heme oxygenase 1/carbon monoxide signaling pathway in the central nervous system of mice with neuropathic pain. *Biochem. Pharmacol.* 148, 52–63. doi: 10.1016/j.bcp.2017.12.007

Singh, K., Connors, S. L., Macklin, E. A., Smith, K. D., Fahey, J. W., Talalay, P., et al. (2014). Sulforaphane treatment of autism spectrum disorder (ASD). *Proc. Natl. Acad. Sci. U.S.A.* 111, 15550–15555. doi: 10.1073/pnas.1416940111

Steru, L., Chermat, R., Thierry, B., and Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology* 85, 367–370.

Walf, A. A., and Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protoc.* 2, 322–328.

Wang, C., and Wang, C. (2017). Anti-nociceptive and anti-inflammatory actions of sulforaphane in chronic constriction injury-induced neuropathic pain mice. *Inflammopharmacology* 25, 99–106. doi: 10.1007/s10787-016-0307-y

Wu, S., Gao, Q., Zhao, P., Gao, Y., Xi, Y., Wang, X., et al. (2016). Sulforaphane produces antidepressant- and anxiolytic-like effects in adult mice. *Behav. Brain Res.* 301, 55–62. doi: 10.1016/j.bbr.2015.12.030

Yanaka, A. (2018). Daily intake of broccoli sprouts normalizes bowel habits in human healthy subjects. *J. Clin. Biochem. Nutr.* 62, 75–82. doi: 10.3164/jcbn.17-42

Yao, W., Zhang, J., Ishima, T., Ren, Q., Yang, C., Dong, C., et al. (2016). Antidepressant effects of TBE-31 and MCE-1, the novel Nrf2 activators, in an inflammation model of depression. *Eur. J. Pharmacol.* 793, 21–27. doi: 10.1016/j.ejphar.2016.10.037

Zhang, J. C., Yao, W., Dong, C., Yang, C., Ren, Q., Ma, M., et al. (2017). Prophylactic effects of sulforaphane on depression-like behavior and dendritic changes in mice after inflammation. *J. Nutr. Biochem.* 39, 134–144. doi: 10.1016/j.jnutbio.2016.10.004

Zhang, Y., Kensler, T. W., Cho, C. G., Posner, G. H., and Talalay, P. (1994). Anticarcinogenicactivities of sulforaphane and structurally related synthetic norbornylisothiocyanates. *Proc. Natl. Acad. Sci. U.S.A.* 91, 3147–3150.

Zhao, X., Wang, C., Zhang, J. F., Liu, L., Liu, A. M., Ma, Q., et al. (2014). Chronic curcumin treatment normalizes depression-like behaviors in mice with mononeuropathy: involvement of supraspinal serotonergic system and GABAA receptor. *Psychopharmacology* 231, 2171–2187. doi: 10.1007/s00213-013-3368-2

Zychowska, M., Rojewska, E., Kreiner, G., Nalepa, I., Przewlocka, B., and Mika, J. (2013). Minocycline influences the anti-inflammatory interleukins and enhances the effectiveness of morphine under mice diabetic neuropathy. *J. Neuroimmunol.* 262, 35–45. doi: 10.1016/j.jneuroim.2013.06.005

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REVIEW
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The Role of Nrf2 in Liver Disease: Novel Molecular Mechanisms and Therapeutic Approaches

Dongwei Xu[1†], Min Xu[1†], Seogsong Jeong[1], Yihan Qian[2], Hailong Wu[3], Qiang Xia[1] and Xiaoni Kong[1*]*

[1] Department of Liver Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, [2] School of Pharmacy, Fudan University, Shanghai, China, [3] Shanghai Key Laboratory for Molecular Imaging, Collaborative Research Center, Shanghai University of Medicine and Health Sciences, Shanghai, China

Oxidative stress and inflammation are the most important pathogenic events in the development and progression of liver diseases. Nuclear erythroid 2-related factor 2 (Nrf2) is the master regulator of the cellular protection *via* induction of anti-inflammatory, antioxidant, and cyto-protective genes expression. Multiple studies have shown that activation or suppression of this transcriptional factor significantly affect progression of liver diseases. Comprehensive understanding the roles of Nrf2 activation/expression and the outcomes of its activators/inhibitors are indispensable for defining the mechanisms and therapeutic strategies against liver diseases. In this current review, we discussed recent advances in the function and principal mechanisms by regulating Nrf2 in liver diseases, including acute liver failure, hepatic ischemia–reperfusion injury (IRI), alcoholic liver disease (ALD), viral hepatitis, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and hepatocellular carcinoma (HCC).

Keywords: Nrf2, oxidative stress, cytoprotective genes, acute liver injury, viral hepatitis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, hepatocellular carcinoma

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Pallavi R. Devchand, University of Calgary, Canada

****Correspondence:***
Qiang Xia
xiaqiang@shsmu.edu.cn
Xiaoni Kong
xiaoni-kong@126.com

† These authors shared co-first authorship

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INTRODUCTION

Oxidative stress and inflammation are the most important pathogenic events in liver diseases. During liver injuries, the unregulated production of free radicals and/or ROS leads to damage of important biomolecules and cells and generation of proinflammatory genes. Antioxidant and anti-inflammatory therapy has been considered to be beneficial in liver diseases. Nrf2 is the master regulator of the primary means of cellular defense through mediation of antioxidant response, anti-inflammatory and cytoprotective properties, and dysregulation of Nrf2 activity has been revealed to correlated with the development of chronic inflammatory diseases (Alam et al., 1999; Vomund et al., 2017; Bellezza et al., 2018; Hennig et al., 2018). The protective effects of Nrf2 signaling pathway has been identified in a number of disease models, including acute kidney, lung or neurons

Abbreviations: ALD, alcoholic liver disease; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CDDO-Im, CDDO-imidazolide; CYP2E1, cytochrome P450 2E1; D3T, 3H-1,2 dithiole-3-thione; D-GalN, D-galactosamine; DAMP, damage-associated molecular pattern; DILI, drug-induced liver injury; FGF19, fibroblast growth factor 19; GST, glutathione S-transferase; HBV, hepatitis B virus; HBx, HBV stimulates by its X protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRI, ischemia–reperfusion injury; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NQO1, NAD(P)H quinone dehydrogenase one; Nrf2, nuclear erythroid 2-related factor 2; NS, non-structural; PBC, primary biliary cholangitis; PPARγ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; VLDLR, very-low density lipoprotein receptor.

injury, emphysema, and sepsis (Thimmulappa et al., 2007; Reddy et al., 2009; Sussan et al., 2009; Zhang et al., 2012; Liu et al., 2014). Accumulating evidence also has implicated this transcription factor in various liver diseases, including acute hepatoxicity, NAFLD, NASH, ALD, DILI, viral hepatitis, liver fibrosis, hepatic IRI, and primary hepatic malignancies (Klaassen and Reisman, 2010; Tang et al., 2014). Under acute and chronic oxidative stress and inflammatory conditions, Nrf2 is activated and prevents oxidative and inflammatory diseases by modulating genes expression of cytoprotective proteins and enzymes, which decreases ROS levels, inflammation, and cell death (Bataille and Manautou, 2012). However, the function of Nrf2 is not always protective in diseases, recent studies have identified that the gene expression of Nrf2 was associated with the pathogenesis, progression, and metastasis of cancer, resistance to cancer therapy, and the regulation of cancer cells metabolism, thereby suggesting that Nrf2 is a pleiotropic transcriptional factor (Karin and Dhar, 2016; Rojo de la Vega et al., 2018). In this review, we summarized up-to-date studies in the understanding of the roles and mechanisms of Nrf2 and the therapeutic approaches by targeting Nrf2 in liver diseases.

ACTIVATION OF Nrf2 ATTENUATES ACUTE LIVER INJURY

Study has shown that activation of Nrf2 attenuates acute liver injury. Wu et al. (2012) compared serum ALT, LDH, hepatic hemorrhage, and necrosis levels between Nrf2-null and Nrf2-enhanced mice in cadmium-induced acute liver injury mice model; they found that Nrf2-enhanced mice were associated with lower ALT and LDH levels and with fewer morphological alterations. The mRNA levels of cytoprotective genes, including sulfiredoxin-1, glutamate-cysteine ligase, and glutathione peroxidase-2 were expressed only in Nrf2-enhanced mice, suggesting that Nrf2 activation prevents oxidative stress and acute liver injury through modulation of antioxidant defense-associated genes (**Figure 1**). Subsequently, the protective effects of Nrf2 was tested in LPS and D-GalN-induced liver injury mouse models by treatment with mangiferin, which could upregulate the gene expression of Nrf2 in a dose-dependent manner (Pan et al., 2016). Mangiferin treatment suppressed serum levels of ALT, AST, IL-1β, TNF-α, and ROS levels, adding evidences that activation of Nrf2 pathway protects against acute liver injury. Biochanin A, morin, curcumin, andrographolide, oxymatrine, and madecassoside were also found to play a protective role *via* activation of Nrf2 in LPS and D-GalN-induced acute liver injury in mice (Liu et al., 2016; Pan et al., 2017; Tian et al., 2017; Xie et al., 2017; Wang et al., 2018). In addition, the antioxidant pathway of Nrf2 was further tested and found to be effective in carbon tetrachloride-induced and acetaminophen-induced mouse acute liver injury models (Huang et al., 2016; Cao et al., 2017; Peng et al., 2018; Shen Z. et al., 2018). The role of Nrf2 in hepatic IRI was also identified by several studies (Ke et al., 2013; Kudoh et al., 2014; Rao et al., 2015; Ge et al., 2017; Xu et al., 2017). Ke et al. (2013) showed that the Keap1–Nrf2 complex could alleviate oxidative injury in mouse orthotopic liver transplantation through Keap1 signaling (**Figure 1**). The protective effects were identified by limiting hepatic inflammatory responses and hepatocellular necrosis. Recently, our research identified cytoprotective effects of CDDO-Im, a potent activator of the Nrf2 pathway, in hepatic IRI, through inducing Nrf2 target gene HO-1 expression leads to enhanced autophagy in hepatocytes, which results in increased clearance of damaged mitochondria, reduced mtDNA release and ROS production leading to reductions in DAMP release-induced inflammatory responses and subsequent secondary hepatocyte injury (Xu et al., 2017). Despite accumulating evidences, Nrf2-based treatment is yet to enter clinical trials in the USA[1] for patients with acute liver failure.

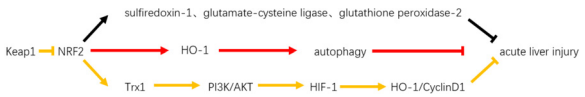

FIGURE 1 | Role of Nrf2 in acute liver injury. The protective effects of Nrf2 in acute liver injury, one is through regulating antioxidant defense-related genes, including sulfiredoxin-1, glutamate-cysteine ligase, and glutathione peroxidase-2, and the other pathway is by promoting its target gene HO-1 and then enhanced autophagy. While its negative regulator-keap1, which by binding to it inhibits Nrf2 activation and Trx1-PI3K/AKT-HIF1-HO-1/CyclinD1 signal pathway and promotes liver injury.

ACTIVATION OF Nrf2 AMELIORATES ALCOHOLIC LIVER DISEASE

Alcohol consumption has been revealed to be significantly associated with the development and progression of liver diseases over decades (Shepard et al., 2010). Alcohol metabolism in the liver includes ethanol oxidation by alcohol dehydrogenase in hepatocytes and microsomal oxidation promoted by CYP2E1 (Bae et al., 2011; Wang et al., 2014a). Alcohol dehydrogenase-associated ethanol metabolism results in acetaldehyde, which gives rise to some downstream effects, such as depletion of glutathione, lipid peroxidation, and generation of ROS (Dey and Cederbaum, 2006). In addition, the dysregulation of antioxidant glutathione by Nrf2-dependent regulation was found to contribute to the development of ALD by providing pathological conditions, whereas the Nrf2-mediated antioxidant response provided protection against alcohol-induced oxidative stress by regulating glutathione metabolism (Harvey et al., 2009; Lu, 2013; Rejitha et al., 2015). Furthermore, the oxidative stress-induced upregulation of Nrf2 is considered to positively modulate expression of VLDLR, which contributes to ALD (Wang et al., 2014b).

In ethanol-exposed mice, the role of Nrf2-induced antioxidant factors was first tested by the Nrf2 inducer D3T (Dong et al., 2008). Upregulation of Nrf2 by D3T treatment has significantly decreased generation of ethanol-induced ROS and apoptosis, which indicated that the activation of Nrf2 could diminish ethanol-induced apoptosis and ameliorate the disease status.

[1] clinicaltrials.gov

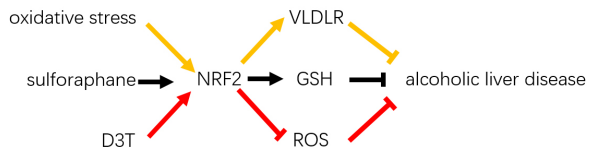

FIGURE 2 | Role of Nrf2 in alcoholic liver disease. Studies shown that oxidative stress promotes Nrf2 activation-induced hepatic VLDLR overexpression and ameliorates ALD. Nrf2 activator-Sulforaphane protects alcoholic fatty liver disease by activating Nrf2 signaling pathway and GSH levels. D3T through upregulate Nrf2 protein levels and decreased ROS levels and liver injuries.

Moreover, Zhou et al. (2014) verified that Nrf2-mediated cytoprotective enzymes could ameliorate alcohol-induced liver steatosis both in *in vivo* and *in vitro* models. They further administered sulforaphane, which is an activator of Nrf2 and present in considerable quantities in brassica vegetables including broccoli, cabbage, and kale, and found it to be effective in improving alcohol-induced liver steatosis (**Figure 2**). Furthermore, recent advances indicated that activation of the Nrf2 pathway was protective in alcohol-induced liver fibrosis and hepatotoxicity, whereas knockdown of Nrf2 was associated with enhanced alcohol-induced hepatocyte necroptosis (Song et al., 2015; Lu et al., 2016; Ni et al., 2017). By contrast, a more recent study demonstrated that ethyl pyruvate, which has multi-effects including antibacterial, anti-inflammatory, antiviral, vasodilatory, antioxidant, and antiapoptotic effects, decreases ALT, AST, hepatic morphological changes, triglycerides, free fatty acids, and the expression of proinflammatory factors and increases the expression of anti-inflammatory factors and peroxisome proliferator-activated receptor-α mRNA which through downregulation of the ROS–Nrf2 signaling pathway, thereby alleviating ALD in mice (Fawcett et al., 1999; Harding et al., 2000; Shen F. et al., 2018). Taken together, these evidences showed that Nrf2 activation plays essential protective role in the development of ALD and that simultaneous downregulation of Nrf2 with ROS and VLDLR may also be effective in the amelioration of ALD (**Figure 2**). Further studies are required to demonstrate the extent of amelioration between upregulation and downregulation of Nrf2 when ROS and VLDLR expression levels are downregulated in ALD.

PROTECTIVE EFFECTS OF Nrf2 ON VIRAL HEPATITIS-INFECTED CELLS AGAINST OXIDATIVE DAMAGE

Oxidative stress has been shown to be implicated in viral hepatitis-associated liver diseases, including HBV and HCV infections (Bolukbas et al., 2005; Ivanov et al., 2013). A previous study indicated that HCV could mediate the phosphorylation and activation of Nrf2, which was regulated by the mitogen-activated protein kinases. The authors further suggested that the activation of Nrf2-derived survival of HCV-infected cells may provide favorable circumstances for carcinogenesis (Burdette et al., 2010). Another study showed that the inhibition of Nrf2 and antioxidant response elements is regulated by the core proteins of HCV-replicating cell-triggered delocalization of small Maf proteins, which were bound to NS proteins NS3, thus reducing the expression of cytoprotective genes (Carvajal-Yepes et al., 2011). From the authors' point of view, inhibition of Nrf2 and antioxidant response element-regulated genes may contribute to HCV-associated pathogenesis due to impaired induction of reactive oxygen intermediates caused by cytoprotective genes, which giving rise to host cell DNA damage and promoting the genetic variability of the viral genome. Moreover, Ivanov et al. (2011) found that the antioxidant-protective Nrf2/antioxidant response element pathway is activated by HCV proteins, including core, E1, E2, NS5A, and NS4B, in an ROS-dependent and -independent manners (**Figure 3**). In addition, a strong upregulation of the antioxidant-protective system was modulated in the earliest stage, indicating that Nrf2 is activated to protect against HCV-induced oxidative stress in the acute stage of HCV infection. In addition, replication of HCV has been reported to be suppressed by Nrf2-mediated heme oxygenase-1 (HO-1) inducible factor, which is a phytocompound isolated from *Lindera erythrocarpa* Makino fruits (lucidone), and a quinone methide triterpene isolated from *Tripterygium wilfordii* root extract (celastrol) (Chen et al., 2013; Tseng et al., 2017). Furthermore, an *in vitro* cell line study from Japan found that knockdown of Nrf2 significantly reduced HCV infection and steatosis (Sugiyama et al., 2014). Most recently, the authors further confirmed that an Nrf2 inhibitor (brusatol) had anti-HCV effects *in vitro* (Murakami et al., 2018) (**Figure 3**).

Hepatitis B virus infection, which causes acute or chronic liver inflammation and contributes to the development of HCC, has been shown to induce activation of Nrf2 and antioxidative response elements *in vivo* and *in vitro* by HBV-regulatory proteins, including HBx and large surface proteins, *via* c-Raf and mitogen-activated protein kinase (Hildt et al., 2002; Schaedler et al., 2010). In addition, the HBx protein-mediated activation of Nrf2 has been introduced to trigger the upregulation of glucose-6-phosphate dehydrogenase, thereby reprogramming metabolism of glucose, and may participate in the development of HCC (Liu et al., 2015). Therefore, Nrf2 is not only a crucial factor that is activated to defend against viral hepatitis-induced

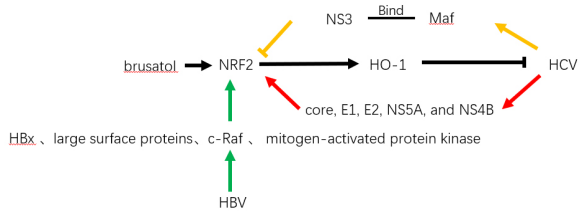

FIGURE 3 | Role of Nrf2 in viral hepatitis-associated liver diseases. Hepatitis C virus was reported that impaired the protection of Nrf2 by promoting sMaf bound to NS3 and brusatol suppressed HCV infection by promoting Nrf2 pathway. HBV-regulatory proteins, including its X protein (HBx) and large surface proteins were reported to activate Nrf2 and antioxidative responses *via* c-Raf and mitogen-activated protein kinase.

oxidative stress but also a protective factor that is involved in the survival of viral hepatitis-infected cells and may contribute to hepatocarcinogenesis.

PROTECTIVE IMPACT OF Nrf2 IN NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease is a progressive disease arising from the accumulation of lipids in hepatocytes and has an increasing incidence worldwide (Satapathy and Sanyal, 2015). Approximately one-third of patients with NAFLD progress to severe NASH, which is linked with inflammation and cirrhosis (Tarantino and Finelli, 2013; Dietrich and Hellerbrand, 2014). Recent studies indicated that ROS and electrophiles are associated with the pathogenesis of NASH; thus, induction of Nrf2 seemed to be promising in the prevention and treatment of NAFLD (Chambel et al., 2015). Du et al. (2016), explored the therapeutic impact of Nrf2 activation by using osteocalcin, and found that it could improve NAFLD by ameliorating oxidative stress and inhibiting the JNK pathway, which is an important pathway involved in the pathogenesis of NAFLD. A recent study demonstrated that scutellarin, a flavonoid glycoside that has an antioxidative stress effect, significantly reduced blood lipid levels and enhanced the antioxidative capacity by activating PPARγ and its coactivator-1α, Nrf2, HO-1, GST, and NQO1, and suppressing nuclear factor κB and Keap1 at the mRNA and protein levels, thus ameliorating NAFLD (Zhang et al., 2018) (**Figure 4**). In addition, a modulator of PPARγ, apigenin, was also revealed to attenuate NAFLD by Nrf2-associated regulation of oxidative stress and hepatocyte lipid metabolism (Feng et al., 2017). Moreover, for the prevention of NAFLD, scutellarin, which is a natural drug with active components of breviscapine, was shown to be effective by enhancing the Nrf2-mediated antioxidant system in high-fat diet- and chronic stress-subjected rats (Fan et al., 2017).

Nuclear erythroid 2-related factor 2 has been found to be a key regulator in the protection against NASH (Gupte et al., 2013). By contrast, loss of Nrf2 or deletion of Nrf2 has been found to cause benign steatosis to develop into NASH and contribute to exacerbation of disease status (Chowdhry et al., 2010; Wang et al., 2013). Ramadori et al. (2017) indicated that overactivation of Nrf2 suppressed the hepatocyte-specific c-met deletion (an accelerative factor for NASH)-induced deleterious impact on the progression of NASH and suggested that Nrf2 repaired liver damage in hepatocyte-specific c-met-deficient mice *via* maintaining balance in cellular redox homeostasis. To date, green tea extract and ezetimibe (an inhibitor of Niemann-Pick-C1-Like 1) have been revealed to promote the protective impact of Nrf2 against lipid accumulation and the inflammatory response during NASH (Lee et al., 2016; Li et al., 2016) (**Figure 4**). However, Nrf2-associated therapeutic approaches for NASH remain to be implemented in a real-world clinical manner in the near future.

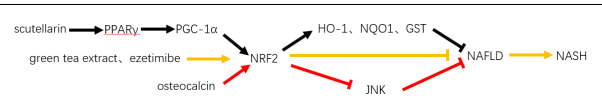

FIGURE 4 | Role of Nrf2 in NAFLD. Multiple studies have shown that several pathways could activate Nrf2 and inhibit NAFLD and NASH including scutellarin-PPARγ/PGC1α-Nrf2-HO-1/NQO1/GST pathway; green tea extract and ezetimibe activated Nrf2 pathway; osteocalcin activated Nrf2 and subsequently inhibited JNK pathway.

Nrf2 IN PRIMARY LIVER CANCER

Hepatocellular carcinoma is the most common primary liver cancer, accounting for more than 80% of all hepatic malignancies (Forner et al., 2018), with molecular alterations in HCC arising in the very early stage of carcinogenesis (Pitot, 2007). Among the changes, activation of Nrf2 was found to be the prominent pathway that contributes to the progression of preneoplastic lesion to malignancy, which was confirmed by *in vivo* detection of the inhibition of the Nrf2 pathway that accompanied the regression of cytokeratin 19-positive nodules (Petrelli et al., 2014). The persistent activation of this transcription factor was found to be associated with the accumulation of p62, thus participating in the development of HCC (Inami et al., 2011). This finding was further supported by Saito et al. (2016) who confirmed the promotive impact of p62 in HCV-positive HCC through Nrf2-dependent metabolic reprogramming. In addition, Nrf2 was also found to participate in protection of HCC cells by facilitating the survival response of FGF19 to endoplasmic reticulum stress (Teng et al., 2017; Tian et al., 2018) (**Figure 5**). Thus, advances were made to regulate the Nrf2 pathway in HCC, including identification of miR-340, miR-144, camptothecin, and valproic acid, which were revealed to be effective in suppressing the Nrf2-dependent pathway, thereby sensitizing HCC cells to anticancer treatments (Shi et al., 2014; Zhou et al., 2016; Chen et al., 2017; Yu et al., 2017) (**Figure 5**). Moreover, indazolo[3,2-b]quinazolinones were revealed to attack HCC cells by suppressing Nrf2/antioxidative response elements and inducing mitochondrial-dependent apoptosis simultaneously (Zhang et al., 2016). In a clinical retrospective study, patients with high expression levels of Nrf2 (n = 48) had significantly reduced overall (median, 13.87 months) and disease-free survival (median, 11.24 months) compared with patients with low expression levels of Nrf2 (n = 17), who exhibited median overall survival of 30.40 months and

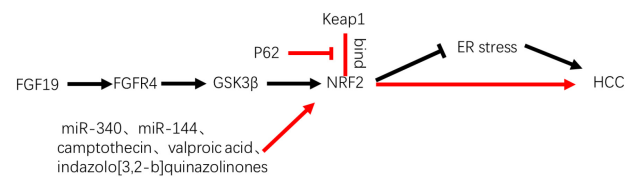

FIGURE 5 | Role of Nrf2 in HCC. P62 and FGF19 were reported to activate Nrf2 and promote HCC by competing with Keap1 or through FGFR4–GSK3β signal pathway. MiR-340, miR-144, camptothecin, valproic acid, and indazolo[3,2-b]quinazolinones, which were revealed in suppressing Nrf2-dependent pathway and sensitizing HCC cells to anticancer treatments.

disease-free survival of 24.43 months ($P < 0.01$) (Zhang et al., 2015). The relative risk of high Nrf2 levels in overall survival was 5.96 with 95% confidence interval of 2.46–14.69 ($P < 0.01$). However, regarding the sample size and retrospective nature, a large-sized prospective clinical study is required to confirm the prognostic impact of Nrf2 in patients with HCC.

CONCLUSION

In this review, we briefly summarized the biology characteristics of Nrf2 pathway and discussed the potential therapeutic applications of targeting Nrf2 in liver diseases. To date, there are currently few pharmacological options available to prevent or treat liver diseases. Recently, in clinical trial, NGM282, an engineered FGF19 analog, could significantly reduce liver fat content in patients with NASH and remarkably improve ALP and transaminase levels in patients with PBC (Harrison et al., 2018; Mayo et al., 2018). The small molecule PRI-724 also identified the anti-fibrotic effects in a phase 1 trial in patients with HCV cirrhosis (Kudo et al., 2018).

A link between liver diseases and oxidative stress is indispensable. The Nrf2 antioxidant pathway is activated to protect the liver by modulating defensive genes, which even protect viral hepatitis-infected cells and HCC cells. A number of preclinical studies have detected regulatory factors for Nrf2; however, further identification of Nrf2 activators for liver injury/failure and Nrf2 inhibitors for viral hepatitis, and HCC is promising for the establishment of extensive and effective approaches to improve the prognosis of liver diseases. Regarding the great potential of this transcription factor, there is an unmet need for prospective clinical trials to explore the therapeutic impact of Nrf2 regulation in patients with liver diseases.

AUTHOR CONTRIBUTIONS

DX and MX wrote the manuscript. SJ and YQ wrote some part of the manuscript and made language retouching for our manuscript. HW revised the manuscript. QX and XK designed and revised the manuscript.

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REFERENCES

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L. (1999). Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* 274, 26071–26078. doi: 10.1074/jbc.274.37.26071

Bae, S. H., Sung, S. H., Cho, E. J., Lee, S. K., Lee, H. E., Woo, H. A., et al. (2011). Concerted action of sulfiredoxin and peroxiredoxin I protects against alcohol-induced oxidative injury in mouse liver. *Hepatology (Baltimore, Md)* 53, 945–953. doi: 10.1002/hep.24104

Bataille, A. M., and Manautou, J. E. (2012). Nrf2: a potential target for new therapeutics in liver disease. *Clin. Pharmacol. Ther.* 92, 340–348. doi: 10.1038/clpt.2012.110

Bellezza, I., Giambanco, I., Minelli, A., and Donato, R. (2018). Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim. Biophys. Acta* 1865, 721–733. doi: 10.1016/j.bbamcr.2018.02.010

Bolukbas, C., Bolukbas, F. F., Horoz, M., Aslan, M., Celik, H., and Erel, O. (2005). Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection. *BMC Infect Dis.* 5:95. doi: 10.1186/1471-2334-5-95

Burdette, D., Olivarez, M., and Waris, G. (2010). Activation of transcription factor Nrf2 by hepatitis C virus induces the cell-survival pathway. *J. Gen. Virol.* 91, 681–690. doi: 10.1099/vir.0.014340-0

Cao, M., Wang, H., Guo, L., Yang, S., Liu, C., Khor, T. O., et al. (2017). Dibenzoylmethane protects against CCl4-induced acute liver injury by activating Nrf2 via JNK, AMPK, and calcium signaling. *AAPS J.* 19, 1703–1714. doi: 10.1208/s12248-017-0133-1

Carvajal-Yepes, M., Himmelsbach, K., Schaedler, S., Ploen, D., Krause, J., Ludwig, L., et al. (2011). Hepatitis C virus impairs the induction of cytoprotective Nrf2 target genes by delocalization of small Maf proteins. *J. Biol. Chem.* 286, 8941–8951. doi: 10.1074/jbc.M110.186684

Chambel, S. S., Santos-Goncalves, A., and Duarte, T. L. (2015). The dual role of Nrf2 in nonalcoholic fatty liver disease: regulation of antioxidant defenses and hepatic lipid metabolism. *Biomed. Res. Int.* 2015:597134. doi: 10.1155/2015/597134

Chen, F., Wang, H., Zhu, J., Zhao, R., Xue, P., Zhang, Q., et al. (2017). Camptothecin suppresses NRF2-ARE activity and sensitises hepatocellular carcinoma cells to anticancer drugs. *Br. J. Cancer* 117, 1495–1506. doi: 10.1038/bjc.2017.317

Chen, W. C., Wang, S. Y., Chiu, C. C., Tseng, C. K., Lin, C. K., Wang, H. C., et al. (2013). Lucidone suppresses hepatitis C virus replication by Nrf2-mediated heme oxygenase-1 induction. *Antimicrob. Agents Chemother.* 57, 1180–1191. doi: 10.1128/AAC.02053-12

Chowdhry, S., Nazmy, M. H., Meakin, P. J., Dinkova-Kostova, A. T., Walsh, S. V., Tsujita, T., et al. (2010). Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. *Free Radic. Biol. Med.* 48, 357–371. doi: 10.1016/j.freeradbiomed.2009.11.007

Dey, A., and Cederbaum, A. I. (2006). Alcohol and oxidative liver injury. *Hepatology (Baltimore, Md)* 43, S63–S74. doi: 10.1002/hep.20957

Dietrich, P., and Hellerbrand, C. (2014). Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. *Best Pract. Res. Clin. Gastroenterol.* 28, 637–653. doi: 10.1016/j.bpg.2014.07.008

Dong, J., Sulik, K. K., and Chen, S. Y. (2008). Nrf2-mediated transcriptional induction of antioxidant response in mouse embryos exposed to ethanol in vivo: implications for the prevention of fetal alcohol spectrum disorders. *Antioxid. Redox Signal.* 10, 2023–2033. doi: 10.1089/ars.2007.2019

Du, J., Zhang, M., Lu, J., Zhang, X., Xiong, Q., Xu, Y., et al. (2016). Osteocalcin improves nonalcoholic fatty liver disease in mice through activation of Nrf2 and inhibition of JNK. *Endocrine* 53, 701–709. doi: 10.1007/s12020-016-0926-5

Fan, H., Ma, X., Lin, P., Kang, Q., Zhao, Z., Wang, L., et al. (2017). Scutellarin prevents nonalcoholic fatty liver disease (NAFLD) and hyperlipidemia via PI3K/AKT-dependent activation of nuclear factor (erythroid-derived 2)-like 2 (nrf2) in Rats. *Med. Sci. Monit.* 23, 5599–5612. doi: 10.12659/MSM.907530

Fawcett, T. W., Martindale, J. L., Guyton, K. Z., Hai, T., and Holbrook, N. J. (1999). Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochem. J.* 339(Pt 1), 135–141.

Feng, X., Yu, W., Li, X., Zhou, F., Zhang, W., Shen, Q., et al. (2017). Apigenin, a modulator of PPARgamma, attenuates HFD-induced NAFLD by regulating hepatocyte lipid metabolism and oxidative stress via Nrf2 activation. *Biochem. Pharmacol.* 136, 136–149. doi: 10.1016/j.bcp.2017.04.014

Forner, A., Reig, M., and Bruix, J. (2018). Hepatocellular carcinoma. *Lancet* 391, 1301–1314. doi: 10.1016/S0140-6736(18)30010-2

Ge, M., Yao, W., Yuan, D., Zhou, S., Chen, X., Zhang, Y., et al. (2017). Brg1-mediated Nrf2/HO-1 pathway activation alleviates hepatic ischemia-reperfusion injury. *Cell Death Dis.* 8:e2841. doi: 10.1038/cddis.2017.236

Gupte, A. A., Lyon, C. J., and Hsueh, W. A. (2013). Nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), a key regulator of the antioxidant response to protect against atherosclerosis and nonalcoholic steatohepatitis. *Curr. Diabetes Rep.* 13, 362–371. doi: 10.1007/s11892-013-0372-1

Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol. Cell* 5, 897–904. doi: 10.1016/S1097-2765(00)80330-5

Harrison, S. A., Rinella, M. E., Abdelmalek, M. F., Trotter, J. F., Paredes, A. H., Arnold, H. L., et al. (2018). NGM282 for treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet* 391, 1174–1185. doi: 10.1016/S0140-6736(18)30474-4

Harvey, C. J., Thimmulappa, R. K., Singh, A., Blake, D. J., Ling, G., Wakabayashi, N., et al. (2009). Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radic. Biol. Med.* 46, 443–453. doi: 10.1016/j.freeradbiomed.2008.10.040

Hennig, P., Garstkiewicz, M., and Grossi, S. (2018). The Crosstalk between Nrf2 and Inflammasomes. *Int. J. Mol. Sci.* 19:E562. doi: 10.3390/ijms19020562

Hildt, E., Munz, B., Saher, G., Reifenberg, K., and Hofschneider, P. H. (2002). The PreS2 activator MHBs(t) of hepatitis B virus activates c-raf-1/Erk2 signaling in transgenic mice. *EMBO J.* 21, 525–535. doi: 10.1093/emboj/21.4.525

Huang, Y. J., Chen, P., Lee, C. Y., Yang, S. Y., Lin, M. T., Lee, H. S., et al. (2016). Protection against acetaminophen-induced acute liver failure by omentum adipose tissue derived stem cells through the mediation of Nrf2 and cytochrome P450 expression. *J. Biomed. Sci.* 23:5. doi: 10.1186/s12929-016-0231-x

Inami, Y., Waguri, S., Sakamoto, A., Kouno, T., Nakada, K., Hino, O., et al. (2011). Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. *J. Cell Biol.* 193, 275–284. doi: 10.1083/jcb.201102031

Ivanov, A. V., Bartosch, B., Smirnova, O. A., Isaguliants, M. G., and Kochetkov, S. N. (2013). HCV and oxidative stress in the liver. *Viruses* 5, 439–469. doi: 10.3390/v5020439

Ivanov, A. V., Smirnova, O. A., Ivanova, O. N., Masalova, O. V., Kochetkov, S. N., and Isaguliants, M. G. (2011). Hepatitis C virus proteins activate NRF2/ARE pathway by distinct ROS-dependent and independent mechanisms in HUH7 cells. *PLoS One* 6:e24957. doi: 10.1371/journal.pone.0024957

Karin, M., and Dhar, D. (2016). Liver carcinogenesis: from naughty chemicals to soothing fat and the surprising role of NRF2. *Carcinogenesis* 37, 541–546. doi: 10.1093/carcin/bgw060

Ke, B., Shen, X. D., Zhang, Y., Ji, H., Gao, F., Yue, S., et al. (2013). KEAP1-NRF2 complex in ischemia-induced hepatocellular damage of mouse liver transplants. *J. Hepatol.* 59, 1200–1207. doi: 10.1016/j.jhep.2013.07.016

Klaassen, C. D., and Reisman, S. A. (2010). Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. *Toxicol. Appl. Pharmacol.* 244, 57–65. doi: 10.1016/j.taap.2010.01.013

Kudo, M., Finn, R. S., Qin, S., Han, K. H., Ikeda, K., Piscaglia, F., et al. (2018). Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 391, 1163–1173. doi: 10.1016/S0140-6736(18)30207-1

Kudoh, K., Uchinami, H., Yoshioka, M., Seki, E., and Yamamoto, Y. (2014). Nrf2 activation protects the liver from ischemia/reperfusion injury in mice. *Ann. Surg.* 260, 118–127. doi: 10.1097/SLA.0000000000000287

Lee, D. H., Han, D. H., Nam, K. T., Park, J. S., Kim, S. H., Lee, M., et al. (2016). Ezetimibe, an NPC1L1 inhibitor, is a potent Nrf2 activator that protects mice from diet-induced nonalcoholic steatohepatitis. *Free Radic. Biol. Med.* 99, 520–532. doi: 10.1016/j.freeradbiomed.2016.09.009

Li, J., Sapper, T. N., Mah, E., Rudraiah, S., Schill, K. E., Chitchumroonchokchai, C., et al. (2016). Green tea extract provides extensive Nrf2-independent protection against lipid accumulation and NFkappaB pro- inflammatory responses during nonalcoholic steatohepatitis in mice fed a high-fat diet. *Mol. Nutr. Food Res.* 60, 858–870. doi: 10.1002/mnfr.201500814

Liu, B., Fang, M., He, Z., Cui, D., Jia, S., Lin, X., et al. (2015). Hepatitis B virus stimulates G6PD expression through HBx-mediated Nrf2 activation. *Cell Death Dis.* 6, e1980. doi: 10.1038/cddis.2015.322

Liu, M., Reddy, N. M., Higbee, E. M., Potteti, H. R., Noel, S., Racusen, L., et al. (2014). The Nrf2 triterpenoid activator, CDDO-imidazolide, protects kidneys from ischemia-reperfusion injury in mice. *Kid. Int.* 85, 134–141. doi: 10.1038/ki.2013.357

Liu, X., Wang, T., Liu, X., Cai, L., Qi, J., Zhang, P., et al. (2016). Biochanin A protects lipopolysaccharide/D-galactosamine-induced acute liver injury in mice by activating the Nrf2 pathway and inhibiting NLRP3 inflammasome activation. *Int. Immunopharmacol.* 38, 324–331. doi: 10.1016/j.intimp.2016.06.009

Lu, C., Xu, W., Zhang, F., Shao, J., and Zheng, S. (2016). Nrf2 knockdown disrupts the protective effect of curcumin on alcohol-induced hepatocyte necroptosis. *Mol. Pharm.* 13, 4043–4053. doi: 10.1021/acs.molpharmaceut.6b00562

Lu, S. C. (2013). Glutathione synthesis. *Biochim. Biophys. Acta* 2013, 3143–3153. doi: 10.1016/j.bbagen.2012.09.008

Mayo, M. J., Wigg, A. J., Leggett, B. A., Arnold, H., Thompson, A. J., Weltman, M., et al. (2018). NGM282 for treatment of patients with primary biliary cholangitis: a multicenter, randomized, double-blind, placebo-controlled trial. *Hepatol. Commun.* 2, 1037–1050. doi: 10.1002/hep4.1209

Murakami, Y., Sugiyama, K., Ebinuma, H., Nakamoto, N., Ojiro, K., Chu, P. S., et al. (2018). Dual effects of the Nrf2 inhibitor for inhibition of hepatitis C virus and hepatic cancer cells. *BMC Cancer* 18:680. doi: 10.1186/s12885-018-4588-y

Ni, Y. H., Huo, L. J., and Li, T. T. (2017). Antioxidant axis Nrf2-keap1-ARE in inhibition of alcoholic liver fibrosis by IL-22. *World J. Gastroenterol.* 23, 2002–2011. doi: 10.3748/wjg.v23.i11.2002

Pan, C. W., Pan, Z. Z., Hu, J. J., Chen, W. L., Zhou, G. Y., Lin, W., et al. (2016). Mangiferin alleviates lipopolysaccharide and D-galactosamine-induced acute liver injury by activating the Nrf2 pathway and inhibiting NLRP3 inflammasome activation. *Eur. J. Pharmacol.* 770, 85–91. doi: 10.1016/j.ejphar.2015.12.006

Pan, C. W., Yang, S. X., Pan, Z. Z., Zheng, B., Wang, J. Z., Lu, G. R., et al. (2017). Andrographolide ameliorates d-galactosamine/lipopolysaccharide-induced acute liver injury by activating Nrf2 signaling pathway. *Oncotarget* 8, 41202–41210. doi: 10.18632/oncotarget.17149

Peng, X., Dai, C., Liu, Q., Li, J., and Qiu, J. (2018). Curcumin attenuates on carbon tetrachloride-induced acute liver injury in mice via modulation of the Nrf2/HO-1 and TGF-beta1/Smad3 pathway. *Molecules (Basel, Switzerland)* 23:E215. doi: 10.3390/molecules23010215

Petrelli, A., Perra, A., Cora, D., Sulas, P., Menegon, S., Manca, C., et al. (2014). MicroRNA/gene profiling unveils early molecular changes and nuclear factor erythroid related factor 2 (NRF2) activation in a rat model recapitulating human hepatocellular carcinoma (HCC). *Hepatology (Baltimore, Md)* 59, 228–241. doi: 10.1002/hep.26616

Pitot, H. C. (2007). Adventures in hepatocarcinogenesis. *Annu. Rev. Pathol.* 2, 1–29. doi: 10.1146/annurev.pathol.2.010506.092027

Ramadori, P., Drescher, H., Erschfeld, S., Fragoulis, A., Kensler, T. W., Wruck, C. J., et al. (2017). Genetic Nrf2 overactivation inhibits the deleterious effects induced by hepatocyte-specific c-met deletion during the progression of NASH. *Oxid. Med. Cell. Longev.* 2017:3420286. doi: 10.1155/2017/3420286

Rao, J., Qian, X., Li, G., Pan, X., Zhang, C., Zhang, F., et al. (2015). ATF3-mediated NRF2/HO-1 signaling regulates TLR4 innate immune responses in mouse liver ischemia/reperfusion injury. *Am. J. Transplant* 15, 76–87. doi: 10.1111/ajt.12954

Reddy, N. M., Suryanaraya, V., Yates, M. S., Kleeberger, S. R., Hassoun, P. M., Yamamoto, M., et al. (2009). The triterpenoid CDDO-imidazolide confers potent protection against hyperoxic acute lung injury in mice. *Am. J. Respir. Crit. Care Med.* 180, 867–874. doi: 10.1164/rccm.200905-0670OC

Rejitha, S., Prathibha, P., and Indira, M. (2015). Nrf2-mediated antioxidant response by ethanolic extract of Sida cordifolia provides protection against alcohol-induced oxidative stress in liver by upregulation of glutathione metabolism. *Redox Rep.* 20, 75–80. doi: 10.1179/1351000214Y.0000000108

Rojo de la Vega, M., Chapman, E., and Zhang, D. D. (2018). NRF2 and the hallmarks of cancer. *Cancer Cell* 34, 21–43. doi: 10.1016/j.ccell.2018.03.022

Saito, T., Ichimura, Y., Taguchi, K., Suzuki, T., Mizushima, T., Takagi, K., et al. (2016). p62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming. *Nat Commun.* 7:12030. doi: 10.1038/ncomms12030

Satapathy, S. K., and Sanyal, A. J. (2015). Epidemiology and natural history of nonalcoholic fatty liver disease. *Semin. Liver Dis.* 35, 221–235. doi: 10.1055/s-0035-1562943

Schaedler, S., Krause, J., Himmelsbach, K., Carvajal-Yepes, M., Lieder, F., Klingel, K., et al. (2010). Hepatitis B virus induces expression of antioxidant response element-regulated genes by activation of Nrf2. *J. Biol. Chem.* 285, 41074–41086. doi: 10.1074/jbc.M110.145862

Shen, F., Wang, Z., Liu, W., and Liang, Y. (2018a). Ethyl pyruvate can alleviate alcoholic liver disease through inhibiting Nrf2 signaling pathway. *Exp. Ther. Med.* 15, 4223–4228. doi: 10.3892/etm.2018.5925

Shen, Z., Wang, Y., Su, Z., Kou, R., Xie, K., and Song, F. (2018b). Activation of p62-keap1-Nrf2 antioxidant pathway in the early stage of acetaminophen-induced acute liver injury in mice. *Chem. Biol. Interact.* 282, 22–28. doi: 10.1016/j.cbi.2018.01.008

Shepard, B. D., Tuma, D. J., and Tuma, P. L. (2010). Chronic ethanol consumption induces global hepatic protein hyperacetylation. *Alcohol. Clin. Exp. Res.* 34, 280–291. doi: 10.1111/j.1530-0277.2009.01091.x

Shi, L., Chen, Z. G., Wu, L. L., Zheng, J. J., Yang, J. R., Chen, X. F., et al. (2014). miR-340 reverses cisplatin resistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway. *Asian Pac J. Cancer Prev.* 15, 10439–10444. doi: 10.7314/APJCP.2014.15.23.10439

Song, X., Yin, S., Huo, Y., Liang, M., Fan, L., Ye, M., et al. (2015). Glycycoumarin ameliorates alcohol-induced hepatotoxicity via activation of Nrf2 and autophagy. *Free Radic. Biol. Med.* 89, 135–146. doi: 10.1016/j.freeradbiomed.2015.07.006

Sugiyama, K., Ebinuma, H., Nakamoto, N., Sakasegawa, N., Murakami, Y., Chu, P. S., et al. (2014). Prominent steatosis with hypermetabolism of the cell line permissive for years of infection with hepatitis C virus. *PLoS One* 9:e94460. doi: 10.1371/journal.pone.0094460

Sussan, T. E., Rangasamy, T., Blake, D. J., Malhotra, D., El-Haddad, H., Bedja, D., et al. (2009). Targeting Nrf2 with the triterpenoid CDDO-imidazolide attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice. *Proc. Natl. Acad. Sci. U.S.A.* 106, 250–255. doi: 10.1073/pnas.0804333106

Tang, W., Jiang, Y. F., Ponnusamy, M., and Diallo, M. (2014). Role of Nrf2 in chronic liver disease. *World J. Gastroenterol.* 20, 13079–13087. doi: 10.3748/wjg.v20.i36.13079

Tarantino, G., and Finelli, C. (2013). Pathogenesis of hepatic steatosis: the link between hypercortisolism and non-alcoholic fatty liver disease. *World J. Gastroenterol.* 19, 6735–6743. doi: 10.3748/wjg.v19.i40.6735

Teng, Y., Zhao, H., Gao, L., Zhang, W., Shull, A. Y., and Shay, C. (2017). FGF19 protects hepatocellular carcinoma cells against endoplasmic reticulum stress via activation of FGFR4-GSK3beta-Nrf2 signaling. *Cancer Res.* 77, 6215–6225. doi: 10.1158/0008-5472.CAN-17-2039

Thimmulappa, R. K., Fuchs, R. J., Malhotra, D., Scollick, C., Traore, K., Bream, J. H., et al. (2007). Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. *Antioxid. Redox Signal.* 9, 1963–1970. doi: 10.1089/ars.2007.1745

Tian, B., Lu, Z. N., and Guo, X. L. (2018). Regulation and role of nuclear factor-E2-related factor 2 (Nrf2) in multidrug resistance of hepatocellular carcinoma. *Chem. Biol. Interact.* 280, 70–76. doi: 10.1016/j.cbi.2017.12.014

Tian, Y., Li, Z., Shen, B., Zhang, Q., and Feng, H. (2017). Protective effects of morin on lipopolysaccharide/d-galactosamine-induced acute liver injury by inhibiting TLR4/NF-kappaB and activating Nrf2/HO-1 signaling pathways. *Int. Immunopharmacol.* 45, 148–155. doi: 10.1016/j.intimp.2017.02.010

Tseng, C. K., Hsu, S. P., Lin, C. K., Wu, Y. H., Lee, J. C., and Young, K. C. (2017). Celastrol inhibits hepatitis C virus replication by upregulating heme oxygenase-1 via the JNK MAPK/Nrf2 pathway in human hepatoma cells. *Antiv. Res.* 146, 191–200. doi: 10.1016/j.antiviral.2017.09.010

Vomund, S., Schafer, A., Parnham, M. J., Brüne, B., and von Knethen, A. (2017). Nrf2, the master regulator of anti-oxidative responses. *Int. J. Mol. Sci.* 18:E2772. doi: 10.3390/ijms18122772

Wang, C., Cui, Y., Li, C., Zhang, Y., Xu, S., Li, X., et al. (2013). Nrf2 deletion causes "benign" simple steatosis to develop into nonalcoholic steatohepatitis in mice fed a high-fat diet. *Lipids Health Dis.* 12:165. doi: 10.1186/1476-511X-12-165

Wang, W., Wu, L., Li, Q., Zhang, Z., Xu, L., Lin, C., et al. (2018). Madecassoside prevents acute liver failure in LPS/D-GalN-induced mice by inhibiting p38/NF-kappaB and activating Nrf2/HO-1 signaling. *Biomed. Pharmacother.* 103, 1137–1145. doi: 10.1016/j.biopha.2018.04.162

Wang, Y., Kou, Y., Wang, X., Cederbaum, A., and Wang, R. (2014a). Multifactorial comparative proteomic study of cytochrome P450 2E1 function in chronic alcohol administration. *PLoS One* 9:e92504. doi: 10.1371/journal.pone.0092504

Wang, Z., Dou, X., Li, S., Zhang, X., Sun, X., Zhou, Z., et al. (2014b). Nuclear factor (erythroid-derived 2)-like 2 activation-induced hepatic very-low-density lipoprotein receptor overexpression in response to oxidative stress contributes to alcoholic liver disease in mice. *Hepatology (Baltimore, Md)* 59, 1381–1392. doi: 10.1002/hep.26912

Wu, K. C., Liu, J. J., and Klaassen, C. D. (2012). Nrf2 activation prevents cadmium-induced acute liver injury. *Toxicol. Appl. Pharmacol.* 263, 14–20. doi: 10.1016/j.taap.2012.05.017

Xie, Y. L., Chu, J. G., Jian, X. M., Dong, J. Z., Wang, L. P., Li, G. X., et al. (2017). Curcumin attenuates lipopolysaccharide/d-galactosamine-induced acute liver injury by activating Nrf2 nuclear translocation and inhibiting NF-kB activation. *Biomed. Pharmacother.* 91, 70–77. doi: 10.1016/j.biopha.2017.04.070

Xu, D., Chen, L., Chen, X., Wen, Y., Yu, C., Yao, J., et al. (2017). The triterpenoid CDDO-imidazolide ameliorates mouse liver ischemia-reperfusion injury through activating the Nrf2/HO-1 pathway enhanced autophagy. *Cell Death Dis.* 8:e2983. doi: 10.1038/cddis.2017.386

Yu, J. I., Choi, C., Shin, S. W., Son, A., Lee, G. H., Kim, S. Y., et al. (2017). Valproic acid sensitizes hepatocellular carcinoma cells to proton therapy by suppressing NRF2 activation. *Sci. Rep.* 7:14986. doi: 10.1038/s41598-017-15165-3

Zhang, F., Wang, S., Zhang, M., Weng, Z., Li, P., Gan, Y., et al. (2012). Pharmacological induction of heme oxygenase-1 by a triterpenoid protects neurons against ischemic injury. *Stroke* 43, 1390–1397. doi: 10.1161/STROKEAHA.111.647420

Zhang, M., Zhang, C., Zhang, L., Yang, Q., Zhou, S., Wen, Q., et al. (2015). Nrf2 is a potential prognostic marker and promotes proliferation and invasion in human hepatocellular carcinoma. *BMC Cancer* 15:531. doi: 10.1186/s12885-015-1541-1

Zhang, X., Ji, R., Sun, H., Peng, J., Ma, X., Wang, C., et al. (2018). Scutellarin ameliorates nonalcoholic fatty liver disease through the PPARgamma/PGC-1alpha-Nrf2 pathway. *Free Radic. Res.* 52, 198–211. doi: 10.1080/10715762.2017.1422602

Zhang, Y., Qiao, R., He, D., Zhao, Z., Yang, S., Zou, H., et al. (2016). Indazolo[3,2-b]quinazolinones attack hepatocellular carcinoma Hep3B cells by inducing mitochondrial-dependent apoptosis and inhibition of Nrf2/ARE signaling pathway. *Curr. Mol. Med.* 16, 820–828. doi: 10.2174/1566524016666161128114444

Zhou, R., Lin, J., and Wu, D. (2014). Sulforaphane induces Nrf2 and protects against CYP2E1-dependent binge alcohol-induced liver steatosis. *Biochim. Biophys. Acta* 1840, 209–218. doi: 10.1016/j.bbagen.2013.09.018

Zhou, S., Ye, W., Zhang, Y., Yu, D., Shao, Q., Liang, J., et al. (2016). miR-144 reverses chemoresistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway. *Am. J. Transl. Res.* 8, 2992–3002.

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REVIEW
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Contribution of Nrf2 Modulation to the Mechanism of Action of Analgesic and Anti-inflammatory Drugs in Pre-clinical and Clinical Stages

Larissa Staurengo-Ferrari[1], Stephanie Badaro-Garcia[1], Miriam S. N. Hohmann[1], Marília F. Manchope[1], Tiago H. Zaninelli[1], Rubia Casagrande[2] and Waldiceu A. Verri Jr.[1]†*

[1] Departamento de Patologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Brazil, [2] Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, Brazil

Despite the progress that has occurred in recent years in the development of therapies to treat painful and inflammatory diseases, there is still a need for effective and potent analgesics and anti-inflammatory drugs. It has long been known that several types of antioxidants also possess analgesic and anti-inflammatory properties, indicating a strong relationship between inflammation and oxidative stress. Understanding the underlying mechanisms of action of anti-inflammatory and analgesic drugs, as well as essential targets in disease physiopathology, is essential to the development of novel therapeutic strategies. The Nuclear factor-2 erythroid related factor-2 (Nrf2) is a transcription factor that regulates cellular redox status through endogenous antioxidant systems with simultaneous anti-inflammatory activity. This review summarizes the molecular mechanisms and pharmacological actions screened that link analgesic, anti-inflammatory, natural products, and other therapies to Nrf2 as a regulatory system based on emerging evidences from experimental disease models and new clinical trial data.

Keywords: Nrf2, Keap1, pain, inflammation, oxidative stress, analgesic, anti-inflammatory, antioxidant

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***Correspondence:**
Waldiceu A. Verri Jr.
waldiceujr@yahoo.com.br; waverri@uel.br
orcid.org/0000-0003-2756-9283

†Present address:
Waldiceu A. Verri Jr., Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Brazil

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INTRODUCTION

A cross-talk between varied reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a common feature of inflammatory and painful diseases. These oxidants can be generated by enzymes abundant in immune and non-immune cells (Mittal et al., 2014) as part of protective actions against infections and environmental threats, including microbial or any noxious insults (Janssen-Heininger et al., 2008). The agonist binding to cellular receptors dictates the actions of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and nitric oxide synthases (NOS) to produce superoxide anion and nitric oxide, respectively. Besides the oxidative burst, ROS are also produced in mitochondrial compartments as a result of respiratory chain activity where oxygen consumption is high (Paiva and Bozza, 2014).

Compelling evidence also indicates that oxidative stress is not only related to tissue damage, but also cellular signaling pathways that tightly control cell division, migration and mediator

production that ultimately regulate diverse cellular functions (Finkel, 2003; Janssen-Heininger et al., 2008; Pinho-Ribeiro et al., 2016b). Indeed, ROS/RNS sustain their own production and induce the release of cytokines, adhesion molecules, lipid mediators, inflammasome assembly and cyclooxygenase (COX)-2 expression by mechanisms involving the nuclear factor kappa B (NF-κB) activation (Verri et al., 2012; Wardyn et al., 2015; Hennig et al., 2018). NF-κB also induces mitochondrial activity and NADPH oxidase expression in the context of inflammation (Manea et al., 2007; Mauro et al., 2011; Wardyn et al., 2015). Activation of the nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is central to the disruption of this circle. In fact, *in vivo* studies have shown that Nrf2 signaling has an essential role in limiting neuropathies (Arruri et al., 2017), arthritis (Ferrandiz et al., 2018), colitis (Xu et al., 2018), pneumonia (Athale et al., 2012), pulmonary fibrosis (Yan et al., 2017), skin diseases (Schafer and Werner, 2015), liver (Bae et al., 2013), and kidney damage (Shen et al., 2017), as well as affecting tumor development (Sporn and Liby, 2012). Importantly, the structural features and signaling of Nrf2 protein assign its activity to maintain cellular redox homeostasis (Hayes and Dinkova-Kostova, 2014).

It is well established that Nrf2 activity is controlled, in part, by the cytosolic protein Kelch-like ECH-associated protein 1 (Keap1), as portrayed in **Figure 1**. Under homeostatic conditions, Nrf2 levels and its activation are controlled essentially by Keap1. Two Keap1 molecules maintain Nrf2 attached to its DLG and ETH motifs, which favors CUL3-mediated ubiquitination of Nrf2 and subsequent proteasome degradation (Itoh et al., 1999). A small proportion of Nrf2 escapes the inhibitory complex and accumulates in the nucleus to mediate basal antioxidant responsive element (ARE)-dependent gene expression and maintains cellular homeostasis (Kansanen et al., 2013). Conversely, upon oxidative stress or in the presence of electrophilic or activating compounds, the modification of key Keap1 cysteine residues promotes the dissociation of the inhibitory complex and nuclear translocation of Nrf2. In the nucleus, Nrf2 forms a heterodimer with its partner sMAF (v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog) and binds to ARE, driving the expression of an array of Nrf2-target genes, for example NAD(P)H quinone-oxidoreductase 1 (NQO1), heme-oxygenase 1(HO-1), glutamate-cysteine ligase (GCL), glutathione S-transferases (GSTs), catalase (CAT), superoxide dismutase (SOD) and thioredoxin UDP-glucuronosyltransferase (Nguyen et al., 2009; Ruiz et al., 2013; Hayes and Dinkova-Kostova, 2014). This signaling is defined as the canonical mechanism of Nrf2 pathway (Silva-Islas and Maldonado, 2018). Importantly, this pathway can be modulated by protein kinases involved in signal transduction in the cytosol, such as protein kinase C (PKC), phosphoinositide 3-kinase (PI_3K) and mitogen-activated protein kinase (MAPK) ERK1/2 (Bloom and Jaiswal, 2003; Nguyen et al., 2003; Manandhar et al., 2007).

Alternatively, the interaction of Keap1-Nrf2 can be disrupted by non-canonical mechanisms (**Figure 1**). These independent mechanisms involve the disruption of Keap1/Nrf2 interaction by competitive binding of disrupter proteins p62, p53-induced p21 (Toledano, 2009; Best and Sutherland, 2018); DPP3 (Hast et al., 2013), WTX (Karapetian et al., 2005), Prothymosin α (Karapetian et al., 2005), PALB2 (Ma et al., 2012), or BRCA1 (Gorrini et al., 2013) to Keap1 (Lau et al., 2010). Of note, p62 is particularly interesting, since it is the most studied non-canonical pathway of Nrf2 activation. The deficiency in autophagy upregulates p62, that binds to Keap1, thereby inhibiting the Keap1-Cul3-E3 ubiquitin ligase complex and stabilizing Nrf2 (Lau et al., 2010). In addition, PI3K/Akt signaling pathway can activate serine threonine kinase glycogen synthase kinase 3-beta (GSK-3β), which phosphorylates Nrf2 and, in turn, results in the induction of downstream HO-1, glutathione peroxidase, GST A1, NQO-1 and GCL expression (Salazar et al., 2006; Best and Sutherland, 2018). These recent discoveries on the mechanisms of Nrf2 regulation illustrate possible therapeutic strategies to modulate its activity.

Of particular interest is the notion that pharmacological activation of Nrf2 interferes in inflammation (Hayes and Dinkova-Kostova, 2014). It is also important to mention that Nrf2 is expressed in many tissues, mainly those exposed to the environment or associated with detoxification (Itoh et al., 2010). In other words, it is likely that Nrf2 can be exploited as a target in distinct organs. Thus, in this review we will discuss the involvement of Nrf2 in the mechanisms of action of classic analgesic and anti-inflammatory drugs, as well as natural products and other molecules that modulate Nrf2, in the context of experimental models (summarized in **Table 1**) and human diseases (summarized in **Table 2**).

MODULATION OF NRF2 BY ANALGESIC DRUGS AND SYNERGIC EFFECTS

Despite their deleterious side effects, opioids are one of the most effective analgesic drugs employed in the clinic (Knezevic et al., 2017). Opioids produce their pain-relieving actions by interacting with μ, δ or κ opioid receptors (Bovill, 1997). Growing evidence has shown that Nrf2-activators can synergize with opioids to achieve better analgesic effects. Sulforaphane (SFN), for instance, is an activator of Nrf2 transcription factor that enhances the antiallodynic and antihyperalgesic effects produced by morphine. This effect was attributed to local increase in the expression of μ-opioid receptors, as observed in animals with peripheral inflammation. Notwithstanding, SFN enhances the production of HO-1 and NQO1 in the spinal cord and paw tissue, suggesting that the antiallodynic and antihyperalgesic effects of SFN and morphine are produced by a Nrf2 antioxidant-mediated mechanism (Redondo et al., 2017). Fentanyl, another opioid drug, when combined with butorphanol, activates Nrf2-ARE signaling via kappa-opioid receptor. The activation of this pathway increased the expression of downstream genes NQO1 and HO-1 and prevented oxidative stress in myocardial ischemia-reperfusion (I/R) injury model (Zhang et al., 2016). Corroborating these findings, the induction of Nrf2 can also enhance the antinociceptive effects of delta-opioid receptors in diabetic neuropathy associated to type 2 diabetes (McDonnell et al., 2017). Collectively, these data suggest that the use of

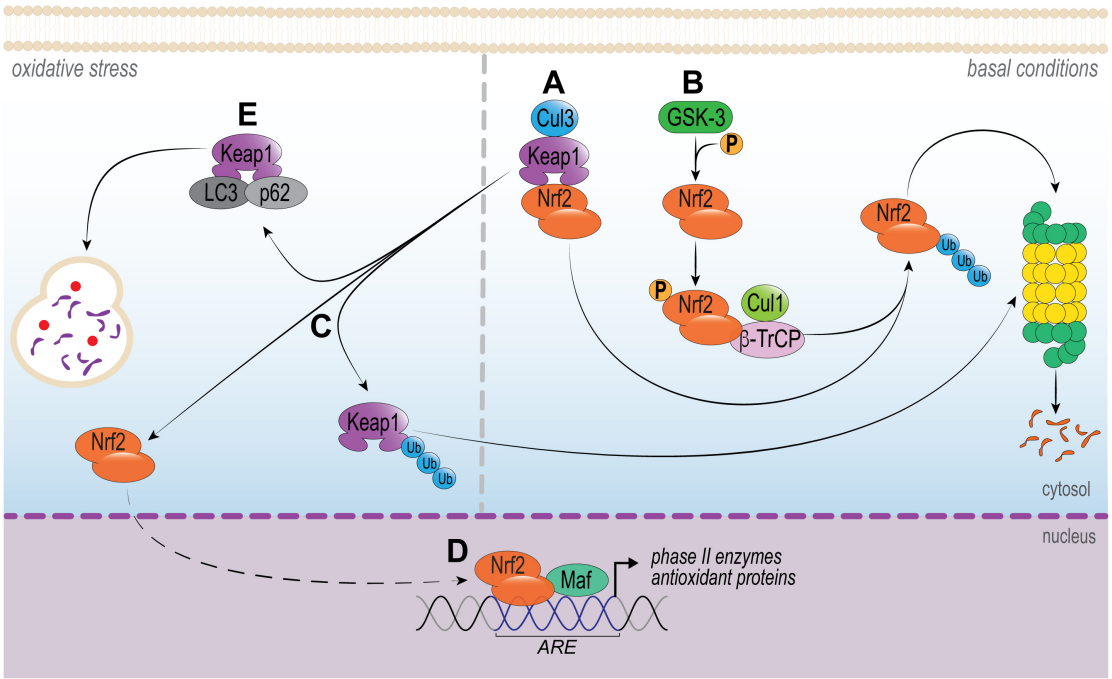

FIGURE 1 | Intracellular signaling pathways that regulate Nrf2. In basal conditions, **(A)** Nrf2 is sequestered in the cytosol by Keap1 by two motifs (ETGE and DLG), which are essential to recruit Nrf2. Keap1 works as a dimeric redox sensitive substrate adaptor for cullin-based E3 ubiquitin ligase, which inhibits the transcriptional activity of Nrf2 via ubiquitination and proteasomal degradation. This signaling is known as canonical pathway. **(B)** Alternatively, Nrf2 is also regulated by a non-canonical pathway. The phosphorylation of Nrf2 by GSK-3 facilitates its recognition by β-TrCP, leading to Cul1-mediated ubiquitination, followed by Nrf2 proteasome degradation. Under oxidative stress or pathological conditions, **(C,D)** Keap1-CUL3 ubiquitin E3 ligase activity decreases and Nrf2 dissociates from Keap1. Nrf2 translocates to the nucleus and heterodimerizes with small musculoaponeurotic fibrosarcoma (Maf) protein and binds to DNA and other transcription partners to setting up a nuclear complex with the ubiquitin-conjugating enzyme UbcM2. These nuclear complexes formed with Nrf2 induce the expression of the ARE-gene battery, such as: NQO1, HMOX1, GCL, GSTs, CAT, SOD, and thioredoxin UDP-glucuronosyltransferase. **(E)** The multifunctional protein p62 and LC3 acts by sequestration of Keap1, which culminates in its autophagic degradation. As a consequence, Nrf2 can translocate to the nucleus and activate ARE.

Nrf2-activators may be beneficial, since lower doses of opioid could be used, thus likely reducing their side effects.

Cannabinoids are molecules that were originally isolated from *Cannabis sativa* and later discovered to be endogenously produced. Cannabinoids represent an alternative pain relief therapy group of drugs with varied origins (Elikkottil et al., 2009). Some molecular effects of cannabinoids depend on Nrf2. One postulated mechanism of action is that endogenous cannabinoids can enhance SOD synthesis and decrease ROS production via Nrf2. These effects were implicated in the neuroprotective effect of cannabinoids in a model of Parkinson's disease, a progressive nervous system disorder with inflammatory features that affects movement (More and Choi, 2015). Anandamide, an endogenous cannabinoid, leads to Nrf2 activation and downstream HO-1 mRNA expression in MCF-7 and MDA-MB-231 breast cancer cell lines (Li H. et al., 2013). Cannabidiol, a natural cannabinoid constituent isolated from cannabis, controls LPS-induced oxidative stress and inflammation in BV2 cells by inducing Nrf2 and ATF4 transcription factors in a mechanism involving Nrf2-Hmox1 and the Nrf2/ATF4 pathways (Juknat et al., 2013).

Desipramine, a tricyclic antidepressant that displays pain-relief and anti-inflammatory effects, is commonly used in low doses to control neuropathic pain (Roumestan et al., 2007; Hearn et al., 2014). It has been demonstrated that desipramine elevates HO-1 expression through ERK and JNK pathways, leading to Nrf2 activation in Mes23.5 dopaminergic neurons. Desipramine-induced high HO-1 expression also protects dopaminergic neurons from cell death, supporting the notion that this drug could be a promising therapeutic approach to treat neurodegenerative disease (Lin et al., 2012).

In summary, the Keap1/Nrf2/HO-1 axis contributes to the analgesic effects of cannabinoids, anticonvulsant, antidepressant, and enhances the analgesic effects of opioids.

MODULATION OF NRF2 BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Non-steroidal anti-inflammatory drugs (NSAIDs) are a broad class of anti-inflammatory drugs commonly prescribed for pain and inflammation (Cashman, 1996). NSAIDs relieve pain by blocking COX subtypes 1 and 2 enzymes, which results in the inhibition of prostanoid production (Simmons et al., 2004) via peripheral and central actions (Cashman, 1996). Depending on the NSAID, its mechanism of action may also include the

TABLE 1 | Summary of evidence on analgesic and anti-inflammatory drugs that modulate Nrf2.

Classification	Compound	Disease or experimental model	Dose/Concentration	Outcome	Reference
Opioid analgesics	Morphine	CFA-induced inflammatory pain	*In vivo:* 50 μg/30 μL, i.pl.	Synergy with the induction of Nrf2 to achieve better analgesic effect	Redondo et al., 2017
	Fentanyl	Myocardial I/R injury	*In vivo:* 50 μg/kg, i.v.	Synergy with butorphanol to activate Nrf2-ARE pathway to reduce oxidative stress	Zhang et al., 2016
	[d-Pen(2),d-Pen(5)]-Enkephalin	db/db mice	*In vivo:* 0.15 mg/kg	Synergy with SFN and induction of Nrf2 activation to enhance antinociceptive effect	McDonnell et al., 2017
	SNC-80	db/db mice	*In vivo:*0.5 mg/kg	Synergy with SFN and induction of Nrf2 activation to enhance antinociceptive effect	McDonnell et al., 2017
Non-opioids analgesics	Anandamide	Breast cancer cells	*In vitro:* 2.5 μM	Activation of Nrf2-ARE pathway to induce HO-1 transcription in breast cancer cells	Li H. et al., 2013
	Cannabidiol	LPS-activated BV2 cells	*In vitro:* 10 mM	Activation of Nrf2-Hmox1 and the Nrf2/ATF4 pathways to control LPS-induced activation of microglial cells	Juknat et al., 2013
	Desipramine	Mes23.5 dopaminergic neurons	*In vitro:* 20 μM	Protection of neuronal cell death through Nrf2 activation	Juknat et al., 2013
NSAIDs	Aspirin	Human melanocytes	*In vitro:* 10–90 μM	Protection of human melanocytes against H_2O_2-induced oxidative stress via Nrf2 activation	Jian et al., 2016
		Spinal cord contusion model in Sprague-Dawley rats	*In vivo:* 20 mg/kg, i.p.	Suppression of neuronal apoptosis and reduction of inflammation through Nrf2/HO-1 signaling pathway	Wei et al., 2017
	Celecoxibe	Human Umbilical Vein Endothelial Cells	*In vitro:* 1–10 μM	Vascular protection via AMPK-CREB-Nrf2 signaling	Al-Rashed et al., 2018
	Diclofenac	Mosquito fish	*In vivo:* exposure of 1.572×10^{-3} μmol – 1.572 μmol	Activation of Nrf2 as a protective mechanism due to large absorption and accumulation of diclofenac	Bao et al., 2017
	Indomethacin	ARPE-19 cells	*In vitro:* 50–250 μM	Inhibition of macrophage infiltration and reduced VEGF levels due to Nrf2 activation	Yoshinaga et al., 2011
	Bromfenac	ARPE-19 cells	*In vitro:* 5–160 μM	Inhibition of macrophage infiltration and reduced VEGF levels due to Nrf2 activation	Yoshinaga et al., 2011
SAIDs	Dexamethasone	Zebrafish larvae	*In vivo:* exposure of 50 nM-50 pM	Nrf2-mediated oxidative stress response	Chen et al., 2017
		Lymphoblastoid cells	*In vitro:*100 nM	Increase of GSH and NADPH levels as well as improved the antioxidant capacity in a Nrf2-dependent manner	Biagiotti et al., 2016
		Human bronchial epithelial cells	*In vitro:* 10^{-6} M	Nrf2/AOX1 pathway enhances airway epithelial barrier integrity	Shintani et al., 2015
	Prednisolone	Zebrafish larvae	*In vivo:* exposure of 50 nM-50 pM	Nrf2-mediated oxidative stress response	Chen et al., 2017
	Triamcinolone	Zebrafish larvae	*In vivo:* exposure of 50 nM-50 pM	Nrf2-mediated oxidative stress response	Chen et al., 2017
	Clobetasol propionate	NSCLC cell lines	*In vivo:* 0.5–1 mg/kg *In vitro:* 10–100 nm	Tumor growth suppression due to high Nrf2 activity	Choi et al., 2017
	Budesonide	Cigarette smoke-, LPS-induced pulmonary	*In vivo:* 1 or 3 mg/kg	Glucocorticoid sensitivity during inflammatory response is dependent on Nrf2-HDAC2 axis	Adenuga et al., 2010
Natural products	Hesperidin-methyl-chalcone	MSU-induced gout arthritis	*In vivo:* 30 mg/kg, p.o.	Inhibition of experimental gout arthritis by decreasing NF-κB activation and inducing Nrf2/HO-1 pathway.	Ruiz-Miyazawa et al., 2018a
	Naringenin	Superoxide anion-induced inflammatory pain	*In vivo:* 50 mg/kg, p.o.	Activation of Nrf2/HO-1 pathway to promote antinociceptive effect	Manchope et al., 2016
		Titanium dioxide (TiO2)-induced chronic arthritis	*In vivo:* 50 mg/kg, p.o.	Activation of Nrf2/HO-1 pathway to promote antinociceptive effect	Manchope et al., 2018
		D-galactose-induced mice brain aging	*In vivo:* 50 mg/kg, p.o.	Nrf2 activation through PI_3K/Akt pathway	Zhang et al., 2017

(Continued)

TABLE 1 | Continued

Classification	Compound	Disease or experimental model	Dose/Concentration	Outcome	Reference
	Quercetin	Titanium dioxide (TiO2)-induced chronic arthritis	*In vivo:* 30 mg/kg, i.p.	Inhibition of inflammation in (TiO2)-induced chronic arthritis by decreasing NF-κB activation and inducing Nrf2/HO-1 pathway.	Borghi et al., 2017
		Human normal liver L-02 cells	*In vitro:* 50 μM	Prevention of hepatotoxicity via interacting with Keap1 and blocking the binding of Keap1 with Nrf2	Ji et al., 2015
	Curcumin	Superoxide anion-induced pain-like behavior	*In vivo:* 10 mg/kg, s.c.	Activation of Nrf2/HO-1 pathway to promote antinociceptive effect	Fattori et al., 2015
		Deprivation/ reoxygenation model	*In vivo:* 300 mg/kg, i.p. *In vitro:*5 μM	Protects neurons against ischemic injury through Akt/Nrf2 pathway.	Wu et al., 2013
	Caffeic acid	Acetaminophen-induced liver injury	*In vitro:* human normal liver L-02 cells and HepG2 cells	Protection of APAP-induced hepatotoxicity by inhibiting the binding of Keap1 to Nrf2, and leading to increased expression of HO-1 and NQO1.	Pang et al., 2016
	Vanillic acid	β-amyloid-induced oxidative stress in mice; HT22 cells	*In vivo:* 30mg/kg, i.p. *In vitro:* 100 μM	Neuroprotective effect of against Aβ1-42-induced neurotoxicity through Nrf2 and HO-1 induction	Amin et al., 2017
	Kaurenoic acid	Acute lung injury	*In vivo:* 3 mg/kg, i.t.	Suppression of neutrophilic lung inflammation via Nrf2 activation	Kim et al., 2016
	Glycyrrhizin	Lipopolysaccharide-activated RAW 264.7 cells	*In vivo:* 200 mg/kg, i.p *In vitro:* 2 mM	Reduction of HMGB1 release by induction of p38MAPK/Nrf2/HO-1 signals	Kim et al., 2015
	Rosmarinic acid	Acute liver damage	*In vivo:* 50 mg/kg, p.o.	Hepatoprotective activity due enhanced Nrf2 and HO-1 expression	Domitrovic et al., 2013
		β-amyloid-induced oxidative stress	*In vitro:*1–10 μM	GSK-3β inactivation via the Akt contributing to Fyn dephosphorylation, leading accumulation of Nrf2 in the nucleus	Rong et al., 2018
	Carnosic acid	Ischemia/reperfusion model	*In vivo:* 1 mg/kg, i.p. *In vitro:* 10 mmol/L	Neurons protection from oxidative stress and excitotoxicity through activation of Keap1/Nrf2 transcriptional pathway	Satoh et al., 2008
	Epigallocatechin gallate	Fluoride-induced renal injury	*In vivo:* 40 mg/kg, p.o.	Attenuation of fluoride-induced oxidative stress, renal inflammation and apoptosis by Nrf2 activation	Thangapandiyan and Miltonprabu, 2014
		PM2.5-induced oxidative stress injury	*In vitro:* 50–200 μM EGCG for 24 h	EGCG protects HUVECs from PM2.5-induced oxidative stress injury by upregulating Nrf2/HO-1 via activation of the p38, MAPK and the ERK1/2 signaling pathways	Yang et al., 2015
	Sulforaphane	Nitroglycerin-induced hyperalgesia	*In vivo:* 5 mg/kg, i.p.	Elevated cellular and nuclear levels of the Nrf2 protein	Di et al., 2016
		Spared nerve injury	*In vivo:* 30 mg/kg, i.p.	Decreased Keap1-Nrf2 signaling in mPFC, hippocampus, and muscle contribute to anhedonia susceptibility post-SNI surgery,	Li S. et al., 2018
		COPD alveolar macrophages	*In vitro:* 10 mM	Inhibition of lung inflammation and improvement of bacterial clearance through Nrf2 activation and its downstream target	Harvey et al., 2011
	Capsaicin	HepG2 cells	*In vitro:* 200 μM	Increased production of ROS, Nrf2 activation and induction of HO-1 expression via the PI_3K/Akt signaling pathways	Joung et al., 2007
Other drugs	Dimethyl fumarate	R6/2 and YAC128 Models of Huntington's Disease	*In vivo:* 30 mg/kg, p.o.	Increased Nrf2 immunoreactivity in neuronal subpopulations	Ellrichmann et al., 2011
		Chronic experimental autoimmune encephalomyelitis	*In vivo:* 15 mg/kg, p.o.	Reduced macrophage inflammation in the spinal cord and increased levels of IL-10.	Schilling et al., 2006
		Myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis	*In vivo:* 15 mg/kg, p.o. *In vitro:* human, rat and mouse astrocytes 100 μM	Increased murine neuronal survival and protected human or rodent astrocytes against oxidative stress. Increased stabilization and activation of Nrf2.	Linker et al., 2011

(Continued)

TABLE 1 | Continued

Classification	Compound	Disease or experimental model	Dose/Concentration	Outcome	Reference
	Trichostatin A	Inflammatory cystic fibrosis lung disease	*In vitro:* 10 μM	Neff activation and downregulation of innate and adaptive immune responses to reduce lung disease	Bodas et al., 2018
	Sodium butyrate	Permanent middle cerebral artery occlusion	*In vivo:* 5 mg/L	Keap1/Nrf2 dissociation followed by Nrf2 translocation and transcription of HO-1, promoting neuroprotection in stroke	Wang et al., 2012
	Auranofin	U937 and HepG2 cells	*In vitro:* 2.5 μM	Activation of Nrf2/small Maf resulting in transcription of NQO1, GCSh, HO-1 genes and downregulation of inflammatory genes involved in rheumatic diseases	Kataoka et al., 2001; Kim N.H et al., 2010
	15d-PGJ_2	Ischemia/reperfusion injury	*In vivo:* 0.3 mg/kg, i.v	Prevention of hepatic I/R injury by activation of Nrf2	Kudoh et al., 2014
		Experimental gouty arthritis induced by monosodium urate	*In vivo:* 30 mg/kg, sc	15d-PGJ2-loaded nanocapsules increase mRNA expression of Nrf2/HO-1 signaling and thereby increase in the antioxidant defenses in a PPAR-γ-dependent manner in experimental gout	Ruiz-Miyazawa et al., 2018b
	DHA	Vascular endothelial cell activation by coplanar polychlorinated biphenyls	*In vitro:* 40 μM	Increased DNA binding of Nrf2 and downstream expression of NAD(P)H:quinone oxidoreductase (NQO1), similarly to the Nrf-2 activator sulforaphane.	Majkova et al., 2011
	Resolvin D1	UV radiation-induced skin inflammation	*In vivo:*30 ng/animal i.p.	RvD1 treatment increased the Nrf2 and its downstream targets NQO1 and HO-1 mRNA expression	Saito et al., 2018
	Lipoxin A4	UV radiation-induced skin inflammation	*In vivo:* 10 ng/animal i.p.	Systemic treatment with LXA4 increases mRNA expression and enhanced nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream target enzyme nicotinamide adenine dinucleotide (phosphate) quinone oxidoreductase (Nqo1) mRNA expression.	Martinez et al., 2018
	DEETGE-CAL-Tat synthetic peptides	Brain injured mice	*In vivo:*15.6 μg/animal i.c.v..	Increase the mRNA levels for Nrf2-driven genes and reduced blood-brain barrier compromise.	Zhao et al., 2011
		Global cerebral ischemia	*In vivo*: arrange of doses, i.c.v. and s.c.	Induced Nrf2 antioxidant/cytoprotective target genes, reduced oxidative stress, and induced strong neuroprotection and marked preservation of hippocampal-dependent cognitive function	Tu et al., 2015
	Head-to-tail cyclic peptide (Peptide 3)	RAW 264.7 cells and LPS (1 μg/mL)	*In vitro*: 1–10 μM	Exhibited anti-inflammatory effects and induced activation of Nrf2-regulated defense system and enhancing the antioxidant capacity.	Lu et al., 2018

inhibition of NFκB activation and induction of pro-resolving lipid mediators (Serhan, 2017). The compounds in willow bark, for example salicylate, provide the basis for aspirin and many other traditional non-selective NSAIDs (Serhan, 2017). Accumulating evidence has shown that various NSAIDs have effects toward the Keap1/Nrf2/ARE pathway (Antman et al., 2007).

Aspirin, the most commonly used NSAID, has free radical scavenging property, specifically the removal of hydrogen peroxide. Aspirin has been reported to have protective effects against H_2O_2 in primary human melanocytes by activating Nrf2-ARE pathway and inducing HO-1 expression (Jian et al., 2016). Taken this into account, the authors suggested aspirin as a new antioxidant for the treatment to vitiligo. Aspirin also displayed neuroprotective effects in spinal cord injury model. This effect was attributed to a Nrf2/NQO1/HO-1 signaling pathway-dependent inhibition of neuronal apoptosis, astrocyte activation, oxidative stress and metabolic dysregulation (Wei et al., 2018).

The COX-2 transcription is activated by JNK2/c-jun pathway, which inhibits PI3-K activity and leads to suppression of Nrf2-ARE transcriptional activity (Healy et al., 2005). Corroborating these data, COX-2 selective NSAIDs activate the Nrf2/ARE pathway. Celecoxib is a selective COX-2 inhibitor (Gong et al., 2012) that has been reported to activate AMPK-CREB-Nrf2-dependent signaling and enhance vascular endothelium protection. Treatment of human endothelial cells with celecoxib leads to COX-2 independent signaling via phosphorylation of AMPK, resulting in the nuclear translocation of Nrf2. Together, CREB and Nrf2 pathways upregulate the expression of the

TABLE 2 | Complete clinical trials related to Nrf2.

Trial registration	Drug	Drug intake	Disease	Enrollment	Study phase	Outcome	Side effects	Country
NCT02023931	Broccoli Sprout Extract	600 μmol systemic delivery or 100 μmol systemic and topical delivery	Healthy subject	10	Early Phase 1	Not provided	Not provided	United States
NCT01335971	Sulforaphane	4.4 and 26.6 mg, daily by mouth	COPD	89	Phase 2	Treatment did not alter the expression of Nrf2 target genes and did not have an effect on levels of other anti-oxidants or markers of inflammation	Nausea (20.69%) Bad taste in mouth (31.03%) Heartburn (24.14%) Bloating/gas (20.69%) Abdominal discomfort (20.69%)	United States
NCT01315665	Broccoli sprouts	100 g of raw broccoli sprouts daily during 5 days	Cystic Fibrosis	15	Not mentioned	Treatment promoted activated Nrf-2 in the cytoplasm of nasal epithelial cells and changes in lymphocyte glutathione levels	Abdominal pain (20%); Back pain (20%); Blood in urine (20%)	United States
NCT01625130	Broccosprouts® (Brassica Protection Products LLC) homogenate	Homogenized with water using a ratio of 1:1.2	Healthy subject	16	Not mentioned	Not provided	Not provided	United States
NCT01715480	Broccosprouts® (Brassica Protection Products LLC) homogenate	Orally daily for 3 weeks	Sickle cell disease	21	Not mentioned	Not provided	Not provided	United States
NCT01845493	Broccosprout homogenate	Orally daily during 3 days	Asthma	16	Phase 1	Not provided	Not provided	United States
NCT02433925	Resveratrol	500 mg per day during 4 weeks	Chronic kidney disease	20	Phase 3	Not provided	Not provided	Brazil
NCT02255422	RTA 408 capsules	Capsules of 2.5, 5, 10, 20, 40, 80, 160 mg, orally	Mitochondrial Myopathy	53	Phase 2	Not provided	Not provided	Denmark United States
NCT02800265	Avmacol	8 tablets every evening for 3 evenings	Healthy subject	10	Not mentioned	Not provided	Not provided	United States
NCT01716858	Sulforaphane-rich Broccoli Sprout Extract	Not mentioned	Schizophrenia	10	Phase 2	Not provided	Not provided	Japan
NCT01269723	Broccoli sprout	Drink the broccoli shake homogenate	Immune Response to Live Attenuated Influenza Virus in Smokers and Non-smokers patients	51	Not applicable	Not provided	Not provided	United States
NCT02592954	Broccoli sprout	500 nM of extract in jojoba oil	Epidermolysis Bullosa Simplex Pachyonychia Congenita	5	Phase 1	Not provided	Not provided	United States

(Continued)

TABLE 2 | Continued

Trial registration	Drug	Drug intake	Disease	Enrollment	Study phase	Outcome	Side effects	Country
NCT02808624	L-carnosine	500 mg per day	Peripheral Neuropathy on Cancer	65	Phase 1 Phase 2	Not provided	Not provided	Egypt
NCT03115034	Melatonin	6mg per day (3 days before operation to 3 days after operation)	Carotid Endarterectomy	60	Phase 4	Not provided	Not provided	China
NCT02683863	BG00012 (dimethyl fumarate) (Tecfidera®)	DMF 120 mg BID for the first 4 weeks of treatment followed by DMF 240 mg BID for 24 weeks	Multiple Sclerosis	20	Phase 4	Not provided	Not provided	United States
NCT03393377	Fluvastatin and Valsartan	Fluvastatin 10 mg and Valsartan 20 mg orally for 30 days	Atherosclerosis	20	Not applicable	Not provided	Not provided	Slovenia
NCT01674231	Grapes in the form of a Freeze-dried Whole Grape Powder	60g freeze-dried whole grape powder with 296 mg polyphenols per day for 4 weeks	Obesity Inflammation Cardiovascular Disease	20	Not mentioned	Treatment enhanced Nrf2 expression in peripheral blood mononuclear cells	Not provided	United States
NCT01802333	Cytarabine Daunorubicin Hydrochloride Idarubicin Vorinostat	Not mentioned	Acute Myeloid Leukemia	756	Phase 3	Not provided	Not provided	Canada United States
NCT01831193	Curcumin	320 mg/day during 8 weeks	Proteinuric Chronic Kidney Disease	120	Phase 3	No effect of CUR was observed on the antioxidant enzymes activities or Nrf2 activation	Not provided	Mexico
UCLA trial	Sulforaphane	25–200 g of broccoli sprout homogenate, daily, during 1–4 days	Healthy smokers	65	Not mentioned	Increased mucosal Phase II enzyme expression in the upper airway of human subjects.	Not provided	United States
NCT00811889	Bardoxolone Methyl	Doses of 25, 75, or 150 mg of Bardoxolone methyl daily, during 24 or 52 weeks	Chronic kidney dis	227	Phase 2	Increased glomerular filtration rate, Effects were maintained for 52 weeks after a 24 weeks administration.	Muscle spasm 42% (25-mg group) 61% (75-mg group) 59% (150-mg group)	United States (Antman et al., 2007)
NCT00529438	Bardoxolone Methyl	Doses of 5, 50, or 100 mg of Bardoxolone methyl daily oral administration during 21 consecutive days of a 28-day cycle for up to 12 cycles.	Advanced Solid Tumors and Lymphomas	47	Phase 1	Increased levels of NQO1 mRNA in PBMCs. Decreased levels of NF-κB and cyclin D1 in tumor biopsies. Increased glomerular filtration rate. Safe with maximum tolerated dose 900 mg/d.	Nausea (>3%) Vomiting (>3%)	United States

(Continued)

TABLE 2 | Continued

Trial registration	Drug	Drug intake	Disease	Enrollment	Study phase	Outcome	Side effects	Country
NCT01351675	Bardoxolone Methyl	Single dose of 20 mg of Bardoxolone methyl daily	Type 2 diabetes mellitus and stage 4 chronic kidney disease patients	2185	Phase 3	Improved glomerular filtration rate for 24 weeks and persisted at 52 weeks.	Heart failure (6%) Coronary artery disorder (5%)	United States European Union Australia Canada Israel Mexico
NCT01373554	Oltipraz	Doses of 30 or 60 mg of Oltipraz or placebo per oral, twice a day during 24 weeks	Non-alcoholic Fatty Liver Disease	60	Phase 2	Reduced liver fat content and body mass indices. Did not effect insulin resistance, liver enzymes, lipids or cytokines levels	Not provided	Republic of Korea
NCT00956098	Oltipraz	Single dose (30–90 mg) and multiple-dose (60 or 90 mg) of Oltipraz	Liver fibrosis Liver cirrhosis	81	Phase 2	Pharmacokinetics studies shown that oltipraz was rapidly absorbed and demonstrate efficacy and safety	Abdominal discomfort (16%) Dizziness (24%) Dyspepsia (24%)	Republic of Korea

antioxidant and anti-inflammatory genes such as HO-1 and H-Ferritin (FHC). In light of this, celecoxib improves endothelial function, minimizing cardiovascular risk in patients (Al-Rashed et al., 2018).

Another group later reported that Mosquito fish (*Gambusia affinis*) exposed to diclofenac for 7 days presented increased expression of *Nrf2* mRNA and its downstream related genes. This response occurred as a protective mechanism due to large absorption and accumulation of diclofenac, which caused the buildup of ROS and induction of the antioxidant responses (Bao et al., 2017). In a model of choroidal neovascularization (CNV), indomethacin or bromfenac showed modulatory activity on Nrf2. In ARPE-19 [human diploid retinal pigment epithelium (RPE)] cells, indomethacin or bromfenac induced the translocation of Nrf2 into the nucleus and high levels of HO-1 in the perinuclear lesion and cytoplasm. *In vivo,* similar therapeutic effects were produced by these NSAIDs. In rat CNV model, indomethacin or bromfenac increased Nrf2 or HO-1 expression, inhibited macrophage infiltration and reduced VEGF levels. These data support NSAIDs treatment as a reasonable therapeutic approach for CNV due to their anti-angiogenic effect and that increasing Nrf2 signaling is a contributing underlying mechanism (Yoshinaga et al., 2011). Overall, these results demonstrate the potential protective effects of a NSAID in inflammatory conditions via the canonical Keap1/Nrf2 pathway.

MODULATION OF NRF2 BY STEROIDAL ANTI-INFLAMMATORY DRUGS (SADs)

Synthetic SADs resemble natural glucocorticoids with peculiar differences in both pharmacodynamics and pharmacokinetics features (Clark and Belvisi, 2012). They possess powerful immunosuppressive actions (Busillo and Cidlowski, 2013) and the most frequently prescribed SADs are prednisone/prednisolone, dexamethasone, and budesonide. Nevertheless, there are several other SADs that are used to treat numerous diseases (Clark and Belvisi, 2012). Synthetic glucocorticoids have been indispensable over the last half-century for treating several inflammatory and autoimmune diseases such as allergies, asthma, rheumatoid arthritis, Graves' disease, psoriasis, sepsis, and transplanted organ rejections. It is noteworthy that the therapeutic benefits of glucocorticoids are limited by severe adverse effects that develop in patients subjected to long-term use. Adverse effects include osteoporosis, skin atrophy, diabetes, abdominal obesity, glaucoma, growth retardation in children, immunosuppression, inhibition of wound repair, and hypertension hypertension (Miner et al., 2005; Rhen and Cidlowski, 2005).

Regarding the mechanism to achieve the pharmacological effects, glucocorticoids transduce their actions by binding to the glucocorticoid receptor (GR) in the cell cytoplasm. Upon ligand binding, the complex glucocorticoid-GR undergoes conformational change that triggers its translocation to the nucleus. In the nucleus, the glucocorticoid-GR complex binds to glucocorticoid responsive elements, which recruit either coactivator or corepressor proteins. This biding results in the modification of chromatin structure, which can facilitate or inhibit the transcription machinery (Hebbar and Archer, 2003; Nagaich et al., 2004). Moreover, the complex can also interact with other transcription factors such as NF-κB (McKay and Cidlowski, 1999; De Bosscher et al., 2003) and Nrf2 (Ki et al., 2005; Kratschmar et al., 2012). Although glucocorticoids exert their actions mainly through genomic (transactivation and transrepression) mechanisms, non-genomic actions have also been described (Groeneweg et al., 2011).

Experimental approaches based on immunoprecipitation of Nrf2 and its interacting proteins identified GR as a novel Nrf2-binding partner (Alam et al., 2017). In agreement, numerous studies have shown that both natural and synthetic glucocorticoids can modulate Nrf2 activity via GR signaling (Alam et al., 2017; Choi et al., 2017). A microarray analysis of developing zebrafish larvae exposed to glucocorticoids (dexamethasone, prednisolone, and triamcinolone) revealed that Nrf2 was among the top perturbed canonical pathways in oxidative stress response. In agreement with these data, GR signaling can block Nrf2-mediated cytoprotection from oxidative stress (Kratschmar et al., 2012; Alam et al., 2017) and inhibition of GR nuclear translocation restores dexamethasone-induced inhibition of Nrf2/HO-1 expression (Singh and Haldar, 2016). Mechanistically, it was demonstrated that dexamethasone enhances GR recruitment to antioxidant response elements (AREs) without affecting chromatin binding of Nrf2, resulting in the inhibition of acetyltransferase CBP (CREB-binding protein) recruitment and histone acetylation at AREs. This repressive effect was inhibited by the addition of histone deacetylase inhibitors, suggesting that the reduction in Nrf2 transcriptional activation by GR signaling is dependent on the inhibition of histone acetylation (Alam et al., 2017).

In contrast to previous data showing that dexamethasone mitigates Nrf2-mediated response during oxidative stress, in Ataxia telangiectasia lymphoblastoid cells, dexamethasone increased GSH and NADPH levels, as well as improved the antioxidant capacity in a Nrf2-dependent manner. Dexamethasone was shown to induce the translocation of Nrf2 from the cytosol to the nucleus, where its accumulation continued up to 24-h after drug administration to sustain phase II antioxidant gene expression (Biagiotti et al., 2016). In experimental autoimmune encephalomyelitis (EAE), dexamethasone also presented antioxidant effect via up-regulation of Nrf2 and antioxidant enzymes and Nrf2-nuclear translocation (Li B. et al., 2013).

Glucocorticoid-mediated Nrf2 activation also plays an important role in various other physiological and pathological settings. In liver regeneration, Nrf2 has been shown to be a key player (Ishtiaq et al., 2018). A variety of factors regulate hepatic tissue regeneration, among them, augmenter of liver regeneration (ALR) (Dayoub et al., 2013). Chronic release of glucocorticoids increases intracellular ROS levels in hepatocytes, which induces Keap1-Nrf2 conformational changes. These events culminate in the release of the activated Nrf2, an important inducer of ALR expression (Adenuga et al., 2010; Dayoub et al., 2013). In the absence of Nrf2, decreased ALR levels and delayed liver regeneration are observed in mice subjected to partial hepatectomy (Zou et al., 2015). Therefore, glucocorticoid effect on liver regeneration is dependent on Nrf2 regulation of ALR expression. In airway epithelial cells, Nrf2 pathway was identified as essential to steroid (dexamethasone)-induced enhancement of airway epithelial barrier. The transfection of cells with specific siRNA reduced the enhancement of airway epithelial barrier integrity and the accumulation of tight junction and adherent junction proteins at sites of cell–cell contact. Moreover, transfecting cells with aldehyde oxidase 1 (AOX1)-specific siRNA, a downstream enzyme of Nrf2, also reduced steroid-induced enhancement of airway epithelial barrier integrity (Shintani et al., 2015). In cancer cells on the other hand, increased Nrf2 activation due to Keap1 or NRF2 mutations occurs frequently and this seems to be important in the maintenance of these cells. Therefore, Nrf2 inhibition could be a promising therapeutic strategy to target these cells (Ma et al., 2018; Rojo de la Vega et al., 2018). An initial screening of compounds with inhibitory effect on Nrf2 revealed the glucocorticoid clobetasol propionate (CP) as one of the most potent Nrf2 inhibitors. CP prevented nuclear accumulation and promoted Beta-transducin repeats-containing proteins (β-TrCP)-dependent degradation of Nrf2 in a GR- and GSK-3-dependent manner. As a result, CP induced oxidative stress in cancer cells, which strongly suppressed the growth of tumors with Keap1 mutation and high Nrf2 activity (Choi et al., 2017).

In addition to the various effects of GR-signaling via Nrf2, an inverse relation between Nrf2 and glucocorticoid activity has been reported. In a lung inflammation model, it was demonstrated that glucocorticoid activity is regulated by Nrf2. Compared to the WT, $Nrf2^{-/-}$ mice subjected to LPS-induced lung inflammation showed decreased HDAC2 levels and increased markers of inflammation, which was not reversed by the glucocorticoid budesonide (Adenuga et al., 2010). Abnormal lung inflammation and oxidant burden was associated with a significant reduction in HDAC2 abundance and glucocorticoid resistance, thus, glucocorticoid sensitivity during inflammatory response can be dependent on Nrf2-HDAC2 axis.

NATURAL PRODUCTS-DERIVED PHARMACOLOGICAL MODULATORS OF NRF2

Natural products have been described as extraordinarily rich sources of Nrf2 activators. Phytochemicals are biologically active compounds found in plants and considered to be the main representatives of Nrf2-activators (Kou et al., 2013; Zhu et al., 2016). In fact, analgesic and anti-inflammatory effects produced by many phytochemicals are considered to occur via Nrf2 pathway (Kumar et al., 2014), secondary to increased phase 2 enzymes, GSH production and turnover [GCL and glutathione reductase (GR)], increase of HO-1 and NQO1 levels, and reduced ROS levels (Ma, 2013; **Figure 2**). It is known that ROS such as superoxide anion induce inflammatory pain (Maioli et al., 2015) via NF-κB activation (Pinho-Ribeiro et al., 2016a) and increased TNFα, IL-1β (Fattori et al., 2015; Yamacita-Borin et al., 2015; Manchope et al., 2016) and endothelin levels (Serafim et al., 2015). These inflammatory peptides sensitize nociceptor sensory neuron terminals that transduce the nociceptive stimuli in peripheral tissue (Pinho-Ribeiro et al., 2017). Thus, the use of phytochemicals to induce Nrf2 pathway and reduce ROS production is a conceivable approach to treat inflammatory and painful conditions.

Nrf2 inducer phytochemicals can be classified into the following distinct classes: Michael acceptors, oxidizable phenols and quinones, isothiocyanates, dithiolethiones, polyenes or

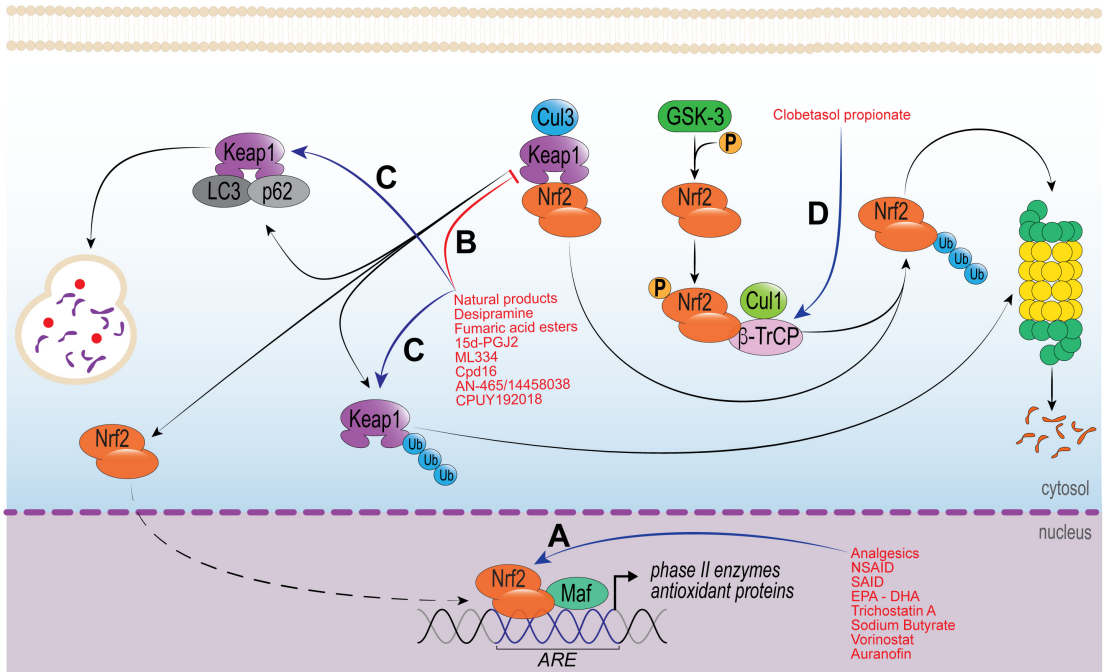

FIGURE 2 | Molecular mechanisms of drugs that modulate Nrf2 activity. **(A)** Group of drugs that increase Nrf2 biding to DNA and/or, Nrf2-Maf affinity to ARE region and/or modulate histone acetyltransferase (HDAC). **(B,C)** Drugs modulating the disruption of Keap1/Nrf2 complex; either **(B)** by protein–protein interaction though Michael addition reaction and/or, **(C)** increasing Keap1 degradation through proteasome or autophagic pathways. **(D)** Drug targeting the degradation of Nrf2 via proteasome by increasing the GSK-3/β-TrCP signaling and reducing Nrf2 activity.

vicinal dimercaptans chemical types (Kumar et al., 2014). The common feature between them is the ability to react with sulfhydryl groups by alkylation, oxidation or reduction. Most phytochemicals are Michael acceptors (olefins or acetylenes-conjugated with electron-withdrawing groups) and undergo a conjugate addition with nucleophilic amino acids (cysteine, lysine, and serine) found in electrophile-sensitive proteins (Powers et al., 2002). Moreover, phytochemicals can react with sulfhydryl groups, acting as nucleophiles in Michael reaction signaling, culminating in the up-regulation of phase 2 enzymes (Dinkova-Kostova et al., 2001; Kumar et al., 2014).

Flavonoids are well known for their analgesic and anti-inflammatory properties. Hesperidin-methyl-chalcone (HMC), naringenin and quercetin, for example, exhibit analgesic and anti-inflammatory effects in acute and chronic inflammatory pain conditions. In fact, HMC, naringenin and quercetin inhibited carrageenan-, LPS-, and superoxide anion-induced inflammatory pain by mitigating leukocyte recruitment, oxidative stress, IL-33, TNFα, IL-1β, IL-6 production and NF-κB activation in mice (Maioli et al., 2015; Pinho-Ribeiro et al., 2015, 2016b,c; Manchope et al., 2016). Both the analgesic and anti-inflammatory actions of the aforementioned molecules have been attributed, at least in part, to Nrf2 pathway activation. The flavonoid HMC inhibited gout-induced inflammatory pain reducing synovitis, leukocyte recruitment, oxidative stress, inflammatory cytokines (TNFα, IL-1β, and IL-6) and inducing Nrf2 activation and HO-1 mRNA expression in mice (Ruiz-Miyazawa et al., 2018a). The flavanone naringenin inhibited titanium dioxide/prosthesis-like-induced chronic arthritis by mitigating leukocyte recruitment, cartilage degradation, bone resorption, oxidative stress, and inflammatory cytokine expression (IL-33, TNF-α, IL-1β, and IL-6), and NF-κB activation (Manchope et al., 2018). It is noteworthy that the chronic effect of naringenin in titanium dioxide/prosthesis-like model may be related to its inhibitory effect on superoxide anion-induced inflammatory pain through Nrf2 activation and increased HO-1 mRNA expression (Manchope et al., 2016). Mechanistically, naringenin increased Nrf2/HO-1 through PI_3K/Akt signaling pathway in D-galactose-induced mice brain aging (Zhang et al., 2017). The flavonoid quercetin has also been reported to inhibit chronic inflammatory pain in titanium dioxide/prosthesis-like-induced chronic arthritis and Ehrlich tumor-induced pain in mice (Calixto-Campos et al., 2015b). The mechanisms attributed to this analgesic effect was reduced leukocyte recruitment, oxidative stress, and inflammatory cytokine production (TNF-α, IL-1β, and IL-6) secondary to increased Nrf2 and phase 2 enzymes HO-1, GPx1 and GR (Calixto-Campos et al., 2015b; Borghi et al., 2017). Quercetin also presents hepatoprotective effect in acetaminophen (APAP)-induced cytotoxicity in human liver L-02 cells by inducing Nrf2 activation and its downstream enzymes HO-1 and GCL. Mechanistically, quercetin-induced Nrf2 activation by Keap1-dependent and Keap1-independent mechanisms. Keap1-dependent mechanism was secondary to the interaction of H-benzene and H-bond of quercetin to Keap-1 Arg415 and Tyr527, and Gly364, respectively. The Keap1-independent mechanism, on the other hand, occurred through JNK MAP

kinase signaling pathway. Interestingly, quercetin also induced hepatoprotection by increasing p62 protein via JNK signaling pathway (Ji et al., 2015). Collectively, the flavonoid induction of Nrf2 and its downstream enzymes may be Keap1-dependent and Keap1-independent, and by the non-canonical pathway via p62 (**Figure 2**). However, further investigations are needed to establish which pathways are being activated by each molecule.

Curcumin, a yellow pigment found in turmeric, inhibited superoxide anion-induced inflammatory pain via the inhibition of leukocyte recruitment, oxidative stress, TNF-α, and IL-1β production and induction of Nrf2 activation and HO-1 expression (Fattori et al., 2015). In addition, this polyphenol inhibited neurological impairment, brain edema and infarction volume, capillary leakage, and oxidative stress in middle cerebral artery occlusion-induced ischemic/reperfusion damage in rats by activating Nrf2 and inhibiting NF-κB (Li et al., 2016). The curcumin induction of Nrf2 expression and its downstream enzymes were dependent of PI_3K/Akt signaling pathway activation in an *in vitro* model of ischemia/reperfusion (Wu et al., 2013). Studies show that curcumin may also exert therapeutic effect as a Michael acceptor by binding to COX-1, COX-2 (Selvam et al., 2005), and GSK-3β (Bustanji et al., 2009). Moreover, in a randomized double-blind placebo-controlled clinical trial, the positive effect of the dietary supplementation with curcumin on the redox status and Nrf2 activation was observed in patients with non-diabetic or diabetic proteinuria in chronic kidney disease (Jimenez-Osorio et al., 2016).

Cinnamic acid and its derivates are one of the simplest phenolic acids found in nature (Anantharaju et al., 2016). Caffeic acid, a cinnamic acid derivate found in thyme and oregano, possesses analgesic activity. This analgesic effect was observed in the formalin- (second phase) and acetic acid-induced writhing tests, as well as, LPS- and carrageenan-induced mechanical hyperalgesia in rats (Mehrotra et al., 2011). Later studies revealed that caffeic acid also has hepatoprotective and anti-inflammatory effects. In APAP-induced liver injury model, this molecule protected mice by reducing neutrophil recruitment and ROS levels and increasing GSH levels in the liver. Interestingly, caffeic acid alone, i.e., independent of APAP-induced injury, boosted hepatic GSH levels (Pang et al., 2016). These effects were attributed to the induction of Nrf2 and its downstream enzymes HO-1 and NQO1 by caffeic acid, as observed in human liver L-02 cells. Mechanistically, caffeic acid decreased Keap1 content through the H-benzene interaction with Arg415 of Keap1. In addition, the hydrogen atom of the hydroxyl at 3-position in caffeic acid formed water-mediated hydrogen bonds with Ser508, and the carbonyl group of CA could form H-bond with Gly603 and Ser363, enhancing the binding affinity between caffeic acid and Keap1 (Pang et al., 2016).

The flavoring agent vanillic acid is also an analgesic and anti-inflammatory. In carrageenan-induced inflammatory pain model, vanillic acid mitigated hyperalgesia, leukocyte recruitment, oxidative stress, IL-33, TNFα, and IL-1β production, and NF-κB activation in mice. Accordingly, the analgesic and anti-inflammatory effects of vanillic acid were related to Nrf2 activation (Calixto-Campos et al., 2015a). In agreement, vanillic acid also inhibited Aβ1-42-induced oxidative stress, neuroinflammation and cognitive impairment in mice through Nrf2 activation and HO-1 expression (Amin et al., 2017). Although the effects were described to be secondary to Nrf-2 pathway activation, these studies did not show how this occurred. Therefore, further investigations demonstrating the precise molecular mechanism involved in vanillic acid activity are necessary.

Terpenoids are naturally occurring organic chemicals derived from five-carbon isoprene unit comprising mono-, di-, and tri-terpenoids. Kaurenoic acid is a diterpene found in *Sphagneticola trilobata* with analgesic and anti-inflammatory properties *in vivo*. Both carrageenan-induced inflammatory pain and TNF-α and IL-1β production were inhibited by kaurenoic acid in mice (Mizokami et al., 2012). It is postulated that the analgesic effect of this compound could be related to Nrf2 activation, since kaurenoic acid attenuated LPS-induced acute lung injury, neutrophil recruitment and inflammatory cytokine gene expression (TNFα and IL-1β) by activating Nrf2 and regulating the expression of phase 2 enzymes NQO-1, HO-1 and glutamate-cysteine ligase catalytic subunit (GCLC) (Kim et al., 2016). Glycyrrhizin a pentacyclic triterpenoid found in licorice root inhibited CFA-induced mechanical and thermal hyperalgesia in mice paw and increased IL-6, TNFα, and IL-1β levels, microglia activation, HMGB1, and NF-κB activation in spinal cord (Sun et al., 2018). Mechanistically, glycyrrhizin inhibited LPS-induced HMGB1 release through p38/Nrf2-dependent induction of HO-1 in Raw 264.7 cells (Kim et al., 2015). *In silico* molecular docking assay showed that glycyrrhizin interacted directly with 16-mer Nrf2 peptide binding site on Keap1, suggesting that the interference in Keap1/Nrf2 binding was involved (Kamble et al., 2017). These findings suggest that Nrf2 activation by glycyrrhizin may be both Keap1 dependent and independent.

Oxidizable phenols and quinones were one of the first classes described as phase 2 inducers before the discovery of Nrf2/ARE pathway (Prochaska et al., 1985; Kumar et al., 2014). Catechol (1,2-diphenol) and hydroquinone (1,4-diphenol) are Nrf2 inducers that undergo oxidation by cytochrome p450 *in vivo*. This reaction results in the formation of its quinone derivate, which contains a Michael acceptor. This quinone can react with critical cysteine residues in Keap1, resulting in Nrf2 activation and increased phase 2 enzyme expression (Bensasson et al., 2008). Rosmarinic acid is a polyphenol-containing a catechol moiety found in rosemary and peppermint. It has been demonstrated that pre- or post-treatment with this compound mitigates chronic constriction injury (CCI)-induced neuropathic pain by inhibiting mechanical and thermal allodynia, oxidative stress, glial cell activation, and TNFα production (Anantharaju et al., 2016). These effects were related to Nrf2 activation. Rosmarinic acid also inhibited carbon tetrachloride-induced liver intoxication in mice by reducing oxidative and nitrosative stress, TNFα, and COX-2 protein expression, and NF-κB activation, as well as inducing Nrf2 activation and HO-1 in the liver (Domitrovic et al., 2013). Importantly, it was demonstrated that Nrf2 activation ocurred through Akt/GSK-3β/Fyn pathway, because the inhibition of β-amyloid-induced oxidative stress by rosmarinic acid was abrogated by Akt inhibitor LY294002,

GSK-3β inhibitor LiCl, Nrf2 shRNA, or Fyn shRNA in PC12 cells (Rong et al., 2018). Nevertheless, it remains to be determined if Nrf2 activation by rosmarinic acid is also dependent on Keap1 interaction. In contrast, the interaction with Keap1 was demonstrated for carsonic acid. This molecule interacts with Keap1 via alkylation of critical cysteine residues. This interaction was central to the inhibition of oxidative stress in immature cortical neurons (Satoh et al., 2008). Moreover, the importance of Nrf2 activation in carsonic acid activity was highlighted by the abrogation of this effect in dominant negative Nrf2 cortical neurons (Satoh et al., 2008). *In vivo*, carsonic acid translocates into the brain, increases the level of reduced glutathione *in vivo*, and protects the brain against middle cerebral artery ischemia/reperfusion (Satoh et al., 2008).

Epigallocatechin gallate (EGCG), a catechin polyphenol found in green tea, inhibited bone cancer-induced pain and neuroinflammation by decreasing TNFα in mice spinal cord (Li and Zhang, 2015). ECCG is protective in fluoride-induced intoxication kidney damage in rats. The mechanism attributed to this effect was decreasing oxidative stress, NFκB activation, and inflammatory cytokine (TNF-α, and IL-6) levels, secondary to Nrf2 activation and upregulation of phase 2 enzymes HO-1, GCL, and GST (Thangapandiyan and Miltonprabu, 2014). Collectively, these data indicate that the analgesic and anti-inflammatory effects described for EGCG could be related to Nrf2 activation. Mechanistically EGCG effects involve the activation of ERK and p38 MAPK signaling pathways. In human umbilical vein endothelial cells, PD98059 (a selective inhibitor of extracellular signal regulated kinase [ERK]-1/2) and SB203580 (a selective inhibitor of p38 MAPK), but not SP600125 [a selective inhibitor of c-jun N-terminal kinase (JNK)], attenuated the EGCG-induced Nrf2 and HO-1 expression. Moreover, silencing Nrf2 abolished EGCG-induced enhancement of cell viability and the upregulation of Nrf2 and HO-1 (Yang et al., 2015). Collectively, the studies discussed above show that oxidizable phenols and quinones also induce Nrf2 and its downstream enzymes through Keap1 dependent and independent mechanisms.

Isothiocyanates are derived from their glucosinolate precursors, which are found in cruciferous plants. Glucosinolates are hydrolyzed by plant enzyme myrosinase or by mammalian gastrointestinal microflora (Fahey et al., 2001; Shapiro et al., 2001) and important biological activity has been attributed to these compounds. Sulforaphane, an isothiocyanate found in broccoli, brussels sprout and cabbage, inhibits CCI-induced neuropathic pain and the expression of inflammatory cytokines, COX-2 and iNOS proteins in the spinal cord. Interestingly, the opioid pathway seemed to be involved in sulforaphane analgesic effect, since naloxone inhibited its anti-allodynic action (Wang and Wang, 2017). Anhedonia (loss of pleasure) is a common feature in patients with neuropathic pain and spared nerve ligation (SNI) in rats, which can induce a depression-like phenotype in some of the animals. This phenotype is related with reduction in Nrf2 levels in medial prefrontal cortex (mPFC), hippocampus, spinal cord and skeletal muscle, but not nucleus accumbens in anhedonia-susceptible compared to anhedonia-resistant and sham rats (Li S. et al., 2018). Sulforaphane pretreatment inhibited SNI-induced mechanical hyperalgesia in anhedonia susceptible and resistant rats and normalized Nrf2 levels in mPFC, hippocampus, spinal cord and skeletal muscle in anhedonia-susceptible rats (Yao et al., 2016). In agreement, sulforaphane also inhibited nitroglycerin-induced hyperalgesia and neuronal activation (c-fos and nNOS immunoreactivity) in trigeminal nucleus caudalis in mice by increasing nuclear Nrf2 and phase 2 proteins HO-1 and NQO-1 in neurons (Di et al., 2016). In other contexts, sulforaphane controls inflammation and improves bacterial clearance in chronic obstructive pulmonary disease (COPD). Mice exposed to cigarette smoke for 6 months and challenged with *Haemophilus influenza* or *Pseudomonas aeruginosa* presented pulmonary inflammation, increased in leukocyte recruitment, and impairment in bacterial clearance by alveolar macrophages. The treatment with sulforaphane ameliorated these events through Nrf2 activation and upregulation of macrophage receptor with collagenous structure (MARCO), its downstream target. In agreement, sulforaphane restored the capacity of bacteria recognition and phagocytosis by alveolar macrophages from COPD patients (Harvey et al., 2011). Mechanistically, sulforaphane seems to induce Nrf2 activation through Keap1 cysteine residue 151 interaction (Hu et al., 2011). Regarding the effects of sulforaphane in humans (**Table 2**), the oral consumption of sulforaphane doses contained in standardized broccoli sprout homogenate increased phase II enzymes in nasal lavage cells in a placebo-controlled dose escalation trial (Riedl et al., 2009). On the other hand, in a randomized double-blind clinical trial, patients with COPD receiving oral sulforaphane treatment did not present significant changes in Nrf2 target genes or markers of inflammation in alveolar macrophages or bronchial epithelial cells (Wise et al., 2016). Interestingly, although similar doses of sulforaphane were tested, these studies reported different outcomes. Differences in the study subjects may account for this. The first trial was conducted with healthy non-smokers, whereas in the second, the subjects were either active or former smokers with COPD. It is plausible that higher doses of sulforaphane may be necessary to produce significant changes in Nrf2 target genes and disease outcome as the disease severity increases. In this case, inhalable formulation of sulforaphane may be more effective in guaranteeing higher delivery in the lungs. Nevertheless, further studies should be conducted to test this hypothesis.

Capsaicin, a transient receptor potential cation channel subfamily V member 1 (TRPV1) agonist, is a compound found in the red pepper. Acutely, capsaicin induces pain, but chronically, it displays analgesic effects when administrated centrally or peripherally by depleting neuropeptides at supraspinal level (Fattori et al., 2016). Unexpectedly, capsaicin induces the production of ROS, which can interact with NQO1. Subsequently, Nrf2-ARE binding can occur; followed by Nrf2 activation and induction of HO-1 expression via the PI_3K/Akt signaling pathways in HepG2 cells (Joung et al., 2007). However, it is unknown whether the analgesic effect of chronic capsaicin treatment depends on Nrf2 induction.

In summary, this section of the review brought data from the key phytochemical classes that have analgesic and anti-inflammatory activity through Nrf2 pathway (**Figure 2**). It is noteworthy that natural products are good candidates to associate

with conventional analgesic and anti-inflammatory therapeutic protocols. The association of these molecules could reduce the dose of drugs such as opioids, non-steroidal and steroidal anti-inflammatory, that have several known adverse reactions. Seeking for novel analgesics and anti-inflammatory drugs with lessened side effects is also important and may produce substitute drugs for cases in which the side effects of current drugs do not allow their therapeutic use.

OTHER THERAPIES MODULATING NRF2

In addition to the previously discussed classes of drugs, many other compounds have shown important biological activity via Nrf2. There are a variety of Nrf2 inducers, most of which are electrophilic molecules. These molecules react with cysteine thiols of Keap1, being Cys151, Cys273, and Cys288 residues the most prone to electrophile reaction. Electrophile adducts can inhibit Keap1 in two different ways. First, by the induction of a conformational change in Keap1, which results in the loss of its binding capacity to Nrf2. Second, by blocking the interaction between Keap1 and CUL3/RBX1, resulting in sequestration of Keap1 with Nrf2 and further stabilization of newly synthetized Nrf2 (reviewed in Cuadrado et al., 2018; **Figure 2**).

Fumaric acid esters, including dimethyl fumarate (DMF) and the monoester form monomethyl fumarate (MMF), are the most prominent examples of Keap1 cysteine residue modifiers (Lin et al., 2011). DMF [Tecfidera® by Biogen] is to date the only Food and Drug Administration approved drug registered as NRF2 activator. DMF and other fumaric acid esters have been used to treat psoriasis for over 50 years, when the role of Nrf2 in disease was still unknown. Nevertheless, clinical trials have demonstrated the effectiveness of these compounds in reducing psoriasis area and severity index (Altmeyer et al., 1994), and treating cases of moderate to severe chronic plaque psoriasis (Mrowietz et al., 2017; Tzaneva et al., 2018; **Table 2**). The mechanisms of action related to these effects include a shift from a T helper (Th)1 toward a Th2 immune response, in addition to the overall decrease in the number of peripheral T cells (Ghoreschi et al., 2011; Tahvili et al., 2015). Considering the immune modulatory actions of fumaric acid esters, it is not surprising that these compounds have also therapeutic effects in other auto-immune diseases such as cutaneous lupus erythematosus (Kuhn et al., 2016; Saracino and Orteu, 2017) and multiple sclerosis (MS) **Table 2**. DMF was approved in 2013 for the treatment of MS (Xu et al., 2015) and is currently used as the first line treatment of relapsing-remitting MS that does not respond to traditional therapies (Bar-Or et al., 2013). Positive results in EAE, a mouse model for MS, were early indications that fumaric acid esters may have beneficial effects in this disease (Schilling et al., 2006). Schilling et al. (2006) reported important therapeutic effects on the course of disease and histology attributed to the reduction in macrophage-mediated inflammation in the spinal cord and increase in systemic IL-10 levels (Schilling et al., 2006). Accordingly, in this same model of MS, DMF-mediated beneficial effects on clinical course and preservation of myelin, axons, and neurons were observed in WT, but not in *Nrf2*$^{-/-}$ mice (Ellrichmann et al., 2011; Linker et al., 2011). In line with the potent anti-inflammatory effects described, DMF also modulates the immune response in dendritic cells and T cells by reducing the release of inflammatory cytokines (Ockenfels et al., 1998). Moreover, DMF prevents endothelial dysfunction and cardiovascular pathologic ROS formation and inflammation, as well as decrease atherosclerosis and kidney dysfunction in diabetic mice (Tan et al., 2014; Sharma et al., 2017). Overall, the anti-inflammatory and immune modulatory activities of fumaric acid esters indicate that these compounds may also be successful in treating other disease in which chronic inflammation and ROS production are important pathological mechanisms.

As previously discussed in this review, Nrf2 acetylation mediated by histone acetyltransferase/HDAC enhances its transcriptional ability and the expression of downstream targets. This is the mechanism of action of the pan-HDAC inhibitor trichostatin A, which protects against cartilage degradation via the reduction in matrix metalloproteinase (MMP)s and proinflammatory cytokines TNF-α, IL-1β, and IL-6 in osteoarthritis (Cai et al., 2015). Moreover, protective effects via Nrf2 in inflammatory cystic fibrosis lung disease and cerebral ischemic damage have also been observed for trichostatin A (Wang et al., 2012; Bodas et al., 2018). Other HDAC inhibitors, for example sodium butyrate and vorinostat, also mitigate inflammation and the up regulation of MMPs and aggrecanase 2 in human osteoarthritis chondrocytes. As a result, HDAC inhibitors have protective effects against cartilage degradation through mechanisms such as Nrf2 activation and the inhibition of NF-κB and MAPK (Khan and Haqqi, 2018). In addition to the drugs discussed in this section, numerous other Nrf2 inducers acting as electrophilic Keap1 modifiers have been or are currently being tested in clinical trials for the treatment of diseases/conditions such as kidney disease, diabetes, liver diseases (non-alcoholic fatty liver disease, and liver fibrosis and cirrhosis), and cancer. Some examples of these molecules are Bardoxolone methyl (Pergola et al., 2011; Hong et al., 2012; de Zeeuw et al., 2013) and Oltipraz (Kim S.G. et al., 2010; Kim et al., 2017a), which are shown in **Table 2**.

Auranofin (2,3,4,6-Tetra-O-acetyl-1-thio-beta-D-glucopyranosato-S [triethylphosphine] gold) is a gold(I)-containing antirheumatic drug that possesses anti-inflammatory properties mainly via HO-1 induction. The antirheumatic gold(I)-containing compound selectively activates the DNA binding of the heterodimer Nrf2 and small Maf. Once bound to the ARE or Maf-recognition element, Nrf2/small Maf induces a range of antioxidative stress genes, including *HO-1* and *γ-glutamylcysteine synthetase*, which contribute to the scavenging of ROS and exert anti-inflammatory effects (Kataoka et al., 2001). Moreover, auranofin can elevate cellular levels of Nrf2 by increasing protein stability. Co-immunoprecipitation and Western blot analysis indicated that auranofin inhibits Nrf2 degradation by inducing the dissociation of the Nrf2-Keap1 complex, resulting in nuclear accumulation of Nrf2. Additionally, mechanistic studies revealed that upregulation of Keap1/Nrf2 signaling and downstream HO-1 by auranofin is dependent on Rac1/iNOS induction and MAPK activation (Kim N.H et al., 2010).

The role of lipid mediators in promoting the resolution of inflammation has been widely demonstrated (Serhan et al., 2008). Strikingly, studies are now showing that these effects might be induced via Nrf2 activation. The pro-resolving lipid mediator 15d-PGJ_2, for example, has been reported to interact with Nrf2. 15d-PGJ_2 forms an adduct to Keap1 and disrupts Nrf2 ubiquitination, leading to the accumulation of Nrf2 in the nucleus (Joo et al., 2017). Numerous studies have demonstrated the anti-inflammatory activity of 15d-PGJ_2 via Nrf2 activation (Kudoh et al., 2014; Li et al., 2015; Ruiz-Miyazawa et al., 2018b). In a model of gout arthritis in mice, for example, 15d-PGJ_2-loaded nanocapsules reduced monosodium urate (MSU)-induced pain, inflammatory cytokine production, and NLRP3 inflammasome and NF-κB activation (Ruiz-Miyazawa et al., 2018b). Treatment with 15d-PGJ_2-loaded NC mitigated oxidative stress and increased both *Nrf2* and *HO1* mRNA expression, which were reverted by the PPAR-γ inhibitor GW9662. These observations suggest that these effects of 15d-PGJ_2-loaded NC are PPAR-γ dependent, which is in line with previous studies showing that PPAR-γ activation results in increased Nrf2/HO-1 signaling and antioxidant defenses (Hsu et al., 2013). Further substantiating the central role of Nrf2 in the anti-inflammatory activity of 15d-PGJ_2, Bretscher et al. (2015) demonstrated that the reduction in IL-6 and IL-12 expression by 15d-PGJ_2 was not present in Nrf2-deficient myeloid cells (Bretscher et al., 2015). Interestingly, 15d-PGJ_2 also potentiates macrophage efferocytosis through Nrf2-mediated upregulation of CD36 and HO-1. Macrophage efferocytosis is central to the clearance of apoptotic neutrophils during the resolution of inflammation, thus 15d-PGJ_2 also promotes the resolution of inflammation (Kim et al., 2017b).

Other lipid mediators that also seem to mediate anti-inflammatory effects via Nrf2 are lipoxin A4 (LXA_4) and Resolvin D1 (RvD1) (Martinez et al., 2018; Saito et al., 2018). In ultraviolet (UV) radiation-induced skin inflammation in mice, LXA_4 and RvD1 greatly diminished inflammation, matrix metalloproteinase 9 expression, and sunburn cell counts (Martinez et al., 2018; Saito et al., 2018). These lipids also induced Nrf2 and Nqo1 expression, as well as mitigated oxidative stress. Skin damage induced by UVB irradiation is highly dependent on ROS; and Nrf2 has an important role in the restorative adaptive response to UV radiation-induced inflammation and sunburn reaction (Patwardhan and Bhatt, 2015). Therefore, it is likely that increased Nrf2 expression is important in the protective effect of LXA_4 and RvD1 against UV radiation-induced skin inflammation. In the same rationale, polyunsaturated fatty acids have also been shown to activate Nrf2 in inflammation. The omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are sources for the production of Protectin and Resolvin D-series, and Resolvin-E series, respectively (Serhan et al., 2008). Therefore it is conceivable that they also may modulate Nrf2 activity. Both DHA and EPA were shown to be cytoprotective against oxidative insults in endothelial cells in a Nrf2-dependent manner (Lee et al., 2015; Sakai et al., 2017). DHA also significantly mitigated the toxicity and increase in MCP-1 levels by organic pollutants such as polychlorinated biphenyls (PCBs) in vascular endothelial cells; and this effect was attributed to increased Nrf2 DNA binding and downstream expression of antioxidants (Majkova et al., 2011). Although several of these studies have reported Nrf2 pathway as being central to the anti-inflammatory and antioxidant effects of the aforementioned lipid mediators and polyunsaturated fatty acids, the precise molecular mechanism involved in the activation of Nrf2 was not demonstrated. In this sense, the interaction between Nrf2 and these molecules is still unclear.

Although Keap1 oxidation by electrophilic compounds (classic inducers) is essential for Nrf2 activation and its downstream effects (canonical pathway), the lack of selectivity of electrophilic Keap1 inhibitors is frequently overlooked and may account for some of the off-target and undesired side effects. Bardoxolone methyl, for example, can interact with over 500 different proteins, including many different transcription factors (Yore et al., 2011; Zhang, 2013). To overcome the lack of selectivity and off-target effects, a new class of NRF2 inducers that prevent the docking of NRF2 to KEAP1 has emerged (Richardson et al., 2015). The Kelch-DC domain of Keap1 binds to Nrf2 via either its DLG or ETGE motif; both of which are thought to be the major targets of small peptides capable of disrupting Keap1-Nrf2 protein–protein interaction (PPI) (Xu et al., 2015; Gazaryan and Thomas, 2016; Jiang et al., 2016; Saito et al., 2016; Dinkova-Kostova et al., 2017). To date, five families of Keap1-Nrf2 PPI inhibitors have been described: tetrahydroisoquinoline, thiopyrimidine, naphthalene, carbazone, and urea derivatives (reviewed recently in Cuadrado et al., 2018) and studies show that these peptides are excellent candidates to activate Nrf2 due to their potent activity and specificity (Hancock et al., 2012, 2013).

The discovery that the DEETGE sequence in Nrf2 is critical for the Keap1-Nrf2 interaction led to the development of DEETGE-CAL-Tat synthetic peptides. These peptides containing the DEETGE sequence, a caplain cleavage site, and a HIV-Tat cell transduction domain, were shown to disrupt Keap1-Nrf2 interaction and induce Nrf2 genes *in vitro* and *in vivo* in brain injured mice (Zhao et al., 2011). A subsequent study revealed that this peptide also had neuroprotective and cognitive-preserving effects in rats subjected to global cerebral ischemia. The administration of the DEETGE-CAL-Tat peptide strongly enhanced nuclear translocation and DNA binding of Nrf2, as well as expression of known Nrf2-regulated target antioxidant/cell-defense proteins in the hippocampal CA1 region. Intracerebroventricular pre-treatment or peripheral post-treatment also induced robust neuroprotection in the hippocampal CA1 region and strongly preserved cognitive function in this model (Tu et al., 2015). In a study by Lu et al. (2018), on the other hand, the head-to-tail cyclic strategy was applied in the development of novel peptide inhibitors (Lu et al., 2018). A novel cyclic peptide 3 showed high binding affinity with Keap1 and potency in Nrf2 activation at cellular level. This peptide exhibited effective anti-inflammatory effects in mouse RAW 264.7 cells by activating the Nrf2-regulated defense system and enhancing the antioxidant capacity (Lu et al., 2018). Importantly, from the large number of compounds indexed, LH601, benzenesulfonyl- pyrimidone 2, N-phenyl-benzenesulfonamide, and a series of 1,4-diphenyl-1,2,3-triazoles seem to be more promising candidates to inhibit the PPI

with KEAP1, due to favorable atomic interaction with KEAP1, affinity, and thermodynamic parameters of binding (Cuadrado et al., 2018). Nevertheless, the effect of these molecules has yet to be tested. Moreover, more studies are necessary to test the effects, potency, and safety and better elucidate the mechanisms of the newly developed small peptides discussed herein.

ROLE OF DRUGS ACTING VIA NRF2 IN CANCER

Chronic inflammation, pain and oxidative stress often accompanies cancer. Nrf2 has been conventionally considered as a tumor suppressor, especially in the early stages of cancer. The definition that Nrf2 is a tumor suppressor comes from its cytoprotective effect against exogenous and endogenous insults such as xenobiotics (Sporn and Liby, 2012). More recently, molecular analysis have stablished that Nrf2 is a oncogenic factor and its activation leads to chemotherapy resistance (Ganan-Gomez et al., 2013). In this context, it is controversial whether the activation of Nrf2 by pharmacological agents with anti-inflammatory and antioxidant properties are useful for the prevention or treatment of cancer.

In experimental studies, the chemopreventive role of Nrf2 inducers was mainly addressed by using the naturally occurring isothiocyanate, sulforaphane. Sulforaphane has received audience because of its ability to simultaneously modulate early stages of carcinogenetic events (initiation) or hamper steps involved in cancer development (Fimognari and Hrelia, 2007; Jiang et al., 2018). Mechanistically, sulforaphane, via Nrf2, promotes DNA protection by inducing phase II enzymes (Morimitsu et al., 2002). This complex association between sulforaphane-Nrf2 is defined by the reversible modification of Keap1 cysteine residues (Hu et al., 2011), interaction with MAPK, phosphatidylinositol 3-kinase (PI_3K), PKC pathways, NF-κB, or epigenetic modifications (Su et al., 2018). Therefore, the modulation of kinases or DNA methyltransferases results in phosphorylation, nuclear accumulation, and increased transcription and stability of Nrf2. In addition, all effects were observed in nanomolar range (Guan et al., 1990; Su et al., 2018).

Besides sulforaphane, others molecules with distinct mechanisms, including phenethyl isothiocyanate, oltipraz, curcumin, resveratrol, fumaric acid and its esters, and synthetic oleanane triterpenoids also have therapeutic effects in cancer by targeting Nrf2 (Gupta et al., 2004). Of note, although all these chemopreventive molecules can interact with proteins other than Nrf2, indicating that Nrf2-independent mechanisms were also present, their beneficial effects were abrogated or decreased in Nrf2 knockouts (Ramos-Gomez et al., 2001). Additionally, ARE reporter mice and measurements of NAD(P)H:quinone oxidoreductase (*NQO1)* and *GST* transcripts levels were also used to confirm the involvement of Nrf2 (Lewis et al., 2006; Sporn and Liby, 2012). Importantly, all these chemopreventive drugs reduce at low doses the uncontrollable oxidative stress generated by carcinogens that would damage DNA and induce persistent inflammation (Hayes et al., 2010; Hu et al., 2010; Sporn and Liby, 2012). So far, the beneficial role of Nrf2 induction has been widely explored at multiple organ sites including skin (Ben Yehuda Greenwald et al., 2017; Alyoussef and Taha, 2018), lungs (Kumar et al., 2011; To et al., 2015; Creelan et al., 2017; Lin et al., 2017), bladder (Iida et al., 2004; Leone et al., 2017), breast (Pledgie-Tracy et al., 2007; Soto-Balbuena et al., 2018; Soundararajan and Kim, 2018), colon (Rajendran et al., 2015; Guo et al., 2018), pancreas (Kallifatidis et al., 2009), stomach (Fahey et al., 2002), and oral cancer (Bauman et al., 2016; Soundararajan and Kim, 2018).

Because Nrf2 promotes cell survival under stress, it is coherent to assume that an increase in Nrf2 could be protective for cancer cells. In this context, the hyperactivation or unbalanced regulation of Nrf2 may participate in tumor growth or be involved in chemoresistance (Ganan-Gomez et al., 2013). However, only a few studies reporting a cancer-promoting role of Nrf2 have been published.

The frequencies of Nrf2 and Keap1 mutation in tumors are often low (Taguchi et al., 2011). Following the discoveries and characterization, it was observed that common oncogenes, such as *KRAS*, *BRAF,* and *MYC*, increase the transcription and activity of Nrf2, resulting in an increase in cytoprotection and, most notably, a decrease in free radical generation (DeNicola et al., 2011; Sutcliffe et al., 2011). Thus, oncogenes may promote tumorigenesis, in part, in a Nrf2-dependent manner by enhancing the survival of tumor cells (DeNicola et al., 2011). Therefore, considering that cells do not appear to become refractory to repeated activation of the NRF2 pathway by drugs, it is possible that cancer cells utilize a non-mutated pathway to support tumor growth in early stages (Perera and Bardeesy, 2011). Despite these effects, studies point out that increased levels of fumarate, due to chronic exposition to DMF, can become carcinogenic to cells (Sporn and Liby, 2012; To et al., 2015). However, additional studies are required to determine whether phytochemicals, synthetic chemopreventive agents or others drugs targeting Nrf2 increase or decrease cancer risk.

Persistent activation of Nrf2 also enhances resistance to etoposide, doxorubicin, tamoxifen and cisplatin. Alongside with this, many Nrf2 downstream genes, in particular, heme oxygenase-1 (HO-1) have been shown to contribute to the observed Nrf2-dependent chemoresistance (Jozkowicz et al., 2007; Jeddi et al., 2017). In this sense, the abrogation of drug-induced ROS by Nrf2 can confer chemoresistance. Moreover, there is no clear understanding between pharmacological agents acting via Nrf2 and direct resistance, since the persistent activation of Nrf2 is usually a result of a genetic mutation (Homma et al., 2009; Kensler and Wakabayashi, 2010).

Overall, the timing of Nrf2 activation is important in the context of cancer, as summarized in **Figure 3**. Enhancing Nrf2 is essential for the prevention of cancer, especially at low doses by drugs that enhance this pathway. On the other hand, in fully malignant cells and in advanced stages of cancer, the enhancement of Nrf2 caused by mutations can protect the tumor microenvironment. At this point, modulating unique redox regulatory mechanism or avoiding use of Nrf2 inducers from general source that greatly accumulate in

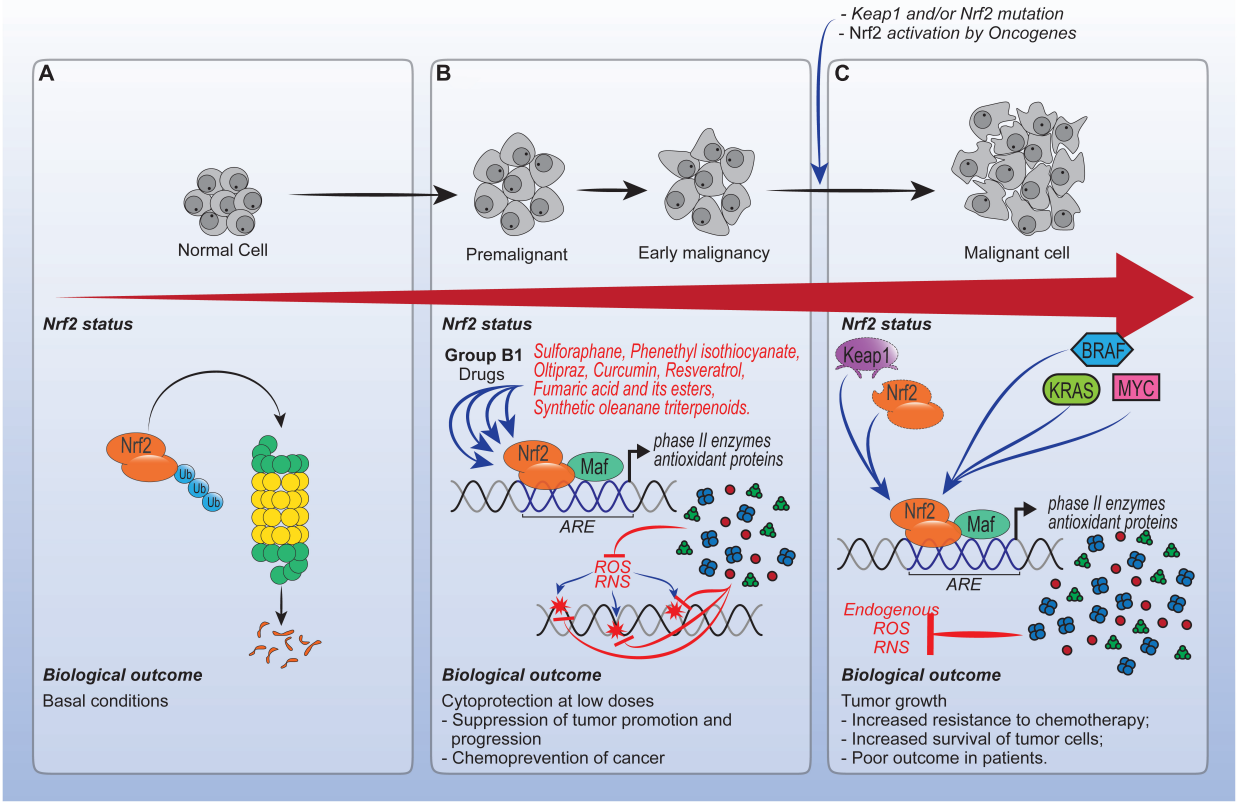

FIGURE 3 | Role of drugs acting via Nrf2 in cancer. **(A)** In normal cells, Nrf2 activity is regulated by canonical and non-canonical pathways, which in the absence of oxidative stress, culminate in Nrf2 proteossomal degradation. **(B)** Enhancing Nrf2 in premalignant and early malignant cells is important to prevent cancer development, specially by low doses of drugs capable of inducing phase II enzymes and antioxidant proteins expression. **(C)** Otherwise, in malignant cells the enhancement of Nrf2 activity caused by mutations such as *Kras*, *Bras* and *Myc*, can protect tumors from the cytotoxic effects of reactive oxygen species (ROS) induced by chemotherapy. However, the effects of drugs that act via Nrf2 at intermediate and chemotherapy stages still need investigation. Overall, the effects of Nrf2 in cancer depend on the biological development stage of tumor cells.

cells might be effective. However, the direct effects of Nrf2 inducers or even palliative drugs acting via Nrf2, for instance, morphine on cells at intermediate stage of cancer, need further investigation.

NON-PHARMACOLOGICAL APPROACHES TARGETING KEAP1-NRF2 PATHWAY

In this section we will discuss data from preclinical studies on non-pharmacological approaches of targeting Keap1-Nrf2 signaling pathway in varied inflammatory disorders. Among the non-pharmacological approaches, the use of interference RNA (iRNA) and genetic constructions targeting the knockdown (KD) of *keap1* gene are widely applied in a variety of *in vivo* and *in vitro* studies. In fact, a *keap1* KD with background on scurfy mice was developed (Suzuki et al., 2017). Scurfy mice are deficient in T_{reg} cells; therefore, they succumb to severe multi-organ inflammation by 4 weeks of age. The systemic activation of Nrf2, by KD of Keap1, considerably ameliorated inflammation and lethality. In addition, increases in Nrf2 activation reduced the number of activated T cells and the amount of pro-inflammatory cytokines (Suzuki et al., 2017).

Similarly, *in vivo* KD of Keap1 decreased fasting-induced steatosis (Xu et al., 2013). The overt-activation of Nrf2 in this particular case decreased the levels of lipid accumulation in the liver and reduced the expression of lipogenic genes and genes related to fatty acid transport. In addition to these findings, the authors propose that Nrf2 plays a role in insulin signaling regulation and enhances insulin sensitivity in skeletal muscle (Xu et al., 2013). The Keap1 KD was also investigated in glomerulosclerosis (Miyazaki et al., 2014). The increase in Nrf2 activity ameliorates podocyte injury caused by an immunotoxin. These findings suggest that the Keap1-Nrf2 system is a promising target in the treatment of chronic liver and kidney diseases.

Although great efforts have been made to study the role of Nrf2 in various diseases, the *in vivo* study of Keap1 is limited to its knockdown, as previously described. Interestingly, Keap1-null mice present postnatal lethality (Wakabayashi et al., 2003). In fact, constitutive activation of Nrf2 culminate in morphological

alterations on esophagus and forestomach, manifesting as hyperkeratosis lesions. Two major hypotheses were postulated to explain the lethality of Keap1-null mice. First, a subset of genes for squamous cell differentiation is within the ARE region, therefore, Nrf2 activation induces their expression. Second, the process of desquamation and keratin degradation is dependent on oxidative stress. In this sense, the high amounts of antioxidant proteins prevent keratin oxidation, avoiding desquamation (Wakabayashi et al., 2003). In spite of the protective role Nrf2 plays in many scenarios of diseases, driving its uncontrolled activation may be a dangerous path, as will be discussed further in this section.

The increase o Nrf2 activity was also investigated on human renal tubular HK-2 cell line by KD *keap1* gene with a short hairpin iRNA. Cells with decreased expression of keap1 showed up-regulation in a set of antioxidant and detoxifying proteins, which increased the resistance to cisplatin and doxorubicin cytotoxicity (Jeong et al., 2015). This work demonstrated the potential of Nrf2 to induce the expression of four renal transporters involved in the excretion of drugs, which in a translational manner suggest the importance of Nrf2 in human xenobiotic-induced nephrotoxicity. In a similar way, the KD of *keap1* in Hep2 cancer cells line increased the expression of antioxidant agents and reduced the apoptosis rate when cells were challenged with H_2O_2 (Li C. et al., 2018). As discussed in this review, the up-regulation of Nrf2 may favor tumor growth by reducing apoptosis and the relation between Nrf2 and cancer cells is contradictory, therefore, genetic interventions that increase Nrf2 activity may not be a suitable therapeutic approach for cancer.

Despite effective, KD methodologies listed above are limited to the basic research level and obviously not reliable to be considered as therapy for humans today. Nevertheless, the effort of these alternative approaches placed Keap1 as an important target to elucidate the function of genes as well as finding new therapeutic interventions.

CONCLUNDING REMARKS

Considering that Nrf2 signaling pathway can regulate at least 600 genes, of which 200 encode cytoprotective proteins that are involved in diseases and the dynamic connections between diseases and drugs, modulating Nrf2 activity is a promising pharmacological approach in inflammatory and painful diseases (Ahmed et al., 2017). Nrf2 activators are commonly naturally occurring and plant-derived, but many others are synthetic compounds as represented by pharmacological classes of analgesics, glucocorticoids, NSAIDs, pro-resolution lipid mediators, electrophilic compounds and others as discussed above. To improve comprehension, we highlighted some examples of drugs with analgesic or anti-inflammatory actions that act via Nrf2 by canonical or non-canonical pathways in **Figure 2**. Moreover, for a comprehensive overview of the current evidence on molecules that mediate analgesic and anti-inflammatory actions via Nrf2 using *in vivo and in vitro* approaches, we include in **Table 1** examples of compounds that belong to the aforementioned pharmacological classes and the main mechanisms demonstrated in experimental models. In **Table 2**, we provide an overview of molecules tested in completed clinical trials that were discussed in this review. Additionally, we have included molecules that were tested, but no outcome was reported following the completion of the study, for example CXA-10, cytarabine, daunorubicin hydrochloride, fluvastatin, grape powder, idarubicin, L-carnosine, melatonin, resveratrol, RTA 408, valsartan. Data from **Table 2** and other clinical trials that were not included herein can be found at: www.clinicaltrials.gov/ct2/results?term=nrf2&Search=Apply&age_v=&gndr=&type=&rslt=.

In conclusion, modulating Nrf2 activity is a promising approach to achieve homeostasis during inflammatory responses where oxidative stress is an essential player. Nevertheless, a better understanding of how the activation or inhibition of Nrf2 can modulate the course and/or outcome of inflammatory diseases is an important strategy for the discovery of new drugs or the repurposing of drugs that target NRF2. Importantly, recent studies focusing on the development of more specific and potent peptides that target Keap1-Nrf2 PPI are promising. However, the effect of many of these molecules have not been tested *in vivo* yet. Therefore, investigations on their effect, mechanisms of action, and safety will be of great value. Further, translational investigations on the therapeutic effect and safety of new or repurposed modulators of Nrf2 pathway in humans is warranted.

AUTHOR CONTRIBUTIONS

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REFERENCES

Adenuga, D., Caito, S., Yao, H., Sundar, I. K., Hwang, J. W., Chung, S., et al. (2010). Nrf2 deficiency influences susceptibility to steroid resistance via HDAC2 reduction. *Biochem. Biophys. Res. Commun.* 403, 452–456. doi: 10.1016/j.bbrc.2010.11.054

Ahmed, S. M., Luo, L., Namani, A., Wang, X. J., and Tang, X. (2017). Nrf2 signaling pathway: pivotal roles in inflammation. *Biochim. Biophys. Acta* 1863, 585–597. doi: 10.1016/j.bbadis.2016.11.005

Alam, M. M., Okazaki, K., Nguyen, L. T. T., Ota, N., Kitamura, H., Murakami, S., et al. (2017). Glucocorticoid receptor signaling represses the antioxidant response by inhibiting histone acetylation mediated by the transcriptional activator NRF2. *J. Biol. Chem.* 292, 7519–7530. doi: 10.1074/jbc.M116.773960

Al-Rashed, F., Calay, D., Lang, M., Thornton, C. C., Bauer, A., Kiprianos, A., et al. (2018). Celecoxib exerts protective effects in the vascular endothelium via COX-2-independent activation of AMPK-CREB-Nrf2 signalling. *Sci. Rep.* 8:6271. doi: 10.1038/s41598-018-24548-z

Altmeyer, P. J., Matthes, U., Pawlak, F., Hoffmann, K., Frosch, P. J., Ruppert, P., et al. (1994). Antipsoriatic effect of fumaric acid derivatives, Results of a multicenter double-blind study in 100 patients. *J. Am. Acad. Dermatol.* 30, 977–981. doi: 10.1016/S0190-9622(94)70121-0

Alyoussef, A., and Taha, M. (2018). Antitumor activity of sulforaphane in mice model of skin cancer via blocking sulfatase-2. *Exp. Dermatol.* doi: 10.1111/exd.13802 [Epub ahead of print].

Amin, F. U., Shah, S. A., and Kim, M. O. (2017). Vanillic acid attenuates Abeta1-42-induced oxidative stress and cognitive impairment in mice. *Sci. Rep.* 7:40753. doi: 10.1038/srep40753

Antman, E. M., Bennett, J. S., Daugherty, A., Furberg, C., Roberts, H., and Taubert, K. A. (2007). Use of nonsteroidal antiinflammatory drugs: an update for clinicians: a scientific statement from the American Heart Association. *Circulation* 115, 1634–1642. doi: 10.1161/CIRCULATIONAHA.106.181424

Anantharaju, P. G., Gowda, P. C., Vimalambike, M. G., and Madhunapantula, S. V. (2016). An overview on the role of dietary phenolics for the treatment of cancers. *Nutr. J.* 15:99. doi: 10.1186/s12937-016-0217-2

Arruri, V., Komirishetty, P., Areti, A., Dungavath, S. K. N., and Kumar, A. (2017). Nrf2 and NF-kappaB modulation by Plumbagin attenuates functional, behavioural and biochemical deficits in rat model of neuropathic pain. *Pharmacol. Rep.* 69, 625–632. doi: 10.1016/j.pharep.2017.02.006

Athale, J., Ulrich, A., Macgarvey, N. C., Bartz, R. R., Welty-Wolf, K. E., Suliman, H. B., et al. (2012). Nrf2 promotes alveolar mitochondrial biogenesis and resolution of lung injury in *Staphylococcus aureus* pneumonia in mice. *Free Radic. Biol. Med.* 53, 1584–1594. doi: 10.1016/j.freeradbiomed.2012.08.009

Bae, S. H., Sung, S. H., Oh, S. Y., Lim, J. M., Lee, S. K., Park, Y. N., et al. (2013). Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. *Cell Metab.* 17, 73–84. doi: 10.1016/j.cmet.2012.12.002

Bao, S., Nie, X., Ou, R., Wang, C., Ku, P., and Li, K. (2017). Effects of diclofenac on the expression of Nrf2 and its downstream target genes in mosquito fish (*Gambusia affinis*). *Aquat. Toxicol.* 188, 43–53. doi: 10.1016/j.aquatox.2017.04.008

Bar-Or, A., Gold, R., Kappos, L., Arnold, D. L., Giovannoni, G., Selmaj, K., et al. (2013). Clinical efficacy of BG-12 (dimethyl fumarate) in patients with relapsing-remitting multiple sclerosis: subgroup analyses of the DEFINE study. *J. Neurol.* 260, 2297–2305. doi: 10.1007/s00415-013-6954-7

Bauman, J. E., Zang, Y., Sen, M., Li, C., Wang, L., Egner, P. A., et al. (2016). Prevention of carcinogen-induced oral cancer by sulforaphane. *Cancer Preven. Res. (Phila)* 9, 547–557. doi: 10.1158/1940-6207.CAPR-15-0290

Yehuda Greenwald, B. M., Frusic-Zlotkin, M., Soroka, Y., Ben Sasson, S., Bitton, R., Bianco-Peled, H., et al. (2017). Curcumin protects skin against UVB-induced cytotoxicity via the keap1-Nrf2 pathway: the use of a microemulsion delivery system. *Oxid. Med. Cell Longev.* 2017:5205471. doi: 10.1155/2017/5205471

Bensasson, R. V., Zoete, V., Dinkova-Kostova, A. T., and Talalay, P. (2008). Two-step mechanism of induction of the gene expression of a prototypic cancer-protective enzyme by diphenols. *Chem. Res. Toxicol.* 21, 805–812. doi: 10.1021/tx7002883

Best, S. A., and Sutherland, K. D. (2018). "Keaping" a lid on lung cancer: the Keap1-Nrf2 pathway. *Cell Cycle* 17, 1696–1707. doi: 10.1080/15384101.2018.1496756

Biagiotti, S., Menotta, M., Orazi, S., Spapperi, C., Brundu, S., Fraternale, A., et al. (2016). Dexamethasone improves redox state in ataxia telangiectasia cells by promoting an NRF2-mediated antioxidant response. *FEBS J.* 283, 3962–3978. doi: 10.1111/febs.13901

Bloom, D. A., and Jaiswal, A. K. (2003). Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J. Biol. Chem.* 278, 44675–44682. doi: 10.1074/jbc.M307633200

Bodas, M., Mazur, S., Min, T., and Vij, N. (2018). Inhibition of histone-deacetylase activity rescues inflammatory cystic fibrosis lung disease by modulating innate and adaptive immune responses. *Respir. Res.* 19:2. doi: 10.1186/s12931-017-0705-8

Borghi, S. M., Mizokami, S. S., Pinho-Ribeiro, F. A., Fattori, V., Crespigio, J., Clemente-Napimoga, J. T., et al. (2017). The flavonoid quercetin inhibits titanium dioxide (TiO2)-induced chronic arthritis in mice. *J. Nutr. Biochem.* 53, 81–95. doi: 10.1016/j.jnutbio.2017.10.010

Bovill, J. G. (1997). Mechanisms of actions of opioids and non-steroidal anti-inflammatory drugs. *Eur. J. Anaesthesiol. Suppl.* 15, 9–15. doi: 10.1097/00003643-199705001-00003

Bretscher, P., Egger, J., Shamshiev, A., Trotzmuller, M., Kofeler, H., Carreira, E. M., et al. (2015). Phospholipid oxidation generates potent anti-inflammatory lipid mediators that mimic structurally related pro-resolving eicosanoids by activating Nrf2. *EMBO Mol. Med.* 7, 593–607. doi: 10.15252/emmm.201404702

Busillo, J. M., and Cidlowski, J. A. (2013). The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. *Trends Endocrinol. Metab.* 24, 109–119. doi: 10.1016/j.tem.2012.11.005

Bustanji, Y., Taha, M. O., Almasri, I. M., Al-Ghussein, M. A., Mohammad, M. K., and Alkhatib, H. S. (2009). Inhibition of glycogen synthase kinase by curcumin: investigation by simulated molecular docking and subsequent in vitro/in vivo evaluation. *J. Enzyme Inhib. Med. Chem.* 24, 771–778. doi: 10.1080/14756360802364377

Cai, D., Yin, S., Yang, J., Jiang, Q., and Cao, W. (2015). Histone deacetylase inhibition activates Nrf2 and protects against osteoarthritis. *Arthritis Res. Ther.* 17:269. doi: 10.1186/s13075-015-0774-3

Calixto-Campos, C., Carvalho, T. T., Hohmann, M. S. N., Pinho-Ribeiro, F. A., Fattori, V., Manchope, M. F., et al. (2015a). Vanillic acid inhibits inflammatory pain by inhibiting neutrophil recruitment, oxidative stress, cytokine production, and NFκB activation in mice. *J. Nat. Prod.* 78, 1799–1808. doi: 10.1021/acs.jnatprod.5b00246

Calixto-Campos, C., Corrêa, M. P., Carvalho, T. T., Zarpelon, A. C., Hohmann, M. S. N., Rossaneis, A. C., et al. (2015b). Quercetin reduces ehrlich tumor-induced cancer pain in mice. *Anal. Cell. Pathol. (Amsterdam)* 2015, 285708–285708. doi: 10.1155/2015/285708

Cashman, J. N. (1996). The mechanisms of action of NSAIDs in analgesia. *Drugs* 52(Suppl. 5), 13–23. doi: 10.2165/00003495-199600525-00004

Chen, Q., Li, C., Gong, Z., Chan, E. C. Y., Snyder, S. A., and Lam, S. H. (2017). Common deregulated gene expression profiles and morphological changes in developing zebrafish larvae exposed to environmental-relevant high to low concentrations of glucocorticoids. *Chemosphere* 172, 429–439. doi: 10.1016/j.chemosphere.2017.01.036

Choi, E. J., Jung, B. J., Lee, S. H., Yoo, H. S., Shin, E. A., Ko, H. J., et al. (2017). A clinical drug library screen identifies clobetasol propionate as an NRF2 inhibitor with potential therapeutic efficacy in KEAP1 mutant lung cancer. *Oncogene* 36, 5285–5295. doi: 10.1038/onc.2017.153

Clark, A. R., and Belvisi, M. G. (2012). Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor. *Pharmacol. Ther.* 134, 54–67. doi: 10.1016/j.pharmthera.2011.12.004

Creelan, B. C., Gabrilovich, D. I., Gray, J. E., Williams, C. C., Tanvetyanon, T., Haura, E. B., et al. (2017). Safety, pharmacokinetics, and pharmacodynamics of oral omaveloxolone (RTA 408), a synthetic triterpenoid, in a first-in-human trial of patients with advanced solid tumors. *Onco Targets Ther.* 10, 4239–4250. doi: 10.2147/OTT.S136992

Cuadrado, A., Manda, G., Hassan, A., Alcaraz, M. J., Barbas, C., Daiber, A., et al. (2018). Transcription factor NRF2 as a therapeutic target for chronic diseases: a systems medicine approach. *Pharmacol. Rev.* 70, 348–383. doi: 10.1124/pr.117.014753

Dayoub, R., Vogel, A., Schuett, J., Lupke, M., Spieker, S. M., Kettern, N., et al. (2013). Nrf2 activates augmenter of liver regeneration (ALR) via antioxidant response element and links oxidative stress to liver regeneration. *Mol. Med.* 19, 237–244. doi: 10.2119/molmed.2013.00027

De Bosscher, K., Vanden Berghe, W., and Haegeman, G. (2003). The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr. Rev.* 24, 488–522. doi: 10.1210/er.2002-0006

de Zeeuw, D., Akizawa, T., Audhya, P., Bakris, G. L., Chin, M., Christ-Schmidt, H., et al. (2013). Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. *N. Engl. J. Med.* 369, 2492–2503. doi: 10.1056/NEJMoa1306033

DeNicola, G. M., Karreth, F. A., Humpton, T. J., Gopinathan, A., Wei, C., Frese, K., et al. (2011). Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475, 106–109. doi: 10.1038/nature10189

Di, W., Shi, X., Lv, H., Liu, J., Zhang, H., Li, Z., et al. (2016). Activation of the nuclear factor E2-related factor 2/anitioxidant response element alleviates the nitroglycerin-induced hyperalgesia in rats. *J. Headache Pain* 17:99. doi: 10.1186/s10194-016-0694-x

Dinkova-Kostova, A. T., Kostov, R. V., and Canning, P. (2017). Keap1, the cysteine-based mammalian intracellular sensor for electrophiles and oxidants. *Arch. Biochem. Biophys.* 617, 84–93. doi: 10.1016/j.abb.2016.08.005

Dinkova-Kostova, A. T., Massiah, M. A., Bozak, R. E., Hicks, R. J., and Talalay, P. (2001). Potency of michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3404–3409. doi: 10.1073/pnas.051632198

Domitrovic, R., Skoda, M., Vasiljev Marchesi, V., Cvijanovic, O., Pernjak Pugel, E., and Stefan, M. B. (2013). Rosmarinic acid ameliorates acute liver damage and fibrogenesis in carbon tetrachloride-intoxicated mice. *Food Chem. Toxicol.* 51, 370–378. doi: 10.1016/j.fct.2012.10.021

Elikkottil, J., Gupta, P., and Gupta, K. (2009). The analgesic potential of cannabinoids. *J. Opioid. Manage.* 5, 341–357. doi: 10.5055/jom.2009.0034

Ellrichmann, G., Petrasch-Parwez, E., Lee, D. H., Reick, C., Arning, L., Saft, C., et al. (2011). Efficacy of fumaric acid esters in the R6/2 and YAC128 models of Huntington's disease. *PLoS One* 6:e16172. doi: 10.1371/journal.pone.0016172

Fahey, J. W., Haristoy, X., Dolan, P. M., Kensler, T. W., Scholtus, I., Stephenson, K. K., et al. (2002). Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[a]pyrene-induced stomach tumors. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7610–7615. doi: 10.1073/pnas.112203099

Fahey, J. W., Zalcmann, A. T., and Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56, 5–51. doi: 10.1016/S0031-9422(00)00316-2

Fattori, V., Hohmann, M. S., Rossaneis, A. C., Pinho-Ribeiro, F. A., and Verri, W. A. (2016). Capsaicin: current understanding of its mechanisms and therapy of pain and other pre-clinical and clinical uses. *Molecules* 21:E844. doi: 10.3390/molecules21070844

Fattori, V., Pinho-Ribeiro, F. A., Borghi, S. M., Alves-Filho, J. C., Cunha, T. M., Cunha, F. Q., et al. (2015). Curcumin inhibits superoxide anion-induced pain-like behavior and leukocyte recruitment by increasing Nrf2 expression and reducing NF-κB activation. *Inflamm. Res.* 64, 993–1003. doi: 10.1007/s00011-015-0885-y

Ferrandiz, M. L., Nacher-Juan, J., and Alcaraz, M. J. (2018). Nrf2 as a therapeutic target for rheumatic diseases. *Biochem. Pharmacol.* 152, 338–346. doi: 10.1016/j.bcp.2018.04.010

Fimognari, C., and Hrelia, P. (2007). Sulforaphane as a promising molecule for fighting cancer. *Mutat. Res.* 635, 90–104. doi: 10.1016/j.mrrev.2006.10.004

Finkel, T. (2003). Oxidant signals and oxidative stress. *Curr. Opin. Cell Biol.* 15, 247–254. doi: 10.1016/S0955-0674(03)00002-4

Ganan-Gomez, I., Wei, Y., Yang, H., Boyano-Adanez, M. C., and Garcia-Manero, G. (2013). Oncogenic functions of the transcription factor Nrf2. *Free Radic. Biol. Med.* 65, 750–764. doi: 10.1016/j.freeradbiomed.2013.06.041

Gazaryan, I. G., and Thomas, B. (2016). The status of Nrf2-based therapeutics: current perspectives and future prospects. *Neural Regen. Res.* 11, 1708–1711. doi: 10.4103/1673-5374.194706

Ghoreschi, K., Bruck, J., Kellerer, C., Deng, C., Peng, H., Rothfuss, O., et al. (2011). Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells. *J. Exp. Med.* 208, 2291–2303. doi: 10.1084/jem.20100977

Gong, L., Thorn, C. F., Bertagnolli, M. M., Grosser, T., Altman, R. B., and Klein, T. E. (2012). Celecoxib pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenet. Genomics* 22, 310–318. doi: 10.1097/FPC.0b013e32834f94cb

Gorrini, C., Baniasadi, P. S., Harris, I. S., Silvester, J., Inoue, S., Snow, B., et al. (2013). BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. *J. Exp. Med.* 210, 1529–1544. doi: 10.1084/jem.20121337

Groeneweg, F. L., Karst, H., De Kloet, E. R., and Joels, M. (2011). Rapid non-genomic effects of corticosteroids and their role in the central stress response. *J. Endocrinol.* 209, 153–167. doi: 10.1530/JOE-10-0472

Guan, D., Ohta, H., Tawil, T., Liddle, R. A., and Green, G. M. (1990). CCK-releasing activity of rat intestinal secretion: effect of atropine and comparison with monitor peptide. *Pancreas* 5, 677–684. doi: 10.1097/00006676-199011000-00007

Guo, Y., Wu, R., Gaspar, J. M., Sargsyan, D., Su, Z. Y., Zhang, C., et al. (2018). DNA methylome and transcriptome alterations and cancer prevention by curcumin in colitis-accelerated colon cancer in mice. *Carcinogenesis* 39, 669–680. doi: 10.1093/carcin/bgy043

Gupta, S., Adhami, V. M., Subbarayan, M., Maclennan, G. T., Lewin, J. S., Hafeli, U. O., et al. (2004). Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model. *Cancer Res.* 64, 3334–3343. doi: 10.1158/0008-5472.CAN-03-2422

Hancock, R., Bertrand, H. C., Tsujita, T., Naz, S., El-Bakry, A., Laoruchupong, J., et al. (2012). Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction. *Free Radic. Biol. Med.* 52, 444–451. doi: 10.1016/j.freeradbiomed.2011.10.486

Hancock, R., Schaap, M., Pfister, H., and Wells, G. (2013). Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction with improved binding and cellular activity. *Org. Biomol. Chem.* 11, 3553–3557. doi: 10.1039/c3ob40249e

Harvey, C. J., Thimmulappa, R. K., Sethi, S., Kong, X., Yarmus, L., Brown, R. H., et al. (2011). Targeting Nrf2 signaling improves bacterial clearance by alveolar macrophages in patients with COPD and in a mouse model. *Sci. Transl. Med.* 3:78ra32. doi: 10.1126/scitranslmed.3002042

Hast, B. E., Goldfarb, D., Mulvaney, K. M., Hast, M. A., Siesser, P. F., Yan, F., et al. (2013). Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination. *Cancer Res.* 73, 2199–2210. doi: 10.1158/0008-5472.CAN-12-4400

Hayes, J. D., and Dinkova-Kostova, A. T. (2014). The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* 39, 199–218. doi: 10.1016/j.tibs.2014.02.002

Hayes, J. D., Mcmahon, M., Chowdhry, S., and Dinkova-Kostova, A. T. (2010). Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. *Antioxid. Redox. Signal.* 13, 1713–1748. doi: 10.1089/ars.2010.3221

Healy, Z. R., Lee, N. H., Gao, X., Goldring, M. B., Talalay, P., Kensler, T. W., et al. (2005). Divergent responses of chondrocytes and endothelial cells to shear stress: cross-talk among COX-2, the phase 2 response, and apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14010–14015. doi: 10.1073/pnas.0506620102

Hearn, L., Moore, R. A., Derry, S., Wiffen, P. J., and Phillips, T. (2014). Desipramine for neuropathic pain in adults. *Cochrane Database Syst. Rev.* 9:CD011003. doi: 10.1002/14651858.CD011003

Hebbar, P. B., and Archer, T. K. (2003). Chromatin remodeling by nuclear receptors. *Chromosoma* 111, 495–504. doi: 10.1007/s00412-003-0232-x

Hennig, P., Garstkiewicz, M., Grossi, S., Di Filippo, M., French, L. E., and Beer, H. D. (2018). The crosstalk between Nrf2 and inflammasomes. *Int. J. Mol. Sci.* 19:E562. doi: 10.3390/ijms19020562

Homma, S., Ishii, Y., Morishima, Y., Yamadori, T., Matsuno, Y., Haraguchi, N., et al. (2009). Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin. Cancer Res.* 15, 3423–3432. doi: 10.1158/1078-0432.CCR-08-2822

Hong, D. S., Kurzrock, R., Supko, J. G., He, X., Naing, A., Wheler, J., et al. (2012). A phase I first-in-human trial of bardoxolone methyl in patients with advanced solid tumors and lymphomas. *Clin. Cancer Res.* 18, 3396–3406. doi: 10.1158/1078-0432.CCR-11-2703

Hsu, W. H., Lee, B. H., Chang, Y. Y., Hsu, Y. W., and Pan, T. M. (2013). A novel natural Nrf2 activator with PPARgamma-agonist (monascin) attenuates the

toxicity of methylglyoxal and hyperglycemia. *Toxicol. Appl. Pharmacol.* 272, 842–851. doi: 10.1016/j.taap.2013.07.004

Hu, C., Eggler, A. L., Mesecar, A. D., and Van Breemen, R. B. (2011). Modification of keap1 cysteine residues by sulforaphane. *Chem. Res. Toxicol.* 24, 515–521. doi: 10.1021/tx100389r

Hu, R., Saw, C. L., Yu, R., and Kong, A. N. (2010). Regulation of NF-E2-related factor 2 signaling for cancer chemoprevention: antioxidant coupled with antiinflammatory. *Antioxid. Redox. Signal.* 13, 1679–1698. doi: 10.1089/ars.2010.3276

Iida, K., Itoh, K., Kumagai, Y., Oyasu, R., Hattori, K., Kawai, K., et al. (2004). Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res.* 64, 6424–6431. doi: 10.1158/0008-5472.CAN-04-1906

Ishtiaq, S. M., Khan, J. A., and Arshad, M. I. (2018). Psychosocial-stress, liver regeneration and weight gain: a conspicuous pathophysiological triad. *Cell Physiol. Biochem.* 46, 1–8. doi: 10.1159/000488378

Itoh, K., Mimura, J., and Yamamoto, M. (2010). Discovery of the negative regulator of Nrf2. Keap1: a historical overview. *Antioxid. Redox. Signal.* 13, 1665–1678. doi: 10.1089/ars.2010.3222

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., et al. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76–86. doi: 10.1101/gad.13.1.76

Janssen-Heininger, Y. M., Mossman, B. T., Heintz, N. H., Forman, H. J., Kalyanaraman, B., Finkel, T., et al. (2008). Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radic. Biol. Med.* 45, 1–17. doi: 10.1016/j.freeradbiomed.2008.03.011

Jeddi, F., Soozangar, N., Sadeghi, M. R., Somi, M. H., and Samadi, N. (2017). Contradictory roles of Nrf2/Keap1 signaling pathway in cancer prevention/promotion and chemoresistance. *DNA Repair (Amst.)* 54, 13–21. doi: 10.1016/j.dnarep.2017.03.008

Jeong, H. S., Ryoo, I. G., and Kwak, M. K. (2015). Regulation of the expression of renal drug transporters in KEAP1-knockdown human tubular cells. *Toxicol. In Vitro* 29, 884–892. doi: 10.1016/j.tiv.2015.03.013

Ji, L. L., Sheng, Y. C., Zheng, Z. Y., Shi, L., and Wang, Z. T. (2015). The involvement of p62-Keap1-Nrf2 antioxidative signaling pathway and JNK in the protection of natural flavonoid quercetin against hepatotoxicity. *Free Radic. Biol. Med.* 85, 12–23. doi: 10.1016/j.freeradbiomed.2015.03.035

Jian, Z., Tang, L., Yi, X., Liu, B., Zhang, Q., Zhu, G., et al. (2016). Aspirin induces Nrf2-mediated transcriptional activation of haem oxygenase-1 in protection of human melanocytes from H2 O2 -induced oxidative stress. *J. Cell Mol. Med.* 20, 1307–1318. doi: 10.1111/jcmm.12812

Jiang, X., Liu, Y., Ma, L., Ji, R., Qu, Y., Xin, Y., et al. (2018). Chemopreventive activity of sulforaphane. *Drug Des. Dev. Ther.* 12, 2905–2913. doi: 10.2147/DDDT.S100534

Jiang, Z. Y., Lu, M. C., and You, Q. D. (2016). Discovery and development of Kelch-like ECH-associated protein 1. nuclear factor erythroid 2-related factor 2 (KEAP1:NRF2) protein-protein interaction inhibitors: achievements, challenges, and future directions. *J. Med. Chem.* 59, 10837–10858. doi: 10.1021/acs.jmedchem.6b00586

Jimenez-Osorio, A. S., Garcia-Nino, W. R., Gonzalez-Reyes, S., Alvarez-Mejia, A. E., Guerra-Leon, S., Salazar-Segovia, J., et al. (2016). The effect of dietary supplementation with curcumin on redox status and Nrf2 activation in patients with nondiabetic or diabetic proteinuric chronic kidney disease: a pilot study. *J. Ren. Nutr.* 26, 237–244. doi: 10.1053/j.jrn.2016.01.013

Joo, M., Lee, J. Y., and Kim, K. H. (2017). 15-deoxy-Δ12,14-prostaglandin J2 enhances the interaction between Keap1 and p62/SQSTM1. *J. Immunol.* 198:222.222.

Joung, E. J., Li, M. H., Lee, H. G., Somparn, N., Jung, Y. S., Na, H. K., et al. (2007). Capsaicin induces heme oxygenase-1 expression in HepG2 cells via activation of PI3K-Nrf2 signaling: NAD(P)H:quinone oxidoreductase as a potential target. *Antioxid. Redox. Signal.* 9, 2087–2098. doi: 10.1089/ars.2007.1827

Jozkowicz, A., Was, H., and Dulak, J. (2007). Heme oxygenase-1 in tumors: is it a false friend? *Antioxid. Redox Signal.* 9, 2099–2117. doi: 10.1089/ars.2007.1659

Juknat, A., Pietr, M., Kozela, E., Rimmerman, N., Levy, R., Gao, F., et al. (2013). Microarray and pathway analysis reveal distinct mechanisms underlying cannabinoid-mediated modulation of LPS-induced activation of BV-2 microglial cells. *PLoS One* 8:e61462. doi: 10.1371/journal.pone.0061462

Kallifatidis, G., Rausch, V., Baumann, B., Apel, A., Beckermann, B. M., Groth, A., et al. (2009). Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. *Gut* 58, 949–963. doi: 10.1136/gut.2008.149039

Kamble, S. M., Patel, H. M., Goyal, S. N., Noolvi, M. N., Mahajan, U. B., Ojha, S., et al. (2017). In silico evidence for binding of pentacyclic triterpenoids to Keap1-Nrf2 protein-protein binding site. *Comb. Chem. High Throughput Screen* 20, 215–234. doi: 10.2174/1386207319666161214111822

Kansanen, E., Kuosmanen, S. M., Leinonen, H., and Levonen, A. L. (2013). The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. *Redox Biol.* 1, 45–49. doi: 10.1016/j.redox.2012.10.001

Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., et al. (2005). Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol.* 25, 1089–1099. doi: 10.1128/MCB.25.3.1089-1099.2005

Kataoka, K., Handa, H., and Nishizawa, M. (2001). Induction of cellular antioxidative stress genes through heterodimeric transcription factor Nrf2/small Maf by antirheumatic gold(I) compounds. *J. Biol. Chem.* 276, 34074–34081. doi: 10.1074/jbc.M105383200

Kensler, T. W., and Wakabayashi, N. (2010). Nrf2: friend or foe for chemoprevention? *Carcinogenesis* 31, 90–99. doi: 10.1093/carcin/bgp231

Khan, N. M., and Haqqi, T. M. (2018). Epigenetics in osteoarthritis: potential of HDAC inhibitors as therapeutics. *Pharmacol. Res.* 128, 73–79. doi: 10.1016/j.phrs.2017.08.007

Ki, S. H., Cho, I. J., Choi, D. W., and Kim, S. G. (2005). Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. *Mol. Cell. Biol.* 25, 4150–4165. doi: 10.1128/MCB.25.10.4150-4165.2005

Kim, K. H., Sadikot, R. T., and Joo, M. (2016). Therapeutic effect of ent-kaur-16-en-19-oic acid on neutrophilic lung inflammation and sepsis is mediated by Nrf2. *Biochem. Biophys. Res. Commun.* 474, 534–540. doi: 10.1016/j.bbrc.2016.04.122

Kim, N. H., Oh, M. K., Park, H. J., and Kim, I. S. (2010). Auranofin, a gold(I)-containing antirheumatic compound, activates Keap1/Nrf2 signaling via Rac1/iNOS signal and mitogen-activated protein kinase activation. *J. Pharmacol. Sci.* 113, 246–254. doi: 10.1254/jphs.09330FP

Kim, S. G., Kim, Y. M., Choi, Y. H., Lee, M. G., Choi, J. Y., Han, J. Y., et al. (2010). Pharmacokinetics of oltipraz and its major metabolite (RM) in patients with liver fibrosis or cirrhosis: relationship with suppression of circulating TGF-beta1. *Clin. Pharmacol. Ther.* 88, 360–368. doi: 10.1038/clpt.2010.89

Kim, W., Kim, B. G., Lee, J. S., Lee, C. K., Yeon, J. E., Chang, M. S., et al. (2017a). Randomised clinical trial: the efficacy and safety of oltipraz, a liver X receptor alpha-inhibitory dithiolethione in patients with non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* 45, 1073–1083. doi: 10.1111/apt.13981

Kim, W., Lee, H. N., Jang, J. H., Kim, S. H., Lee, Y. H., Hahn, Y. I., et al. (2017b). 15-deoxy-delta(12,14)-prostaglandin J2 exerts proresolving effects through nuclear factor E2-related factor 2-induced expression of CD36 and heme oxygenase-1. *Antioxid. Redox Signal.* 27, 1412–1431. doi: 10.1089/ars.2016.6754

Kim, Y. M., Kim, H. J., and Chang, K. C. (2015). Glycyrrhizin reduces HMGB1 secretion in lipopolysaccharide-activated RAW 264.7 cells and endotoxemic mice by p38/Nrf2-dependent induction of HO-1. *Int. Immunopharmacol.* 26, 112–118. doi: 10.1016/j.intimp.2015.03.014

Knezevic, N. N., Yekkirala, A., and Yaksh, T. L. (2017). Basic/translational development of forthcoming opioid- and nonopioid-targeted pain therapeutics. *Anesth. Analg.* 125, 1714–1732. doi: 10.1213/ANE.0000000000002442

Kou, X., Kirbergerb, M., and Chen, Y. N. (2013). Natural products for cancer prevention associated with Nrf2–ARE pathway. *Food Sci. Hum. Wellness* 2, 22–28. doi: 10.1016/j.fshw.2013.01.001

Kratschmar, D. V., Calabrese, D., Walsh, J., Lister, A., Birk, J., Appenzeller-Herzog, C., et al. (2012). Suppression of the Nrf2-dependent antioxidant response by glucocorticoids and 11beta-HSD1-mediated glucocorticoid activation in hepatic cells. *PLoS One* 7:e36774. doi: 10.1371/journal.pone.0036774

Kudoh, K., Uchinami, H., Yoshioka, M., Seki, E., and Yamamoto, Y. (2014). Nrf2 activation protects the liver from ischemia/reperfusion injury in mice. *Ann. Surg.* 260, 118–127. doi: 10.1097/SLA.0000000000000287

Kuhn, A., Landmann, A., Patsinakidis, N., Ruland, V., Nozinic, S., Perusquia Ortiz, A. M., et al. (2016). Fumaric acid ester treatment in cutaneous lupus

erythematosus (CLE): a prospective, open-label, phase II pilot study. *Lupus* 25, 1357–1364. doi: 10.1177/0961203316644335

Kumar, H., Kim, I. S., More, S. V., Kim, B. W., and Choi, D. K. (2014). Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases. *Nat. Prod. Rep*. 31, 109–139. doi: 10.1039/C3NP70065H

Kumar, V., Kumar, S., Hassan, M., Wu, H., Thimmulappa, R. K., Kumar, A., et al. (2011). Novel chalcone derivatives as potent Nrf2 activators in mice and human lung epithelial cells. *J. Med. Chem.* 54, 4147–4159. doi: 10.1021/jm2002348

Lau, A., Wang, X. J., Zhao, F., Villeneuve, N. F., Wu, T., Jiang, T., et al. (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol. Cell. Biol.* 30, 3275–3285. doi: 10.1128/MCB.00248-10

Lee, S. E., Kim, G. D., Yang, H., Son, G. W., Park, H. R., Cho, J. J., et al. (2015). Effects of eicosapentaenoic acid on the cytoprotection through Nrf2-mediated heme oxygenase-1 in human endothelial cells. *J. Cardiovasc. Pharmacol.* 66, 108–117. doi: 10.1097/FJC.0000000000000251

Leone, A., Diorio, G., Sexton, W., Schell, M., Alexandrow, M., Fahey, J. W., et al. (2017). Sulforaphane for the chemoprevention of bladder cancer: molecular mechanism targeted approach. *Oncotarget* 8, 35412–35424. doi: 10.18632/oncotarget.16015

Lewis, P. A., Mullany, D. V., Courtney, M., and Coyer, F. (2006). Australasian trends in intra-aortic balloon counterpulsation weaning: results of a postal survey. *Crit. Care Resuscitation* 8, 361–367.

Li, B., Li, R., Xie, X., Song, X., Zhang, J., Wang, Y., et al. (2013). [Anti-oxidative effects of dexamethasone on mice with experimental autoimmune encephalomyelitis]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 29, 1155–1158.

Li, H., Wood, J. T., Whitten, K. M., Vadivel, S. K., Seng, S., Makriyannis, A., et al. (2013). Inhibition of fatty acid amide hydrolase activates Nrf2 signalling and induces heme oxygenase 1 transcription in breast cancer cells. *Br. J. Pharmacol.* 170, 489–505. doi: 10.1111/bph.12111

Li, C., Cheng, L., Wu, H., He, P., Zhang, Y., Yang, Y., et al. (2018). Activation of the KEAP1NRF2ARE signaling pathway reduces oxidative stress in Hep2 cells. *Mol. Med. Rep.* 18, 2541–2550.

Li, S., Yang, C., Fang, X., Zhan, G., Huang, N., Gao, J., et al. (2018). Role of Keap1-Nrf2 signaling in anhedonia symptoms in a rat model of chronic neuropathic pain: improvement with sulforaphane. *Front. Pharmacol*. 9:887. doi: 10.3389/fphar.2018.00887

Li, Q., and Zhang, X. (2015). Epigallocatechin-3-gallate attenuates bone cancer pain involving decreasing spinal tumor necrosis factor-alpha expression in a mouse model. *Int. Immunopharmacol.* 29, 818–823. doi: 10.1016/j.intimp.2015.08.037

Li, W., Suwanwela, N. C., and Patumraj, S. (2016). Curcumin by down-regulating NF-kB and elevating Nrf2, reduces brain edema and neurological dysfunction after cerebral I/R. *Microvasc. Res.* 106, 117–127. doi: 10.1016/j.mvr.2015.12.008

Li, X. Y., Luo, B. L., Wang, L. J., Zhang, W. D., and Liu, Z. G. (2015). 15-Deoxy-prostaglandin J2 anti-inflammation in a rat model of chronic obstructive pulmonary disease and human bronchial epithelial cells via Nrf2 activation. *Genet. Mol. Res.* 14, 14037–14042. doi: 10.4238/2015.October.29.22

Lin, H. Y., Yeh, W. L., Huang, B. R., Lin, C., Lai, C. H., Lin, H., et al. (2012). Desipramine protects neuronal cell death and induces heme oxygenase-1 expression in Mes23.5 dopaminergic neurons. *PLoS One* 7:e50138. doi: 10.1371/journal.pone.0050138

Lin, K., Yang, R., Zheng, Z., Zhou, Y., Geng, Y., Hu, Y., et al. (2017). Sulforaphane-cysteine-induced apoptosis via phosphorylated ERK1/2-mediated maspin pathway in human non-small cell lung cancer cells. *Cell Death Discov.* 3:17025. doi: 10.1038/cddiscovery.2017.25

Lin, S. X., Lisi, L., Dello Russo, C., Polak, P. E., Sharp, A., Weinberg, G., et al. (2011). The anti-inflammatory effects of dimethyl fumarate in astrocytes involve glutathione and haem oxygenase-1. *ASN Neuro* 3:e00055. doi: 10.1042/AN20100033

Linker, R. A., Lee, D. H., Ryan, S., Van Dam, A. M., Conrad, R., Bista, P., et al. (2011). Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain* 134, 678–692. doi: 10.1093/brain/awq386

Lu, M. C., Jiao, Q., Liu, T., Tan, S. J., Zhou, H. S., You, Q. D., et al. (2018). Discovery of a head-to-tail cyclic peptide as the Keap1-Nrf2 protein-protein interaction inhibitor with high cell potency. *Eur. J. Med. Chem.* 143, 1578–1589. doi: 10.1016/j.ejmech.2017.10.052

Ma, J., Cai, H., Wu, T., Sobhian, B., Huo, Y., Alcivar, A., et al. (2012). PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function. *Mol. Cell. Biol.* 32, 1506–1517. doi: 10.1128/MCB.06271-11

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Ma, S., Paiboonrungruan, C., Yan, T., Williams, K. P., Major, M. B., and Chen, X. L. (2018). Targeted therapy of esophageal squamous cell carcinoma: the NRF2 signaling pathway as target. *Ann. N. Y. Acad. Sci.* 1434, 164–172. doi: 10.1111/nyas.13681

Maioli, N. A., Zarpelon, A. C., Mizokami, S. S., Calixto-Campos, C., Guazelli, C. F. S., Hohmann, M. S. N., et al. (2015). The superoxide anion donor, potassium superoxide, induces pain and inflammation in mice through production of reactive oxygen species and cyclooxygenase-2. *Braz. J. Med. Biol. Res.* 48, 321–331. doi: 10.1590/1414-431X20144187

Majkova, Z., Layne, J., Sunkara, M., Morris, A. J., Toborek, M., and Hennig, B. (2011). Omega-3 fatty acid oxidation products prevent vascular endothelial cell activation by coplanar polychlorinated biphenyls. *Toxicol. Appl. Pharmacol.* 251, 41–49. doi: 10.1016/j.taap.2010.11.013

Manandhar, S., Cho, J. M., Kim, J. A., Kensler, T. W., and Kwak, M. K. (2007). Induction of Nrf2-regulated genes by 3H-1, 2-dithiole-3-thione through the ERK signaling pathway in murine keratinocytes. *Eur. J. Pharmacol.* 577, 17–27. doi: 10.1016/j.ejphar.2007.08.018

Manchope, M. F., Artero, N. A., Fattori, V., Mizokami, S. S., Pitol, D. L., Issa, J. P. M., et al. (2018). Naringenin mitigates titanium dioxide (TiO2)-induced chronic arthritis in mice: role of oxidative stress, cytokines, and NFkappaB. *Inflamm. Res.* 67, 997–1012. doi: 10.1007/s00011-018-1195-y

Manchope, M. F., Calixto-Campos, C., Coelho-Silva, L., Zarpelon, A. C., Pinho-Ribeiro, F. A., Georgetti, S. R., et al. (2016). Naringenin inhibits superoxide anion-induced inflammatory pain: role of oxidative stress, cytokines, Nrf-2 and the NO-cGMP-PKG-KATPChannel signaling pathway. *PLoS One* 11:e0153015. doi: 10.1371/journal.pone.0153015

Manea, A., Manea, S. A., Gafencu, A. V., and Raicu, M. (2007). Regulation of NADPH oxidase subunit p22(phox) by NF-kB in human aortic smooth muscle cells. *Arch. Physiol. Biochem.* 113, 163–172. doi: 10.1080/13813450701531235

Martinez, R. M., Fattori, V., Saito, P., Melo, C. B. P., Borghi, S. M., Pinto, I. C., et al. (2018). Lipoxin A4 inhibits UV radiation-induced skin inflammation and oxidative stress in mice. *J. Dermatol. Sci.* doi: 10.1016/j.jdermsci.2018.04.014 [Epub ahead of print].

Mauro, C., Leow, S. C., Anso, E., Rocha, S., Thotakura, A. K., Tornatore, L., et al. (2011). NF-kappaB controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. *Nat. Cell Biol.* 13, 1272–1279. doi: 10.1038/ncb2324

McDonnell, C., Leanez, S., and Pol, O. (2017). The induction of the transcription factor Nrf2 enhances the antinociceptive effects of delta-opioid receptors in diabetic mice. *PLoS One* 12:e0180998. doi: 10.1371/journal.pone.0180998

McKay, L. I., and Cidlowski, J. A. (1999). Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.* 20, 435–459.

Mehrotra, A., Shanbhag, R., Chamallamudi, M. R., Singh, V. P., and Mudgal, J. (2011). Ameliorative effect of caffeic acid against inflammatory pain in rodents. *Eur. J. Pharmacol.* 666, 80–86. doi: 10.1016/j.ejphar.2011.05.039

Miner, J. N., Hong, M. H., and Negro-Vilar, A. (2005). New and improved glucocorticoid receptor ligands. *Expert Opin. Investig. Drugs* 14, 1527–1545. doi: 10.1517/13543784.14.12.1527

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxid. Redox. Signal.* 20, 1126–1167. doi: 10.1089/ars.2012.5149

Miyazaki, Y., Shimizu, A., Pastan, I., Taguchi, K., Naganuma, E., Suzuki, T., et al. (2014). Keap1 inhibition attenuates glomerulosclerosis. *Nephrol. Dial. Transplant.* 29, 783–791. doi: 10.1093/ndt/gfu002

Mizokami, S. S., Arakawa, N. S., Ambrosio, S. R., Zarpelon, A. C., Casagrande, R., Cunha, T. M., et al. (2012). Kaurenoic acid from *Sphagneticola trilobata* inhibits inflammatory pain: effect on cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. *J. Nat. Prod.* 75, 896–904. doi: 10.1021/np200989t

More, S. V., and Choi, D. K. (2015). Promising cannabinoid-based therapies for Parkinson's disease: motor symptoms to neuroprotection. *Mol. Neurodegener.* 10:17. doi: 10.1186/s13024-015-0012-0

Morimitsu, Y., Nakagawa, Y., Hayashi, K., Fujii, H., Kumagai, T., Nakamura, Y., et al. (2002). A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J. Biol. Chem.* 277, 3456–3463. doi: 10.1074/jbc.M110244200

Mrowietz, U., Szepietowski, J. C., Loewe, R., Van De Kerkhof, P., Lamarca, R., Ocker, W. G., et al. (2017). Efficacy and safety of LAS41008 (dimethyl fumarate) in adults with moderate-to-severe chronic plaque psoriasis: a randomized, double-blind, Fumaderm((R)) – And placebo-controlled trial (BRIDGE). *Br. J. Dermatol.* 176, 615–623. doi: 10.1111/bjd.14947

Nagaich, A. K., Rayasam, G. V., Martinez, E. D., Becker, M., Qiu, Y., Johnson, T. A., et al. (2004). Subnuclear trafficking and gene targeting by steroid receptors. *Ann. N. Y. Acad. Sci.* 1024, 213–220. doi: 10.1196/annals.1321.002

Nguyen, T., Nioi, P., and Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem.* 284, 13291–13295. doi: 10.1074/jbc.R900010200

Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., and Pickett, C. B. (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J. Biol. Chem.* 278, 4536–4541. doi: 10.1074/jbc.M207293200

Ockenfels, H. M., Schultewolter, T., Ockenfels, G., Funk, R., and Goos, M. (1998). The antipsoriatic agent dimethylfumarate immunomodulates T-cell cytokine secretion and inhibits cytokines of the psoriatic cytokine network. *Br. J. Dermatol.* 139, 390–395. doi: 10.1046/j.1365-2133.1998.02400.x

Paiva, C. N., and Bozza, M. T. (2014). Are reactive oxygen species always detrimental to pathogens? *Antioxid. Redox Signal.* 20, 1000–1037. doi: 10.1089/ars.2013.5447

Pang, C., Zheng, Z., Shi, L., Sheng, Y., Wei, H., Wang, Z., et al. (2016). Caffeic acid prevents acetaminophen-induced liver injury by activating the Keap1-Nrf2 antioxidative defense system. *Free Radic. Biol. Med.* 91, 236–246. doi: 10.1016/j.freeradbiomed.2015.12.024

Patwardhan, J., and Bhatt, P. (2015). Ultraviolet-B protective effect of flavonoids from *Eugenia caryophylata* on human dermal fibroblast cells. *Pharmacogn. Mag.* 11, S397–S406. doi: 10.4103/0973-1296.168979

Perera, R. M., and Bardeesy, N. (2011). Cancer: when antioxidants are bad. *Nature* 475, 43–44. doi: 10.1038/475043a

Pergola, P. E., Raskin, P., Toto, R. D., Meyer, C. J., Huff, J. W., Grossman, E. B., et al. (2011). Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N. Engl. J. Med.* 365, 327–336. doi: 10.1056/NEJMoa1105351

Pinho-Ribeiro, F. A., Fattori, V., Zarpelon, A. C., Borghi, S. M., Staurengo-Ferrari, L., Carvalho, T. T., et al. (2016a). Pyrrolidine dithiocarbamate inhibits superoxide anion-induced pain and inflammation in the paw skin and spinal cord by targeting NF-(B and oxidative stress. *Inflammopharmacology* 24, 97–107. doi: 10.1007/s10787-016-0266-3

Pinho-Ribeiro, F. A., Zarpelon, A. C., Fattori, V., Manchope, M. F., Mizokami, S. S., Casagrande, R., et al. (2016b). Naringenin reduces inflammatory pain in mice. *Neuropharmacology* 105, 508–519. doi: 10.1016/j.neuropharm.2016.02.019

Pinho-Ribeiro, F. A., Zarpelon, A. C., Mizokami, S. S., Borghi, S. M., Bordignon, J., Silva, R. L., et al. (2016c). The citrus flavonone naringenin reduces lipopolysaccharide-induced inflammatory pain and leukocyte recruitment by inhibiting NF-(B activation. *J. Nutr. Biochem.* 33, 8–14. doi: 10.1016/j.jnutbio.2016.03.013

Pinho-Ribeiro, F. A., Hohmann, M. S. N., Borghi, S. M., Zarpelon, A. C., Guazelli, C. F. S., Manchope, M. F., et al. (2015). Protective effects of the flavonoid hesperidin methyl chalcone in inflammation and pain in mice: role of TRPV1, oxidative stress, cytokines and NF-κB. *Chem. Biol. Interact* 228, 88–99. doi: 10.1016/j.cbi.2015.01.011

Pinho-Ribeiro, F. A., Verri, W. A., and Chiu, I. M. (2017). Nociceptor sensory neuron–immune interactions in pain and inflammation. *Trends Immunol.* 38, 5–19. doi: 10.1016/j.it.2016.10.001

Pledgie-Tracy, A., Sobolewski, M. D., and Davidson, N. E. (2007). Sulforaphane induces cell type-specific apoptosis in human breast cancer cell lines. *Mol. Cancer Ther.* 6, 1013–1021. doi: 10.1158/1535-7163.MCT-06-0494

Powers, J. C., Asgian, J. L., Ekici, O. D., and James, K. E. (2002). Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem. Rev.* 102, 4639–4750. doi: 10.1021/cr010182v

Prochaska, H. J., De Long, M. J., and Talalay, P. (1985). On the mechanisms of induction of cancer-protective enzymes: a unifying proposal. *Proc. Natl. Acad. Sci. U.S.A.* 82, 8232–8236. doi: 10.1073/pnas.82.23.8232

Rajendran, P., Dashwood, W. M., Li, L., Kang, Y., Kim, E., Johnson, G., et al. (2015). Nrf2 status affects tumor growth, HDAC3 gene promoter associations, and the response to sulforaphane in the colon. *Clin. Epigenet.* 7:102. doi: 10.1186/s13148-015-0132-y

Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., et al. (2001). Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3410–3415. doi: 10.1073/pnas.051618798

Redondo, A., Chamorro, P. A. F., Riego, G., Leanez, S., and Pol, O. (2017). Treatment with sulforaphane produces antinociception and improves morphine effects during inflammatory pain in mice. *J. Pharmacol. Exp. Ther.* 363, 293–302. doi: 10.1124/jpet.117.244376

Rhen, T., and Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. *N. Engl. J. Med.* 353, 1711–1723. doi: 10.1056/NEJMra050541

Richardson, B. G., Jain, A. D., Speltz, T. E., and Moore, T. W. (2015). Non-electrophilic modulators of the canonical Keap1/Nrf2 pathway. *Bioorg. Med. Chem. Lett.* 25, 2261–2268. doi: 10.1016/j.bmcl.2015.04.019

Riedl, M. A., Saxon, A., and Diaz-Sanchez, D. (2009). Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. *Clin. Immunol.* 130, 244–251. doi: 10.1016/j.clim.2008.10.007

Rojo de la Vega, M., Chapman, E., and Zhang, D. D. (2018). NRF2 and the Hallmarks of Cancer. *Cancer Cell* 34, 21–43. doi: 10.1016/j.ccell.2018.03.022

Rong, H., Liang, Y., and Niu, Y. (2018). Rosmarinic acid attenuates beta-amyloid-induced oxidative stress via Akt/GSK-3beta/Fyn-mediated Nrf2 activation in PC12 cells. *Free Radic. Biol. Med.* 120, 114–123. doi: 10.1016/j.freeradbiomed.2018.03.028

Roumestan, C., Michel, A., Bichon, F., Portet, K., Detoc, M., Henriquet, C., et al. (2007). Anti-inflammatory properties of desipramine and fluoxetine. *Respir Res.* 8:35. doi: 10.1186/1465-9921-8-35

Ruiz, S., Pergola, P. E., Zager, R. A., and Vaziri, N. D. (2013). Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. *Kidney Int.* 83, 1029–1041. doi: 10.1038/ki.2012.439

Ruiz-Miyazawa, K. W., Pinho-Ribeiro, F. A., Borghi, S. M., Staurengo-Ferrari, L., Fattori, V., Amaral, F. A., et al. (2018a). Hesperidin methylchalcone suppresses experimental gout arthritis in mice by inhibiting NF-kappaB activation. *J. Agric. Food Chem.* 66, 6269–6280. doi: 10.1021/acs.jafc.8b00959

Ruiz-Miyazawa, K. W., Staurengo-Ferrari, L., Pinho-Ribeiro, F. A., Fattori, V., Zaninelli, T. H., Badaro-Garcia, S., et al. (2018b). 15d-PGJ2-loaded nanocapsules ameliorate experimental gout arthritis by reducing pain and inflammation in a PPAR-gamma-sensitive manner in mice. *Sci. Rep.* 8:13979. doi: 10.1038/s41598-018-32334-0

Saito, P., Melo, C. P. B., Martinez, R. M., Fattori, V., Cezar, T. L. C., Pinto, I. C., et al. (2018). The lipid mediator resolvin D1 reduces the skin inflammation and oxidative stress induced by UV irradiation in hairless mice. *Front. Pharmacol.* 9:1242. doi: 10.3389/fphar.2018.01242

Saito, R., Suzuki, T., Hiramoto, K., Asami, S., Naganuma, E., Suda, H., et al. (2016). Characterizations of three major cysteine sensors of keap1 in stress response. *Mol. Cell. Biol.* 36, 271–284.

Sakai, C., Ishida, M., Ohba, H., Yamashita, H., Uchida, H., Yoshizumi, M., et al. (2017). Fish oil omega-3 polyunsaturated fatty acids attenuate oxidative stress-induced DNA damage in vascular endothelial cells. *PLoS One* 12:e0187934. doi: 10.1371/journal.pone.0187934

Salazar, M., Rojo, A. I., Velasco, D., De Sagarra, R. M., and Cuadrado, A. (2006). Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *J. Biol. Chem.* 281, 14841–14851. doi: 10.1074/jbc.M513737200

Saracino, A. M., and Orteu, C. H. (2017). Severe recalcitrant cutaneous manifestations in systemic lupus erythematosus successfully treated with fumaric acid esters. *Br. J. Dermatol.* 176, 472–480. doi: 10.1111/bjd.14698

Satoh, T., Kosaka, K., Itoh, K., Kobayashi, A., Yamamoto, M., Shimojo, Y., et al. (2008). Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. *J. Neurochem.* 104, 1116–1131. doi: 10.1111/j.1471-4159.2007.05039.x

Schafer, M., and Werner, S. (2015). Nrf2–A regulator of keratinocyte redox signaling. *Free Radic. Biol. Med.* 88, 243–252. doi: 10.1016/j.freeradbiomed.2015.04.018

Schilling, S., Goelz, S., Linker, R., Luehder, F., and Gold, R. (2006). Fumaric acid esters are effective in chronic experimental autoimmune encephalomyelitis and suppress macrophage infiltration. *Clin. Exp. Immunol.* 145, 101–107. doi: 10.1111/j.1365-2249.2006.03094.x

Selvam, C., Jachak, S. M., Thilagavathi, R., and Chakraborti, A. K. (2005). Design, synthesis, biological evaluation and molecular docking of curcumin analogues as antioxidant, cyclooxygenase inhibitory and anti-inflammatory agents. *Bioorg. Med. Chem. Lett.* 15, 1793–1797. doi: 10.1016/j.bmcl.2005.02.039

Serafim, K. G. G., Navarro, S. A., Zarpelon, A. C., Pinho-Ribeiro, F. A., Fattori, V., Cunha, T. M., et al. (2015). Bosentan, a mixed endothelin receptor antagonist, inhibits superoxide anion-induced pain and inflammation in mice. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 388, 1211–1221. doi: 10.1007/s00210-015-1160-z

Serhan, C. N. (2017). Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *FASEB J.* 31, 1273–1288. doi: 10.1096/fj.201601222R

Serhan, C. N., Yacoubian, S., and Yang, R. (2008). Anti-inflammatory and proresolving lipid mediators. *Annu. Rev. Pathol.* 3, 279–312. doi: 10.1146/annurev.pathmechdis.3.121806.151409

Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., and Talalay, P. (2001). Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. *Cancer Epidemiol. Biomarkers. Preven.* 10, 501–508.

Sharma, A., Rizky, L., Stefanovic, N., Tate, M., Ritchie, R. H., Ward, K. W., et al. (2017). The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator dh404 protects against diabetes-induced endothelial dysfunction. *Cardiovasc. Diabetol.* 16:33. doi: 10.1186/s12933-017-0513-y

Shen, X., Hu, B., Xu, G., Chen, F., Ma, R., Zhang, N., et al. (2017). Activation of Nrf2/HO-1 pathway by glycogen synthase kinase-3beta inhibition attenuates renal ischemia/reperfusion injury in diabetic rats. *Kid. Blood Press. Res.* 42, 369–378. doi: 10.1159/000477947

Shintani, Y., Maruoka, S., Gon, Y., Koyama, D., Yoshida, A., Kozu, Y., et al. (2015). Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates airway epithelial barrier integrity. *Allergol. Int.* 64(Suppl.), S54–S63. doi: 10.1016/j.alit.2015.06.004

Silva-Islas, C. A., and Maldonado, P. D. (2018). Canonical and non-canonical mechanisms of Nrf2 activation. *Pharmacol. Res.* 134, 92–99. doi: 10.1016/j.phrs.2018.06.013

Simmons, D. L., Botting, R. M., and Hla, T. (2004). Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol. Rev.* 56, 387–437. doi: 10.1124/pr.56.3.3

Singh, A. K., and Haldar, C. (2016). Melatonin modulates glucocorticoid receptor mediated inhibition of antioxidant response and apoptosis in peripheral blood mononuclear cells. *Mol. Cell. Endocrinol.* 436, 59–67. doi: 10.1016/j.mce.2016.07.024

Soto-Balbuena, C., Rodriguez, M. F., Escudero Gomis, A. I., Ferrer Barriendos, F. J., Le, H. N., and Pmb-Huca, G. (2018). Incidence, prevalence and risk factors related to anxiety symptoms during pregnancy. *Psicothema* 30, 257–263.

Soundararajan, P., and Kim, J. S. (2018). Anti-carcinogenic glucosinolates in cruciferous vegetables and their antagonistic effects on prevention of cancers. *Molecules* 23:E2983. doi: 10.3390/molecules23112983

Sporn, M. B., and Liby, K. T. (2012). NRF2 and cancer: the good, the bad and the importance of context. *Nat. Rev. Cancer* 12, 564–571. doi: 10.1038/nrc3278

Su, X., Jiang, X., Meng, L., Dong, X., Shen, Y., and Xin, Y. (2018). Anticancer activity of sulforaphane: the epigenetic mechanisms and the Nrf2 signaling pathway. *Oxid. Med. Cell Longev.* 2018:5438179. doi: 10.1155/2018/5438179

Sun, X., Zeng, H., Wang, Q., Yu, Q., Wu, J., Feng, Y., et al. (2018). Glycyrrhizin ameliorates inflammatory pain by inhibiting microglial activation-mediated inflammatory response via blockage of the HMGB1-TLR4-NF-kB pathway. *Exp. Cell Res.* 369, 112–119. doi: 10.1016/j.yexcr.2018.05.012

Sutcliffe, J. G., Hedlund, P. B., Thomas, E. A., Bloom, F. E., and Hilbush, B. S. (2011). Peripheral reduction of beta-amyloid is sufficient to reduce brain beta-amyloid: implications for Alzheimer's disease. *J. Neurosci. Res.* 89, 808–814. doi: 10.1002/jnr.22603

Suzuki, T., Murakami, S., Biswal, S. S., Sakaguchi, S., Harigae, H., Yamamoto, M., et al. (2017). Systemic activation of NRF2 alleviates lethal autoimmune inflammation in scurfy mice. *Mol. Cell. Biol.* 37:e00063-17. doi: 10.1128/MCB.00063-17

Taguchi, K., Motohashi, H., and Yamamoto, M. (2011). Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells* 16, 123–140. doi: 10.1111/j.1365-2443.2010.01473.x

Tahvili, S., Zandieh, B., and Amirghofran, Z. (2015). The effect of dimethyl fumarate on gene expression and the level of cytokines related to different T helper cell subsets in peripheral blood mononuclear cells of patients with psoriasis. *Int. J. Dermatol.* 54, e254–e260. doi: 10.1111/ijd.12834

Tan, S. M., Sharma, A., Stefanovic, N., Yuen, D. Y., Karagiannis, T. C., Meyer, C., et al. (2014). Derivative of bardoxolone methyl, dh404, in an inverse dose-dependent manner lessens diabetes-associated atherosclerosis and improves diabetic kidney disease. *Diabetes Metab. Res. Rev.* 63, 3091–3103. doi: 10.2337/db13-1743

Thangapandiyan, S., and Miltonprabu, S. (2014). Epigallocatechin gallate supplementation protects against renal injury induced by fluoride intoxication in rats: role of Nrf2/HO-1 signaling. *Toxicol. Rep.* 1, 12–30. doi: 10.1016/j.toxrep.2014.01.002

To, C., Ringelberg, C. S., Royce, D. B., Williams, C. R., Risingsong, R., Sporn, M. B., et al. (2015). Dimethyl fumarate and the oleanane triterpenoids, CDDO-imidazolide and CDDO-methyl ester, both activate the Nrf2 pathway but have opposite effects in the A/J model of lung carcinogenesis. *Carcinogenesis* 36, 769–781. doi: 10.1093/carcin/bgv061

Toledano, M. B. (2009). The guardian recruits cops: the p53-p21 axis delegates prosurvival duties to the Keap1-Nrf2 stress pathway. *Mol. Cell.* 34, 637–639. doi: 10.1016/j.molcel.2009.06.005

Tu, J., Zhang, X., Zhu, Y., Dai, Y., Li, N., Yang, F., et al. (2015). Cell-permeable peptide targeting the Nrf2-keap1 interaction: a potential novel therapy for global cerebral ischemia. *J. Neurosci.* 35, 14727–14739. doi: 10.1523/JNEUROSCI.1304-15.2015

Tzaneva, S., Geroldinger, A., Trattner, H., and Tanew, A. (2018). Fumaric acid esters in combination with a 6-week course of narrowband ultraviolet B provides an accelerated response compared with fumaric acid esters monotherapy in patients with moderate-to-severe plaque psoriasis: a randomized prospective clinical study. *Br. J. Dermatol.* 178, 682–688. doi: 10.1111/bjd.16106

Verri, W. A., Vicentini, F. T. M. C., Baracat, M. M., Georgetti, S. R., Cardoso, R. D., Cunha, T. M., et al. (2012). "Flavonoids as anti-inflammatory and analgesic drugs: mechanisms of action and perspectives in the development of pharmaceutical forms," in *Studies in Natural Products Chemistry*, ed. Atta-U-Rahman (Amsterdam: Elsevier). doi: 10.1016/B978-0-444-53836-9.00026-8

Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., et al. (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* 35, 238–245. doi: 10.1038/ng1248

Wang, B., Zhu, X., Kim, Y., Li, J., Huang, S., Saleem, S., et al. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Radic. Biol. Med.* 52, 928–936. doi: 10.1016/j.freeradbiomed.2011.12.006

Wang, C., and Wang, C. (2017). Anti-nociceptive and anti-inflammatory actions of sulforaphane in chronic constriction injury-induced neuropathic pain mice. *Inflammopharmacology* 25, 99–106. doi: 10.1007/s10787-016-0307-y

Wardyn, J. D., Ponsford, A. H., and Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-kappaB response pathways. *Biochem. Soc. Trans.* 43, 621–626. doi: 10.1042/BST20150014

Wei, J., Zhu, H., Lord, G., Bhattachayya, M., Jones, B. M., Allaway, G., et al. (2017). Nrf2 exerts cell-autonomous antifibrotic effects: compromised function in systemic sclerosis and therapeutic rescue with a novel heterocyclic chalcone derivative. *Transl. Res.* 183:e71. doi: 10.1016/j.trsl.2016.12.002

Wei, W., Shurui, C., Zipeng, Z., Hongliang, D., Hongyu, W., Yuanlong, L., et al. (2018). Aspirin suppresses neuronal apoptosis, reduces tissue inflammation, and restrains astrocyte activation by activating the Nrf2/HO-1 signaling pathway. *Neuroreport* 29, 524–531. doi: 10.1097/WNR.0000000000000969

Wise, R. A., Holbrook, J. T., Criner, G., Sethi, S., Rayapudi, S., Sudini, K. R., et al. (2016). Lack of effect of oral sulforaphane administration on Nrf2 expression in COPD: a randomized, double-blind, placebo controlled trial. *PLoS One* 11:e0163716. doi: 10.1371/journal.pone.0175077

Wu, J., Li, Q., Wang, X., Yu, S., Li, L., Wu, X., et al. (2013). Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway. *PLoS One* 8:e59843. doi: 10.1371/journal.pone.0059843

Xu, J., Donepudi, A. C., Moscovitz, J. E., and Slitt, A. L. (2013). Keap1-knockdown decreases fasting-induced fatty liver via altered lipid metabolism and decreased fatty acid mobilization from adipose tissue. *PLoS One* 8:e79841. doi: 10.1371/journal.pone.0079841

Xu, L. L., Liu, T., Wang, L., Li, L., Wu, Y. F., Li, C. C., et al. (2018). 3-(1H-Benzo[d]imidazol-6-yl)-5-(4-fluorophenyl)-1,2,4-oxadiazole (DDO7232), a Novel Potent Nrf2/ARE inducer, ameliorates DSS-induced murine colitis and protects NCM460 cells against oxidative stress via ERK1/2 phosphorylation. *Oxid. Med. Cell. Longev.* 2018:3271617. doi: 10.1155/2018/3271617

Xu, Z., Zhang, F., Sun, F., Gu, K., Dong, S., and He, D. (2015). Dimethyl fumarate for multiple sclerosis. *Cochrane Database Syst. Rev.* 4:CD11076. doi: 10.1002/14651858.CD011076.pub2

Yamacita-Borin, F. Y., Zarpelon, A. C., Pinho-Ribeiro, F. A., Fattori, V., Alves-Filho, J. C., Cunha, F. Q., et al. (2015). Superoxide anion-induced pain and inflammation depends on TNF(/TNFR1 signaling in mice. *Neurosci. Lett.* 605, 53–58. doi: 10.1016/j.neulet.2015.08.015

Yan, B., Ma, Z., Shi, S., Hu, Y., Ma, T., Rong, G., et al. (2017). Sulforaphane prevents bleomycininduced pulmonary fibrosis in mice by inhibiting oxidative stress via nuclear factor erythroid 2related factor2 activation. *Mol. Med. Rep.* 15, 4005–4014. doi: 10.3892/mmr.2017.6546

Yang, G. Z., Wang, Z. J., Bai, F., Qin, X. J., Cao, J., Lv, J. Y., et al. (2015). Epigallocatechin-3-gallate protects HUVECs from PM2.5-induced oxidative stress injury by activating critical antioxidant pathways. *Molecules* 20, 6626–6639. doi: 10.3390/molecules20046626

Yao, W., Zhang, J. C., Ishima, T., Dong, C., Yang, C., Ren, Q., et al. (2016). Role of Keap1-Nrf2 signaling in depression and dietary intake of glucoraphanin confers stress resilience in mice. *Sci. Rep.* 6:30659. doi: 10.1038/srep30659

Yore, M. M., Kettenbach, A. N., Sporn, M. B., Gerber, S. A., and Liby, K. T. (2011). Proteomic analysis shows synthetic oleanane triterpenoid binds to mTOR. *PLoS One* 6:e22862. doi: 10.1371/journal.pone.0022862

Yoshinaga, N., Arimura, N., Otsuka, H., Kawahara, K., Hashiguchi, T., Maruyama, I., et al. (2011). NSAIDs inhibit neovascularization of choroid through HO-1-dependent pathway. *Lab. Invest.* 91, 1277–1290. doi: 10.1038/labinvest.2011.101

Zhang, D. D. (2013). Bardoxolone brings Nrf2-based therapies to light. *Antioxid. Redox Signal.* 19, 517–518. doi: 10.1089/ars.2012.5118

Zhang, X., Li, J., Yang, J., and Huang, Z. (2016). Fentanyl combined with butorphanol protects myocardial ischemia/reperfusion injury via κ-opioid receptor-mediated Nrf2-ARE signaling. *Int. J. Clin. Exp. Med.* 9, 2500–2506.

Zhang, Y., Liu, B., Chen, X., Zhang, N., Li, G., Zhang, L. H., et al. (2017). Naringenin ameliorates behavioral dysfunction and neurological deficits in a d-galactose-induced aging mouse model through activation of PI3K/Akt/Nrf2 pathway. *Rejuvenation Res.* 20, 462–472. doi: 10.1089/rej.2017.1960

Zhao, J., Redell, J. B., Moore, A. N., and Dash, P. K. (2011). A novel strategy to activate cytoprotective genes in the injured brain. *Biochem. Biophys. Res. Commun.* 407, 501–506. doi: 10.1016/j.bbrc.2011.03.046

Zhu, J., Wang, H., Chen, F., Fu, J., Xu, Y., Hou, Y., et al. (2016). An overview of chemical inhibitors of the Nrf2-ARE signaling pathway and their potential applications in cancer therapy. *Free Radic. Biol. Med.* 99, 544–556. doi: 10.1016/j.freeradbiomed.2016.09.010

Zou, Y., Hu, M., Lee, J., Nambiar, S. M., Garcia, V., Bao, Q., et al. (2015). Nrf2 is essential for timely M phase entry of replicating hepatocytes during liver regeneration. *Am. J. Physiol. Gastrointest. Liver Physiol.* 308, G262–G268. doi: 10.1152/ajpgi.00332.2014

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Treatment With Naringenin Elevates the Activity of Transcription Factor Nrf2 to Protect Pancreatic β-Cells From Streptozotocin-Induced Diabetes *in vitro* and *in vivo*

Rashmi Rajappa[1], Dornadula Sireesh[2], Magesh B. Salai[1], Kunka M. Ramkumar[2], Suryanarayanan Sarvajayakesavulu[1] and SubbaRao V. Madhunapantula[3]†*

[1] Department of Water & Health, Faculty of Life Sciences, JSS Academy of Higher Education and Research, Mysuru, India, [2] SRM Institute of Science and Technology, Chennai, India, [3] Center of Excellence in Molecular Biology & Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru, India

Chronic hyperglycemia and unusually high oxidative stress are the key contributors for diabetes in humans. Since nuclear factor E2-related factor 2 (Nrf2) controls the expression of antioxidant- and detoxification genes, it is hypothesized that targeted activation of Nrf2 using phytochemicals is likely to protect pancreatic β-cells, from oxidative damage, thereby mitigates the complications of diabetes. Naringenin is one such activator of Nrf2. However, it is currently not known whether the protective effect of naringenin against streptozotocin (STZ) induced damage is mediated by Nrf2 activation. Hence, the potential of naringenin to activate Nrf2 and protect pancreatic β-cells from STZ-induced damage in MIN6 cells is studied. In MIN6 cells, naringenin could activate Nrf2 and its target genes GST and NQO1, thereby inhibit cellular apoptosis. In animals, administration of 50 mg/kg body weight naringenin, for 45 days, significantly decreased STZ-induced blood glucose levels, normalized the lipid profile, and augmented the levels of antioxidants in pancreatic tissues. Immunohistochemical analysis measuring the number of insulin-positive cells in pancreas showed restoration of insulin expression similar to control animals. Furthermore, naringenin promoted glycolysis while inhibiting gluconeogenesis. In conclusion, naringenin could be a good anti-diabetic agent, which works by promoting Nrf2 levels and by decreasing cellular oxidative stress.

Keywords: diabetes, streptozotocin, naringenin, Nrf2, MIN6 cells, apoptosis

Abbreviations: BSA, Bovine Serum Albumin; CAT, catalase; GPX, glutathione peroxidase; GSH, glutathione; IDF, International Diabetes Federation; IPGTT, Intraperitoneal Glucose Tolerance Test; Keap1, Kelch-like ECH-Associated Protein-1; MAPK, Mitogen-Activated Protein Kinase; MLD-STZ, Mutiple Low Dose Streptozotocin; NF-Kb, nuclear Factor kappa-light-chain-enhancer of activated B cells; NQO1, NAD(P)H Quinone Oxidoreductase 1; Nrf2, Nuclear factor E2-related factor 2; ROS, Reactive Oxygen Species; SDS-PAGE, Sodium Dodecyl Sulfate- Polyacrylamide gel electrophoresis; SOD, superoxide dismutase.

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Águeda González Rodríguez, Instituto de Investigación Sanitaria del Hospital Universitario de La Princesa, Spain

Reviewed by:
Carlota Recio, Universidad de Las Palmas de Gran Canaria, Spain
Claudio Ferrante, Università degli Studi "G. d'Annunzio" Chieti - Pescara, Italy

****Correspondence:***
SubbaRao V. Madhunapantula
mvsstsubbarao@jssuni.edu.in; madhunapantulas@yahoo.com

†Present address:
SubbaRao V. Madhunapantula, Special Interest Group on Cancer Biology and Cancer Stem Cells, JSS Academy of Higher Education & Research, Mysuru, India

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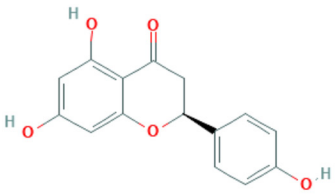
INTRODUCTION

Diabetes is a non-communicable disease with multiple etiological factors resulting from a defect in insulin secretion, insulin action, or both, leads to chronic hyperglycemia with disturbances in the metabolism of carbohydrates, lipids, and proteins (American Diabetes and Association, 2010). Despite various cost-effective treatment strategies and public campaigns highlighting the key risk factors for diabetes, the incidence and burden are increasing at alarming rates with an estimated 422 million individuals presently suffering from this disease globally. According to the IDF, the number of diabetics is predicted to increase to 642 million by 2040 (American Diabetes and Association, 2015). A decrease in the number of insulin-producing functional β-cells and alterations in β-cell mass contributes to the pathophysiology of both type1 and type 2 diabetes (Cerf, 2013). Recent studies have identified that oxidative stress, caused by excess ROSs, is one of the most important causing factors for diabetes complications (Forbes and Cooper, 2013). Moreover, since pancreatic β-cells express very low antioxidant defense enzymes, they are more susceptible to oxidative stress caused by (a) free radicals; (b) misfolded proteins; and (c) endoplasmic reticulum hyperactivity (Lenzen et al., 1996). As a result of this cellular stress, β-cells undergo apoptosis, culminating in pancreatic dysfunction (Kajimoto and Kaneto, 2004). Therefore, promoting the expression of genes coding for antioxidant enzymes appears to be a possible therapeutic approach against stress-associated cell damage in pancreatic β-cell (Qin and Hou, 2016).

Nuclear factor erythroid 2-related factor-2 (Nrf2) is a key leucine zipper transcription factor that regulates the expression of intracellular antioxidant enzymes thereby prevent the loss of cells due to oxidative stress (Lacher et al., 2015). Under basal conditions, Nrf2 exists in its inactive state due to its association with Keap1 (Kelch-like erythroid-cell-derived protein with CNC homology [ECH]-associated protein) (Ma and He, 2012). Mechanistically, Keap1 anchors Nrf2 in the cytoplasm and target it for proteasomal degradation by promoting its association with the Cullin-3 (Cul3)/Ring box-1 (Rbx1) E3 ligase system (Ma and He, 2012). However, when cells are exposed to oxidative or electrophilic stress, the reactive cysteines of Keap1 undergo modification, causing dissociation of the Nrf2 from Keap1 complex, allowing its' translocation into the nucleus. Nuclear Nrf2 binds to antioxidant response elements (ARE) sequences and trigger the expression of genes involved in combating cellular stress (Jung and Kwak, 2010). These stress-response genes include NADPH quinone oxidoreductase (NQO1), Heme oxygenase-1 (HO-1), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and γ-glutamylcysteine synthetase (GCS) (Jung and Kwak, 2010). Since Nrf2-dependent cellular defense response can protect organs, activation of Nrf2 using phytochemical has been implicated as a strategy to combat diseases such as diabetes (Lu et al., 2016).

Naringenin (**Figure 1**) is a bioactive flavonoid predominantly present in citrus fruits such as grapes, blood orange, lemons, and tomatoes (Sumathi et al., 2015). Prior studies have demonstrated that naringenin treatment could offer protection against



Common Name: Naringenin
IUPAC Name: 5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one
Molecular Weight: 272.26

FIGURE 1 | Chemical structure of naringenin. Naringenin is a flavonoid present in citrus species fruits.

(a) ethanol-induced hepatotoxicity and (b) cisplatin-induced nephrotoxicity (Sumathi et al., 2015). For instance, naringenin has been shown to down-regulate the phosphorylation of MAPK and nuclear factor kappaB (NFκB) subunit p65 in daunorubicin-induced nephrotoxicity by preventing the epidermal growth factor receptor (EGFR)-phosphoinositide-3 kinase (PI3K)-Akt/extracellular signal regulated kinase (ERK) MAPK signaling (Karuppagounder et al., 2015). Recently, naringenin has been shown to activate Nrf2 (Esmaeili and Alilou, 2014). Esmaeili and Alilou (2014) reported that naringenin suppressed the hepatic inflammation by stimulating the Nrf2 pathway in carbon tetrachloride treated rats. In addition, naringenin provided protection against 6-hydroxy dopamine-induced oxidative stress in SH-SY5Y cells (Lou et al., 2014). However, not much is known about whether naringenin reduces oxidative stress in MIN6 pancreatic β-cells through Nrf2 signaling. Therefore, in the current study, we have determined the Nrf2 activation potential of naringenin in MIN6 cells *in vitro*; and assessed its protective effect against streptozotocin (STZ)-induced pancreatic β-cell apoptosis. Furthermore, the anti-diabetic and anti-oxidative effects of naringenin were studied using male Wistar albino mice treated with multiple low doses of STZ. In conclusion, data from our studies report the ability of naringenin to activate Nrf2 thereby provide protection against STZ-induced cell death *in vitro* as well as in experimental animal models.

MATERIALS AND METHODS

Culturing of MIN6 Cells

MIN6 is a mouse insulinoma cell line obtained from National Centre for Cell Science (NCCS), Pune, India. MIN6 display many important characteristics that are similar to pancreatic islets (Ishihara et al., 1993). For example, MIN6 cells exhibit glucose-stimulated insulin secretion (GSIS) (Cheng et al., 2012). MIN6 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.0 mM glutamine (Purchased from GE Healthcare, Little Chalfont, United Kingdom) in a carbon dioxide incubator maintained at 37°C with 5% CO_2. MIN6 cells with passage

number between 5 and 20 were used for all the experiments (Elango et al., 2016).

Determination of Cell Viability Using MTT Assay

The effect of naringenin on the viability of MIN6 cells was measured using an MTT assay (Mosmann, 1983). Experimentally, first, MIN6 cells (2×10^4 cells/well) were plated in 96-well plates and allowed to grow for 24 h in a CO_2 incubator. Next, the growing cells were exposed to increasing concentrations (0–200 μM) of naringenin (Sigma Chemical Company, St. Louis, MO, United States) for 24 h at 37°C. After treatment, cells were replenished with 90 μL phenol-red free media containing 10 μL MTT (5 mM) and incubated for additional 3 h in the CO_2 incubator. Media was aspirated, the precipitate was dissolved in 50 μL DMSO, and the absorbance measured at 540 nm using a plate reader (Infinite 1000, Tecan, Mannedorf, Switzerland). The experiments were performed in triplicate. The relative cell viability (%) compared to control cells treated with DMSO was calculated using: Cell viability (%) = $(A_{sample}\text{-}A_{blank})/A_{control}\text{-}A_{blank}) \times 100$. Since an about 35% cell death was observed at 200 μM, subsequent studies were conducted with naringenin concentrations < 200 μM.

To study the protective role of naringenin on STZ-induced cytotoxicity, first, the MIN6 cells were pretreated with increasing concentration of naringenin (0–100 μM) for 24 h. Next, the naringenin-treated cells were exposed to 10 mM STZ (Primary stock of 1.0M was prepared by dissolving in 0.1M Citrate buffer pH 4.5 followed by the addition of DMSO) for 1 h and the number of viable cells estimated using MTT assay. All experiments were performed in triplicates.

Evaluation of the Potential of Naringenin to Activate Nrf2 Using Nrf2-Keap1 Complementation System

2×10^4 MIN6 cells/ml were transiently transfected with Nrf2-Keap1 complementation system in a 12-well plate using Lipofectamine-2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, United States). Six hours after transfection, the media was replaced with a fresh batch of medium, and cells treated with naringenin (25, 50, 100 μM) for 24 h. Control and treated cells were lysed in 1X lysis buffer (pH 7.8; Promega, Madison, WI, United States), protein lysates collected, and the debris separated by centrifugation at 10,000 *g* at 4°C for 5 min. Total protein was estimated using the Bradford reagent (Bio-Rad Laboratories Inc, Hercules, CA, United States). Next, 100 μL luciferase substrate (prepared by mixing 10 ml of luciferase assay buffer with the lyophilized Luciferin; Promega, Madison, WI, United States) was added to the 20 μL of supernatant containing 175.0 μg of total protein and the luciferase activity measured using a luminometer (Promega, Madison, WI, United States). The developed sensor system detects the potential of naringenin to stimulate the Nrf2-Keap1 complex dissociation. A fall in luciferase signal is inversely proportional to the activation of Nrf2. The results were presented as fold change of three independent experiments.

Separation of Nuclear and Cytosolic Fractions Using Pierce NE-PER Kit

To check the effect of naringenin on Nrf2 translocation, nuclear and cytoplasmic extracts were separated using Pierce NE-PER® kit according to the manufacturer's guidelines (Pierce, Rockford, IL, United States). In brief, cells (2×10^4 MIN6 cells/ml) were homogenized in CER-I buffer vigorous vortexing in the pre-extraction buffer and incubated on ice for 15 min. The cellular homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C and the supernatant containing the cytoplasmic fraction separated. Next, nuclei present in the pellet were suspended in NER buffer (provided with protease inhibitors) and the nuclear fraction generated by thorough vortex mixing for 1 min with a break for every 10 min for 40 min. The vortexed samples were centrifuged at 16,000 × *g* for 15 min at 4°C, to separate the supernatant containing a nuclear fraction. The separated nuclear fraction was used for western blotting.

Estimation of Total Protein and Western Blotting

Total protein content in the cytoplasmic and nuclear fractions was quantified with the Bradford method using BSA as a standard (Kruger, 2009). Samples (100 μg) were denatured using sample buffer at 95°C for 5 min, proteins were separated on 4–12% SDS-PAGE gel (Bio-Rad, Philadelphia, PA, United States) and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, United States). Primary and secondary antibodies against Nrf2, caspase-3, β-actin and lamin-B (Santa Cruz Biotechnology, Santa Cruz, CA, United States) were used to detect corresponding proteins. The blot was developed using ECL (Bio-Rad, Philadelphia, PA, United States) and the signals captured using a gel documentation system (GBOX, Syngene, United Kingdom).

ARE-Luciferase Reporter Gene Assay

NQO1-ARE-Luc and GST-ARE-Luc reporter constructs, given by Donna D. Zhang (College of Pharmacy, University of Arizona, Tucson, AZ, United States) to Dr. Ramkumar, were used for cell-based reporter gene assay. For more details about the construction of these reporter plasmids, readers can refer to (Ramkumar et al., 2013). ARE-Luc constructs (500 ng/well) were transiently transfected into MIN6 cells in 12-well plates using Lipofectamine-2000 as described previously (Ramkumar et al., 2013). After 6 h of transfection, the media was changed and the cells exposed to 25, 50, and 100 μM naringenin for 24 h. The cell lysates were collected and the luminometric assay was carried out according to Ramkumar et al. (2013). The increase in the luciferase activity compared to control DMSO-treated cells was represented. The results were represented as the averages of at least three independent experiments.

Measurement of Intracellular Reactive Oxygen Species

Levels of intracellular ROS were determined by flow cytometry using an oxidation-sensitive fluorescent dye, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Eruslanov

and Kusmartsev, 2010). Experimentally, first, MIN6 cells (2×10^4 cells/well in 100 μL) were treated with 50 and 100 μM naringenin in complete medium for 24 h. Next, naringenin-treated cells were exposed to 10 mM STZ for 1 h and the cells were incubated with 20 μM H_2DCFDA (10 μL) for 30 min at 37°C. The reaction was terminated by washing with phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS). Cells were centrifuged at x800 *g* for 10 min, washed, and resuspended in 1ml PBS. The fluorescence intensity was measured using FACS (BD Biosciences, San Jose, CA, United States). Data were analyzed using BD Cell QuestTM Pro Analysis software and the shift in fluorescence intensity caused by DCF production, which is an indicator of ROS generation, represented as a histogram.

Annexin V-FITC/PI Double Staining and Analysis by Flow Cytometry

To assess the efficacy of naringenin for protecting MIN6 cells from STZ-induced cell death, the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen), was used (Hingorani et al., 2011). Experimentally, MIN6 cells were cultured in 6-well plates and treated with naringenin (50 and 100 μM) for 24 h. Next, naringenin-treated cells were exposed to 10 mM STZ. After 1 h, the cells were trypsinized, collected and washed with cold PBS. The supernatant was discarded and the cells resuspended in 200 μL of the 1X binding buffer, containing 50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4. To 100 μL cell suspension, 5 μL annexin-V-FITC and 1 μL of PI solution (100 μg/mL) were added. Then, the cells were incubated for 15 min at room temperature in dark, and 400 μL of 1X annexin-binding buffer added. The stained cells were examined by flow cytometry using Cell QuestTM Pro Analysis software.

Determination of Protective Effects of Naringenin in Mice

Animal experiments were conducted after receiving approval from the JSS Medical College Institutional Animal Ethics Committee (JSSMC/IAEC/18/5675/DEC2013), Mysuru, India. In brief, male albino mice of 4–6 weeks old, weighing about 25–30 g, were procured from the Central Animal Facility, JSS Medical College and were maintained under standard laboratory conditions: viz., temperature (25 ± 2°C) and humidity with an alternating 12 h-12 h light/dark cycles.

Experimental Induction of Diabetes

Experimental diabetes was induced by multiple low dose streptozotocin (MLD-STZ) injections according to Elango et al. (2016). In brief, STZ was dissolved in 0.1M citrate buffer (pH-4.5) and injected 50 mg/kg/day intraperitoneally for 5 successive days. Blood (50 μL) was collected from the tail vein and used for estimating glucose using a glucometer (Accu-Check Active). Mice with blood glucose concentration > 250 mg/dl were selected for testing the potency of naringenin for mitigating STZ-induced cell damage. First, 24 STZ treated and 12 normal mice were divided into a total of 6 groups as shown in **Table 1**. Next, naringenin 50 mg/kg and 100 mg/kg body weight (prepared in 0.5% carboxymethyl cellulose) was administered orally to control and experimental groups for 45 days. At the end of the experiment (after 24 h of last naringenin administration), animals were deprived of food overnight and euthanized using chloroform and sacrificed by decapitation (Cenedella et al., 1975). Blood was collected by cardiac puncture in all experimental animals and allowed for clotting. Serum was separated by centrifuging at 3000 rpm for 15 min at room temperature and stored at −20°C until analysis. Glibenclimide (600 μg/kg body weight; prepared in 0.5% carboxymethyl cellulose) was used as a reference drug in this study.

Analytical Procedure

Fasting blood glucose levels were monitored using Accu-Chek Active Glucometer every 3rd day. IPGTT was carried out at the completion of the experimental period (Goren et al., 2004). Briefly, after 12 h fasting, the mice were intraperitoneally injected with glucose (1 g/kg b.w) and blood glucose measured at 30, 60, 90, and 120 min. Serum insulin level was determined using mouse ELISA kit (Crystal Chem Inc.).

The collected blood was used for the estimation of total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL)-cholesterol using kits available from Coral Clinical System, Goa, India. Phospholipids and free fatty acids were determined according to the protocols described by Falholt et al. (1973) and Stewart (1980). Very low-density lipoprotein (VLDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol were calculated by Friedwald formula. VLDL = TG/5; LDL = TC-(HDL + VLDL).

The level of lipid peroxidation, in terms of TBARS formed, and hydroperoxides content was measured using the methods described by Niehaus and Samuelsson (1968) and Jiang et al. (1992), respectively. Further, antioxidant enzymes (a) SOD by Kakkar et al. (1984); (b) CAT by Sinha (1972); (c) GPX by Rotruck et al. (1973); and GSH S-Transferase by Habig et al. (1974) and antioxidant molecules such as reduced GSH by Ellman (1959) were measured in the pancreatic tissues.

Estimation of Carbohydrate Metabolic Enzymes and Glycogen Content in Liver of Control and Experimental Mice

Hexokinase, Glucose-6-phosphatase (G6-P) and Fructose-1,6-bisphosphatase (F1,6-BP) were assayed according to the protocols described by Brandstrup et al. (1957); Koide and Oda (1959), and Gancedo and Gancedo (1971) respectively. The inorganic phosphate (Pi) liberated was estimated by the method of Fiske and Subbarow (1925). Glucose-6-phosphate dehydrogenase (G6-PD) was determined by the method of Ells and Kirkman (1961). Glycogen content was estimated by the method of Morales et al. (1973) and protein content in tissue homogenates was measured by the method of Lowry et al. (1951).

Histology of Pancreas

Pancreatic tissues were fixed in 10% buffered neutral formalin for 24 h and dehydrated using ethanol and xylene for

TABLE 1 | Control and experimental groups of mice.

Group	No. of mice	Category	Treatment agent	Dose and frequency of the treatment agent (mg/kg b.w.)	Route of administration	Comment
I	6	Control	Vehicle: Carboxy methyl cellulose (CMC)	0.5%, Every day for 45 days	Oral	Vehicle control
II	6		Naringenin* (NAR)	100 mg/kg, every day for 45 days	Oral	Naringenin control
III	6	STZ	Streptozotocin (STZ)	50 mg/kg, 5 consecutive days	Intra peritoneal	STZ control
IV	6		STZ, followed by NAR*	STZ – 50 mg/kg, 5 consecutive days NAR – 50 mg/kg, every day for 45 days	Oral	STZ mice treated with low dose naringenin
V	6		STZ, followed by NAR*	STZ – 50 mg/kg, 5 consecutive days NAR – 100 mg/kg, every day for 45 days	Oral	STZ mice treated with high dose naringenin
VI	6		STZ, followed by Glibenclamide*	STZ – 50 mg/kg, 5 consecutive days GLC – 600 μg/kg, every day for 45 days	Oral	A positive control group

**Naringenin (NAR) and glibenclamide (GLC) were suspended in 0.5% CMC.*

30 min each. Tissues were then incubated in liquid paraffin (58°C) twice for 60 min each. Tissue blocks were sectioned (5 μm thickness) and stained using hematoxylin and eosin (Feldman and Wolfe, 2014). The pancreatic sections were analyzed for morphological changes using a microscope (Carl Zeiss, Thornwood, NY, United States). The photomicrographs representing the overall assessment were captured and showed in the figures.

Immunohistochemical Staining for Insulin

To examine the expression of insulin in pancreatic tissues, an immunohistochemical examination was carried out with insulin antibody (SC-9168; Santa Cruz Biotechnology, Heidelberg, Germany). The 5 μm thick paraffin sections were deparaffinized in xylene and hydrated with ethanol. Then the hydrated sections were treated with 3% H_2O_2 in methanol for 30 min to block any endogenous peroxidase. The sections were washed at least three times with 0.01M phosphate buffer (pH 7.4) for 10 min; and processed further by an indirect immunoperoxidase technique using a One-Step Polymer-HRP Detection kit (Leica Biosystems, Newcastle, United Kingdom) with secondary antibodies. The sections were counterstained with hematoxylin. The stained sections were observed under microscope and representative images captured.

Statistical Analysis

All the experiments were carried out with at least three replicate measurements (intra experimental) and two independent analysis (duplicate analysis) Average values were calculated and the results expressed as mean ± SEM. The statistical significance was performed using one-way ANOVA, followed by Tukey's *post hoc* test using SPSS software 20 (SPSS, Cary, NC, United States); $P < 0.05$ was considered significant.

RESULTS

Activation of Nrf2 by naturally occurring antioxidants such as naringenin is one of the potentially viable strategies to treat diseases where Nrf2 plays a key role in disease management.

Naringenin Upregulated the Expression of Nrf2 in MIN6 Cells

In order to determine whether naringenin has the potential to activate master regulator of antioxidant proteins, i.e., Nrf2, the level of activated Nrf2 was measured using a complementation system in MIN6 cells (Ramkumar et al., 2013). MIN6 cells are known to contain very low endogenous antioxidant machinery compared to many other cells and express minimal Nrf2 (Grankvist et al., 1981). In addition, MIN6 cells are very sensitive to changes in antioxidant levels in the surroundings, hence, they respond quickly to various stress signals (Cheng et al., 2012). Therefore, MIN6 cells are the best suitable cells to study the effect of ROS inducers as well as to determine the ability of antioxidant molecules to trigger Nrf2 transcription factors (Elango et al., 2016). Experimentally, the ability of naringenin (25, 50, and 100 μM for 24 h) to dissociate the Nrf2-Keap1 complex was measured using the Nrf2-Keap1 complementation system in MIN6 cells (Elango et al., 2016). After treatment with naringenin, MIN6 cells were harvested and luciferase activity measured using a luminometer. The developed sensor system detects the potential of naringenin to stimulate the Nrf2-Keap1 complex dissociation. A fall in luciferase signal is inversely proportional to the activation of Nrf2 (Ramkumar et al., 2013). The data showed a dose-dependent increase in the Nrf2 activity (**Figure 2A**). For example, at 50 and 100 μM concentration of naringenin an about 3.9- and 4.8- fold increase, respectively, was observed (**Figure 2A**). At these concentrations of naringenin showed no cytotoxic symptoms (**Figure 2B**).

Naringenin Promoted the Translocation of Nrf2 to Nucleus and Increased the Expression of Nrf2 Target Genes, NQO1 and GST in MIN6 Cells

Since naringenin exhibited the ability to activate Nrf2 in complementation assay, next, we have determined the efficacy of naringenin to promote the translocation of Nrf2 by isolating the nuclear and cytoplasmic fractions of MIN6 cells exposed

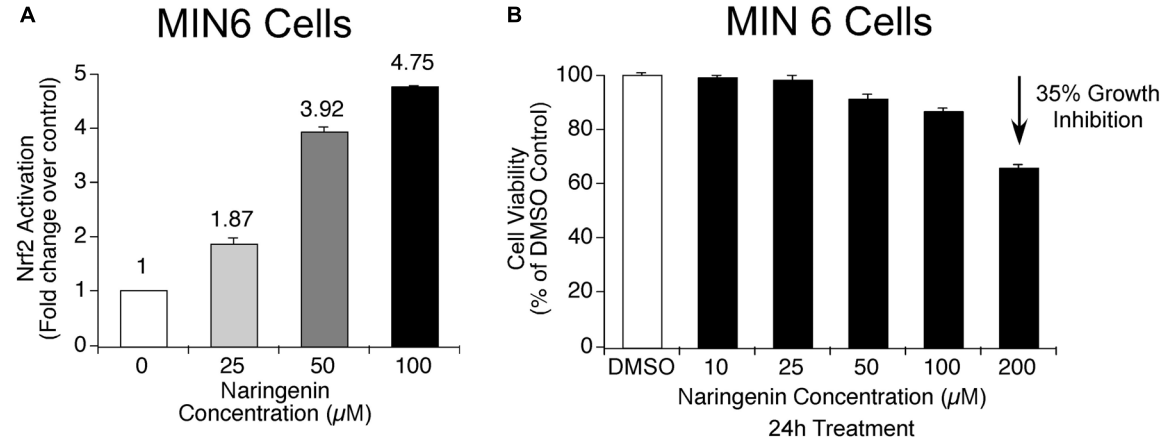


FIGURE 2 | Naringenin activates Nrf2 in MIN6 cells. **(A)** Nrf2 activation potential of naringenin was measured using Nrf2-Keap1 complementation system at 25.0 μM, 50.0 and 100 μM concentrations in MIN6 cells (Ramkumar et al., 2013). The sensor system identifies the potential of naringenin to promote the dissociation of the Nrf2-Keap1 complex. A drop in luciferase signal is inversely proportional to Nrf2 activation. A concentration-dependent activation of Nrf2 by naringenin was observed indicating the dissociation of the complex. **(B)** The concentrations of naringenin used to treat MIN6 cells found not toxic. In order to determine whether the concentration of naringenin used to treat MIN6 cells had any impact on the viability of cells, an MTT assay was carried out as detailed in experimental methods section. Data represents mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's *post hoc* test. *Significant compared with untreated control.

to 25, 50, and 100 μM naringenin for 24 h. The amount of Nrf2 in each fraction was measured using western blotting and normalized to the respective loading controls (Elango et al., 2016). The data showed a concentration-dependent increase in the nuclear Nrf2 with a concomitant decrease in the cytosolic fraction (**Figures 3A,B**).

Next, to further determine whether the translocated Nrf2 is functionally active and elevates the expression of target genes NQO1 and GST, a luciferase-based reporter assay was carried out using ARE-LUC-NQO1 and ARE-LUC-GST constructs (Ramkumar et al., 2013). MIN6 cells were transfected with luciferase-expressing ARE-NQO1 and ARE-GST constructs and the transfected cells treated with 25, 50, and 100 μM naringenin for 24 h. Cell lysates were collected and analyzed for the luciferase activity. Analysis of the data revealed a dose-dependent increase in the luciferase activity in cells harboring NQO1 and GST constructs indicating that naringenin-induced Nrf2 is functionally active and promotes the expression of its target genes NQO1 and GST (**Figure 3C**).

Streptozotocin Induced Apoptosis in MIN6 Pancreatic β-Cells by Elevating Caspase-3 Expression and Increasing ROS Levels

Streptozotocin (STZ) is a naturally occurring toxin produced by *Streptomyces achromogenes*. STZ is known to induces diabetes by specifically destroying pancreatic β-cells (Schnedl et al., 1994). Mechanistically STZ inhibits DNA synthesis, induce the generation of free radicals and nitric oxide in pancreatic β-cells thereby promotes pancreatic β-cells death (Schnedl et al., 1994). As a result of pancreatic β-cells destruction, the animals exposed to STZ develop diabetes (Eleazu et al., 2013). Since naringenin potentially upregulates the expression of functionally active Nrf2 in MIN6 cells, its' administration is predicted to reduce the cellular oxidative stress thereby protect β-cells from undergoing apoptosis (induced by STZ). To test this hypothesis, first, the levels of apoptosis induced by STZ in MIN6 cells were measured and, next, the ability of naringenin to protect MIN6 cells from STZ-induced apoptosis was quantitated.

Addition of STZ Induced ROS Production in MIN6 Cells

In order to elucidate the effect of STZ on ROS levels, MIN6 cells were treated with 10 mM STZ for 1 h and the cells subjected to FACS analysis to estimate H_2DCFDA-fluorescence (Kang et al., 2011). Principally, the non-fluorescent H_2DCFDA undergoes de-esterification in the cells and form a highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Cohn et al., 2008). The resulted DCF was detected using a fluorescence analyzer. Analysis of the data showed increased intracellular ROS levels with a significant shift of the peak (shift in relative fluorescence intensity) in MIN6 cells (**Figure 4A**). A significant shift in the fluorescent peak was observed upon treatment of MIN6 cells with 10mM STZ compared to control cells treated with DMSO (**Figure 4A**). Therefore, the addition of STZ to MIN6 cells triggered ROS generation and oxidative stress.

Addition of STZ to MIN6 Cells Increased Apoptosis

The ability of STZ to induce apoptosis in MIN6 cells was estimated by staining with Annexin-V and PI. The ratio of apoptotic and necrotic cells was determined and the results represented in **Figure 4B**. Analysis of the results showed a significant increase in the number of apoptotic cells upon treatment of MIN6 cells with STZ. Compared to the control treatment, the percentage of early apoptotic cells increased to

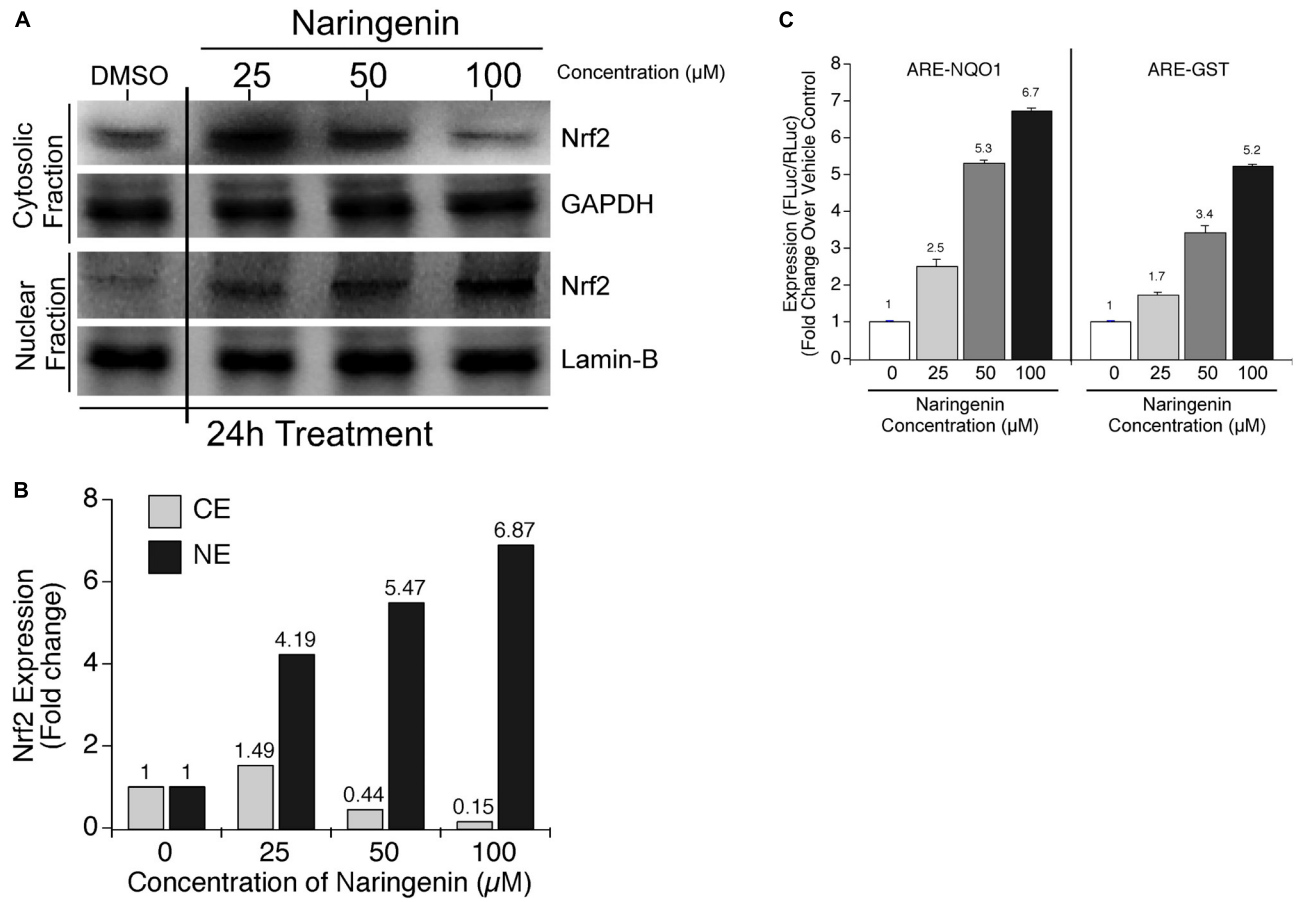


FIGURE 3 | Naringenin induced nuclear translocation of Nrf2 promoted the transcription of NQO1 and GST in MIN6 cells. **(A,B)** *Naringenin-induced Nrf2 is predominantly localized in the nucleus of MIN6 cells.* Analysis of the protein lysates collected from control and naringenin treated cells by western blotting showed a significant increase in nuclear Nrf2 upon exposure of MIN6 cells to 50 and 100 μM naringenin. **(C)** *Naringenin augmented the ARE-driven expression of Nrf2 target genes, NQO1, and GST, in MIN6 cells.* MIN6 cells were transfected with ARE-hNQO1 or ARE-GST luciferase constructs as detailed in Section "Materials and Methods" (Elango et al., 2016). Transfected cells were treated with 25, 50, and 100 μM naringenin for 24 h and luciferase assay performed. Luciferase activity represented as fold induction relative to control cells. Data showed was the average of 3 independent experiments with mean ± SEM. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's *post hoc* test. *Significant compared with untreated control, #Significant compared with respective control; $P < 0.05$.

13.56%, late apoptotic cells increased to 59.26%, and necrotic cells increased to 3.76% (**Figure 4B**). Taken together, STZ significantly induced apoptosis in MIN6 cells.

STZ Induced Caspase-3 Expression in MIN6 Cells

Apoptosis induced by STZ was further verified by measuring the caspase-3 expression using western blot analysis (Porter and Jänicke, 1999). MIN6 cells were exposed to 10 mM of STZ for 1 h. After treatment, cells were washed with ice-cold PBS, lysed, centrifuged, and analyzed for total protein by the Bradford assay. Samples containing 30 μg of total protein were assayed for caspase-3 expression using western blotting. The data showed a significant increase in the caspase-3 expression (1.46 fold), compared to control treatment (1.0) (**Figure 4C**). Therefore, it is concluded that STZ is a good inducer of apoptosis in MIN6 cells.

Naringenin Protected MIN6 Cells From Streptozotocin-Induced Apoptotic Cell Death by Upregulating Nrf2

Naringenin Protected MIN6 Cells From STZ-Induced Cell Death

To check whether antioxidant naringenin could protect MIN6 cells from STZ-induced cell death, the viability of MIN6 cells was estimated using MTT assay. Experimentally, MIN6 cells were first pre-treated with non-cytotoxic concentrations of naringenin (0–100 μM) for 24 h and then exposed to 10 mM STZ for 1 h. Cell viability was then measured using the MTT assay and the percentage growth inhibition calculated by comparing with vehicle-treated cells (Mosmann, 1983). Cells pre-treated with naringenin showed increased viability of 47.95, 72.88, and 76.24% at 25, 50, and 100 μM concentrations,

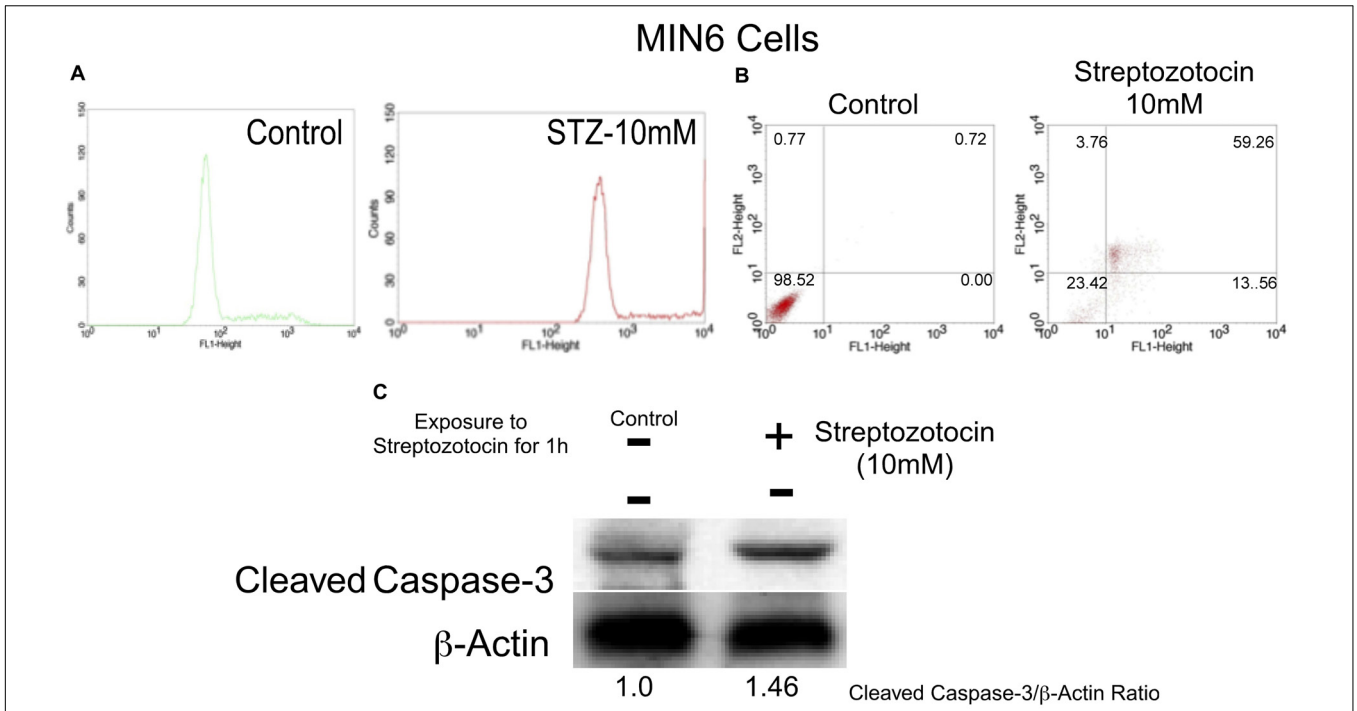


FIGURE 4 | Streptozotocin induced apoptosis in MIN6 cells by elevating caspase-3 and ROS levels. **(A)** *STZ treatment increased the levels of intracellular ROS in MIN6 cells.* MIN6 cells were treated with STZ (10 mM) for 1 h and then H_2DCFDA (20 μM) was added. The cells were further incubated for 30 min at 37°C and subjected to H_2DCFDA-fluorescence analysis. The fluorescent peak in STZ-treated cells shifted compared to the peak in control treated cells indicating the production of high levels of ROS. **(B)** *STZ induced apoptosis in MIN6 cells*. First, MIN6 cells were treated with STZ (10 mM) for 1 h. Next, cells were incubated with Annexin-V/PI and analyzed by Flow Cytometry. The scatter plots were divided into four quadrants: UL (Upper Left quadrant representing Annexin-V negative, PI positive), UR (Upper Right quadrant representing both Annexin V and PI positive), LL (Lower Left quadrant representing both Annexin-V and PI negative), LR (Lower Right quadrant representing Annexin-V positive and PI negative). The results showed that STZ treatment resulted in apoptotic cell death. **(C)** *STZ enhanced the expression of caspase-3 in MIN6 cells*. Level of caspase-3 expression in control and STZ treated cells was determined using western blotting. First, MIN6 cells were treated with STZ (10 mM) for 1 h, and cells lysed, centrifuged, and analyzed for total protein using Bradford assay. Next, quantitated protein was separated on polyacrylamide gels and transferred to nitrocellulose membranes. The level of caspase-3 was detected using a primary antibody specific for caspase-3. Analysis of the results showed that STZ treatment increased the caspase-3 expressions by 1.46 fold, compared with the control treatment (1.0).

respectively, compared to the 10 mM STZ treated cells (38.43%) (**Figure 5A**).

Treatment With Naringenin Reduced STZ-Induced ROS Production in MIN6 Cells

Reactive oxygen species play a significant role in the pathogenesis of STZ-induced diabetes (Asmat et al., 2016). Therefore, to determine the potency of naringenin to mitigate the levels of STZ-induced ROS, MIN6 cells were first treated with naringenin (0–100 μM) for 24 h, followed by exposing the cells to 10 mM STZ treatment for 1 h. These cells were subjected to H_2DCFDA-fluorescence analysis. Mechanistically, the non-fluorescent H_2DCFDA gets de-esterified intra-cellularly to produce fluorescent 2′,7′-dichlorofluorescein (DCF) (Cohn et al., 2008). Compared to control untreated naringenin, exposure to STZ for 1 h significantly induced ROS production, i.e., the fluorescent peak in STZ-treated cells shifted toward the right (**Figure 5B**). However, STZ-induced ROS production was reduced by naringenin treatment in a dose-dependent manner (**Figure 5B**). The shift in peak, toward untreated control peak, upon naringenin treatment (50 and 100 μM) indicates a diminished ROS level as shown by a change in the fluorescence intensity when compared to STZ treatment (**Figure 5B**). These results indicate that naringenin inhibits ROS generation thereby minimizing the damage caused to MIN6 cells.

Prior Exposure of MIN6 Cells to Naringenin Could Decrease the Apoptosis Inducing Effect of STZ

To assess the cytoprotective effect of naringenin against STZ treatment-induced toxicity in MIN6 cells, the levels of apoptosis was estimated by FACS using Annexin-V/PI staining (Hingorani et al., 2011). Flipping of phosphatidylserine of the plasma membrane from the inner surface to the outer surface is an early event in apoptosis (Hingorani et al., 2011). Annexin-V binds to the phosphatidylserine, hence, labeled Annexin-V helps in the detection of cells undergoing apoptosis (Hingorani et al., 2011). Propidium iodide is used in combination with labeled Annexin-V, to measure the cell membrane integrity as the propidium iodide is impermeable if the cells are viable, but can enter the cells that are undergoing apoptosis or necrosis (Cohn et al., 2008). Experimentally, MIN6 cells were pretreated with naringenin (0–100 μM) for 24 h, followed by treatment

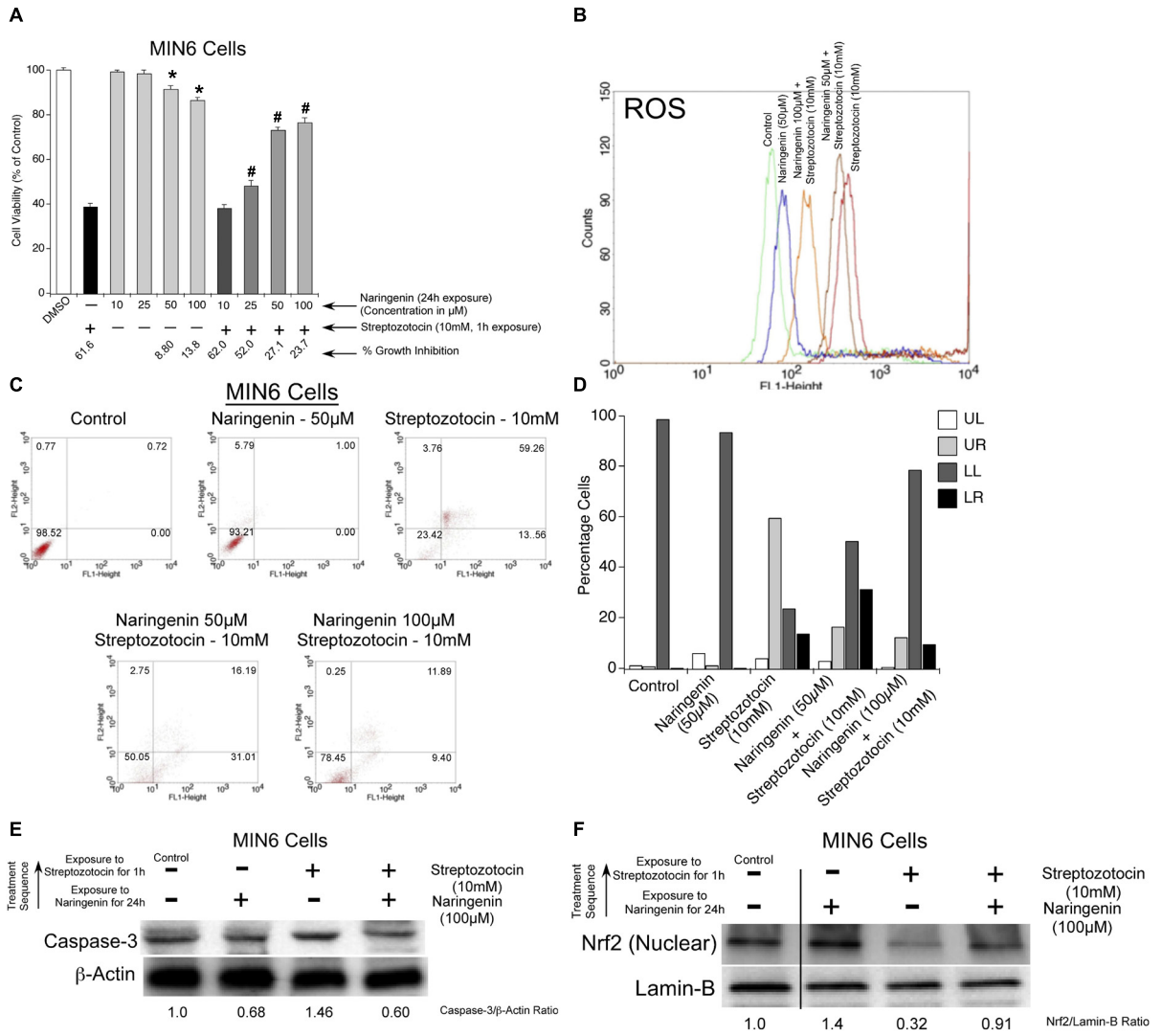


FIGURE 5 | Naringenin protected MIN6 cells from streptozotocin induced apoptotic cell death. **(A)** *Naringenin protected MIN6 cells from STZ-induced cytotoxicity.* To determine the protective effect of naringenin on STZ-induced cytotoxicity, a cell viability assay was carried out using MTT reagent. MIN6 cells were treated with naringenin (0–100 μM) alone, and naringenin for 24 h followed by STZ (10 mM) for 1 h. Naringenin-pretreated cells showed increased viability compared to the STZ (10 mM) alone treated cells. Data represented is the mean ± SEM of three separate experiments. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's *post hoc* test. *Significant compared with DMSO-treated control, #Significant compared with STZ treated group; $P < 0.05$. **(B)** *Naringenin inhibited the STZ-treatment induced ROS accumulation in MIN6 cells:* Intracellular ROS levels were estimated in MIN6 cells by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) staining followed by analysis of stained cells using FACS. MIN6 cells were pretreated with naringenin (0–100 μM) for 24 h, followed by exposing the cells to 10 mM STZ for 1 h and then subjected to H_2DCFDA-fluorescence analysis. A greater shift in the peak position implies the higher amount of DCF and greater ROS generation. **(C,D)** *Naringenin could mitigate the apoptosis inducing effect of STZ in MIN6 cells:* MIN6 cells were, first, pretreated with naringenin (0–100 μM) for 24 h, followed by STZ treatment (10 mM) for 1 h. Analysis of Annexin and PI stained cell showed decrease in early apoptotic cells with naringenin (100 μM) treatment. **(E)** *STZ-treatment induced caspase-3 was reduced by pretreatment with naringenin in MIN6 cells*. MIN6 cells were pretreated with naringenin (0–100 μM) for 24 h, followed by exposure to 10 mM STZ for 1 h. Cell lysates were collected and analyzed by SDS-PAGE followed by Western blotting to detect caspase-3 expression using specific antibody. Analysis of the blots showed a significant increase in the caspase 3 (1.46 fold) upon exposure to STZ. **(F)** *Naringenin upregulated Nrf2 in STZ-treated MIN6 cells.* To check whether naringenin treatment increased the Nrf2 level in MIN6 cells incubated with STZ, the nuclear fractions were collected and analyzed for Nrf2 expression using western blotting. The authenticity of nuclear fraction was confirmed by probing with laminin-B. A significant decrease in nuclear Nrf2 was observed in STZ treated cells.

with 10 mM STZ treatment for 1 h. Next, cells were treated with Annexin-V and Propidium Iodide and analyzed by flow cytometry. The collected data was analyzed using cell quest pro-software. Analysis of the results showed no major cell death with 50 μM naringenin treatment (**Figures 5C,D**). However, treatment with STZ (10 mM for 1 h) induced apoptosis in MIN6 cells (**Figures 5C,D**). A significant increase in early apoptotic cells (13.56%) and late apoptotic cells (59.26%) along with 3.76% necrotic cells was observed in STZ treated cells. Prior treatment with 50 and 100 μM naringenin could reduce the apoptosis

compared to STZ treated cells (**Figures 5C,D**). For instance, 100 μM naringenin treatment reduced the percentage of early apoptotic cells to 9.40% from 13.56% (10 mM STZ treated), late apoptotic cells reduced to 11.89% from 59.26% (10mM STZ treated) and necrotic cells reduced to 0.25% from 3.76% (10 mM STZ treated). Taken together, this data showed the ability of naringenin to mitigate the effect of STZ-induced apoptosis in MIN6 cells.

Naringenin Decreased the Caspase-3 Expression in STZ-Treated MIN6 Cells

To further determine the anti-apoptotic effects of naringenin, MIN6 cells were pretreated with naringenin (0–100 μM) for 24 h, followed by exposure to 10 mM STZ treatment for 1 h. Clear cell lysates were collected by centrifugation and total protein quantitated by the Bradford method (Kruger, 2009). About 30 μg of total protein was used to study the expression of caspase-3 by probing with a specific antibody. Exposure of MIN6 cells to STZ caused an increase in caspase-3 expression compared to control cells without treatment. Prior exposure of cells to naringenin decreased the STZ-induced caspase-3 expression (**Figure 5E**).

Cytoprotective Effect of Naringenin Is Mediated by the Upregulation of Nrf2 in MIN6 Cells

Since naringenin exhibited the activation of cytoprotective Nrf2, next, the effect of naringenin against STZ induced toxicity was estimated. In order to study this hypothesis, first, MIN6 cells were treated with naringenin and cytosolic and nuclear fractions separated using a commercially available nuclear extraction kit (Pierce NE-PER®) as per the manufacturer's instructions (Pierce, Rockford, IL, United States). The total protein content in the separated fractions was estimated using the Bradford method and level of Nrf2 expression analyzed using western blotting. Analysis of the data showed a significant increase in the Nrf2 level upon treatment of MIN6 cells with 100 μM naringenin, which protected MIN6 cells against STZ-induced toxicity when compared to untreated and STZ-treated cells (**Figure 5F**).

Oral Administration of Naringenin Reduced the STZ-Induced Fasting Blood Glucose Levels, Improved Glucose Tolerance (IPGTT) and Response to Insulin in STZ-Treated Mice

Since naringenin showed a better cytoprotective effect in the *in vitro* experiments, next, its ability to decrease the complications of diabetes, especially blood glucose, was tested in preclinical animal models. Experimentally, first, diabetes was induced by MLD-STZ administration (i.p.; 50 mg/kg body weight/day) for 5 days. As shown in **Figure 6A**, all mice showed hyperglycemia with a serum glucose level of 357 ± 13.6 mg/dl in comparison with control mice exposed to vehicle 0.5% CMC (99.5 ± 4.9 mg/dl). Oral administration of naringenin for 45 days caused a significant ($p < 0.05$) dose-dependent (50 and 100 mg/kg b.w.) reduction in blood glucose levels in STZ mice. For example, blood glucose levels of STZ mice were reduced from 357 ± 13.6 mg/dl to 141.25 ± 10.6 mg/dl after administration of naringenin at the dose of 100 mg/kg b.w. These results were comparable with positive control glibenclamide at 600 μg/kg b.w (**Figure 6A**). However, naringenin (100 mg/kg b.w.) treated control mice did not show any statistically significant ($p < 0.05$) difference when compared to that of control vehicle-treated mice (**Figure 6A**). In summary, naringenin could reduce the STZ-induced hyperglycemia in mice.

Oral Administration of Naringenin Could Improve the Glucose Tolerance in STZ-Treated Mice

In order to assess whether naringenin administration helps to improve the ability of mice to process ingested glucose, IPGTT was carried out as detailed in methods section (Goren et al., 2004). After 45 days of treatment with naringenin, mice fasted for 12 h and saline glucose solution at 1 g/kg b.w. injected by intraperitoneal route (Goren et al., 2004). Glucose levels were measured in the blood collected from the tail vein at 30, 60, 90, and 120 min after the glucose load. All the groups showed an initial sharp increase of glucose up to 30 min. Administration of 50 and 100 mg/kg b.w. of naringenin and 600 μg/kg b.w. of glibenclamide (positive control) showed a progressive decrease in the glucose level from 60 to 120 min that was comparable with the control 0.5% CMC treated group (**Figure 6B**). But, in the STZ group, the blood glucose levels remained high even after 120 min (430.75 ± 16.3 mg/dl) (**Figure 6B**). Therefore, the glucose tolerance was significantly improved by naringenin treatment. Control animals administered with 100 mg/kg b.w. naringenin showed a normal pattern of glucose tolerance (**Figure 6B**).

Oral Administration of Naringenin Improved Insulin Secretion in STZ-Treated Mice

In order to evaluate β-cell function in terms of insulin release, serum insulin level was measured after 45 days of treatment. STZ mice showed a significant ($p < 0.05$) decrease in serum insulin (from 13.16 ± 0.2 μIU/ml to 4.1 ± 0.2 μIU/ml) compared to vehicle-treated control mice (**Figure 6C**). Oral administration of naringenin for a period of 45 days, dose-dependently increased the levels of insulin in STZ treated mice. Administration of 50 mg/kg b.w. and 100 mg/kg b.w. naringenin increased the insulin levels to 8.8 ± 0.2 μIU/ml and 11.1 ± 0.4 μIU/ml, respectively (**Figure 6C**). The positive control 600 μg/kg b.w. glibenclamide augmented the insulin levels up to 12.8 ± 0.1 μIU/ml, which is similar to control animals (**Figure 6C**). Treatment of control animals (exposed to vehicle 0.5% CMC) with 100 mg/kg b.w. naringenin did not change insulin levels (**Figure 6C**). The increased insulin level with naringenin treatment could be due to the insulinotropic effect, which helps in the protection and/or regeneration of β-cells so that they produce sufficient insulin.

Oral Administration of Naringenin Restored the STZ-Induced Altered Lipid Profile in Mice

The anti-hyperlipidemic efficacy of naringenin was investigated in STZ treated mice by measuring the levels of lipids in serum (de Almeida et al., 2012) (**Table 2**). STZ administered animals showed

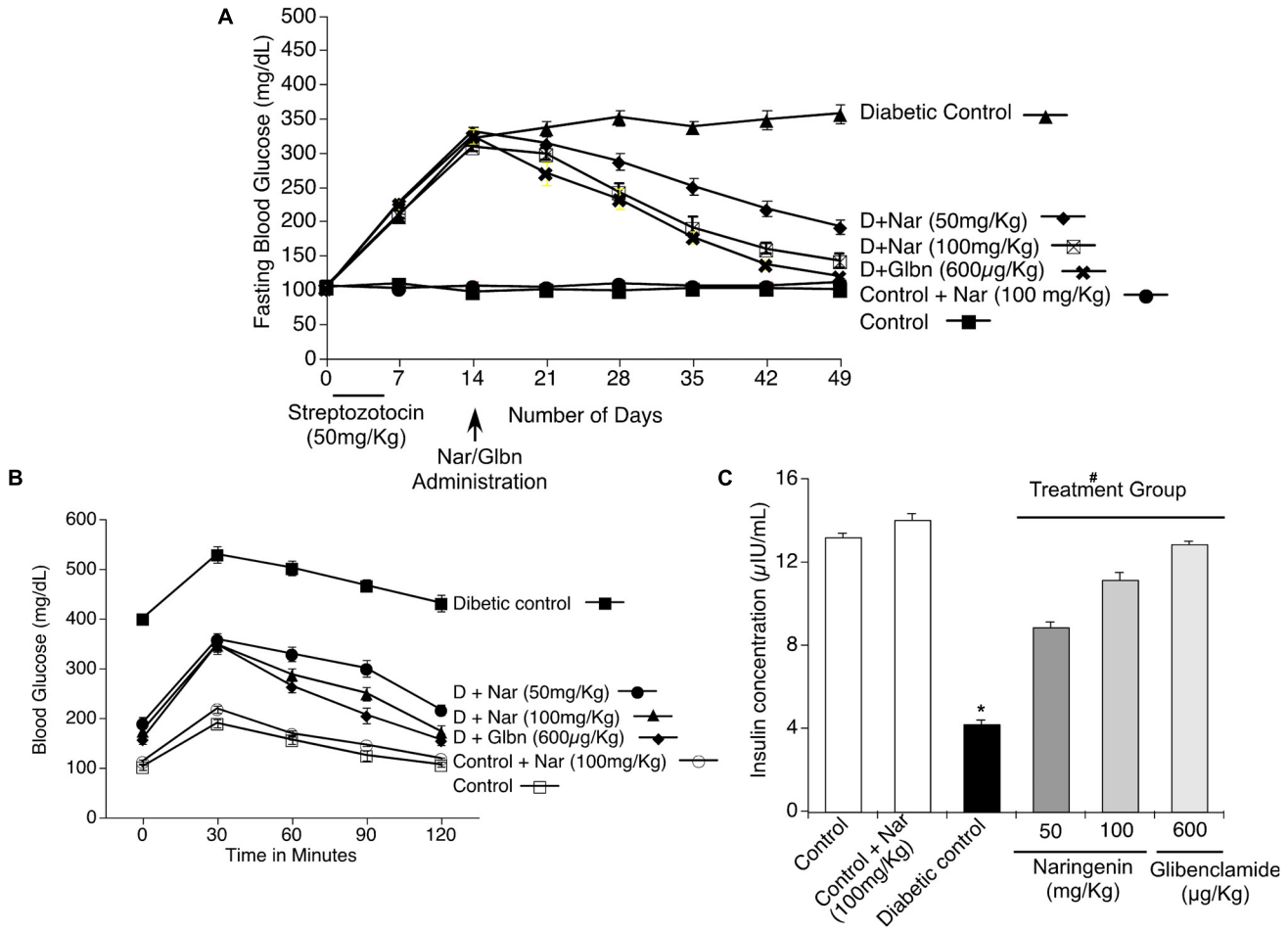


FIGURE 6 | Effects of naringenin on STZ induced hyperglycemia, serum insulin level and intra peritoneal glucose tolerance test (IPGTT) in control and experimental mice. **(A)** *Naringenin administration could decrease the STZ-induced glucose levels in mice.* Streptozotocin, a glucopyranose derivative of N-methyl-N-nitrosourea, is a unique diabetogenic compound known to induce diabetes in mice. Administration of 50 and 100 mg/kg b.w. of naringenin showed a significant dose-dependent reduction in blood glucose level compared to STZ control group. Similarly, 600 μg/kg b.w. glibenclamide showed a marked reduction in blood glucose level compared to STZ control group. **(B)** *Naringenin could improve the response of mice to glucose load in intra peritoneal glucose tolerance test (IPGTT).* The anti-hyperglycemic effect of naringenin was conformed by assessing the IPGTT in STZ induced diabetic mice. **(C)** *Naringenin could improve the serum insulin levels in STZ-induced diabetic mice*: Data are mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey *post hoc* test. *STZ control mice were compared with control mice. #STZ + NAR (50 and 100 mg/kg b.w.) and STZ + GLC (600 μg/kg b.w.) treated mice were compared with STZ control mice; $P < 0.05$.

significantly ($P < 0.05$) increased serum (a) total cholesterol (TC) from 75.03 ± 6.2 mg/dl to 181 ± 2.0 mg/dl, (b) LDL from 16.6 ± 8.1 mg/dl to 127.2 ± 2.2 mg/dl, (c) VLDL from 10.26 ± 0.5 mg/dl to 27.06 ± 1.3 mg/dl, (d) Triglycerides (TG) from 51.33 ± 2.5 mg/dl to 135.3 ± 6.5 mg/dl, (e) Phospholipids from 82.33 ± 4.8 mg/dl to 114.5 ± 7.8 mg/dl, (f) Free fatty acids (FFA) from 2.43 ± 0.4 mg/dl to 5.56 ± 0.2 mg/dl, and significantly decreased HDL level from 48.16 ± 3.1 mg/dl to 26.7 ± 1.24mg/dl (**Table 2**). Oral administration of naringenin (50 and 100 mg/kg b.w.) showed a significant ($P < 0.05$) reduction in TC, LDL, VLDL, TG, Phospholipids, and FFA. In addition, a significant ($p < 0.05$) increase in the concentration of HDL compared to STZ controls was noted (**Table 2**). For instance, TC was decreased from 181 ± 2.0 mg/dl to 106.6 ± 4.9 mg/dl. Likewise serum LDL was reduced from 127.2 ± 2.2 mg/dl to 47.56 ± 6.1 mg/dl and VLDL from 27.06 ± 1.3 mg/dl to 17.33 ± 1.1 mg/dl. Similarly, while triglyceride content was reduced from 135.3 ± 6.5 mg/dl to 86.66 ± 5.5 mg/dl the phospholipid level decreased from 114.5 ± 7.8 mg/dl to 87.05 ± 7.2 mg/dl upon exposure to naringenin. Furthermore, whereas free fatty acids showed a significant decrease from 5.56 ± 0.2 mg/dl to 2.76 ± 0.2 mg/dl, the level of HDL content increased from 26.7 ± 1.24 mg/dl to 41.76 ± 1.2 mg/dl with naringenin administration (**Table 2**). Administration of the positive control glibenclamide (600 μg/kg b.w.) to STZ-induced diabetic mice has restored the lipid profile to normal levels (**Table 2**). The observed anti-hyperlipidemic effect of naringenin could be due to either its insulin stimulatory effect on pancreatic β-cells or due to decreased cholesterogenesis and fatty acid synthesis.

TABLE 2 | Effect of naringenin on serum lipid profile in control and STZ-treated mice.

Groups	Serum Lipid Profile						
	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)	Phospholipids (mg/dl)	Free Fatty Acids (mg/dl)
Control	75.03 ± 6.2	51.33 ± 2.5	48.16 ± 3.1	16.6 ± 8.1	10.26 ± 0.5	82.33 ± 4.8	2.43 ± 0.4
NAR control (100 mg/kg)	73.46 ± 5.8	48.33 ± 3.5	50.73 ± 2.6	13.0 ± 8.17	9.66 ± 0.7	77.90 ± 3.7	1.93 ± 0.5
STZ control	181 ± 2.0*	135.3 ± 6.5*	26.7 ± 1.24*	127.2 ± 2.2*	27.06 ± 1.3*	114.5 ± 7.8*	5.56 ± 0.2*
STZ + NAR (50 mg/kg)	138.4 ± 1.8#	111.6 ± 6.1#	37.33 ± 3.9#	78.76 ± 2.7#	22.33 ± 1.2#	94.15 ± 5.6#	3.16 ± 0.3#
STZ + NAR (100 mg/kg)	106.6 ± 4.9#	86.66 ± 5.5#	41.76 ± 1.2#	47.56 ± 6.1#	17.33 ± 1.1#	87.05 ± 7.2#	2.76 ± 0.2#
STZ + GLC (600 μg/kg)	95.7 ± 2.8#	72.33 ± 4.1#	45.2 ± 3.79#	36.03 ± 5.8#	14.46 ± 0.8#	85.07 ± 6.8#	2.53 ± 0.1#

*HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol. Injection of STZ to mice not only increased the levels of blood glucose but also elevated the serum lipids as evidenced by high TG, TC, LDL, VLDL, phospholipids, free fatty acids and reduced HDL (Krishnakumari et al., 2011). Treating these diabetic animals with naringenin (50 and 100 mg/kg b.w.) for 45 days significantly ($p < 0.05$) reduced the elevated lipids. The positive control 600 μg/kg b.w. glibenclamide also showed significant reduction in the serum lipids. Data represents mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's post hoc test. *STZ mice were compared with control mice. #STZ + NAR (50 and 100 mg/kg b.w.) and STZ + GLC (600 μg/kg b.w.) treated mice were compared with STZ control mice; $P < 0.05$.*

Oral Administration of Naringenin Retarded the Formation of TBARS and Hydroperoxides in STZ-Treated Pancreatic Tissues

Lipid peroxidation and formation of hydroperoxides are key indicators of tissue damage, which occurs due oxidative stress (El-Aal, 2012). Since STZ is known to promote cellular oxidative stress, first, the levels of lipid peroxides were measured by boiling 0.1 ml tissue homogenate with 2.0 ml of TBA:TCA:HCl reagent for 15 min followed by cooling, and centrifuging at 10,000 rpm for 5 min to separate clear supernatant. The levels of TBARS (mM/100 g tissue) were estimated by reading the absorption at 535 nm (Niehaus and Samuelsson, 1968). **Table 3** shows a significant increase in the concentration of TBARS in pancreatic tissue from about 2 mM/100 g tissue to ∼4.2 mM/100 g tissue upon STZ treatment. Administration of 50 mg/kg b.w. and 100 mg/kg b.w. naringenin significantly reduced the TBARS in pancreatic tissue to ∼3.6 mM/100 g tissue and ∼2.8 mM/100 g tissue, respectively (**Table 3**). The positive control 600 μg/kg b.w. glibenclamide reduced the TBARS levels to ∼2.5 mM/100 g tissue (**Table 3**). Treatment of control animals (exposed to vehicle 0.5% CMC) with 100 mg/kg b.w. naringenin did not change TBARS levels (**Table 3**).

To estimate the concentration of hydroperoxides, 0.2 ml of tissue homogenate was incubated with 1.8 ml of Fox reagent at room temperature for 30 min and the absorbance measured at 560 nm (Jiang et al., 1992). The results were expressed as mM hydroperoxides produced per 100 g tissues. The STZ-treatment significantly elevated the tissue pancreatic hydroperoxides from 16 mM/100 g tissue to ∼23 mM/100 g tissue (**Table 3**). Elevated pancreatic hydroperoxides were brought down to about ∼20 mM/100 g tissue and ∼17 mM/100 g tissue, respectively upon 50 mg/kg b.w. and 100 mg/kg b.w. naringenin administration (**Table 3**). Oral administration of glibenclamide (600 μg/kg b.w.) decreased hydroperoxides levels (to ∼16 mM/100 g) that are similar to control animals (**Table 3**). Treatment of control animals with 100 mg/kg b.w. naringenin did not change hydroperoxides levels (**Table 3**).

TABLE 3 | Changes in the levels of TBARS and hydroperoxides in pancreatic tissues of control and experimental mice.

Groups	TBARS (mM/100 g tissue)	Hydroperoxides (mM/100 g tissue)
Control	2.16 ± 0.15	16.40 ± 0.18
NAR control (100 mg/kg)	1.97 ± 0.09	16.12 ± 0.19
STZ control	4.22 ± 0.20*	22.96 ± 1.19*
STZ + NAR (50 mg/kg)	3.68 ± 0.07#	20.67 ± 0.49#
STZ + NAR (100 mg/kg)	2.82 ± 0.09#	17.66 ± 0.98#
STZ + GLC (600 μg/kg)	2.56 ± 0.20#	16.41 ± 0.20#

*The levels of TBARS and hydroperoxide concentration were determined using colorimetric assays as described in materials and methods section (Niehaus and Samuelsson, 1968). Analysis of the results showed a significant increase in the levels of TBARS and hydroperoxides ($p < 0.05$) upon administering STZ to mice. Oral administration of 50 and 100 mg/kg b.w. of naringenin and 600 μg/kg b.w. of glibenclamide (positive control) showed a significant decrease in TBARS and hydroperoxides levels. Data represents mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's post hoc test. *STZ control mice were compared with control mice. #STZ + NAR (50 and 100 mg/kg b.w.) and STZ + GLC (600 μg/kg b.w.) treated mice were compared with STZ control mice; $P < 0.05$.*

Oral Administration of Naringenin Enhanced the Expression of Nrf2 and Thereby Increased the Activities of SOD, CAT, GPX, GST Enzymes and the Levels of GSH in the Pancreatic Tissues of STZ-Treated Mice

Since, *in vitro* studies have demonstrated the Nrf2 activation potential of naringenin, we have hypothesized that the cytoprotective effects of naringenin might be mediated through similar mechanisms even in mice. Therefore, protein lysates were collected from the pancreatic tissues of control and experimental animals and subjected for western blotting. Analysis of the data showed an about 2.5-fold increase in the Nrf2 expression compared to control or STZ treated mice (**Figure 7**). However, interestingly, a slight non-significant increase in the Nrf2 expression (compared to control mice) was observed in STZ

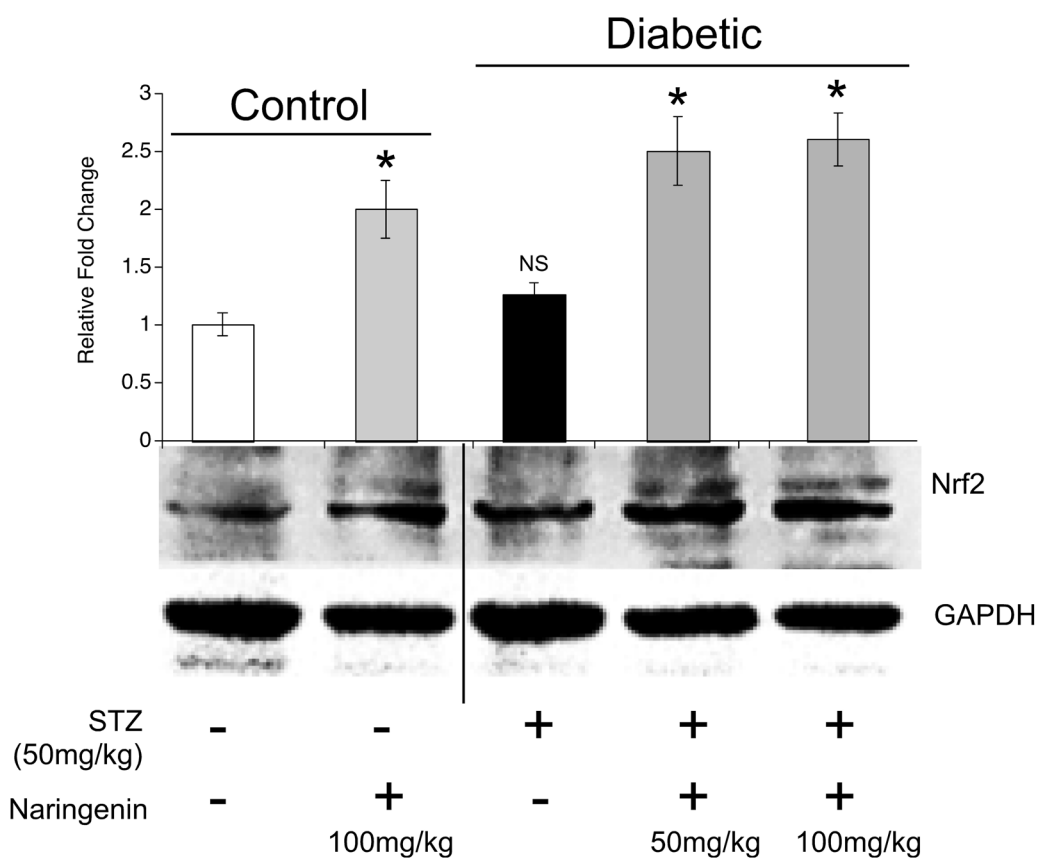


FIGURE 7 | Naringenin upregulated the expression of Nrf2 in pancreatic tissue: Pancreatic tissue lysates were generated and analyzed by western blotting to detect Nrf2 and the loading control GAPDH. Analysis of the data showed a significant increase in the Nrf2 expression in tissues collected from animals treated with naringenin compared to vehicle and streptozotocin administered ones. Interestingly, a slight non-significant increase in Nrf2 was observed even in the STZ administered animals. *Naringenin (100 mg/kg b.w.) and STZ C NAR (50 and 100 mg/kg b.w.) treated mice were compared with vehicle control and STZ control mice; $P < 0.05$ (One-way ANOVA).

treated mice (**Figure 7**). It is well known that Nrf2 regulates the cellular oxidative stress by increasing the expression of its target genes NQO1, SOD, CAT, GPX, and GSH transferase (Jung and Kwak, 2010). Therefore, we have measured the activities of these Nrf2 target genes in the pancreatic tissue (**Table 4**). SOD is an enzyme, which catalyzes the dis-mutation of superoxide radicals to H_2O_2 thus decreasing the possibility of superoxide anion interacting with nitric oxide that forms reactive peroxynitrite (Fukai and Ushio-Fukai, 2011). The SOD activity in the tissues was assayed according to the method developed by Kakkar et al. (1984). The reaction was initiated by addition of NADH. After incubation for 90 s, the glacial acetic acid was added to stop the reaction and the colored complex formed was extracted into butanol layer and measured at 560 nm. The SOD activity was significantly ($p < 0.05$) decreased in pancreatic tissue from about ∼6.4 units/min/mg protein to ∼2.1 units/min/mg protein upon STZ injection (**Table 4**). Administration of 50 mg/kg b.w. and 100 mg/kg b.w. naringenin significantly augmented the SOD levels in pancreatic tissue to ∼4.5 units/min/mg protein and ∼5.5 units/min/mg protein, respectively (**Table 4**). The positive control 600 μg/kg b.w. glibenclamide augmented the SOD levels to ∼6.1 units/min/mg protein (**Table 4**). Treatment of control animals with 100 mg/kg b.w. naringenin did not change SOD levels (**Table 4**).

Catalase is a tetrameric hemin-enzyme, which decomposes hydrogen peroxide into water and molecular oxygen (Kirkman and Gaetani, 1984). The CAT activity (μmoles of H_2O_2 consumed/min/mg protein) in the tissues was assayed by the method developed by Sinha (1972). Dichromate is converted to chromic acetate after heating in the presence of hydrogen peroxide with the formation of an intermediate perchromic acid. The produced chromic acetate was measured at 590 nm. The CAT activity significantly decreased in pancreatic tissue from about 18 units in control animals to 7.8 units; upon STZ injection (**Table 4**). Administration of 50 mg/kg b.w. naringenin and 100 mg/kg b.w. naringenin significantly augmented the CAT activity in pancreatic tissue to ∼13.8 and ∼18.0 units, respectively (**Table 4**). The positive control 600 μg/kg b.w.

TABLE 4 | Effect of naringenin on the activities of SOD, CAT, GPX, GST, and GSH in the pancreatic tissues of control and experimental mice.

Groups	SOD (Units/mg Protein)	CAT (Units/mg Protein)	GPX (Units/mg Protein)	GST (Units/mg Protein)	GSH (mg/100 g tissue)
Control	6.41 ± 0.20	18.76 ± 1.11	5.46 ± 0.20	4.175 ± 0.2	11.28 ± 0.12
NAR control (100 mg/kg)	6.52 ± 0.37	18.90 ± 0.75	5.64 ± 0.35	4.332 ± 0.2	12.03 ± 0.37
STZ control	2.12 ± 0.10*	7.83 ± 0.56*	2.81 ± 0.19*	1.392 ± 0.1*	6.18 ± 0.133*
STZ + NAR (50 mg/kg)	4.59 ± 0.11#	13.82 ± 0.99#	4.83 ± 0.07#	3.007 ± 0.1#	8.60 ± 0.385#
STZ + NAR (100 mg/kg)	5.56 ± 0.16#	18.05 ± 1.01#	5.47 ± 0.12#	3.312 ± 0.1#	10.73 ± 0.46#
STZ + GLC (600 μg/kg)	6.16 ± 0.28#	18.54 ± 1.33#	5.83 ± 0.16#	3.957 ± 0.1#	11.24 ± 0.26#

*Persistent hyperglycemia modulates the levels of free radicals, antioxidant enzymes and their substrates (Birben et al., 2012). Administration of STZ to mice showed a significant ($p < 0.05$) decrease in the levels of antioxidants compared to control group (**Table 4**). Oral administration of 50 and 100 mg/kg b.w. of naringenin and 600 μg/kg b.w. of glibenclamide (a positive control) showed a significant increase in the antioxidants to near normal levels in pancreatic tissues of control mice (**Table 4**). Data are expressed as mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's post hoc test. *STZ control mice were compared with control mice. #STZ + NAR (50 and 100 mg/kg b.w.) and STZ + GLC (600 μg/kg b.w.) treated mice were compared with STZ control mice; $P < 0.05$.*

glibenclamide significantly elevated the CAT activity, which is similar to the activity of control animals (**Table 4**). No significant change was observed in mice treated with 100 mg/kg b.w. naringenin in comparison to that of control group administered with 0.5% carboxymethyl cellulose demonstrating that naringenin administration has no major impact on CAT activity (**Table 4**).

GPX is a selenium-containing peroxidase, which decomposes H_2O_2 and lipid peroxides through GSH, as a hydrogen donor, into the water and protects the cells from free radicals (Lubos et al., 2011). The activity of GPX was determined by the method of Rotruck et al. (1973). The GPX activity (μmoles of GSH oxidized/min/mg protein) was significantly decreased in pancreatic tissue from about from 5.4 μmoles in control animals to ∼2.8 μmoles; upon STZ treatment (**Table 4**). Oral administration of 50 mg/kg b.w. naringenin and 100 mg/kg b.w. naringenin significantly augmented the GPX activity in pancreatic tissue to ∼4.8 and ∼5.4 μmoles; respectively (**Table 4**). The positive control 600 μg/kg b.w. glibenclamide significantly augmented the GPX activity in pancreatic tissue up to 5.8 μmoles compared to STZ control animals (**Table 4**). No significant change was observed even in non-diabetic mice treated with naringenin in comparison to that of the control group demonstrating that naringenin administration did not affect the GPX levels (**Table 4**).

GST is a GSH-dependent cytosolic enzyme, which protects cells from the damage caused by ROS (Hayes and McLELLAN, 1999). In order to determine whether administration of naringenin restored the GST activity, a method developed by Habig et al. (1974) was used and the results expressed as μmoles of CDNB conjugate formed /min/mg protein. Analysis of the data represented in **Table 4** showed about 75% decrease in GST activity in the pancreatic tissue, compared to vehicle-treated mice, in STZ injected animals. Oral administration of naringenin (50 mg/kg and 100 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) significantly ($p < 0.05$) increased the GST activity levels. For example, administration of 100 mg/kg body weight naringenin elevated the pancreatic GST activity to ∼3.3 units (**Table 4**). Similarly, 600 μg/kg body weight glibenclamide elevated the pancreatic GST activity to ∼3.9 units (**Table 4**). No significant change in GST activity was observed between vehicle control and 100 mg/kg body weight naringenin-treated non-diabetic mice (**Table 4**).

GSH is a potent free-radical scavenger and co-substrate for GPX activity (Sies, 1999). Several studies have shown the ability of STZ to decrease cellular GSH levels (Birben et al., 2012). Therefore, to determine whether administration of naringenin could mitigate the effect of STZ-induced GSH depletion effect, pancreatic tissues were collected and processed to estimate GSH content using Ellman's assay (Ellman, 1959). The data in **Table 4** shows a significant decrease in pancreatic tissue GSH levels upon administering mice with STZ (from ∼11.28 mg/100 g tissue to ∼6 mg/100 g tissue). However, when naringenin or glibenclamide was administered the levels had gone up to the ones present in control animals (**Table 4**). However, no significant changes were observed in non-diabetic mice treated with naringenin in comparison to that of a control group of mice (**Table 4**).

Oral Administration of Naringenin Restored the Changes in Carbohydrate Metabolizing Enzymes in STZ-Injected Liver Tissues

Oral Administration of Naringenin Enhanced the Activity of Hexokinase and Glucose-6-Phosphate Dehydrogenase

The first step in the glycolysis is the fixation of glucose by phosphorylation into glucose-6-phosphate by Hexokinase enzyme (Guo et al., 2012). Hexokinase functions as a glucose sensor of insulin-producing pancreatic β-cells and regulates the glycogen synthesis and hepatic glucose production (Guo et al., 2012). The hexokinase activity in the liver tissue was determined by the method of Brandstrup et al. (1957). Hexokinase converts D-glucose and ATP to glucose 6-phosphate and ADP. The residual glucose was reacted with an *o*-toluidine reagent to form a green color, which was measured at 640 nm. The hexokinase activity was significantly ($p < 0.05$) reduced in liver tissues of STZ control mice from 87.71 ± 3.4 units/mg protein to 60.04 ± 0.9 units/mg protein (**Table 5**). Oral administration of 50 mg/kg b.w. and 100 mg/kg b.w. naringenin significantly augmented the activity of hexokinase to 74.12 ± 1.0 units/mg

TABLE 5 | Changes in the activities of key enzymes of carbohydrate metabolism and glycogen in the liver of control and experimental mice.

Groups	Hexokinase ($IU^{@}$/g protein)	G6-PD (10^4mIU/mg protein)	G6-P (IU*mg protein)	F1,6-BP ($IU^{\$}$/mg protein)	Glycogen (mg/100 g Tissue)
Control	87.61 ± 3.4	4.29 ± 3.70	0.178 ± 0.004	0.189 ± 0.025	32.44 ± 2.04
NAR control (100mg/kg)	88.97 ± 0.4	4.47 ± 0.27	0.174 ± 0.020	0.184 ± 0.024	32.61 ± 0.91
STZ control	60.04 ± 0.9*	2.61 ± 0.03*	0.297 ± 0.010*	0.440 ± 0.003*	21.27 ± 1.44*
STZ+NAR (50mg/kg)	74.12 ± 1.0#	3.62 ± 0.14#	0.255 ± 0.005#	0.316 ± 0.015#	27.88 ± 1.60#
STZ+NAR (100mg/kg)	82.54 ± 3.5#	4.16 ± 0.09#	0.231 ± 0.010#	0.267 ± 0.027#	29.88 ± 0.98#
STZ+GLC (600μg/kg)	85.06 ± 2.4#	4.06 ± 0.16#	0.202 ± 0.016#	0.223 ± 0.026#	28.05 ± 0.30#

*$^{@}$IU-moles of glucose phosphorylated per min, *IU-moles of inorganic phosphate liberated per min, $^{\$}$IU-moles of inorganic phosphate liberated per h. G6-PD, Glucose-6-phosphate dehydrogenase; G6-P, Glucose-6-phophatase; F1,6-BP, Fructose-1,6-bisphosphatase. Diabetes inducing agent Streptozotocin decreased the activities of hexokinase and glucose-6-phosphate dehydrogenase, while promoting the activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase. Oral administration of naringenin (50 and 100 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) significantly ($p < 0.05$) increased the activity of hexokinase and glucose-6-phosphate dehydrogenase in liver of STZ-induced diabetic mice indicating improved utilization of glucose by liver in diabetic mice. The activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase were decreased leading to low gluconeogenesis. In addition, oral administration of naringenin (50 and 100 mg/kg b.w.) and glibenclamide (600 μg/kg b.w.) significantly ($p < 0.05$) increased the concentration of liver glycogen. Data are expressed as mean ± SE from six mice in each group. Statistical analysis was performed by one-way ANOVA ($P < 0.05$), followed by Tukey's post hoc test. *Significantly different from control at $P < 0.05$. #Significantly different from diabetes group at $P < 0.05$.*

protein and 82.54 ± 3.5 units/mg protein, respectively (**Table 5**). The positive control 600 μg/kg b.w. glibenclamide increased the activity to 85.06 ± 2.4 units/mg protein (**Table 5**). However, treatment of control non-diabetic animals with 100 mg/kg b.w. naringenin did not show any difference (**Table 5**).

Glucose-6-phosphate dehydrogenase (G6-PD) is the first regulating enzyme of the pentose phosphate pathway of glucose metabolism that produces (a) ribose-5-phosphate; (b) reducing equivalent, NADPH and a variety of sugars with carbon chain length beginning from 3C to 7C (Cappai et al., 2011). A method developed by Ells (1961) was used to measure the G6-PD activity in liver tissue (Ells and Kirkman, 1961). The increase in absorbance when NADP reduced to NADPH was measured. The reduction of NADP occurs due to the transfer of electrons from glucose-6-phosphate to NADP, which is catalyzed by G6-PD. The STZ control animals showed a significant ($p < 0.05$) decrease in the activity of G6-PD in liver tissue (4.29 ± 0.3 units/mg protein VS 2.61 ± 0.1 units/mg protein) (**Table 5**). Oral administration of 50 mg/kg b.w. naringenin and 100 mg/kg b.w. naringenin significantly augmented the G6-PD activity to 3.62 ± 0.1 units/mg protein and 4.16 ± 0.1 units/mg protein, respectively (**Table 5**). 600 μg/kg b.w. of glibenclamide also augmented the activity, which is similar to control animals exposed to vehicle 0.5% CMC (**Table 5**). Treatment of non-diabetic animals with 100 mg/kg b.w. naringenin did not change G6-PD activity compared to vehicle 0.5% CMC (**Table 5**).

Oral Administration of Naringenin Decreased the Activities of Glucose-6-Phosphatase and Fructose-1, 6-bisphosphatase in STZ-Injected Liver Tissues

Glucose-6-phosphatase (G6-P), a key gluconeogenic enzyme, catalyzes the dephosphorylation of glucose-6-phosphate to glucose (van Schaftingen and Gerin, 2002). Under normal conditions, insulin suppresses the activity of this enzyme (Shulman, 2000). However, in diabetics, the levels of G6-P get elevated to provide glucose to starving hepatocytes (Roden and Bernroider, 2003). Therefore, in order to test whether STZ induced animals also exhibit elevated G6-P activity, the method developed by Hikaru and Toshitsugu (1959) was adopted to measure the G6-P activity in the liver tissue (Koide, 1959). The inorganic phosphorus liberated during this conversion was estimated using Fiske and Subbarow (1925). The G6-P activity was significantly ($p < 0.05$) elevated in liver tissue of STZ control mice from 0.178 ± 0.02 units/mg protein to 0.297 ± 0.01 units/mg protein (**Table 5**). Elevated G6-P activity was reduced to about 0.255 ± 0.05 units/mg protein and 0.231 ± 0.01 units/mg protein, respectively upon 50 mg/kg b.w. and 100 mg/kg b.w. naringenin administration (**Table 5**). Oral administration of 600 μg/kg b.w. of glibenclamide decreased the activity to 0.202 ± 0.02 units/mg protein (**Table 5**). No significant change in G6-P activity was observed between vehicle control and 100 mg/kg b.w. naringenin-treated non-diabetic mice (**Table 5**).

Fructose-1, 6-bisphosphatase (F1,6-BP) is another key gluconeogenic enzyme effected by diabetes (van Schaftingen and Gerin, 2002). This enzyme is a rate-limiting enzyme in the gluconeogenic pathway and catalyzes the dephosphorylation of fructose-1,6-bisphosphate to fructose-6-phosphate (van Schaftingen and Gerin, 2002). The F1,6-BP activity in the liver tissue was determined by the method of Gancedo (Gancedo and Gancedo, 1971). The inorganic phosphorus liberated was estimated using (Fiske and Subbarow, 1925). **Table 5** shows a significant increase in the activity of F1,6-BP from 0.189 ± 0.02 units/mg protein to 0.440 ± 0.03 units/mg protein upon STZ administration. Oral administration of 50 mg/kg b.w. naringenin and 100 mg/kg b.w. naringenin reduced the STZ induced F1,6-BP activity to 0.316 ± 0.01 units/mg protein and 0.267 ± 0.02 units/mg protein, respectively (**Table 5**). The positive control 600 μg/kg b.w. glibenclamide brought down the fructose-1, 6-bisphosphatase activity to 0.223 ± 0.02 units/mg protein (**Table 5**). However, treatment of control non-diabetic animals exposed to vehicle 0.5% CMC or treated with 100 mg/kg body weight naringenin did not change F1,6-BP activity (**Table 5**).

Oral Administration of Naringenin Increased the Liver Glycogen Levels in STZ-Administered Mice

Glycogen, a polymer of glucose residues produced by the glycogen synthase enzyme, is the key storage form of glucose and its amount in liver and muscle is an indication of insulin activity (Pederson et al., 2005). A method devolved by Morales et al. (1973) was used to extract and estimate the liver glycogen. Glucose is dehydrated by sulphuric acid to furfural derivative which then complexes with anthrone to give a green colored complex, which is read at 620 nm. The glycogen level was significantly ($p < 0.05$) decreased in liver tissues of STZ control mice from 32.44 ± 2.0 units to 21.27 ± 1.4 units (**Table 5**). Oral administration of 50 mg/kg b.w. naringenin and 100 mg/kg b.w. naringenin significantly augmented the glycogen content to 27.88 ± 1.6 units and 29.88 ± 0.9 units, respectively (**Table 5**). 600 μg/kg b.w. of glibenclamide also augmented to 28.05 ± 0.3 units. However, 100 mg/kg b.w naringenin-treated animals did not change liver glycogen compared to vehicle control treated non-diabetic mice (**Table 5**).

Oral Administration of Naringenin Restored the Altered Morphology of Pancreatic Tissues Observed in Mice Administered With STZ

Since STZ administration is known to damage pancreas by reducing the size and number of functionally active β-cells, an attempt was made to check whether naringenin and the positive control glibenclamide restored these altered morphological features (Bâlici et al., 2015). Control and naringenin (100 mg/kg b.w.) treated non-diabetic mice showed a normal pattern with clear and well-structured pancreatic islets (**Figure 8**). However, the pancreatic sections of STZ control mice displayed severe necrotic changes, exclusively in the center of pancreatic islets. In addition, disappearing of the nucleus with a comparative reduction in size was observed (**Figure 8**). Furthermore, pancreatic islets of STZ animals treated with naringenin (50 mg/kg b.w.) exhibited moderate expansion (**Figure 8**). Likewise, STZ animals treated with naringenin (100 mg/kg b.w.) showed a significant improvement in the pancreatic islet with distinct cellularity changes and increase in granulation, compared to the STZ control mice (**Figure 8**). The positive control 600 μg/kg b.w. glibenclamide showed healthy pancreas (**Figure 8**).

Oral Administration of Naringenin Restored the Insulin Secreting Cells in Mice Administered With STZ

The immunohistochemical method was used to study the distribution pattern as well as number of functional β-cells in pancreatic islets of mice (Kim et al., 2009). Control (vehicle treated) and naringenin control (100 mg/kg b.w.) groups' showed islets with a comparatively larger region of positive immunoreactivity, demonstrating the existence of healthy cells secreting insulin in the pancreas (**Figure 9**). However, the STZ control islet cells showed distorted morphology with very few insulin-positive cells compared to vehicle controls (**Figure 9**). While 50 mg/kg b.w. naringenin showed a moderate increase in

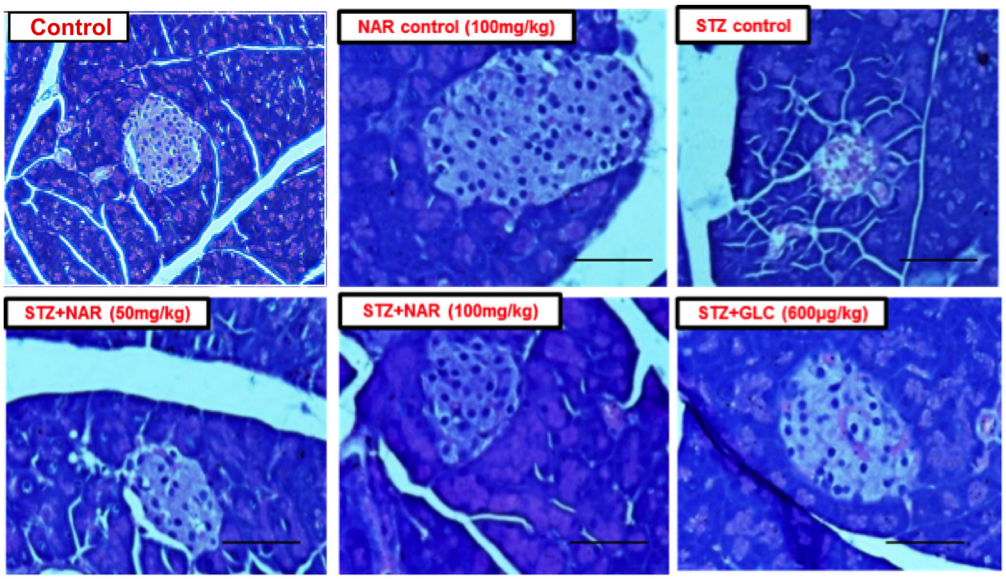


FIGURE 8 | Administration of naringenin restored the normal structure of pancreas (Magnification: 40X, scale 100 μm). Control and naringenin treated animals exhibited normal pancreatic islet cells. However, animals administered with STZ showed a reduction in the size of islets, damaged β-cell population, and necrotic tissue. Analysis of pancreas collected from STZ administered mice subsequently treated with naringenin (50 mg/kg b.w.) exhibited moderate expansion of pancreatic islets, while other animals treated with 100 mg/kg b.w. naringenin restored necrotic and fibrotic changes and increased number and size of the islets. STZ mice treated with glibenclamide (600 μg/kg b.w.) showed absence of necrosis and fibrotic changes and increased number and size of the islets, indicating the restoration of normal morphological features.

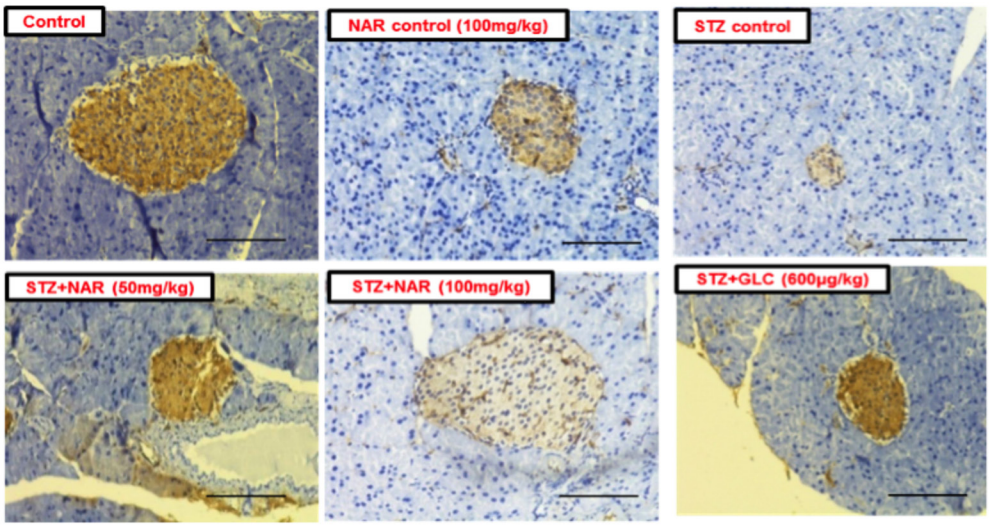


FIGURE 9 | Immunohistochemical analysis of insulin secreting cells in pancreatic islets of normal and experimental mice (Magnification-400X, Scale-100 μm). Sections of pancreas collected from control and naringenin-treated non-diabetic animals showed the presence of normal morphology of islets and β-cells; however, STZ control showed a significant decrease in insulin immunoreactivity and a number of immunoreactive β-cells. While diabetic mice (STZ mice) treated with naringenin 50 mg/kg b.w. showed a moderate increase in insulin immunoreactivity, the ones administered with 100 mg/kg b.w. naringenin and 600 μg/kg b.w. glibenclamide showed a significant increase in insulin immunoreactivity and number of immunoreactive β-cells.

insulin immuno-reactivity, the ones received 100 mg/kg b.w. naringenin showed the relatively large area of positive immuno-reactivity with numerous brown insulin granules in the β-cells of pancreatic islets (**Figure 9**). Pancreatic section of STZ mice treated with positive control compound glibenclamide showed normal histo-architecture of the pancreas with insulin cells (**Figure 9**). Collectively, these observations conform the role of naringenin in increasing the insulin-positive cells in the pancreas.

DISCUSSION

Oxidative stress, which results from the imbalance due to: (a) overproduction of oxygen/nitrogen radicals; (b) loss/decrease of antioxidant enzymes; (c) low levels of natural endogenous antioxidants; (d) changes in transcription factors/transcription factor-associated protein(s) controlling the expression of endogenous radical scavenging enzymes (Rahal et al., 2014) results in the generation of oxidized lipids, broken DNA, modified proteins and carbohydrates (Birben et al., 2012). As a result of these changes in macromolecules: (a) the normal cells undergo transformation into cancer cells; (b) cells might lose normal functions such as on-demand proliferation and differentiation; (c) cells may undergo apoptosis or autophagic death, and (d) cells may lose the ability to execute repair processes (Klaunig et al., 2011). Therefore, oxidative stress is responsible for the origin of various diseases that include diabetes (Jiménez-Osorio et al., 2015). Hence, controlling oxidative stress is key to prevent diabetes.

Several lines of evidences suggest that phytochemicals such as flavonoids not only act as antioxidants to inhibit ROS but also trigger the expression of cyto-protective transcription factors such as Nrf2, which helps in the transcription of genes that include NQO1, SOD, GST, HO1 and many more (Stefanson and Bakovic, 2014). Among various flavonoids known to activate Nrf2 signaling resveratrol and curcumins have been well studied for treating diabetes (Tabatabaei-Malazy et al., 2015). Despite its potent role in Nrf2 activation, resveratrol suffers from low bioavailability, which limited its usage in the clinic (Elango et al., 2016). Hence, in this study, we made an attempt to investigate the Nrf2 activation potential of naringenin in STZ-exposed MIN6 cells and mice.

Pancreatic β-cells are highly susceptible to oxidative stress, which appears to be in part due to the lack of robust antioxidant capacity (Lenzen et al., 1996). Experimental studies have shown that adenoviral-mediated overexpression of antioxidant enzymes *in vitro* in β-cells, as well as exogenous treatment with antioxidants *in vivo* in animals, safeguards pancreatic β-cells from such insults (Lei and Vatamaniuk, 2011). Hence, initially, we checked whether naringenin has the potential to activate Nrf2 in MIN6 cell line using the Nrf2-Keap1 complementation system. The sensor system recognizes the potential of naringenin to promote Nrf2-Keap1 complex dissociation (Ramkumar et al., 2013). Treatment of MIN6 cell line with naringenin-induced endogenous as well as ectopically expressed Nrf2 in a dose-dependent manner indicating the potential of this compound to reduce oxidative stress. In addition, our data showed naringenin-induced activation and subsequent nuclear translocation of Nrf2 at a dosage of 50 and 100 μM. Further, when the MIN6 cells were transfected with luciferase-expressing ARE-NQO1 and ARE-GST constructs and, subsequently treated with naringenin, a dose-dependent increase in the luciferase activity in both NQO1 and GST was observed indicating that the naringenin

treatment induced Nrf2, thereby elevated the levels of NQO1 and GST. Hence we further evaluated the antioxidant potential of naringenin in STZ induced diabetic models.

Streptozotocin, a derivative of *N*-methyl-*N*-nitrosourea, stands unique for its diabetogenic potential in β-cells (Schnedl et al., 1994). Mechanistically STZ induces diabetes by transferring the methyl group to the DNA molecule thereby triggering damage and fragmentation (Bennett and Pegg, 1981). DNA strand breaks induced by STZ treatment direct the cells to synthesize more poly (ADP-ribose) polymerase (PARP) to circumvent the STZ-induced apoptotic effects (Bennett and Pegg, 1981). However, over-stimulated PARP causes the reduction of intracellular NAD^+ and ATP, which ultimately leads to β-cells apoptosis/necrosis (Szkudelski, 2001). Alternatively, it is also been proposed that STZ act as an intracellular nitric oxide donor, which stimulate the generation of ROS (Szkudelski, 2001). In the current study, MIN6 cells were exposed to a 10 mM concentration of STZ for 1 h to induce apoptosis (Kang et al., 2011). Since caspases-3 is involved in the PARP fragmentation, we have assessed the caspases-3 expression using western blotting. STZ induced the expression of caspases-3, however, naringenin attenuated the STZ induced apoptosis by suppressing the expression of cleaved caspases-3. Sustained production of ROSs is a primary stimulant of apoptosis. In the STZ induced MIN6 cells, we found elevated intracellular ROSs. Naringenin could decrease ROSs to a normal level by promoting the expression of NQO1 and GST via Nrf2 pathway.

In vivo, administration of naringenin significantly ameliorated the metabolic effects in STZ-induced diabetic mice. The raised blood glucose levels in diabetic mice were returned to near normal levels in diabetic mice treated with naringenin (**Figure 6A**). Likewise, the naringenin administration significantly enhanced the serum insulin level by stimulating the remaining pancreatic β-cells to produce more insulin to regulate the glucose level (**Figure 6C**). Further, naringenin showed dose-dependent improvement in the intraperitoneal glucose tolerance of the diabetic mice, which is comparable with the anti-diabetic drug, glibenclamide, a known stimulator of insulin (**Figure 6B**). Moreover, STZ-induced diabetic mice displayed significant abnormalities in lipid metabolism, which leads to significant elevation of serum cholesterol, triglycerides, LDL and VLDL and reduction in HDL levels. Administration of naringenin for 45 days showed a significant reduction in the serum cholesterol, triglycerides, LDL and VLDL and increased HDL levels in diabetic mice (**Table 2**). These results specify the lipid-lowering effect of naringenin on diabetic mice also the efficacy is comparative to the dose of naringenin.

Under physiological conditions, liver maintains normoglycemia by controlling blood glucose through glycolysis, gluconeogenesis, and glycogen synthesis processes (Han et al., 2016). However, due to insulin deficiency in diabetes, the above-stated functions are impaired and liver produces further glucose (Ozougwu et al., 2013). Few *in vivo* studies have shown that glucose homeostasis can be restored by phenolic compounds through changes in the activities of enzymes of carbohydrate metabolism in the liver of diabetic rats (Hanhineva et al., 2010; Chandramohan et al., 2015). In agreement with their study, in the current study, naringenin decreased the activities of key enzymes of gluconeogenesis such as glucose 6-phosphatase and fructose 1, 6-bisphosphatase in diabetic mice, increased the activity of glycolytic enzyme hexokinases, the activity of glucose 6-phosphate dehydrogenase and liver glycogen content. These modifications point the shifting of the metabolic pathways to reduced glucose production by the liver (Chandramohan et al., 2015). In addition, the effects of naringenin in the liver are accompanied by an increase in insulin expression as demonstrated by IHC, highlighting that insulin is responsible for these modifications. Furthermore, few recent studies have also shown that Nrf2 can reprogram the cells in such a way that the cell promotes synthesis (anabolism) rather than degradation (catabolism). Since naringenin increases Nrf2, the cells might have reoriented the cellular metabolism to synthesize more glycogen from glucose, leading to a significant decrease in blood glucose levels (Elango et al., 2016). In conclusion, results of our study clearly demonstrate that naringenin helps in the maintenance of glucose homeostasis by regulating key enzymes involved in the glucose metabolism, hence, naringenin may be considered as a good candidate drug for diabetes management.

CONCLUSION

The *in vitro* and *in vivo* results of this study highlight the potential of naringenin to activate Nrf2 and protect the pancreatic β-cells from the oxidative damage caused by STZ. Above findings provide key evidences to demonstrate the anti-diabetic potential of naringenin. However, further studies testing the safety and efficacy of naringenin in higher animals are required to bring this natural product to the clinic. Additionally, strategies for improving the delivery of naringenin are also warranted to reduce the dose as well as to further enhance the potency.

AUTHOR CONTRIBUTIONS

RR conducted most of the experiments, analyzed the data, and wrote the manuscript. DS and MS performed and analyzed the experiments. SM, KR, and SS contributed to the experimental design and data and statistical analysis. All authors contributed to reviewing the results, writing the manuscript, and approved the final version of the manuscript.

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REFERENCES

American Diabetes and Association (2010). Diagnosis and classification of diabetes mellitus. *Diabetes Care* 33(Suppl. 1), S62–S69. doi: 10.2337/dc10-S062

American Diabetes Association (2015). 2 Classification and diagnosis of diabetes. *Diabetes Care* 38(Suppl. 1), S8–S16. doi: 10.2337/dc15-S005

Asmat, U., Abad, K., and Ismail, K. (2016). Diabetes mellitus and oxidative stress—a concise review. *Saudi Pharm. J.* 24, 547–553. doi: 10.1016/j.jsps.2015.03.013

Bâlici, Ş, Wankeu-Nya, M., Rusu, D., Nicula, G. Z., Rusu, M., Florea, A., et al. (2015). Ultrastructural analysis of in vivo hypoglycemiant effect of two polyoxometalates in rats with streptozotocin-induced diabetes. *Microsc. Microanal.* 21, 1236–1248. doi: 10.1017/S1431927615015020

Bennett, R. A., and Pegg, A. E. (1981). Alkylation of DNA in rat tissues following administration of streptozotocin. *Cancer Res.* 41, 2786–2790.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., and Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organ. J.* 5:9. doi: 10.1097/WOX.0b013e3182439613

Brandstrup, N., Kirk, J. E., and Bruni, C. (1957). The hexokinase and phosphoglucoisomerase activities of aortic and pulmonary artery tissue in individuals of various ages. *J. Gerontol.* 12, 166–171. doi: 10.1093/geronj/12.2.166

Cappai, G., Songini, M., Doria, A., Cavallerano, J., and Lorenzi, M. (2011). Increased prevalence of proliferative retinopathy in patients with type 1 diabetes who are deficient in glucose-6-phosphate dehydrogenase. *Diabetologia* 54, 1539–1542. doi: 10.1007/s00125-011-2099-3

Cenedella, R., Galli, C., and Paoletti, R. (1975). Brain free fatty acid levels in rats sacrificed by decapitation versus focused microwave irradiation. *Lipids* 10, 290–293. doi: 10.1007/BF02532702

Cerf, M. E. (2013). Beta cell dysfunction and insulin resistance. *Front. Endocrinol.* 4:37. doi: 10.3389/fendo.2013.00037

Chandramohan, R., Pari, L., Rathinam, A., and Sheikh, B. A. (2015). Tyrosol, a phenolic compound, ameliorates hyperglycemia by regulating key enzymes of carbohydrate metabolism in streptozotocin induced diabetic rats. *Chem. Biol. Interact.* 229, 44–54. doi: 10.1016/j.cbi.2015.01.026

Cheng, K., Delghingaro-Augusto, V., Nolan, C. J., Turner, N., Hallahan, N., Andrikopoulos, S., et al. (2012). High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. *PLoS One* 7:e40868. doi: 10.1371/journal.pone.0040868

Cohn, C. A., Simon, S. R., and Schoonen, M. A. (2008). Comparison of fluorescence-based techniques for the quantification of particle-induced hydroxyl radicals. *Part. Fibre Toxicol.* 5:2. doi: 10.1186/1743-8977-5-2

de Almeida, D. A. T., Braga, C. P., Novelli, E. L. B., and Fernandes, A. A. H. (2012). Evaluation of lipid profile and oxidative stress in STZ-induced rats treated with antioxidant vitamin. *Braz. Arch. Biol. Technol.* 55, 527–536. doi: 10.1590/S1516-89132012000400007

El-Aal, H. A. H. M. A. (2012). "Lipid peroxidation end-products as a key of oxidative stress: effect of antioxidant on their production and transfer of free radicals," in *Lipid Peroxidation*, ed. A. Catala (London: InTech).

Elango, B., Dornadula, S., Paulmurugan, R., and Ramkumar, K. M. (2016). Pterostilbene ameliorates streptozotocin-induced diabetes through enhancing antioxidant signaling pathways mediated by Nrf2. *Chem. Res. Toxicol.* 29, 47–57. doi: 10.1021/acs.chemrestox.5b00378

Eleazu, C. O., Eleazu, K. C., Chukwuma, S., and Essien, U. N. (2013). Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *J. Diabetes Metab. Disord.* 12:60. doi: 10.1186/2251-6581-12-60

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70–77. doi: 10.1016/0003-9861(59)90090-6

Ells, H. A., and Kirkman, H. (1961). A colorimetric method for assay of erythrocytic glucose-6-phosphate dehydrogenase. *Proc. Soc. Exp. Biol. Med.* 106, 607–609. doi: 10.3181/00379727-106-26418

Eruslanov, E., and Kusmartsev, S. (2010). "Identification of ROS using oxidized DCFDA and flow-cytometry," in *Advanced Protocols in Oxidative Stress II*, ed. D. Armstrong (Berlin: Springer), 57–72. doi: 10.1007/978-1-60761-411-1_4

Esmaeili, M. A., and Alilou, M. (2014). Naringenin attenuates CC l4-induced hepatic inflammation by the activation of an Nrf2-mediated pathway in rats. *Clin. Exp. Pharmacol. Physiol.* 41, 416–422. doi: 10.1111/1440-1681.12230

Falholt, K., Lund, B., and Falholt, W. (1973). An easy colorimetric micromethod for routine determination of free fatty acids in plasma. *Clin. Chim. Acta* 46, 105–111. doi: 10.1016/0009-8981(73)90016-8

Feldman, A. T., and Wolfe, D. (2014). "Tissue processing and hematoxylin and eosin staining," in *Histopathology Methods and Protocols, Methods in Molecular Biology*, ed. C. E. Day (Berlin: Springer), 31–43.

Fiske, C. H., and Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375–400.

Forbes, J. M., and Cooper, M. E. (2013). Mechanisms of diabetic complications. *Physiol. Rev.* 93, 137–188. doi: 10.1152/physrev.00045.2011

Fukai, T., and Ushio-Fukai, M. (2011). Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid. Redox Signal.* 15, 1583–1606. doi: 10.1089/ars.2011.3999

Gancedo, J. M., and Gancedo, C. (1971). Fructose-1, 6-diphosphatase, phosphofructokinase and glucose-6-phosphate dehydrogenase from fermenting and non fermenting yeasts. *Arch. Mikrobiol.* 76, 132–138. doi: 10.1007/BF00411787

Goren, H. J., Kulkarni, R. N., and Kahn, C. R. (2004). Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57BLKS/6, DBA/2, and 129X1. *Endocrinology* 145, 3307–3323. doi: 10.1210/en.2003-1400

Grankvist, K., Marklund, S. L., and Taljedal, I. B. (1981). CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem. J.* 199, 393–398. doi: 10.1042/bj1990393

Guo, X., Li, H., Xu, H., Woo, S., Dong, H., Lu, F., et al. (2012). Glycolysis in the control of blood glucose homeostasis. *Acta Pharm. Sin. B* 2, 358–367. doi: 10.1016/j.apsb.2012.06.002

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.

Han, H.-S., Kang, G., Kim, J. S., Choi, B. H., and Koo, S.-H. (2016). Regulation of glucose metabolism from a liver-centric perspective. *Exp. Mol. Med.* 48:e218. doi: 10.1038/emm.2015.122

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H., et al. (2010). Impact of dietary polyphenols on carbohydrate metabolism. *Int. J. Mol. Sci.* 11, 1365–1402. doi: 10.3390/ijms11041365

Hayes, J. D., and McLELLAN, L. I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* 31, 273–300. doi: 10.1080/10715769900300851

Hikaru, K., and Toshitsugu, O. (1959). Pathological occurrence of glucose-6-phosphatase in serum in liver diseases. *Clin. Chim. Acta* 4, 554–561. doi: 10.1016/0009-8981(59)90165-2

Hingorani, R., Deng, J., Elia, J., McIntyre, C., and Mittar, D. (2011). *Detection of Apoptosis Using the BD Annexin V FITC Assay on the BD FACS Verse™ System*. San Diego, CA: BD Biosciences.

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., et al. (1993). Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* 36, 1139–1145. doi: 10.1007/BF00401058

Jiang, Z.-Y., Hunt, J. V., and Wolff, S. P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* 202, 384–389. doi: 10.1016/0003-2697(92)90122-N

Jiménez-Osorio, A. S., González-Reyes, S., and Pedraza-Chaverri, J. (2015). Natural Nrf2 activators in diabetes. *Clin. Chim. Acta* 448, 182–192. doi: 10.1016/j.cca.2015.07.009

Jung, K.-A., and Kwak, M.-K. (2010). The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules* 15, 7266–7291. doi: 10.3390/molecules15107266

Kajimoto, Y., and Kaneto, H. (2004). Role of oxidative stress in pancreatic β-cell dysfunction. *Ann. N. Y. Acad. Sci.* 1011, 168–176. doi: 10.1196/annals.1293.017

Kakkar, P., Das, B., and Viswanathan, P. (1984). A modified spectrophotometric assay of superoxide dismutase. *Indian J. Biochem. Biophys.* 21, 130–132.

Kang, K. A., Kim, J. S., Zhang, R., Piao, M. J., Maeng, Y. H., Kang, M. Y., et al. (2011). KIOM-4 protects against oxidative stress-induced mitochondrial damage in pancreatic β-cells via its antioxidant effects. *Evid. Based Complement. Alternat. Med.* 2011:978682. doi: 10.1093/ecam/neq007

Karuppagounder, V., Arumugam, S., Thandavarayan, R. A., Pitchaimani, V., Sreedhar, R., Afrin, R., et al. (2015). Naringenin ameliorates daunorubicin induced nephrotoxicity by mitigating AT1R, ERK1/2-NFκB p65 mediated inflammation. *Int. Immunopharmacol.* 28, 154–159. doi: 10.1016/j.intimp.2015.05.050

Kim, A., Miller, K., Jo, J., Kilimnik, G., Wojcik, P., and Hara, M. (2009). Islet architecture: a comparative study. *Islets* 1, 129–136. doi: 10.4161/isl.1.2.9480

Kirkman, H. N., and Gaetani, G. F. (1984). Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH. *Proc. Natl. Acad. Sci. U.S.A.* 81, 4343–4347. doi: 10.1073/pnas.81.14.4343

Klaunig, J. E., Wang, Z., Pu, X., and Zhou, S. (2011). Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol. Appl. Pharmacol.* 254, 86–99. doi: 10.1016/j.taap.2009.11.028

Koide, H. (1959). Pathological occurrence of glucose-6-phosphatase in serum in liver disease. *Clin. Chim. Acta* 4, 554–561. doi: 10.1016/0009-8981(59)90165-2

Koide, H., and Oda, T. (1959). Pathological occurrence of glucose-6-phosphatase in serum in liver diseases. *Clin. Chim. Acta* 4, 554–561. doi: 10.1016/0009-8981(59)90165-2

Krishnakumari, S., Bhuvaneswari, P., and Rajeswari, P. (2011). Ameliorative potential of *Coccinia grandis* extract on serum and liver marker enzymes and lipid profile in streptozotocin induced diabetic rats. *Anc. Sci. Life* 31, 26–30.

Kruger, N. J. (2009). "The bradford method for protein quantitation," in *The Protein Protocols Handbook*, ed. J. M. Walker (Berlin: Springer), 17–24. doi: 10.1007/978-1-59745-198-7_4

Lacher, S. E., Lee, J. S., Wang, X., Campbell, M. R., Bell, D. A., and Slattery, M. (2015). Beyond antioxidant genes in the ancient Nrf2 regulatory network. *Free Radic. Biol. Med.* 88, 452–465. doi: 10.1016/j.freeradbiomed.2015.06.044

Lei, X. G., and Vatamaniuk, M. Z. (2011). Two tales of antioxidant enzymes on β cells and diabetes. *Antioxid. Redox Signal.* 14, 489–503. doi: 10.1089/ars.2010.3416

Lenzen, S., Drinkgern, J., and Tiedge, M. (1996). Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic. Biol. Med.* 20, 463–466. doi: 10.1016/0891-5849(96)02051-5

Lou, H., Jing, X., Wei, X., Shi, H., Ren, D., and Zhang, X. (2014). Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway. *Neuropharmacology* 79, 380–388. doi: 10.1016/j.neuropharm.2013.11.026

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.

Lu, M. C., Ji, J. A., Jiang, Z. Y., and You, Q. D. (2016). The Keap1–Nrf2–ARE pathway as a potential preventive and therapeutic target: an update. *Med. Res. Rev.* 36, 924–963. doi: 10.1002/med.21396

Lubos, E., Loscalzo, J., and Handy, D. E. (2011). Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxid. Redox Signal.* 15, 1957–1997. doi: 10.1089/ars.2010.3586

Ma, Q., and He, X. (2012). Molecular basis of electrophilic and oxidative defense: promises and perils of Nrf2. *Pharmacol. Rev.* 64, 1055–1081. doi: 10.1124/pr.110.004333

Morales, M., Jobaggy, A., and Terenzi, H. (1973). Mutations affecting accumulation of glycogen. *Fungal Genetics Rep.* 20:22. doi: 10.4148/1941-4765.1830

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63. doi: 10.1016/0022-1759(83)90303-4

Niehaus, Jr, and Samuelsson, B. (1968). Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur. J. Biochem.* 6, 126–130. doi: 10.1111/j.1432-1033.1968.tb00428.x

Ozougwu, J., Obimba, K., Belonwu, C., and Unakalamba, C. (2013). The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *J. Physiol. Pathophysiol.* 4, 46–57. doi: 10.5897/JPAP2013.0001

Pederson, B. A., Schroeder, J. M., Parker, G. E., Smith, M. W., DePaoli-Roach, A. A., and Roach, P. J. (2005). Glucose metabolism in mice lacking muscle glycogen synthase. *Diabetes Metab. Res. Rev.* 54, 3466–3473. doi: 10.2337/diabetes.54.12.3466

Porter, A. G., and Jänicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* 6:99. doi: 10.1038/sj.cdd.4400476

Qin, S., and Hou, D. X. (2016). Multiple regulations of Keap1/Nrf2 system by dietary phytochemicals. *Mol. Nutr. Food Res.* 60, 1731–1755. doi: 10.1002/mnfr.201501017

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., et al. (2014). Oxidative stress, prooxidants, and antioxidants: the interplay. *Biomed. Res. Int.* 2014:761264. doi: 10.1155/2014/761264

Ramkumar, K. M., Sekar, T. V., Foygel, K., Elango, B., and Paulmurugan, R. (2013). Reporter protein complementation imaging assay to screen and study Nrf2 activators in cells and living animals. *Anal. Chem.* 85, 7542–7549. doi: 10.1021/ac401569j

Roden, M., and Bernroider, E. (2003). Hepatic glucose metabolism in humans—its role in health and disease. *Best Pract. Res. Clin. Endocrinol. Metab.* 17, 365–383.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A., Hafeman, D. G., and Hoekstra, W. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science* 179, 588–590. doi: 10.1126/science.179.4073.588

Schnedl, W. J., Ferber, S., Johnson, J. H., and Newgard, C. B. (1994). STZ transport and cytotoxicity: specific enhancement in GLUT2-expressing cells. *Diabetes Metab. Res. Rev.* 43, 1326–1333.

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. *J. Clin. Invest.* 106, 171–176. doi: 10.1172/JCI10583

Sies, H. (1999). Glutathione and its role in cellular functions. *Free Radic. Biol. Med.* 27, 916–921. doi: 10.1016/S0891-5849(99)00177-X

Sinha, A. K. (1972). Colorimetric assay of catalase. *Anal. Biochem.* 47, 389–394. doi: 10.1016/0003-2697(72)90132-7

Stefanson, A. L., and Bakovic, M. (2014). Dietary regulation of Keap1/Nrf2/ARE pathway: focus on plant-derived compounds and trace minerals. *Nutrients* 6, 3777–3801. doi: 10.3390/nu6093777

Stewart, J. C. M. (1980). Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.* 104, 10–14. doi: 10.1016/0003-2697(80)90269-9

Sumathi, R., Tamizharasi, S., and Sivakumar, T. (2015). Bio-dynamic activity of naringenin–a review. *Int. J. Curr. Adv. Res.* 4, 234–236.

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol. Res.* 50, 537–546.

Tabatabaei-Malazy, O., Larijani, B., and Abdollahi, M. (2015). Targeting metabolic disorders by natural products. *J. Diabetes Metab. Disord.* 14:57. doi: 10.1111/j.1753-0407.2010.00090.x

van Schaftingen, E., and Gerin, I. (2002). The glucose-6-phosphatase system. *Biochem. J.* 362(Pt 3), 513–532. doi: 10.1042/bj3620513

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REVIEW
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Nrf2/ARE Pathway Modulation by Dietary Energy Regulation in Neurological Disorders

Andrea Rodrigues Vasconcelos[1†], Nilton Barreto dos Santos[2†], Cristoforo Scavone[1] and Carolina Demarchi Munhoz[2]*

[1] Laboratory of Molecular Neuropharmacology, Department of Pharmacology, Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil, [2] Laboratory of Neuroendocrinopharmacology and Immunomodulation, Department of Pharmacology, Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil

Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the expression of an array of enzymes with important detoxifying and antioxidant functions. Current findings support the role of high levels of oxidative stress in the pathogenesis of neurological disorders. Given the central role played by Nrf2 in counteracting oxidative damage, a number of studies have targeted the modulation of this transcription factor in order to confer neuroprotection. Nrf2 activity is tightly regulated by oxidative stress and energy-based stimuli. Thus, many dietary interventions based on energy intake regulation, such as dietary energy restriction (DER) or high-fat diet (HFD), modulate Nrf2 with consequences for a variety of cellular processes that affect brain health. DER, by either restricting calorie intake or meal frequency, activates Nrf2 thereby triggering its protective effects, whilst HFD inhibit this pathway, thereby exacerbating oxidative stress. Consequently, DER protocols can be valuable strategies in the management of central nervous system (CNS) disorders. Herein, we review current knowledge of the role of Nrf2 signaling in neurological diseases, namely Alzheimer's disease, Parkinson's disease, multiple sclerosis and cerebral ischemia, as well as the potential of energy intake regulation in the management of Nrf2 signaling.

Keywords: Nrf2, dietary energy restriction, high-fat diet, aging, Alzheimer's disease, Parkinson's disease, multiple sclerosis, cerebral ischemia

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***Correspondence:**
Cristoforo Scavone
cristoforoscavone@gmail.com; cristoforo.scavone@gmail.com

[†]These authors have contributed equally to this work

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INTRODUCTION

It is widely recognized that oxidative stress plays a key role in CNS physiology and pathophysiology (Patel, 2016). Free radicals are constantly produced and are required at physiological levels for signaling and plasticity in the healthy brain. Conversely, their accumulation due to impaired cellular antioxidant defenses or excessive production that exceeds the cell's antioxidant capability can result

Abbreviations: 6-OHDA, 6-hydroxydopamine; Aβ, Amyloid β; AD, Alzheimer's disease; ARE, antioxidant response element; BBB, blood-brain barrier; BMI, body mass index; CAT, catalase; CNS, central nervous system; CR, calorie restriction; DAMPS, damage-associated molecular patterns; DCs, dendritic cells; DER, dietary energy restriction; EAE, experimental autoimmune encephalomyelitis; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; HFD, high-fat diet; HO-1, heme oxygenase 1; IF, intermittent fasting; KEAP1, Kelch-like ECH associated protein 1; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MnSOD, manganese superoxide dismutase; MPTP, methyl-4-phenyl-1, 2, 5, 6-tetrahydropyridine; MS, multiple sclerosis; NQO1, NADPH quinine oxidoreductase 1; Nrf2, Nuclear factor erythroid 2-related factor 2; OGDR, oxygen-glucose deprivation/reoxygenation; OPCs, oligodendrocytes and oligodendrocytes precursor cells; PD, Parkinson's disease; ROS, reactive oxygen species; RRMS, relapsing-remitting multiple sclerosis; SN, substantia nigra; SOD, superoxide dismutase; SPMS, secondary progressive multiple sclerosis; T2D, type 2 diabetes; tMCAO, transient middle cerebral artery occlusion; VEGF, vascular endothelial growth factor.

in neurotoxicity and cell death, which if continued will ultimately lead to pathological processes (Cai et al., 2011). For this reason, oxidative stress has been extensively studied as a therapeutic target to treat brain diseases (Patel, 2016).

It is important to note that the brain can be highly susceptible to oxidative damage, due, in part, to its elevated oxygen demand, the presence of high amounts of polyunsaturated fatty acids that are easily targeted by free radicals, and lower levels of antioxidant enzymes compared to other organs (Ahmad et al., 2017; Mecocci et al., 2018). Some stable products of lipid peroxidation in the CNS are substantial oxidative stress biomarkers largely used in studies involving neurological and neurodegenerative disorders. These oxidation products include isoprostanes and neuroprostanes, derived from the non-enzymatic oxidation of arachidonic acid and decosahexanoic acid, respectively (Reed, 2011).

Aging leads to a gradual increase in brain oxidative stress, which is accompanied by reduced antioxidant defenses and lower levels of neurogenesis (Uttara et al., 2009). Aging is the main risk factor for neurodegenerative disorders (Niccoli and Partridge, 2012), which accounts for 12% of total deaths worldwide (World Health Organization [WHO], 2011; Chen et al., 2016b). Both acute and chronic inflammatory processes reciprocally interact with oxidative stress, with these factors being important to the etiology and course of a wide array of neurological conditions, such as AD, PD, MS, and stroke, as well as to the process of aging *per se* (Sandberg et al., 2014).

Aging is also associated to a progressive reduction in Nrf2 activity (Cuadrado, 2016). Interestingly, long-lived animal species have higher Nrf2 signaling levels, highlighting the importance of Nrf2 protection against aging and aging-related diseases (Bruns et al., 2015). Nrf2 is pivotal in the regulation of cellular redox status, modulating the expression of more than 200 downstream genes encoding Phase II response enzymes during oxidative challenge, including HO-1, GST, CAT, SOD, and NQO1 (Nguyen et al., 2009; Sun et al., 2017). The phase II response in an evolutionary conserved adaption to a broad range of stressors and is intimately linked to the organism's antioxidant defenses, detoxification, and cellular resilience (Hine and Mitchell, 2012). A broad array of published data show that the upregulation of Nrf2 target genes in the CNS can render neurons more resistant to excitotoxic and oxidative insults (Chen et al., 2000; Satoh et al., 2006; Giordano et al., 2007; Tanito et al., 2007; Lim et al., 2008). Nrf2 not only modulates antioxidant defense genes, but also genes that have autophagic and anti-inflammatory properties as well as glucose and lipid metabolism effects (Bruns et al., 2015; Tebay et al., 2015). Nrf2 activation leads to its translocation to the cell nucleus where it triggers the expression of target genes that contain the ARE DNA regulatory sequence in their promoter region (Jaiswal, 2004). The Nrf2/ARE pathway is modulated by the KEAP1. In basal conditions, this protein acts as a Nrf2 repressor, binding to Nrf2 and maintaining it in the cell cytoplasm (Satoh et al., 2006). This regulatory protein also directs Nrf2 to ubiquitination and degradation by proteasomes, thereby limiting its basal cellular levels (Sun et al., 2017) (**Figure 1**).

Many dietary interventions modulate Nrf2. DER and high energy consumption are two of the most studied strategies for energy status regulation, and both act to modulate tNrf2 activity. DER increases Nrf2 activity, in contrast to high energy consumption. DER, induced by chronically or intermittently restricted calorie consumption, subjects neurons to an energetic stress that triggers the Nrf2/ARE pathway and thereby induces many beneficial effects on health and longevity, including the prevention of neurological diseases (Mattson, 2012). In contrast, a plethora of animal and human studies show that a HFD, and associated obesity, enhance inflammation and oxidative stress, resulting in a raised overall mortality and higher incidence of many neurological disorders (Dorrance et al., 2014; Michel, 2016; Mazon et al., 2017; Alfredsson and Olsson, 2018) (**Figure 2**). This chapter focuses on the Nrf2/ARE pathway regulation by dietary interventions and its protective role in the CNS against metabolic, excitotoxic, and oxidative insults, with relevance to AD, PD, MS, and cerebral ischemia.

Nrf2/ARE PATHWAY AND NEUROLOGICAL DISEASES

Both *in vitro* and *in vivo* neurodegenerative models have demonstrated that Nrf2 activation promotes neuroprotective effects (Calkins et al., 2009). However, as noted above, Nrf2 activity declines with age, consequently decreasing the protection afforded by antioxidant enzymes activity. Such Nrf2/ARE signaling impairment renders the organism and the brain more susceptible to oxidative injury, abnormal protein aggregation and neurodegeneration (Itoh et al., 1997; Suh et al., 2004; Shih and Yen, 2007; Morrison et al., 2010; Safdar et al., 2010; Cuadrado, 2016). A number of studies have underscored the importance of the Nrf2/ARE pathway to the pathogenesis and treatment of neurological disorders, including PD, AD, MS, and cerebral ischemia, as described below.

Alzheimer's Disease

Alzheimer's disease is the leading cause of senile dementia, characterized by progressive cognitive impairment and memory loss. In the United States, it is estimated that 5.7 million people have AD in 2018 and this number is predicted to reach 14 million by 2050, due to rises in longevity (Alzheimer's Association, 2018). Classically, AD has been thought to be driven by the accumulation of amyloid-β peptide (Aβ) aggregates and neurofibrillary tangles composed of hyperphosphorylated tau proteins (Arriagada et al., 1992; Selkoe, 1994). However, there is a growing appreciation of a role for other processes in AD etiology and course, including changes in oxidative stress (Mecocci et al., 2018). Oxidative stress markers have long been shown to be increased in AD. Patients with mild cognitive impairment, often a forerunner of AD, also show raised levels of oxidative damage and reduced antioxidant defenses, in comparison to healthy controls (Migliore et al., 2005; Bermejo et al., 2008; Mangialasche et al., 2009; Padurariu et al., 2010). Markers of oxidative stress are all increased in AD, including 4-hydroxynonenal (a product of lipid peroxidation) (Bradley et al., 2012; Di Domenico et al., 2017), and protein nitration and carbonylation (Perluigi et al., 2009;

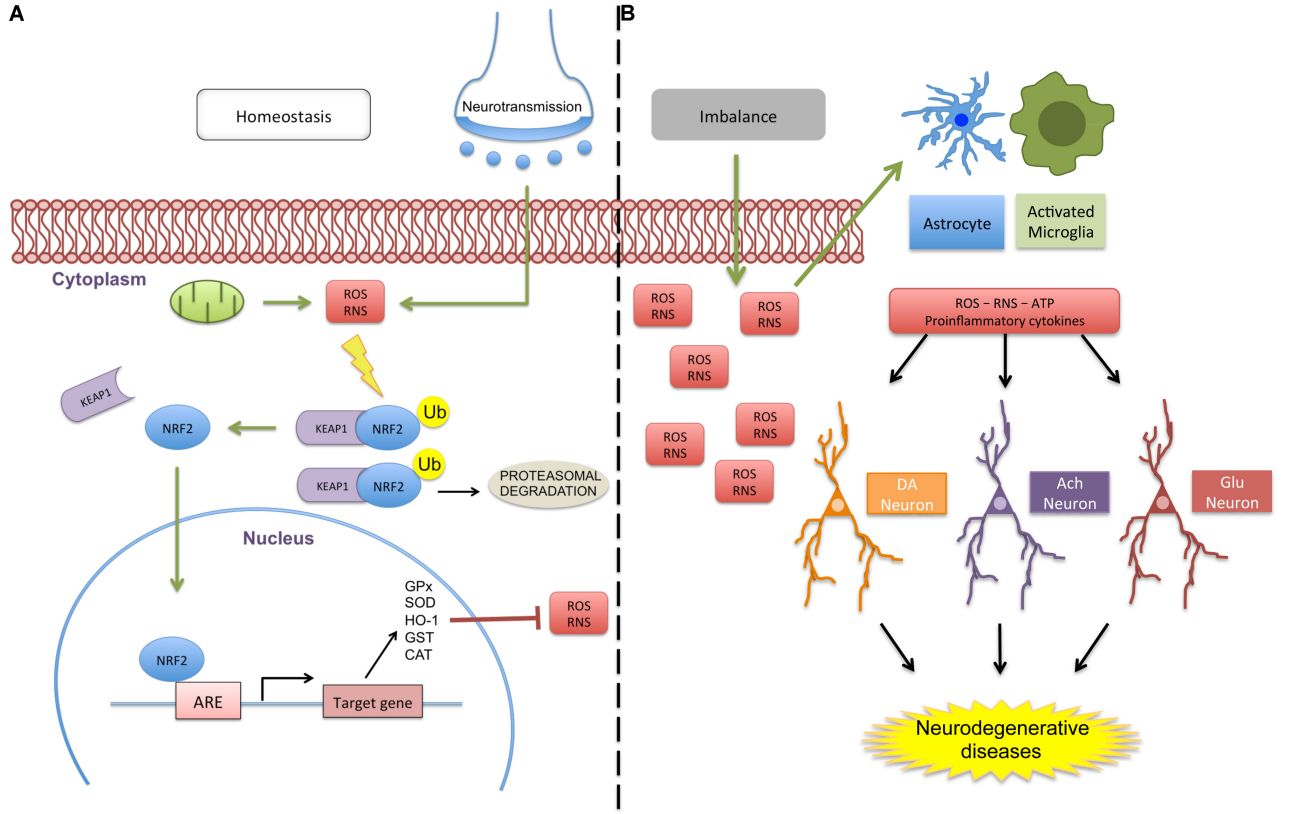

FIGURE 1 | Schematic representation of Nrf2 signaling in homeostasis and a deregulated environment. **(A)** Oxidative molecules (e.g., ROS and RNS) produced by cellular respiration or neurotransmission activate the protective antioxidant pathway by dissociation of the Nrf2/KEAP1 complex. When dissociated from the cytosolic protein KEAP1, Nrf2 translocates to the cell nucleus, triggering the expression of several homeostatic genes with the ARE sequence in their promoters, including GPx, SOD, HO-1, GST, and CAT. When inactivated, Nrf2 is sequestered by KEAP1 and targeted for ubiquitination and proteasomal degradation. **(B)** Altered homeostasis promotes excessive ROS/RNS production that can activate glial cells (astrocytes and microglia) that release proinflammatory and danger molecules patterns, which disrupts neuronal communication and the nature of glial activities. Green arrows represent activation and truncated red lines, inhibition (abbreviations: ACh, acetylcoline; DA, dopamine; CAT, catalase; Glu, glutamate; GPx, Glutathione Peroxidase; GST, glutathione S-transferase; HO-1, heme oxigenase 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; Ub, ubiquitin; ATP, adenosine triphosphate).

Aluise et al., 2011). These changes are often accompanied by a decline in antioxidant defenses, which contributes to such heightened oxidative stress in AD patients (Kim et al., 2006).

Nrf2 levels are decreased in the AD brain (Kanninen et al., 2008). This is supported by data from preclinical models of AD, which indicate reduced brain Nrf2 expression levels (Carvalho et al., 2015; Liu et al., 2016; Manczak et al., 2018), with Nrf2 activation mitigating neuronal apoptosis and spatial memory impairment (Dong F. et al., 2017). Several *in vivo* and *in vitro* studies also show that the activation of Nrf2/HO-1 signaling cascade by flavonoids and microRNA-302 affords protection against neuronal toxicity induced by Aβ (Zou et al., 2013; Kwon et al., 2015; Li H.H. et al., 2016; Wang et al., 2016). Moreover, Nrf2 knockout mice have an impaired clearance of phosphorylated tau by autophagy, contributing to heightened tau aggregation and accumulation in the brain, a well-known hallmark of AD (Jo et al., 2014). In an AD murine model, Nrf2 deletion also results in impaired autophagy, and therefore a decrease in the ability of a cell to clear debris (Joshi et al., 2015). These findings highlight the importance of Nrf2 in AD pathophysiology as well as indicating its potential therapeutic utility in AD.

In contrast, Raina et al. (1999) reported increased levels of NQO1 in AD human brains, suggesting Nrf2 activation. Similarly, three common Nrf2 target genes (p62, HO-1, and GCLM) are upregulated in AD brains (Tanji et al., 2013). Such contrasting results may be due to differential effects on Nrf2 levels at different AD stages, perhaps indicative of an upregulation of antioxidant defenses to counteract oxidative stress in early AD stages, whilst the loss of endogenous antioxidants and Nrf2/ARE pathway activity may be more evident in latter phases of the disease (Sun et al., 2017).

Parkinson's Disease

After AD, PD is the second most prevalent neurodegenerative disease. PD is typically characterized by progressive movement disorders (rigidity, resting tremor, postural instability, hypokinesia, and bradykinesia) as well as by variable degrees of cognitive dysfunction and dementia (Goris et al., 2007; Tufekci et al., 2011). A number of different brain regions show

atrophy in PD, with classic PD symptomatology mediated by the loss of dopamine neurons in the SN. An accumulation of α-synuclein protein is common, in association with proteasome and mitochondria impairment (Mattson, 2012).

As with AD, the etiology of PD still awaits clarification, with a number of studies indicating that oxidative stress is an important contributor to PD pathogenesis and course, including via increased membrane lipid peroxidation and protein damage (Duan and Mattson, 1999; Jenner, 2007; Cuadrado et al., 2009). Sources of oxidative stress in PD brain include chronic neuroinflammation, the metabolism of dopamine producing cytotoxic ROS, and mitochondrial impairment (Blesa et al., 2015). Nrf2 is an emerging target to counteract PD-related neuronal cell death, given its regulation of a plethora of cytoprotective genes with anti-inflammatory and antioxidant properties, as well as its role in optimizing mitochondria biogenesis (Tufekci et al., 2011). Data from human post-mortem PD brains and Nrf2 knockout mice indicate an association between Nrf2/ARE pathway dysfunction and PD pathogenesis (Tufekci et al., 2011). Such data shows that Nrf2 deficiency increases the sensitivity of mice to PD neurotoxins (Jakel et al., 2007; Chen et al., 2009; Colton, 2009), whereas the induction of this transcription factor in astrocytes exerts a protective effect against brain damage in the 6-OHDA model of PD (Jakel et al., 2007). In post-mortem brains of PD patients, the proteins p62 and NQO1 were partially sequestered in Lewy bodies, indicating a compromised neuroprotective capacity of Nrf2 (Lastres-Becker et al., 2016). In the same study, Nrf2 pharmacological activation by dimethyl fumarate in a murine PD model protected SN neurons against α-synuclein toxicity, an effect not evident in Nrf2-knockout mice (Lastres-Becker et al., 2016). Jazwa et al. (2011) also showed that Nrf2 activation upregulates brain HO-1 and NQO1 expression and prevents SN neuronal death as induced by MPTP, a neurotoxin PD model. Furthermore, the link between Nrf2 and PD is also supported by studies showing that antiparkinsonian drugs (e.g., apomorphine, deprenyl, and bromocriptine) can activate the Nrf2/ARE pathway and Nrf2-dependent gene expression, preventing cell oxidative damage and neurodegeneration (Hara et al., 2006; Nakaso et al., 2006; Lim et al., 2008; Kabel et al., 2018).

In human PD brain tissue, Ramsey et al. (2007) showed that hippocampal neurons and glia from CA1 region had lower nuclear Nrf2 when compared to age-matched healthy controls. In contrast, SN neurons of PD patients had higher Nrf2 nuclear localization, indicative of Nrf2 activation, although not sufficient to counteract neurodegeneration in these individuals (Ramsey et al., 2007). Similarly, Nrf2-dependent gene expression is decreased in the striatum but increased in the SN of the MPTP model (Ramsey et al., 2007). A recent study also reported increased Nrf2 activation in SN of mice treated with MPTP, prior to the onset of neurodegeneration (Rizzi et al., 2018).

Further research is required to clarify why the increased Nrf2-dependent gene expression and nuclear translocation in the SN reported in the aforementioned studies is incapable of protecting neurons against oxidative damage. A number of studies indicate a crucial role for Nrf2 activation in glial cells (Jakel et al., 2007; Chen et al., 2009), suggesting that Nrf2 may need to be activated in glia in order to exert its protective effects in PD and PD models. Ramsey study did not report glial Nrf2 nuclear translocation in the SN of PD brains (Ramsey et al., 2007) and *in vitro* studies indicate that neuronal Nrf2 activation, even in the absence of glia, induces neuroprotection against oxidative damage triggered by parkinsonism-inducing neurotoxins (Lee et al., 2003; Cao et al., 2005; Hara et al., 2006; Jakel et al., 2007; Wruck et al., 2007; Hwang and Jeong, 2008; MacKenzie et al., 2008; Satoh et al., 2009; Niso-Santano et al., 2010). Overall, such work indicates that increased Nrf2 activity in both glia and neurons is important to neuronal survival in PD (Tufekci et al., 2011).

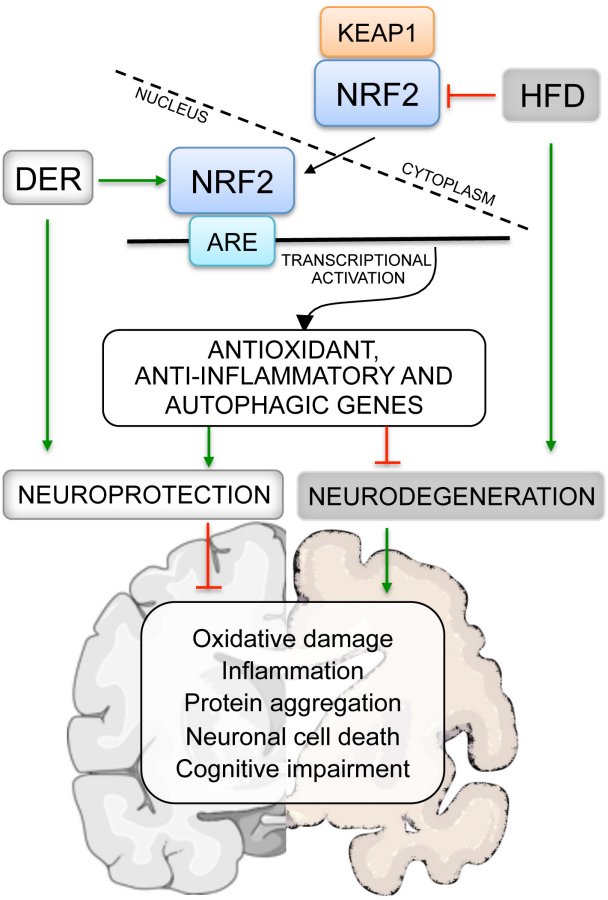

FIGURE 2 | The role of Nrf2 modulation by dietary interventions on brain health. HFD inhibit Nrf2 in the brain, whereas DER is able to activate this transcription factor. When activated, Nrf2 triggers the expression of several neuroprotective genes that counteract oxidative stress and neuroinflammation, preventing the onset of neurological disorders and underlying pathological processes. Green arrows represent activation and truncated red lines, inhibition (abbreviations: DER, dietary energy restriction; HFD, high-fat diet).

Cerebral Ischemia

Cerebrovascular diseases are the second leading cause of death worldwide. Without intervention, the cases of deaths worldwide are estimated to increase from 6.5 million in 2015 to 7–8 million in 2030 (Strong et al., 2007). Ischemic stroke is characterized by decreased blood flow in the brain, causing injury to brain

tissues and impairing normal neurological functioning (Jauch et al., 2013; Ding et al., 2017).

The reduced delivery of nutrients and oxygen after stroke decreases tissue pH, resulting in impairment of the mitochondrial electron transport chain activity and subsequent diminished ATP production. Subsequently, a cascade of events follows that culminates in raised intracellular Na^+, Ca^{2+}, Cl^- concentrations, and K^+ efflux. The neuronal cations increase leads to excessive depolarization and the release of excitatory neurotransmitters, such as glutamate, causing excitotoxicity and triggering oxidative and cell death pathways (Nicholls, 2009).

Increased oxidative stress is evident in stroke. Murine models, subjected to ischemia-reperfusion, show a high concentration of superoxide and peroxynitrite, as well as metalloproteinase-9 production and BBB breakdown (Gursoy-Ozdemir et al., 2004). The anti-apoptotic and anti-oxidative effects of the Nrf2 signaling pathway in stroke have been noted. Nrf2 activators are able to reduce oxidative stress and exert protective roles in models of stroke (Alfieri et al., 2011; Yang et al., 2018). Astrocyte Nrf2 activation is important to the release of antioxidants, such as glutathione, which protect neurons from free radicals produced during ischemia (Alfieri et al., 2011).

Recent work shows Nrf2 to induce angiogenesis via HO-1 (Ding et al., 2014) and VEGF signaling (Huang Y. et al., 2018), which is supported by the work of Bai and coworkers who showed that epigallocatechin-3-gallate, the major effective polyphenol in green tea, promotes angiogenesis and decreases oxidative stress via MAPK/Nrf2/VEGF activation, following tMCAO (Bai et al., 2017; Li L. et al., 2016).

In addition to antioxidant effects, Nrf2/ARE activation can afford protection against the neuroinflammation evident following ischemia. In the ischemic brain, ROS, as with DAMPS, can activate the NLRP3 inflammasome, thereby converting pro-interleukin (IL)-18 and pro-IL-1β into mature IL-18 and IL-1β forms, which are then released (Inoue et al., 2012). Glial cells are important drivers of neuroinflammation and NLRP3 activation (Frank et al., 2007; Gavillet et al., 2008). Xu et al. (2018) showed that the Nrf2/ARE pathway protects against the oxidative stress-induced NLRP3 activation in the BV2 microglial cell line exposed to OGDR. Moreover, neonatal rats treated with LPS 24 h prior to hypoxia-ischemia, were protected against neuropathological effects, whereas LPS administration 72 h before hypoxia-ischemia increased brain damage, which was prevented by treatment with *N*-acetylcysteine, a glutathione precursor (Wang et al., 2007). Such data support the idea that Nrf2 upregulation varies according to the time of ischemia exposure, as well as cell type (glial cells or neurons) and anti-oxidant reserve. Such data suggests complex effects, although it is clear that Nrf2-related pathways are important molecular targets for pharmacologic intervention in the management of ischemic stroke.

Multiple Sclerosis

Multiple sclerosis is the most frequent chronic neuroinflammatory disease of the CNS, characterized by demyelination, as well as focal inflammatory lesions in the brain and spinal cord, which culminates in axonal damage (Lassmann, 2011b). MS is also associated with gray matter synaptic loss and decreased neurogenesis, which contributes to cognitive impairment. MS clinical symptoms include sensory or motor impairment, fatigue, ataxia, and spasticity, as well as cognitive impairment (Chiaravalloti and DeLuca, 2008; Lublin et al., 2014). EAE is the most common experimental model of MS (Simmons et al., 2013). Activated macrophages and microglial cells can produce an array of neurotoxic factors, including proteases, nitric oxide, and ROS, with this being evident in both MS and the EAE model (Glass et al., 2010). Generation of ROS and other free radicals by macrophages is involved in demyelination and axonal damage in EAE. Evidence of lipid peroxidation, as a consequence of oxidative stress, can be detected in the exhaled breath samples of MS patients. Additional studies have shown decreased levels of antioxidant enzymes in blood and cerebrospinal fluid of MS patients (Lassmann, 2011a,b, 2014; Witte et al., 2014). Such data highlights the importance of heightened levels of immune-inflammatory activity and oxidative stress in MS.

Therapeutic strategies in the treatment of MS have primarily focused on dampening the heightened immune-inflammatory activity that is present, including by the use of: imunossupressors, such as synthetic glucocorticoids (e.g., dexamethasone, prednisone, and methylprednisone); monoclonal antibodies (Natalizumab); inhibitors of cell egress from lymphoid organs (Fingolimod), modulators of pro- and anti-inflammatory cytokines (interferon-beta); and microglial inhibitors (acetate glatiramer). However, all of these treatments have shown limited efficacy. Antioxidant therapies have proved to have some utility when used as adjuvants in MS treatment, with endogenous Nrf2 being an important treatment target for the induction of endogenous antioxidants (Kappos et al., 2010; Lim et al., 2014).

Classical treatment in MS has emphasized the role of heightened immune-inflammatory processes. As noted above, such processes are intimately linked to alterations in redox regulation. There is now a growing appreciation of the role the oxidant/antioxidant balance in MS. A growing body of data indicates an important role for ROS in MS, including: a central role in MS lesion development and progression and an initial contribution to the BBB breakdown and leukocyte infiltration that is crucial in early phases of lesion formation in MS (Van der Goes et al., 2001; Schreibelt et al., 2006). ROS can cause oxidative injury in CNS cells, including during demyelination, oligodendrocyte injury, and axonal degeneration. OPCs are highly vulnerable to oxidative injury due to their low levels of endogenous antioxidant enzymes and the relatively high levels of polyunsaturated fatty acids in the myelin sheets, which are more susceptible to lipid peroxidation (Juurlink et al., 1998; French et al., 2009). Furthermore, free radicals are able to impair the maturation of OPCs into myelin-forming mature cells by modulating the genes driving maturation and differentiation.

Antioxidant molecules and oxidative stress are upregulated in active brain lesions in MS patients, but not to a sufficient level to counteract the oxidative stress evident in MS brain tissue. NADPH oxidase subunits are strongly upregulated in the macrophages and microglia in active MS lesions, suggesting

an important role for heightened ROS, including as mediated by an oxidative burst, in the pathophysiology of active lesions. Contributing to such damage are increased expression of inducible nitric oxide synthase (Bo et al., 1994; De Groot et al., 1997; Liu et al., 2001) and myeloperoxidase (Marik et al., 2007; Chen et al., 2008; Gray et al., 2008a,b; Wang et al., 2016) that are evident in the brains of MS patients.

Although a number of different cell types, such as macrophages, can have an antigen presenting capacity, DCs are the body's major antigen presenting cells. There is growing body of data indicating that Nrf2 may regulate the differentiation and function of macrophages and DCs, and therefore in antigen presentation and the regulation of adaptive immune response. In fact, Nrf2 deficiency modifies the phenotype and function of DCs by increasing the expression of co-stimulatory molecules and consequently the antigen-specific T cell reactivity (Al-Huseini et al., 2013). Some of these Nrf2 effects may be mediated via alterations in mitochondrial functioning, as indicated above, including in the mitochondrial regulation of immune cell responses.

Nrf2 is strongly upregulated in active MS lesions, with the expression of Nrf2-responsive genes being predominantly found in areas of initial myelin destruction (Licht-Mayer et al., 2015). Analyses of MS inflammatory lesions showed upregulation of Nrf2 and its downstream antioxidant enzymes, HO-1 and NQO1. In support of the importance of such Nrf2-mediated changes, experimental studies in Nrf2-deficient EAE mice show an increase in disease signals and peripheral cell infiltration (Lim et al., 2014). Accordingly, Johnson et al. (2010) showed that the absence of Nrf2 exacerbates the development of EAE. Part of the effects associated with Nrf2 deficiency may be related to the reduced levels of HO-1. Indeed, mice with a myeloid-specific HO-1 deficiency exhibit a higher incidence of lesions, accompanied by activation of antigen-presenting cells and the infiltration of the pro-inflammatory t helper (Th)17 cells and myelin-specific T cells (Tzima et al., 2009). Knockdown of KEAP1 (Kobayashi et al., 2016) or treatment with a wide range of small molecules that activate Nrf2 (Buendia et al., 2016) inhibits the development and severity of EAE.

ROLE OF Nrf2/ARE PATHWAY MODULATION BY DIETARY INTERVENTIONS IN NEUROLOGICAL DISEASES

A growing literature shows Nrf2 to regulate the expression of genes that reduce gluconeogenesis and lipogenesis, whilst also increasing fatty acids β-oxidation and mitochondria activity. Such data indicates that Nrf2 activity intimately interacts with cellular nutritional and energetic status (Holmstrom et al., 2013; Ludtmann et al., 2014; Tebay et al., 2015). The multiple molecular pathways that may underpin such interactions have been reviewed previously (Tebay et al., 2015). The present article reviews the current knowledge of dietary interventions, namely DER and HDF, on Nrf2 and the relevance of this to neurological disorders.

Dietary Energy Restriction

Dietary energy restriction, the decrease of food intake without malnutrition, is the most powerful intervention known to delay aging processes and extend lifespan (Pearson et al., 2008; Hine and Mitchell, 2012). Several studies have comprehensively highlighted the beneficial effects of DER on cognitive function, metabolic health, and longevity, which have been especially associated with the two main DER protocols: caloric restriction (CR) and IF (Horne et al., 2015; Vasconcelos et al., 2018). In CR protocol, calorie consumption is chronically decreased in 20–40% of the *ad libitum* intake, whilst IF involves a restriction in the frequency of food intake, giving periods of free food intake coupled to fasting periods. These protocols were shown to extend life- and health-span and to counteract numerous age-related diseases. The vulnerability of the CNS to age progression is frequently expressed in neurological disorders, such as AD, PD, and stroke (Martin et al., 2006; Logsdon et al., 2017).

Dietary energy restriction, as a mild stress of energy restriction to the organism, is considered a hormetic stimulus, which is defined as a low dose stressor that induces adaptive responses able to improve resistance to more severe stressors and diseases. Within this context, the energetic challenge to the brain induced by DER triggers beneficial outcomes, including neurogenesis, synapses strengthening, and new synapse formation (Calabrese et al., 2010; Hine and Mitchell, 2012; Horne et al., 2015). Molecularly, DER induces the activation of protective transcription factors, such as Nrf2, that activate the expression of Phase II detoxifying enzymes, thereby increasing neuronal resistance to oxidative stress and death, and hence lowering the risk of neurodegenerative disorders. DER, especially IF, upregulates genes encoding the antioxidant enzymes that are modulated by Nrf2, including GPx, SOD2, and HO-1 (Hine and Mitchell, 2012; Mattson, 2012).

Some of the benefits of CR protocol are associated with Nrf2/ARE pathway activation (Pearson et al., 2008; Martin-Montalvo et al., 2011; Bruns et al., 2015). One of the proposed mechanisms of DER utility is via a transient ROS increase to hormetic levels that may activate Nrf2 (Hine and Mitchell, 2012). For instance, fasting, and its consequential effects on insulin levels, results in a small, transient increase in oxidative stress, triggering activation of the Nrf2/ARE pathway and the upregulation of its target genes (Kim and Novak, 2007).

Various age-related diseases, including AD and PD, are linked to decreased Nrf2 activity and display symptom improvement after Nrf2 activation by DER (Hine and Mitchell, 2012). CR can counteract the age-related loss of cellular antioxidant defenses, partly by promoting the up-regulation of Nrf2/ARE-driven genes, including GST and NQO1, in a variety of body tissues and organs, including the liver and brain (Chen et al., 1994; Hyun et al., 2006). Furthermore, a recent study showed that 30% CR for 12 weeks can prevent neurotoxicity, oxidative damage, and cognitive impairment induced by acrolein. Acrolein has been proposed to be involved in AD etiology, with the efficacy of CR

mediated, at least partly, through the amelioration of acrolein-induced depletion of hippocampal SOD levels, indicating a positive effect of this protocol on Nrf2 signaling (Huang Y.J. et al., 2018).

The age-induced Nrf2 dysfunction in BBB endothelial cells is also prevented by CR, shedding light in the cerebrovascular protective effects of this DER protocol (Csiszar et al., 2014). Importantly, these age-dependent endothelial alterations are thought to play a role in both vascular cognitive impairment and AD (Gorelick et al., 2011; Zlokovic, 2011; Lin et al., 2013). Accordingly, numerous substances proposed as "DER mimetics" (i.e., compounds shown to promote the beneficial effects of DER in the absence of food intake restriction), such as curcumin, resveratrol, and quercetin (Ingram et al., 2006), have been shown to increase longevity and slow down the aging process, at least in part via Nrf2 activation (Balogun et al., 2003; Chen et al., 2005; Hsieh et al., 2006; Tanigawa et al., 2007; Calabrese et al., 2010).

Several studies in murine and primate models of PD have demonstrated that DER can protect dopaminergic neurons, decrease motor dysfunction, and alleviate PD symptoms (Duan and Mattson, 1999; Maswood et al., 2004; Qiu et al., 2012; Griffioen et al., 2013), even when the DER protocol is initiated after the induction of PD by MPTP (Holmer et al., 2005). Published data also indicate that energy consumption profoundly impacts the progression of AD (Mattson, 2012), with DER decreasing brain Aβ accumulation in the APP transgenic murine model of AD (Patel et al., 2005; Wang et al., 2005) and aged primates (Qin et al., 2006). Furthermore, in a triple transgenic AD mice, both IF and CR protocols, when starting at 3 months of age (before symptoms onset), can counteract age-related cognitive impairment (Halagappa et al., 2007). Interestingly, in this study, CR, but not IF, reduced Aβ deposition in the brain. The authors suggest that the IF mechanism of action may involve the prevention of Aβ-mediated negative effects on cognitive function.

Glutathione is an important reducing agent of the phase II antioxidant response. Many genes involved in glutathione metabolism are modulated by Nrf2, including glutathione synthesis genes (GCLM, GCLC), GST, GPx, and GR. Several studies have reported an age-related disruption of the glutathione antioxidant system in rodents and humans, which may result in increased susceptibility to PD and AD (Rao et al., 1990; Samiec et al., 1998; Cho et al., 2003; Suh et al., 2004; Kennedy et al., 2005; Ballatori et al., 2009; Hine and Mitchell, 2012). Cho et al. (2003) showed that CR in rodents is able to prevent the age-related decrease in the levels of glutathione and glutathione-related enzyme activities. Furthermore, many studies showed that GST and GPx levels and activities can be augmented by both fasting and CR (Leakey et al., 1989; Cho et al., 2003; Pearson et al., 2008; Mitchell et al., 2010; Vazquez-Medina et al., 2011). In one of these studies, these effects were evident in mice subjected to 30% CR for 2–4 weeks or short periods of fasting, following ischemia reperfusion injury (Mitchell et al., 2010). Results from the CALERIE Trial of Human Caloric Restriction also showed that GPx activity is increased by 10–30% CR over 6 months in overweight individuals (Meydani et al., 2011).

NQO1 is another important endogenous antioxidant defense enzyme modulated by Nrf2. Altered expression of NQO1 is correlated with many pathologies, including AD and PD (Lastres-Becker, 2017; Chhetri et al., 2018). Long term DR can increase NQO1 expression, resulting in enhanced antioxidant defenses in the brain and liver of aged rats (De Cabo et al., 2004; Hyun et al., 2006). This effect was also observed in studies using DER mimetics (Zhu et al., 2005; Higgins et al., 2009; Son et al., 2010).

DER and its mimetics can also counteract damage following ischemia reperfusion injury (Go et al., 1988; Khan et al., 2006; Saleh et al., 2010; Peng et al., 2012). After experimental stroke in rodents, 70 days of DER leads to a substantial attenuation of cognitive dysfunction as well as increasing hippocampal cell survival (Roberge et al., 2008). These effects seem, in part, to involve the Nrf2-triggering effect of DER (Mattson, 2012). Fasting for up to 4 days or 30% CR for 2–4 weeks results in augmented HO-1 expression and attenuates ischemic damage of the brain, liver, and kidney in rodents (Go et al., 1988; Mitchell et al., 2010; Verweij et al., 2011). Moreover, *in vivo* or *in vitro* data shows that DER mimetics, such as curcumin and plumbagin, also increase HO-1 expression and render rodents more resistant to acute stressors and oxidative damage (Farombi et al., 2008; Son et al., 2010). In one of these studies, plumbagin pre-treatment in a murine model of focal ischemic stroke led to the attenuation of brain injury and neurological deficits. These effects were attributed to Nrf2/ARE activation, given that Nrf2 knockdown prevents such neuroprotective effects (Son et al., 2010).

Current MS treatments have poor efficacy, both for symptom relief and disease progression (Lublin et al., 2014). Genetic risk factors do not fully explain the development of MS, with a number of environmental factors, including infections, smoking, low vitamin D levels and obesity, associated with increased incidence of MS (Ascherio, 2013). A number of studies show childhood/young adulthood obesity to be a risk factor for MS (Munger et al., 2009; Hedstrom et al., 2012, 2014; Langer-Gould et al., 2013). The chronic inflammatory state that is evident in obesity can promote autoimmunity though adipokine production (Calder et al., 2011). The effects of diet on the gut microbiome is thought to contribute to this, via the regulation of pro- and anti-inflammatory responses that regulate DC activation, MHC II presentation, and T cell differentiation in the gut (Goto et al., 2014; Furusawa et al., 2015). The gut microbiome in both RRMS patients and EAE models is altered compared to healthy controls (Chen et al., 2016a; Jangi et al., 2016). Several dietary habits such as high salt intake or long chain fatty acid intake, have been recently recognized as environmental contributors to the pathogenesis of MS and EAE, by expanding TH1 and TH17 cells (Berer et al., 2011; Kleinewietfeld et al., 2013).

Chronic CR has a potent anti-inflammatory effect, protecting against EAE symptoms (Piccio et al., 2008; Meydani et al., 2016). In these studies, CR reduced inflammation, demyelination, and neurodegeneration ameliorating relapsing-remitting EAE in SJL mice and progressive EAE in C57BL/6 mice. CR lowers plasma IL-6 concentration in the course of EAE accompanied by a decrease in leptin, suggesting CR-mediated alterations in the gut-brain axis and associated changes in gut-mediated inflammatory processes (Piccio et al., 2008). As leptin induces Th1 cell differentiation, such changes are likely to lower the levels of proinflammatory cytokine production (Matarese et al., 2005).

Accordingly, MS patients showed an increased concentration of leptin in the serum and cerebrospinal fluid, associated with reduced levels of CD4 (+) CD25+ regulatory T cells and augmented INF-γ release (Matarese et al., 2005).

Recent studies indicate that MS show characteristics of metabolic disease, with SPMS patients showing elevated peroxisomal metabolites (PlsEtn) and increased mitochondrial stress metabolites (VLCFA-PtdEtn), when compared to control patients (Senanayake et al., 2015). Furthermore, SPMS patients showed reduced seric anti-inflammatory hydroxylated long-chain fatty acids called gastro-intestinal tract acids (GTAs), suggesting a diminished protection against MS-related inflammation. In addition, the oxidative stress-induced mitochondrial dysfunction in MS may provide biomarkers for the susceptibility to, and progression of, MS.

Importantly, although Nrf2/ARE signaling plays a critical role in cellular detoxification responses and prevention of age-related diseases, excessive Nrf2 activation has deleterious consequences. This is supported by studies showing KEAP1 knockout to be lethal in mice as a consequence of excessive constitutive Nrf2 activation (Wakabayashi et al., 2003), with Nrf2 overexpression (DeNicola et al., 2011; Lister et al., 2011) or mutation of KEAP2 (Zhang et al., 2010) promoting tumorigenesis. Consequently, pharmacological activation of Nrf2 may be dangerous, with the physiological activation of Nrf2 by DER likely to be a safer alternative.

High Energy Consumption

The Western lifestyle are frequently more sedate with greater levels of over-eating, characterizing a condition of chronic positive energy balance (Martin et al., 2009; Mattson, 2012). In contrast to DER, high-energy consumption is associated with many negative impacts on overall health and longevity, resulting in increased morbidity and mortality (Maffei et al., 1995; Caro et al., 1996; Herrmann et al., 2001). Consequently, being overweight or obesity has reached epidemic proportions, being now classed as the fifth largest cause of death worldwide (Razay et al., 2006; Procaccini et al., 2016). In fact, the World Health Organization estimated that the global burden of obesity and overweight was over 300 million and 1 billion of adults, respectively (World Health Organization [WHO], 2011). Obesity, in turn, is an important risk factor for diabetes, a metabolic disease associated to chronic hyperglycemia and an array of other complications (Tebay et al., 2015). A HFD (typically 40–60% of total calorie intake from fat) in humans and animals results in an augmented vulnerability to an array of medical conditions, including many psychiatric disorders where risk positively correlates with BMI (Lopes et al., 2001; Degirmenci et al., 2015). High BMI in human subjects is also linked to reduced blood flow in brain regions important for cognitive function (Willeumier et al., 2011) and reduced brain integrity (Gazdzinski et al., 2008; Stanek et al., 2011). Even a short-term HFD, for only 7 days, can cause cognitive impairment in humans (Edwards et al., 2011).

Accumulating data clearly shows that obesity is a risk factor for cognitive decline, dementia and neurodegenerative diseases, such as AD and PD (Mazon et al., 2017). One of the mechanisms by which diet-induced obesity can lead to neurological disorders is through increased neuroinflammation and ROS production and the downregulation of endogenous antioxidant enzymes, resulting in increased oxidative damage to the CNS (Edwards et al., 2011; Matsuda and Shimomura, 2013; Guillemot-Legris and Muccioli, 2017; Mazon et al., 2017). Diet-induced obesity can also lead to neurological disorders through microglia activation and BBB disruption, which can trigger neuroinflammation and synaptic impairment, thereby resulting in cognitive decline and neurodegeneration (Pistell et al., 2010; Zlokovic, 2011; Knight et al., 2014; Tucsek et al., 2014) (**Figure 3**).

Nrf2 activation can improve energy expenditure and prevent weight gain in HFD mice (Shin et al., 2009). Moreover, Nrf2/HO-1 pathway activation can ameliorate long-term HFD-induced cognitive impairment, inflammation, as well as the accumulation of Aβ and hyperphosphorilated tau in the hippocampus (FangFang et al., 2017). In young subjects, Nrf2 can counteract the vascular oxidative damage and augmented ROS production associated with obesity (Ungvari et al., 2011a; Csiszar et al., 2015). However, aging is associated with Nrf2/ARE signaling pathway dysfunction, increasing the susceptibility of the elderly to obesity-driven oxidative stress. As oxidative stress is already exacerbated in these individuals by various other factors, the dysfunctioning of Nrf2 signaling will further aggravate this pro-oxidative scenario and contribute to the development of neuropsychiatric disorders in the aging brain (Morrison et al., 2010; Ungvari et al., 2011b,c).

Morrison et al. (2010) showed that 20-month old male mice fed a HFD composed of 60% fat, but not 41% fat, presented increased hippocampal oxidative stress and cognitive decline after 4 months, when compared to controls. These changes were associated with a decrease in Nrf2 levels and activity, suggesting that Nrf2 signaling impairment may be a mechanism of HFD-induced cognitive dysfunction in the aging brain.

Interestingly, numerous studies have demonstrated that DER can counteract the negative effects of HFD and promote a wide array of beneficial effects on the health of overweight individuals (Mattson, 2012). For instance, obese or overweight individuals show improved cognitive function and mood regulation following 12 months of DER (low carbohydrate or fat intake) (Brinkworth et al., 2009).

A HFD can result in neuronal insulin resistance, a marker of diabetes, contributing to cognitive impairment. Moreover, AD risk is positively correlated to brain insulin resistance and diabetes (Pipatpiboon et al., 2012; Tucsek et al., 2014; FangFang et al., 2017). Studies in an AD murine model show that, unlike DER, HFD and T2D symptoms can aggravate Aβ deposition in the brain and contribute to cognitive impairment (Ho et al., 2004; Takeda et al., 2010). In an AD post-mortem study, insulin resistance positively correlated with Aβ plaques and negatively correlated with last recorded cognitive function (Talbot et al., 2012).

A recent study in triple transgenic AD mice showed that HFD (60% fat) for 4 months resulted in cognitive impairment, coupled to increased oxidative stress and neuronal cell death. These changes were attributed to suppressed Nrf2/ARE pathway activation along with reduced expression of the Nrf2 target genes

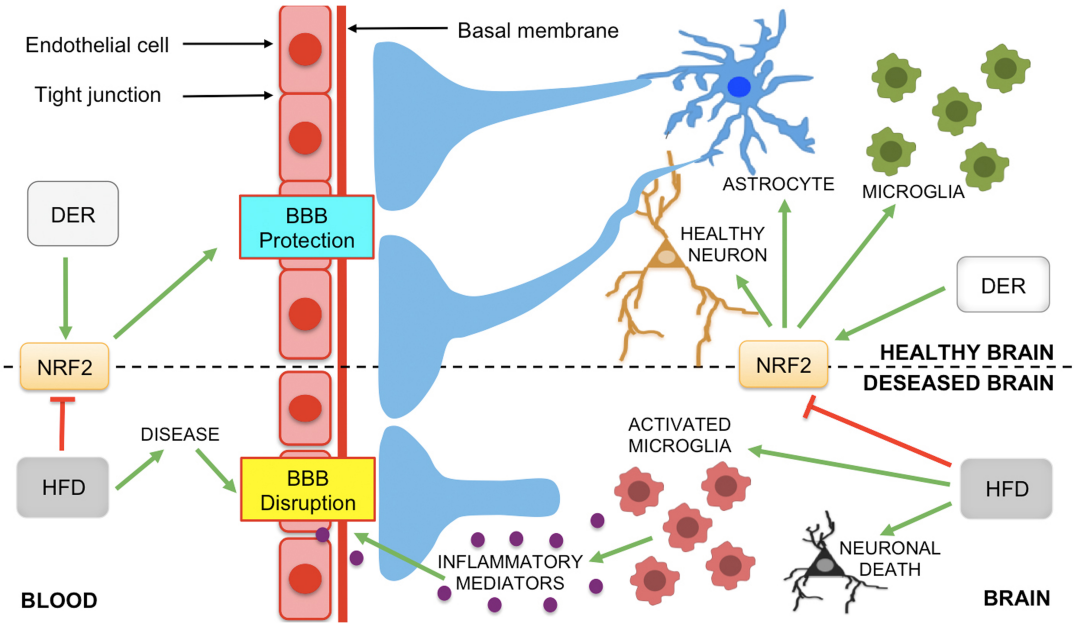

FIGURE 3 | The role of NRF2 and dietary interventions (DER and HFD) on the modification of the BBB. Neurological diseases, such as AD, PD, MS, and ischemic stroke, can lead to BBB disruption. However, Nrf2 protects neurons and the BBB against oxidative stress and inflammation-induced damage. Astrocytes, microglia and neurons produce Nrf2 that activates the expression of antioxidant and anti-inflammatory genes to the maintenance of neuronal health and BBB integrity. Nrf2 protective effects on BBB can be modulated by DER and HFD, modifying the release of inflammatory mediators by glial cells in neurodegenerative diseases. Green arrows represent activation and truncated red lines, inhibition (abbreviations: BBB, blood-brain barrier; DER, dietary energy restriction; HFD, high-fat diet, MS, multiple sclerosis; PD, Parkinson's disease; DER, dietary energy restriction; HFD, high-fat diet).

HO-1 and manganese SOD (MnSOD) (Sah et al., 2017). Tarantini et al. (2018) fed Nrf2 knockout mice a HFD (60% fat) for 5 months, with Nrf2 deficiency significantly increasing HFD-induced brain inflammation, oxidative stress, synaptic disruption and BBB permeability, as well as raising levels of amyloid precursor protein (APP) gene expression, the proteolysis of which produces Aβ. The authors concluded that Nrf2 dysfunction exacerbates the obesity-induced adverse effects in the brain and plays a role in vascular cognitive impairment and AD (Tarantini et al., 2018).

As with AD, calorie intake throughout life can influence the incidence of PD in some individuals (Mattson, 2012). Several lines of evidence have shown that diet, adiposity and T2D are linked to PD (Logroscino et al., 1996; Anderson et al., 1999; Johnson et al., 1999; Abbott et al., 2002; Hu et al., 2006, 2007; Morris et al., 2011; Schernhammer et al., 2011; Xu et al., 2011). All these factors are correlated to a HFD, given that over 80% of T2D patients are overweight or obese, whilst a HFD is frequently used as a model of diabetes (Centers for Disease Control and Prevention (CDC), 2004; Morris et al., 2011).

Many studies have shown that a HFD renders SN neurons more vulnerable to PD neurotoxins and increased ROS levels (Choi et al., 2005; Morris et al., 2010, 2011; Bousquet et al., 2012). In one of these works, mice fed a HFD or control diet for 5 weeks were treated with the PD-related neurotoxin 6-OHDA, with the HFD mice presenting with higher oxidative stress and neurodegeneration (dopamine depletion in the striatum and SN). These poorer outcomes were all correlated with heightened levels of adiposity and insulin resistance (Morris et al., 2010).

Innate and adaptive immune systems have a pivotal role in MS pathogenesis. Nrf2 agonists are promising candidates in the treatment of MS, since CDDO-Im 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole, a Nrf2 activator, promoting the differentiation of the less inflammatory Th2 t cell phenotype in stimulated splenocytes of C57BL/6 mice, thereby lowering INFγ and TNFα production as well as NF-κB DNA binding (Zagorski et al., 2018). However, it has more recently been proposed that inflammatory processes are secondary to primary cytodegenerative processes in some neural cells, such as oligodendrocytes and neurons, with some studies strongly suggesting that alterations in mitochondrial functioning, and consequent increase ROS, can drive the initial neurodegeneration in MS (Witte et al., 2014). Furthermore, increased oxidative stress is correlated with decreased Complex IV electron transport chain gene expression and Nrf2 activity in the non-lesioned gray matter of the frontal and parietal cortex, from post-mortem MS and control groups, suggesting that mitochondrial function is correlated with, if not regulated by, Nrf2 in MS (Pandit et al., 2009).

Epidemiological studies also indicate a positive correlation between MS severity and fatty acids intake, with a long-term, higher level saturated fat consumption being associated with an increased frequency of MS as well as augmented EAE symptomatology (Schwarz and Leweling, 2005; Thompson, 2008; Winer et al., 2009). Moreover, overweight and obese 20-year olds

have a higher risk of developing MS compared to those of normal weight (Hedstrom et al., 2014). Likewise, several other dietary compounds like tea, coffee, alcohol and sweets are connected with MS incidence (Antonovsky et al., 1965; Berr et al., 1989; Sepcic et al., 1993; Tola et al., 1994). Timmermans and colleagues showed an increased clinical score and proinflammatory genes (IL6, INFγ, and IL1β), as well as higher levels of infiltrating CD3+ T cells in the CNS of female animals subjected to a HFD. Similarly, Fernandez-Real and Pickup (Fernandez-Real and Pickup, 2008) showed an increase in inflammatory cytokines, soluble adhesion molecules, and chemokines in the blood of obese people. Accordingly, the Nrf2 activator, CDDO-IM, prevented the body weight gain in animals submitted to HFD, partly by regulating expression of fatty acid synthesis and oxidation enzymes in the liver (Shin et al., 2009).

As in MS, a HFD also is a risk factor for cardiovascular diseases and cerebral ischemia. Recent research has revealed an increase in the prevalence of acute ischemic stroke in children and young adults, correlated with such risk factors, as obesity and lipid disorders (George et al., 2011). Impairment in vascular function is evident in male Wistar rats submitted to 8-week HFD before transient middle cerebral artery occlusion (MCAO), with HFD significantly increasing not only body weight and adiposity as well as associated processes, but also augmenting the infarct size in rats (Li et al., 2013). Such data highlights how alterations in the regulation of energy intake are intimately linked to levels of Nrf2, in the regulation of susceptibility to, and severity of, a wide array of seemingly distinct medical conditions.

Interestingly, both HFD and lack of Nrf2 can increase infarct area after cerebral ischemia. Deutsch et al. (2009) demonstrated that HFD-rats submitted to cerebral ischemia by MCAO showed smaller lumens and thicker MCA walls, when compared to normal diet controls. This is attributed to increased expression of metalloproteinase-2 expression and collagen-1 deposition, suggesting impairment on neurovascular functions (Deutsch et al., 2009; Osmond et al., 2010; Li et al., 2013). In another study, the volume infarct of Nrf2-deficient mice subjected to MCAO for 90 min and 24 h of reperfusion was increased when compared to the control group (Shah et al., 2007). Intracerebral ventricular pre-administration of tert-butylhydroquinone reduced the infarct area in the MCAO mouse brain (Shih et al., 2005). Corroborating these findings, pharmacological agonism of Nrf2, by dimethyl fumarate, reduced NF-κB activation and protected the calcium-activated potassium (BK) channel-mediated coronary vasodilatation in HFD mice (Lu et al., 2017). These findings suggest that both Nrf2 and HFD have important roles in neurovascular and cerebral ischemia modulation.

It is also of note that dietary impacts on the composition of the gut microbiome may be of some relevance to the data presented above. There is considerable interest in the role of gut microbiome changes in the etiology, course and treatment of AD, PD, and MS (Anderson and Maes, 2017), mediated via changes in the gut-brain and gut-liver axes. By increasing gut permeability, gut bacteria and tiny fragments of partially digested food can trigger an immune reaction, with a wide array of consequences, including the possible production of α-synuclein in the gut, and its transport via neurons to the brain in the etiology of PD (Chen et al., 2018). Generally, an increase in the gut bacteria producing the small chain fatty acid, butyrate, is beneficial across a wide array of medical conditions. It is of note that butyrate increases levels of Nrf2 (Anderson et al., 2016), suggesting that some of the benefits of butyrate may be mediated not only by its histone deacetylase inhibitor capacity and its induction of melatonin, but also by its induction of Nrf2 (Dong W. et al., 2017).

CONCLUSION

It is widely accepted that oxidative stress plays a central role in neurological disorders. This underpins the importance of targeting Nrf2 to counteract such oxidative stress and associated brain diseases (Patel, 2016). Dietary interventions such as DER protocols, in contrast to a HFD, can promote small energetic challenges to the brain that enhance Nrf2/ARE pathway activation (Mattson, 2012). Nrf2 up-regulates the expression of several pro-survival genes and counteracts oxidative damage to the CNS, thereby preventing neurodegeneration and obesity-related brain disorders (Calkins et al., 2009). Hence, DER may be a valuable treatment option for brain disorders, including as adjunct therapy with other known Nrf2 activators. However, controversial reports showed that NRF2 activation can result in drug resistance and oncogenic effects (Sporn and Liby, 2012). Thus, additional large-scale studies are warranted to further explore the effects of dietary interventions in Nrf2/ARE signaling and to establish the best dietary protocols for humans to optimize the beneficial effects of Nrf2 for the prevention and/or early intervention in the etiology and course of neurological disorders.

AUTHOR CONTRIBUTIONS

AV, NdS, CS, and CM contributed to the design of the manuscript, literature review, writing of the manuscript, and creation of the figures.

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REFERENCES

Abbott, R. D., Ross, G. W., White, L. R., Nelson, J. S., Masaki, K. H., Tanner, C. M., et al. (2002). Midlife adiposity, and the future risk of Parkinson's disease. *Neurology* 59, 1051–1057. doi: 10.1212/WNL.59.7.1051

Ahmad, W., Ijaz, B., Shabbiri, K., Ahmed, F., and Rehman, S. (2017). Oxidative toxicity in diabetes and Alzheimer's disease: mechanisms behind ROS/ RNS generation. *J. Biomed. Sci.* 24:76. doi: 10.1186/s12929-017-0379-z

Alfieri, A., Srivastava, S., Siow, R. C., Modo, M., Fraser, P. A., and Mann, G. E. (2011). Targeting the Nrf2-Keap1 antioxidant defence pathway for neurovascular protection in stroke. *J. Physiol.* 589, 4125–4136. doi: 10.1113/jphysiol.2011.210294

Alfredsson, L., and Olsson, T. (2018). Lifestyle and environmental factors in multiple sclerosis. *Cold Spring Harb. Perspect. Med.* doi: 10.1101/cshperspect.a028944 [Epub ahead of print].

Al-Huseini, L. M., Aw Yeang, H. X., Sethu, S., Alhumeed, N., Hamdam, J. M., Tingle, Y., et al. (2013). Nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (Nrf2) modulates dendritic cell immune function through regulation of p38 MAPK-cAMP-responsive element binding protein/activating transcription factor 1 signaling. *J. Biol. Chem.* 288, 22281–22288. doi: 10.1074/jbc.M113.483420

Aluise, C. D., Robinson, R. A., Cai, J., Pierce, W. M., Markesbery, W. R., and Butterfield, D. A. (2011). Redox proteomics analysis of brains from subjects with amnestic mild cognitive impairment compared to brains from subjects with preclinical Alzheimer's disease: insights into memory loss in MCI. *J. Alzheimers Dis.* 23, 257–269. doi: 10.3233/JAD-2010-101083

Alzheimer's Association (2018). Alzheimer's disease facts and figures. *Alzheimers Dement.* 14, 367–429.

Anderson, C., Checkoway, H., Franklin, G. M., Beresford, S., Smith-Weller, T., and Swanson, P. D. (1999). Dietary factors in Parkinson's disease: the role of food groups and specific foods. *Mov. Disord.* 14, 21–27. doi: 10.1002/1531-8257(199901)14:1<21::AID-MDS1006>3.0.CO;2-Y

Anderson, G., and Maes, M. (2017). How immune-inflammatory processes link CNS and psychiatric disorders: classification and treatment implications. *CNS Neurol. Disord. Drug Targets* 16, 266–278. doi: 10.2174/1871527315666161122144659

Anderson, G., Seo, M., Berk, M., Carvalho, A. F., and Maes, M. (2016). Gut permeability and microbiota in Parkinson's disease: role of depression, tryptophan catabolites, oxidative and nitrosative stress and melatonergic pathways. *Curr. Pharm. Des.* 22, 6142–6151. doi: 10.2174/1381612822666160906161513

Antonovsky, A., Leibowitz, U., Smith, H. A., Medalie, J. M., Balogh, M., Kats, R., et al. (1965). Epidemiologic study of multiple sclerosis in Israel. I. An overall review of methods and findings. *Arch. Neurol.* 13, 183–193. doi: 10.1001/archneur.1965.00470020073010

Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* 42(3 Pt 1), 631–639. doi: 10.1212/WNL.42.3.631

Ascherio, A. (2013). Environmental factors in multiple sclerosis. *Expert Rev. Neurother.* 13(Suppl. 12), 3–9. doi: 10.1586/14737175.2013.865866

Bai, J., Yu, X. J., Liu, K. L., Wang, F. F., Jing, G. X., Li, H. B., et al. (2017). Central administration of tert-butylhydroquinone attenuates hypertension via regulating Nrf2 signaling in the hypothalamic paraventricular nucleus of hypertensive rats. *Toxicol. Appl. Pharmacol.* 333, 100–109. doi: 10.1016/j.taap.2017.08.012

Ballatori, N., Krance, S. M., Notenboom, S., Shi, S., Tieu, K., and Hammond, C. L. (2009). Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* 390, 191–214. doi: 10.1515/BC.2009.033

Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C. J., Foresti, R., et al. (2003). Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. J.* 371(Pt 3), 887–895. doi: 10.1042/BJ20021619

Berer, K., Mues, M., Koutrolos, M., Rasbi, Z. A., Boziki, M., Johner, C., et al. (2011). Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 479, 538–541. doi: 10.1038/nature10554

Bermejo, P., Martin-Aragon, S., Benedi, J., Susin, C., Felici, E., Gil, P., et al. (2008). Peripheral levels of glutathione and protein oxidation as markers in the development of Alzheimer's disease from Mild Cognitive Impairment. *Free Radic. Res.* 42, 162–170. doi: 10.1080/10715760701861373

Berr, C., Puel, J., Clanet, M., Ruidavets, J. B., Mas, J. L., and Alperovitch, A. (1989). Risk factors in multiple sclerosis: a population-based case-control study in Hautes-Pyrenees, France. *Acta Neurol. Scand.* 80, 46–50. doi: 10.1111/j.1600-0404.1989.tb03841.x

Blesa, J., Trigo-Damas, I., Quiroga-Varela, A., and Jackson-Lewis, V. R. (2015). Oxidative stress and Parkinson's disease. *Front. Neuroanat.* 9:91. doi: 10.3389/fnana.2015.00091

Bo, L., Dawson, T. M., Wesselingh, S., Mork, S., Choi, S., Kong, P. A., et al. (1994). Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. *Ann. Neurol.* 36, 778–786. doi: 10.1002/ana.410360515

Bousquet, M., St-Amour, I., Vandal, M., Julien, P., Cicchetti, F., and Calon, F. (2012). High-fat diet exacerbates MPTP-induced dopaminergic degeneration in mice. *Neurobiol. Dis.* 45, 529–538. doi: 10.1016/j.nbd.2011.09.009

Bradley, M. A., Xiong-Fister, S., Markesbery, W. R., and Lovell, M. A. (2012). Elevated 4-hydroxyhexenal in Alzheimer's disease (AD) progression. *Neurob. Aging* 33, 1034–1044. doi: 10.1016/j.neurobiolaging.2010.08.016

Brinkworth, G. D., Buckley, J. D., Noakes, M., Clifton, P. M., and Wilson, C. J. (2009). Long-term effects of a very low-carbohydrate diet and a low-fat diet on mood and cognitive function. *Arch. Intern. Med.* 169, 1873–1880. doi: 10.1001/archinternmed.2009.329

Bruns, D. R., Drake, J. C., Biela, L. M., Peelor, F. F. III, Miller, B. F., and Hamilton, K. L. (2015). Nrf2 signaling and the slowed aging phenotype: evidence from long-lived models. *Oxid. Med. Cell. Longev.* 2015:732596. doi: 10.1155/2015/732596

Buendia, I., Michalska, P., Navarro, E., Gameiro, I., Egea, J., and Leon, R. (2016). Nrf2-ARE pathway: an emerging target against oxidative stress and neuroinflammation in neurodegenerative diseases. *Pharmacol. Ther.* 157, 84–104. doi: 10.1016/j.pharmthera.2015.11.003

Cai, Z., Zhao, B., and Ratka, A. (2011). Oxidative stress and beta-amyloid protein in Alzheimer's disease. *Neuromol. Med.* 13, 223–250. doi: 10.1007/s12017-011-8155-9

Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., Calabrese, E. J., and Mattson, M. P. (2010). Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. *Antioxid. Redox Signal.* 13, 1763–1811. doi: 10.1089/ars.2009.3074

Calder, P. C., Ahluwalia, N., Brouns, F., Buetler, T., Clement, K., Cunningham, K., et al. (2011). Dietary factors and low-grade inflammation in relation to overweight and obesity. *Br. J. Nutr.* 106(Suppl. 3), S5–S78. doi: 10.1017/S0007114511005460

Calkins, M. J., Johnson, D. A., Townsend, J. A., Vargas, M. R., Dowell, J. A., Williamson, T. P., et al. (2009). The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. *Antioxid. Redox Signal.* 11, 497–508. doi: 10.1089/ARS.2008.2242

Cao, T. T., Ma, L., Kandpal, G., Warren, L., Hess, J. F., and Seabrook, G. R. (2005). Increased nuclear factor-erythroid 2 p45-related factor 2 activity protects SH-SY5Y cells against oxidative damage. *J. Neurochem.* 95, 406–417. doi: 10.1111/j.1471-4159.2005.03377.x

Caro, J. F., Sinha, M. K., Kolaczynski, J. W., Zhang, P. L., and Considine, R. V. (1996). Leptin: the tale of an obesity gene. *Diabetes* 45, 1455–1462. doi: 10.2337/diab.45.11.1455

Carvalho, C., Santos, M. S., Oliveira, C. R., and Moreira, P. I. (2015). Alzheimer's disease and type 2 diabetes-related alterations in brain mitochondria, autophagy and synaptic markers. *Biochim. Biophys. Acta* 1852, 1665–1675. doi: 10.1016/j.bbadis.2015.05.001

Centers for Disease Control and Prevention (CDC) (2004). Prevalence of overweight and obesity among adults with diagnosed. (diabetes)–United States, 1988-1994 and 1999-2002. *MMWR Morb. Mortal. Wkly. Rep.* 53, 1066–1068. doi: 10.1016/j.bbrc.2005.03.237

Chen, C. Y., Jang, J. H., Li, M. H., and Surh, Y. J. (2005). Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem. Biophys. Res. Commun.* 331, 993–1000. doi: 10.1016/j.bbrc.2005.03.237

Chen, J., Chia, N., Kalari, K. R., Yao, J. Z., Novotna, M., Paz Soldan, M. M., et al. (2016a). Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci. Rep.* 6:28484. doi: 10.1038/srep28484

Chen, J. W., Breckwoldt, M. O., Aikawa, E., Chiang, G., and Weissleder, R. (2008). Myeloperoxidase-targeted imaging of active inflammatory lesions in murine experimental autoimmune encephalomyelitis. *Brain* 131(Pt 4), 1123–1133. doi: 10.1093/brain/awn004

Chen, K., Gunter, K., and Maines, M. D. (2000). Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. *J. Neurochem.* 75, 304–313. doi: 10.1046/j.1471-4159.2000.0750304.x

Chen, L. H., Hu, N., and Snyder, D. L. (1994). Effects of age and dietary restriction on liver glutathione transferase activities in Lobund-Wistar rats. *Arch. Gerontol. Geriatr.* 18, 191–205. doi: 10.1016/0167-4943(94)90013-2

Chen, P. C., Vargas, M. R., Pani, A. K., Smeyne, R. J., Johnson, D. A., Kan, Y. W., et al. (2009). Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: critical role for the astrocyte. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2933–2938. doi: 10.1073/pnas.0813361106

Chen, W. W., Zhang, X., and Huang, W. J. (2016b). Role of neuroinflammation in neurodegenerative diseases (Review). *Mol. Med. Rep.* 13, 3391–3396. doi: 10.3892/mmr.2016.4948

Chen, Y., Shao, Q., Yuan, Y. H., and Chen, N. H. (2018). Prion-like propagation of alpha-synuclein in the gut-brain axis. *Brain Res. Bull.* 140, 341–346. doi: 10.1016/j.brainresbull.2018.06.002

Chhetri, J., King, A. E., and Gueven, N. (2018). Alzheimer's Disease and NQO1: is there a Link? *Curr. Alzheimer Res.* 15, 56–66. doi: 10.2174/1567205014666170203095802

Chiaravalloti, N. D., and DeLuca, J. (2008). Cognitive impairment in multiple sclerosis. *Lancet Neurol.* 7, 1139–1151. doi: 10.1016/S1474-4422(08)70259-X

Cho, C. G., Kim, H. J., Chung, S. W., Jung, K. J., Shim, K. H., Yu, B. P., et al. (2003). Modulation of glutathione and thioredoxin systems by calorie restriction during the aging process. *Exp. Gerontol.* 38, 539–548. doi: 10.1016/S0531-5565(03)00005-6

Choi, J. Y., Jang, E. H., Park, C. S., and Kang, J. H. (2005). Enhanced susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in high-fat diet-induced obesity. *Free Radic. Biol. Med.* 38, 806–816. doi: 10.1016/j.freeradbiomed.2004.12.008

Colton, C. A. (2009). Heterogeneity of microglial activation in the innate immune response in the brain. *J. Neuroimmune Pharmacol.* 4, 399–418. doi: 10.1007/s11481-009-9164-4

Csiszar, A., Csiszar, A., Pinto, J. T., Gautam, T., Kleusch, C., Hoffmann, B., et al. (2015). Resveratrol encapsulated in novel fusogenic liposomes activates Nrf2 and attenuates oxidative stress in cerebromicrovascular endothelial cells from aged rats. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 70, 303–313. doi: 10.1093/gerona/glu029

Csiszar, A., Gautam, T., Sosnowska, D., Tarantini, S., Banki, E., Tucsek, Z., et al. (2014). Caloric restriction confers persistent anti-oxidative, pro-angiogenic, and anti-inflammatory effects and promotes anti-aging miRNA expression profile in cerebromicrovascular endothelial cells of aged rats. *Am. J. Physiol. Heart Circ. Physiol.* 307, H292–H306. doi: 10.1152/ajpheart.00307.2014

Cuadrado, A. (2016). NRF2 in neurodegenerative diseases. *Curr. Opin. Toxicol.* 1, 46–53. doi: 10.1016/j.cotox.2016.09.004

Cuadrado, A., Moreno-Murciano, P., and Pedraza-Chaverri, J. (2009). The transcription factor Nrf2 as a new therapeutic target in Parkinson's disease. *Expert Opin. Ther. Targets* 13, 319–329. doi: 10.1517/13543780802716501

De Cabo, R., Cabello, R., Rios, M., Lopez-Lluch, G., Ingram, D. K., Lane, M. A., et al. (2004). Calorie restriction attenuates age-related alterations in the plasma membrane antioxidant system in rat liver. *Exp. Gerontol.* 39, 297–304. doi: 10.1016/j.exger.2003.12.003

De Groot, C. J., Ruuls, S. R., Theeuwes, J. W., Dijkstra, C. D., and Van der Valk, P. (1997). Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J. neuropathol. Exp. Neurol.* 56, 10–20. doi: 10.1097/00005072-199701000-00002

Degirmenci, T., Kalkan-Oguzhanoglu, N., Sozeri-Varma, G., Ozdel, O., and Fenkci, S. (2015). Psychological symptoms in obesity and related factors. *Noro Psikiyatr. Ars.* 52, 42–46. doi: 10.5152/npa.2015.6904

DeNicola, G. M., Karreth, F. A., Humpton, T. J., Gopinathan, A., Wei, C., Frese, K., et al. (2011). Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475, 106–109. doi: 10.1038/nature10189

Deutsch, C., Portik-Dobos, V., Smith, A. D., Ergul, A., and Dorrance, A. M. (2009). Diet-induced obesity causes cerebral vessel remodeling and increases the damage caused by ischemic stroke. *Microvasc. Res.* 78, 100–106. doi: 10.1016/j.mvr.2009.04.004

Di Domenico, F., Tramutola, A., and Butterfield, D. A. (2017). Role of 4-hydroxy-2-nonenal (HNE) in the pathogenesis of alzheimer disease and other selected age-related neurodegenerative disorders. *Free Radic. Biol. Med.* 111, 253–261. doi: 10.1016/j.freeradbiomed.2016.10.490

Ding, Y., Ren, D., Xu, H., Liu, W., Liu, T., Li, L., et al. (2017). Antioxidant and pro-angiogenic effects of corilagin in rat cerebral ischemia via Nrf2 activation. *Oncotarget* 8, 114816–114828. doi: 10.18632/oncotarget.22023

Ding, Y., Zhang, B., Zhou, K., Chen, M., Wang, M., Jia, Y., et al. (2014). Dietary ellagic acid improves oxidant-induced endothelial dysfunction and atherosclerosis: role of Nrf2 activation. *Int. J. Cardiol.* 175, 508–514. doi: 10.1016/j.ijcard.2014.06.045

Dong, F., Wang, S., Wang, Y., Yang, X., Jiang, J., Wu, D., et al. (2017). Quercetin ameliorates learning and memory via the Nrf2-ARE signaling pathway in d-galactose-induced neurotoxicity in mice. *Biochem. Biophys. Res. Commun.* 491, 636–641. doi: 10.1016/j.bbrc.2017.07.151

Dong, W., Jia, Y., Liu, X., Zhang, H., Li, T., Huang, W., et al. (2017). Sodium butyrate activates NRF2 to ameliorate diabetic nephropathy possibly via inhibition of HDAC. *J. Endocrinol.* 232, 71–83. doi: 10.1530/JOE-16-0322

Dorrance, A. M., Matin, N., and Pires, P. W. (2014). The effects of obesity on the cerebral vasculature. *Curr. Vasc. Pharmacol.* 12, 462–472. doi: 10.2174/1570161112666140423222411

Duan, W., and Mattson, M. P. (1999). Dietary restriction and 2-deoxyglucose administration improve behavioral outcome and reduce degeneration of dopaminergic neurons in models of Parkinson's disease. *J. Neurosci. Res.* 57, 195–206. doi: 10.1002/(SICI)1097-4547(19990715)57:2<195::AID-JNR5>3.0.CO;2-P

Edwards, L. M., Murray, A. J., Holloway, C. J., Carter, E. E., Kemp, G. J., Codreanu, I., et al. (2011). Short-term consumption of a high-fat diet impairs whole-body efficiency and cognitive function in sedentary men. *FASEB J.* 25, 1088–1096. doi: 10.1096/fj.10-171983

FangFang, Li, H., Qin, T., Li, M., and Ma, S. (2017). Thymol improves high-fat diet-induced cognitive deficits in mice via ameliorating brain insulin resistance and upregulating NRF2/HO-1 pathway. *Metab. Brain Dis.* 32, 385–393. doi: 10.1007/s11011-016-9921-z

Farombi, E. O., Shrotriya, S., Na, H. K., Kim, S. H., and Surh, Y. J. (2008). Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1. *Food Chem. Toxicol.* 46, 1279–1287. doi: 10.1016/j.fct.2007.09.095

Fernandez-Real, J. M., and Pickup, J. C. (2008). Innate immunity, insulin resistance and type 2 diabetes. *Trends Endocrinol. Metab.* 19, 10–16. doi: 10.1016/j.tem.2007.10.004

Frank, M. G., Baratta, M. V., Sprunger, D. B., Watkins, L. R., and Maier, S. F. (2007). Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. *Brain Behav. Immun.* 21, 47–59. doi: 10.1016/j.bbi.2006.03.005

French, H. M., Reid, M., Mamontov, P., Simmons, R. A., and Grinspan, J. B. (2009). Oxidative stress disrupts oligodendrocyte maturation. *J. Neurosci. Res.* 87, 3076–3087. doi: 10.1002/jnr.22139

Furusawa, Y., Obata, Y., and Hase, K. (2015). Commensal microbiota regulates T cell fate decision in the gut. *Semin. Immunopathol.* 37, 17–25. doi: 10.1007/s00281-014-0455-3

Gavillet, M., Allaman, I., and Magistretti, P. J. (2008). Modulation of astrocytic metabolic phenotype by proinflammatory cytokines. *Glia* 56, 975–989. doi: 10.1002/glia.20671

Gazdzinski, S., Kornak, J., Weiner, M. W., and Meyerhoff, D. J. (2008). Body mass index and magnetic resonance markers of brain integrity in adults. *Ann. Neurol.* 63, 652–657. doi: 10.1002/ana.21377

George, M. G., Tong, X., Kuklina, E. V., and Labarthe, D. R. (2011). Trends in stroke hospitalizations and associated risk factors among children and young adults, 1995-2008. *Ann. Neurol.* 70, 713–721. doi: 10.1002/ana.22539

Giordano, G., White, C. C., Mohar, I., Kavanagh, T. J., and Costa, L. G. (2007). Glutathione levels modulate domoic acid induced apoptosis in mouse cerebellar granule cells. *Toxicol. Sci.* 100, 433–444. doi: 10.1093/toxsci/kfm236

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., and Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell* 140, 918–934. doi: 10.1016/j.cell.2010.02.016

Go, K. G., Prenen, G. H., and Korf, J. (1988). Protective effect of fasting upon cerebral hypoxic-ischemic injury. *Metab. Brain Dis.* 3, 257–263. doi: 10.1007/BF00999535

Gorelick, P. B., Scuteri, A., Black, S. E., Decarli, C., Greenberg, S. M., Iadecola, C., et al. (2011). Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American heart association/american stroke association. *Stroke* 42, 2672–2713. doi: 10.1161/STR.0b013e3182299496

Goris, A., Williams-Gray, C. H., Clark, G. R., Foltynie, T., Lewis, S. J., Brown, J., et al. (2007). Tau and alpha-synuclein in susceptibility to, and dementia in, Parkinson's disease. *Ann. Neurol.* 62, 145–153. doi: 10.1002/ana.21192

Goto, Y., Panea, C., Nakato, G., Cebula, A., Lee, C., Diez, M. G., et al. (2014). Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity* 40, 594–607. doi: 10.1016/j.immuni.2014.03.005

Gray, E., Thomas, T. L., Betmouni, S., Scolding, N., and Love, S. (2008a). Elevated activity and microglial expression of myeloperoxidase in demyelinated cerebral cortex in multiple sclerosis. *Brain Pathol.* 18, 86–95. doi: 10.1111/j.1750-3639.2007.00110.x

Gray, E., Thomas, T. L., Betmouni, S., Scolding, N., and Love, S. (2008b). Elevated myeloperoxidase activity in white matter in multiple sclerosis. *Neurosci. Lett.* 444, 195–198. doi: 10.1016/j.neulet.2008.08.035

Griffioen, K. J., Rothman, S. M., Ladenheim, B., Wan, R., Vranis, N., Hutchison, E., et al. (2013). Dietary energy intake modifies brainstem autonomic dysfunction caused by mutant alpha-synuclein. *Neurobiol. Aging* 34, 928–935. doi: 10.1016/j.neurobiolaging.2012.07.008

Guillemot-Legris, O., and Muccioli, G. G. (2017). Obesity-induced neuroinflammation: beyond the hypothalamus. *Trends Neurosci.* 40, 237–253. doi: 10.1016/j.tins.2017.02.005

Gursoy-Ozdemir, Y., Can, A., and Dalkara, T. (2004). Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia. *Stroke* 35, 1449–1453. doi: 10.1161/01.STR.0000126044.83777.f4

Halagappa, V. K., Guo, Z., Pearson, M., Matsuoka, Y., Cutler, R. G., Laferla, F. M., et al. (2007). Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of Alzheimer's disease. *Neurobiol. Dis.* 26, 212–220. doi: 10.1016/j.nbd.2006.12.019

Hara, H., Ohta, M., and Adachi, T. (2006). Apomorphine protects against 6-hydroxydopamine-induced neuronal cell death through activation of the Nrf2-ARE pathway. *J. Neurosci. Res.* 84, 860–866. doi: 10.1002/jnr.20974

Hedstrom, A. K., Lima Bomfim, I., Barcellos, L., Gianfrancesco, M., Schaefer, C., Kockum, I., et al. (2014). Interaction between adolescent obesity and HLA risk genes in the etiology of multiple sclerosis. *Neurology* 82, 865–872. doi: 10.1212/WNL.0000000000000203

Hedstrom, A. K., Olsson, T., and Alfredsson, L. (2012). High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. *Mult. Scler.* 18, 1334–1336. doi: 10.1177/1352458512436596

Herrmann, T. S., Bean, M. L., Black, T. M., Wang, P., and Coleman, R. A. (2001). High glycemic index carbohydrate diet alters the diurnal rhythm of leptin but not insulin concentrations. *Exp. Biol. Med.* 226, 1037–1044. doi: 10.1177/153537020122601111

Higgins, L. G., Kelleher, M. O., Eggleston, I. M., Itoh, K., Yamamoto, M., and Hayes, J. D. (2009). Transcription factor Nrf2 mediates an adaptive response to sulforaphane that protects fibroblasts in vitro against the cytotoxic effects of electrophiles, peroxides and redox-cycling agents. *Toxicol. Appl. Pharmacol.* 237, 267–280. doi: 10.1016/j.taap.2009.03.005

Hine, C. M., and Mitchell, J. R. (2012). NRF2 and the phase II response in acute stress resistance induced by dietary restriction. *J. Clin. Exp. Pathol.* S4:7329. doi: 10.4172/2161-0681.S4-004

Ho, L., Qin, W., Pompl, P. N., Xiang, Z., Wang, J., Zhao, Z., et al. (2004). Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *FASEB J.* 18, 902–904. doi: 10.1096/fj.03-0978fje

Holmer, H. K., Keyghobadi, M., Moore, C., Menashe, R. A., and Meshul, C. K. (2005). Dietary restriction affects striatal glutamate in the MPTP-induced mouse model of nigrostriatal degeneration. *Synapse* 57, 100–112. doi: 10.1002/syn.20163

Holmstrom, K. M., Baird, L., Zhang, Y., Hargreaves, I., Chalasani, A., Land, J. M., et al. (2013). Nrf2 impacts cellular bioenergetics by controlling substrate availability for mitochondrial respiration. *Biol. Open* 2, 761–770. doi: 10.1242/bio.20134853

Horne, B. D., Muhlestein, J. B., and Anderson, J. L. (2015). Health effects of intermittent fasting: hormesis or harm? A systematic review. *Am. J. Clin. Nutr.* 102, 464–470. doi: 10.3945/ajcn.115.109553

Hsieh, T. C., Lu, X., Wang, Z., and Wu, J. M. (2006). Induction of quinone reductase NQO1 by resveratrol in human K562 cells involves the antioxidant response element ARE and is accompanied by nuclear translocation of transcription factor Nrf2. *Med. Chem.* 2, 275–285. doi: 10.2174/157340606776930709

Hu, G., Jousilahti, P., Bidel, S., Antikainen, R., and Tuomilehto, J. (2007). Type 2 diabetes and the risk of Parkinson's disease. *Diabetes Care* 30, 842–847. doi: 10.2337/dc06-2011

Hu, G., Jousilahti, P., Nissinen, A., Antikainen, R., Kivipelto, M., and Tuomilehto, J. (2006). Body mass index, and the risk of Parkinson disease. *Neurology* 67, 1955–1959. doi: 10.1212/01.wnl.0000247052.18422.e5

Huang, Y., Mao, Y., Li, H., Shen, G., and Nan, G. (2018). Knockdown of Nrf2 inhibits angiogenesis by downregulating VEGF expression through PI3K/Akt signaling pathway in cerebral microvascular endothelial cells under hypoxic conditions. *Biochem. Cell Biol.* 96, 475–482. doi: 10.1139/bcb-2017-0291

Huang, Y. J., Zhang, L., Shi, L. Y., Wang, Y. Y., Yang, Y. B., Ke, B., et al. (2018). Caloric restriction ameliorates acrolein-induced neurotoxicity in rats. *Neurotoxicology* 65, 44–51. doi: 10.1016/j.neuro.2018.01.003

Hwang, Y. P., and Jeong, H. G. (2008). The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress. *FEBS Lett.* 582, 2655–2662. doi: 10.1016/j.febslet.2008.06.045

Hyun, D. H., Emerson, S. S., Jo, D. G., Mattson, M. P., and de Cabo, R. (2006). Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19908–19912. doi: 10.1073/pnas.0608008103

Ingram, D. K., Zhu, M., Mamczarz, J., Zou, S., Lane, M. A., Roth, G. S., et al. (2006). Calorie restriction mimetics: an emerging research field. *Aging Cell* 5, 97–108. doi: 10.1111/j.1474-9726.2006.00202.x

Inoue, M., Williams, K. L., Gunn, M. D., and Shinohara, M. L. (2012). NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10480–10485. doi: 10.1073/pnas.1201836109

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* 236, 313–322. doi: 10.1006/bbrc.1997.6943

Jaiswal, A. K. (2004). Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radic. Biol. Med.* 36, 1199–1207. doi: 10.1016/j.freeradbiomed.2004.02.074

Jakel, R. J., Townsend, J. A., Kraft, A. D., and Johnson, J. A. (2007). Nrf2-mediated protection against 6-hydroxydopamine. *Brain Res.* 1144, 192–201. doi: 10.1016/j.brainres.2007.01.131

Jangi, S., Gandhi, R., Cox, L. M., Li, N., von Glehn, F., Yan, R., et al. (2016). Alterations of the human gut microbiome in multiple sclerosis. *Nat. Commun.* 7:12015. doi: 10.1038/ncomms12015

Jauch, E. C., Saver, J. L., Adams, H. P. Jr., Bruno, A., Connors, J. J., Demaerschalk, B. M., et al. (2013). Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* 44, 870–947. doi: 10.1161/STR.0b013e318284056a

Jazwa, A., Rojo, A. I., Innamorato, N. G., Hesse, M., Fernandez-Ruiz, J., and Cuadrado, A. (2011). Pharmacological targeting of the transcription factor Nrf2 at the basal ganglia provides disease modifying therapy for experimental parkinsonism. *Antioxid. Redox Signal.* 14, 2347–2360. doi: 10.1089/ars.2010.3731

Jenner, P. (2007). Oxidative stress and Parkinson's disease. *Handb. Clin. Neurol.* 83, 507–520. doi: 10.1016/S0072-9752(07)83024-7

Jo, C., Gundemir, S., Pritchard, S., Jin, Y. N., Rahman, I., and Johnson, G. V. (2014). Nrf2 reduces levels of phosphorylated tau protein by inducing autophagy adaptor protein NDP52. *Nat. Commun.* 5:3496. doi: 10.1038/ncomms4496

Johnson, C. C., Gorell, J. M., Rybicki, B. A., Sanders, K., and Peterson, E. L. (1999). Adult nutrient intake as a risk factor for Parkinson's disease. *Int. J. Epidemiol.* 28, 1102–1109. doi: 10.1093/ije/28.6.1102

Johnson, D. A., Amirahmadi, S., Ward, C., Fabry, Z., and Johnson, J. A. (2010). The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. *Toxicol. Sci.* 114, 237–246. doi: 10.1093/toxsci/kfp274

Joshi, G., Gan, K. A., Johnson, D. A., and Johnson, J. A. (2015). Increased Alzheimer's disease-like pathology in the APP/ PS1DeltaE9 mouse model lacking Nrf2 through modulation of autophagy. *Neurobiol. Aging* 36, 664–679. doi: 10.1016/j.neurobiolaging.2014.09.004

Juurlink, B. H., Thorburne, S. K., and Hertz, L. (1998). Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress. *Glia* 22, 371–378. doi: 10.1002/(SICI)1098-1136(199804)22:4<371::AID-GLIA6>3.0.CO;2-6

Kabel, A. M., Omar, M. S., Alhadhrami, A., Alharthi, S. S., and Alrobaian, M. M. (2018). Linagliptin potentiates the effect of l-dopa on the behavioural, biochemical and immunohistochemical changes in experimentally-induced Parkinsonism: role of toll-like receptor 4, TGF-beta1, NF-kappaB and glucagon-like peptide 1. *Physiol. Behav.* 188, 108–118. doi: 10.1016/j.physbeh.2018.01.028

Kanninen, K., Malm, T. M., Jyrkkanen, H. K., Goldsteins, G., Keksa-Goldsteine, V., Tanila, H., et al. (2008). Nuclear factor erythroid 2-related factor 2 protects against beta amyloid. *Mol. Cell. Neurosci.* 39, 302–313. doi: 10.1016/j.mcn.2008.07.010

Kappos, L., Radue, E. W., O'Connor, P., Polman, C., Hohlfeld, R., Calabresi, P., et al. (2010). A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N. Engl. J. Med.* 362, 387–401. doi: 10.1056/NEJMoa0909494

Kennedy, B. P., Rao, F., Botiglieri, T., Sharma, S., Lillie, E. O., Ziegler, M. G., et al. (2005). Contributions of the sympathetic nervous system, glutathione, body mass and gender to blood pressure increase with normal aging: influence of heredity. *J. Hum. Hypertens.* 19, 951–969. doi: 10.1038/sj.jhh.1001912

Khan, S., Salloum, F., Das, A., Xi, L., Vetrovec, G. W., and Kukreja, R. C. (2006). Rapamycin confers preconditioning-like protection against ischemia-reperfusion injury in isolated mouse heart and cardiomyocytes. *J. Mol. Cell. Cardiol.* 41, 256–264. doi: 10.1016/j.yjmcc.2006.04.014

Kim, S. K., and Novak, R. F. (2007). The role of intracellular signaling in insulin-mediated regulation of drug metabolizing enzyme gene and protein expression. *Pharmacol. Ther.* 113, 88–120. doi: 10.1016/j.pharmthera.2006.07.004

Kim, T. S., Pae, C. U., Yoon, S. J., Jang, W. Y., Lee, N. J., Kim, J. J., et al. (2006). Decreased plasma antioxidants in patients with Alzheimer's disease. *Int. J. Geriatr. Psychiatry* 21, 344–348. doi: 10.1002/gps.1469

Kleinewietfeld, M., Manzel, A., Titze, J., Kvakan, H., Yosef, N., Linker, R. A., et al. (2013). Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* 496, 518–522. doi: 10.1038/nature11868

Knight, E. M., Martins, I. V., Gumusgoz, S., Allan, S. M., and Lawrence, C. B. (2014). High-fat diet-induced memory impairment in triple-transgenic Alzheimer's disease (3xTgAD) mice is independent of changes in amyloid and tau pathology. *Neurobiol. Aging* 35, 1821–1832. doi: 10.1016/j.neurobiolaging.2014.02.010

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., et al. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat. Commun.* 7:11624. doi: 10.1038/ncomms11624

Kwon, S. H., Ma, S. X., Hwang, J. Y., Lee, S. Y., and Jang, C. G. (2015). Involvement of the Nrf2/HO-1 signaling pathway in sulfuretin-induced protection against amyloid beta25-35 neurotoxicity. *Neuroscience* 304, 14–28. doi: 10.1016/j.neuroscience.2015.07.030

Langer-Gould, A., Brara, S. M., Beaber, B. E., and Koebnick, C. (2013). Childhood obesity and risk of pediatric multiple sclerosis and clinically isolated syndrome. *Neurology* 80, 548–552. doi: 10.1212/WNL.0b013e31828154f3

Lassmann, H. (2011). A dynamic view of the blood-brain barrier in active multiple sclerosis lesions. *Ann. Neurol.* 70, 1–2. doi: 10.1002/ana.22494

Lassmann, H. (2011). Pathophysiology of inflammation and tissue injury in multiple sclerosis: what are the targets for therapy. *J. Neurol. Sci.* 306, 167–169. doi: 10.1016/j.jns.2010.07.023

Lassmann, H. (2014). Mechanisms of white matter damage in multiple sclerosis. *Glia* 62, 1816–1830. doi: 10.1002/glia.22597

Lastres-Becker, I. (2017). Role of the transcription factor Nrf2 in Parkinson's disease: new insights. *J. Alzheimers Dis. Parkinsonism* 7:340.

Lastres-Becker, I., Garcia-Yague, A. J., Scannevin, R. H., Casarejos, M. J., Kugler, S., Rabano, A., et al. (2016). Repurposing the NRF2 activator dimethyl fumarate as therapy against synucleinopathy in Parkinson's disease. *Antioxid. Redox Signal.* 25, 61–77. doi: 10.1089/ars.2015.6549

Leakey, J. E., Cunny, H. C., Bazare, J., Webb, P. J., Feuers, R. J., Duffy, P. H., et al. (1989). Effects of aging and caloric restriction on hepatic drug metabolizing enzymes in the Fischer 344 rat. I: the cytochrome P-450 dependent monooxygenase system. *Mech. Ageing Dev.* 48, 145–155. doi: 10.1016/0047-6374(89)90046-8

Lee, J. M., Shih, A. Y., Murphy, T. H., and Johnson, J. A. (2003). NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. *J. Biol. Chem.* 278, 37948–37956. doi: 10.1074/jbc.M305204200

Li, L., Pan, H., Wang, H., Li, X., Bu, X., Wang, Q., et al. (2016). Interplay between VEGF and Nrf2 regulates angiogenesis due to intracranial venous hypertension. *Sci. Rep.* 6:37338. doi: 10.1038/srep37338

Li, H. H., Lin, S. L., Huang, C. N., Lu, F. J., Chiu, P. Y., Huang, W. N., et al. (2016). miR-302 attenuates amyloid-beta-induced neurotoxicity through activation of akt signaling. *J. Alzheimers Dis.* 50, 1083–1098. doi: 10.3233/JAD-150741

Li, W., Prakash, R., Chawla, D., Du, W., Didion, S. P., Filosa, J. A., et al. (2013). Early effects of high-fat diet on neurovascular function and focal ischemic brain injury. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 304, R1001–R1008. doi: 10.1152/ajpregu.00523.2012

Licht-Mayer, S., Wimmer, I., Traffehn, S., Metz, I., Bruck, W., Bauer, J., et al. (2015). Cell type-specific Nrf2 expression in multiple sclerosis lesions. *Acta Neuropathol.* 130, 263–277. doi: 10.1007/s00401-015-1452-x

Lim, J. H., Kim, K. M., Kim, S. W., Hwang, O., and Choi, H. J. (2008). Bromocriptine activates NQO1 via Nrf2-PI3K/Akt signaling: novel cytoprotective mechanism against oxidative damage. *Pharmacol. Res.* 57, 325–331. doi: 10.1016/j.phrs.2008.03.004

Lim, J. L., Wilhelmus, M. M., de Vries, H. E., Drukarch, B., Hoozemans, J. J., and van Horssen, J. (2014). Antioxidative defense mechanisms controlled by Nrf2: state-of-the-art and clinical perspectives in neurodegenerative diseases. *Arch. Toxicol.* 88, 1773–1786. doi: 10.1007/s00204-014-1338-z

Lin, A. L., Zheng, W., Halloran, J. J., Burbank, R. R., Hussong, S. A., Hart, M. J., et al. (2013). Chronic rapamycin restores brain vascular integrity and function through NO synthase activation and improves memory in symptomatic mice modeling Alzheimer's disease. *J. Cereb. Blood Flow Metab.* 33, 1412–1421. doi: 10.1038/jcbfm.2013.82

Lister, A., Nedjadi, T., Kitteringham, N. R., Campbell, F., Costello, E., Lloyd, B., et al. (2011). Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy. *Mol. Cancer* 10:37. doi: 10.1186/1476-4598-10-37

Liu, J. S., Zhao, M. L., Brosnan, C. F., and Lee, S. C. (2001). Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am. J. Pathol.* 158, 2057–2066. doi: 10.1016/S0002-9440(10)64677-9

Liu, Y., Deng, Y., Liu, H., Yin, C., Li, X., and Gong, Q. (2016). Hydrogen sulfide ameliorates learning memory impairment in APP/PS1 transgenic mice: a novel mechanism mediated by the activation of Nrf2. *Pharmacol. Biochem. Behav.* 150–151, 207–216. doi: 10.1016/j.pbb.2016.11.002

Logroscino, G., Marder, K., Cote, L., Tang, M. X., Shea, S., and Mayeux, R. (1996). Dietary lipids and antioxidants in Parkinson's disease: a population-based, case-control study. *Ann. Neurol.* 39, 89–94. doi: 10.1002/ana.410390113

Logsdon, A. F., Lucke-Wold, B. P., Rosen, C. L., and Huber, J. D. (2017). "Risk factors: aging," in *Primer on Cerebrovascular Diseases*, 2 Edn, eds L. R. Caplan, J. Biller, M. C. Leary, E. H. Lo, A. J. Thomas, M. Yenari, et al. (New York, NY: Elsevier), 162–167. doi: 10.1016/B978-0-12-803058-5.00033-3

Lopes, I. M., Forga, L., and Martinez, J. A. (2001). Effects of leptin resistance on acute fuel metabolism after a high carbohydrate load in lean and overweight young men. *J. Am. Coll. Nutr.* 20, 643–648. doi: 10.1080/07315724.2001.10719162

Lu, T., Sun, X., Li, Y., Chai, Q., Wang, X. L., and Lee, H. C. (2017). Role of Nrf2 signaling in the regulation of vascular BK channel beta1 subunit expression and BK channel function in high-fat diet-induced diabetic mice. *Diabetes* 66, 2681–2690. doi: 10.2337/db17-0181

Lublin, F. D., Reingold, S. C., Cohen, J. A., Cutter, G. R., Sorensen, P. S., Thompson, A. J., et al. (2014). Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* 83, 278–286. doi: 10.1212/WNL.0000000000000560

Ludtmann, M. H., Angelova, P. R., Zhang, Y., Abramov, A. Y., and Dinkova-Kostova, A. T. (2014). Nrf2 affects the efficiency of mitochondrial fatty acid oxidation. *Biochem. J.* 457, 415–424. doi: 10.1042/BJ20130863

MacKenzie, E. L., Ray, P. D., and Tsuji, Y. (2008). Role and regulation of ferritin H in rotenone-mediated mitochondrial oxidative stress. *Free Radic. Biol. Med.* 44, 1762–1771. doi: 10.1016/j.freeradbiomed.2008.01.031

Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., et al. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat. Med.* 1, 1155–1161. doi: 10.1038/nm1195-1155

Manczak, M., Kandimalla, R., Yin, X., and Reddy, P. H. (2018). Hippocampal mutant APP and amyloid beta-induced cognitive decline, dendritic spine loss, defective autophagy, mitophagy and mitochondrial abnormalities in a mouse model of Alzheimer's disease. *Hum. Mol. Genet.* 27, 1332–1342. doi: 10.1093/hmg/ddy042

Mangialasche, F., Polidori, M. C., Monastero, R., Ercolani, S., Camarda, C., Cecchetti, R., et al. (2009). Biomarkers of oxidative and nitrosative damage in Alzheimer's disease and mild cognitive impairment. *Ageing Res. Rev.* 8, 285–305. doi: 10.1016/j.arr.2009.04.002

Marik, C., Felts, P. A., Bauer, J., Lassmann, H., and Smith, K. J. (2007). Lesion genesis in a subset of patients with multiple sclerosis: a role for innate immunity? *Brain* 130(Pt 11), 2800–2815. doi: 10.1093/brain/awm236

Martin, B., Golden, E., Carlson, O. D., Pistell, P., Zhou, J., Kim, W., et al. (2009). Exendin-4 improves glycemic control, ameliorates brain and pancreatic pathologies, and extends survival in a mouse model of Huntington's disease. *Diabetes* 58, 318–328. doi: 10.2337/db08-0799

Martin, B., Mattson, M. P., and Maudsley, S. (2006). Caloric restriction and intermittent fasting: two potential diets for successful brain aging. *Ageing Res. Rev.* 5, 332–353. doi: 10.1016/j.arr.2006.04.002

Martin-Montalvo, A., Villalba, J. M., Navas, P., and de Cabo, R. (2011). NRF2, cancer, and calorie restriction. *Oncogene* 30, 505–520. doi: 10.1038/onc.2010.492

Maswood, N., Young, J., Tilmont, E., Zhang, Z., Gash, D. M., Gerhardt, G. A., et al. (2004). Caloric restriction increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in a primate model of Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 101, 18171–18176. doi: 10.1073/pnas.0405831102

Matarese, G., Carrieri, P. B., La Cava, A., Perna, F., Sanna, V., De Rosa, V., et al. (2005). Leptin increase in multiple sclerosis associates with reduced number of CD4(+) CD25+ regulatory T cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5150–5155. doi: 10.1073/pnas.0408995102

Matsuda, M., and Shimomura, I. (2013). Increased oxidative stress in obesity: implications for metabolic syndrome, diabetes, hypertension, dyslipidemia, atherosclerosis, and cancer. *Obes. Res. Clin. Pract.* 7, e330–e341. doi: 10.1016/j.orcp.2013.05.004

Mattson, M. P. (2012). Energy intake and exercise as determinants of brain health and vulnerability to injury and disease. *Cell Metab.* 16, 706–722. doi: 10.1016/j.cmet.2012.08.012

Mazon, J. N., de Mello, A. H., Ferreira, G. K., and Rezin, G. T. (2017). The impact of obesity on neurodegenerative diseases. *Life Sci.* 182, 22–28. doi: 10.1016/j.lfs.2017.06.002

Mecocci, P., Boccardi, V., Cecchetti, R., Bastiani, P., Scamosci, M., Ruggiero, C., et al. (2018). A long journey into aging, brain aging, and Alzheimer's disease following the oxidative stress tracks. *J. Alzheimers Dis.* 62, 1319–1335. doi: 10.3233/JAD-170732

Meydani, M., Das, S., Band, M., Epstein, S., and Roberts, S. (2011). The effect of caloric restriction and glycemic load on measures of oxidative stress and antioxidants in humans: results from the CALERIE Trial of Human Caloric Restriction. *J. Nutr. Health Aging* 15, 456–460. doi: 10.1007/s12603-011-0002-z

Meydani, S. N., Das, S. K., Pieper, C. F., Lewis, M. R., Klein, S., Dixit, V. D., et al. (2016). Long-term moderate calorie restriction inhibits inflammation without impairing cell-mediated immunity: a randomized controlled trial in non-obese humans. *Aging* 8, 1416–1431. doi: 10.18632/aging.100994

Michel, J. P. (2016). Is it possible to delay or prevent age-related cognitive decline? *Korean J. Fam. Med.* 37, 263–266. doi: 10.4082/kjfm.2016.37.5.263

Migliore, L., Fontana, I., Trippi, F., Colognato, R., Coppede, F., Tognoni, G., et al. (2005). Oxidative DNA damage in peripheral leukocytes of mild cognitive impairment and AD patients. *Neurobiol. Aging* 26, 567–573. doi: 10.1016/j.neurobiolaging.2004.07.016

Mitchell, J. R., Verweij, M., Brand, K., van de Ven, M., Goemaere, N., van den Engel, S., et al. (2010). Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell* 9, 40–53. doi: 10.1111/j.1474-9726.2009.00532.x

Morris, J. K., Bomhoff, G. L., Gorres, B. K., Davis, V. A., Kim, J., Lee, P. P., et al. (2011). Insulin resistance impairs nigrostriatal dopamine function. *Exp. Neurol.* 231, 171–180. doi: 10.1016/j.expneurol.2011.06.005

Morris, J. K., Bomhoff, G. L., Stanford, J. A., and Geiger, P. C. (2010). Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299, R1082–R1090. doi: 10.1152/ajpregu.00449.2010

Morrison, C. D., Pistell, P. J., Ingram, D. K., Johnson, W. D., Liu, Y., Fernandez-Kim, S. O., et al. (2010). High fat diet increases hippocampal oxidative stress and cognitive impairment in aged mice: implications for decreased Nrf2 signaling. *J. Neurochem.* 114, 1581–1589. doi: 10.1111/j.1471-4159.2010.06865.x

Munger, K. L., Chitnis, T., and Ascherio, A. (2009). Body size and risk of MS in two cohorts of US women. *Neurology* 73, 1543–1550. doi: 10.1212/WNL.0b013e3181c0d6e0

Nakaso, K., Nakamura, C., Sato, H., Imamura, K., Takeshima, T., and Nakashima, K. (2006). Novel cytoprotective mechanism of anti-parkinsonian drug deprenyl: PI3K and Nrf2-derived induction of antioxidative proteins. *Biochem. Biophys. Res. Commun.* 339, 915–922. doi: 10.1016/j.bbrc.2005.11.095

Nguyen, T., Nioi, P., and Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem.* 284, 13291–13295. doi: 10.1074/jbc.R900010200

Niccoli, T., and Partridge, L. (2012). Ageing as a risk factor for disease. *Curr. Biol.* 22, R741–R752. doi: 10.1016/j.cub.2012.07.024

Nicholls, D. G. (2009). Spare respiratory capacity, oxidative stress and excitotoxicity. *Biochem. Soc. Trans.* 37(Pt 6), 1385–1388. doi: 10.1042/BST0371385

Niso-Santano, M., Gonzalez-Polo, R. A., Bravo-San Pedro, J. M., Gomez-Sanchez, R., Lastres-Becker, I., Ortiz-Ortiz, M. A., et al. (2010). Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death: modulation by the Nrf2/Trx axis. *Free Radic. Biol. Med.* 48, 1370–1381. doi: 10.1016/j.freeradbiomed.2010.02.024

Osmond, J. M., Mintz, J. D., and Stepp, D. W. (2010). Preventing increased blood pressure in the obese Zucker rat improves severity of stroke. *Am. J. Physiol. Heart Circ. Physiol.* 299, H55–H61. doi: 10.1152/ajpheart.01111.2009

Padurariu, M., Ciobica, A., Hritcu, L., Stoica, B., Bild, W., and Stefanescu, C. (2010). Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease. *Neurosci. Lett.* 469, 6–10. doi: 10.1016/j.neulet.2009.11.033

Pandit, A., Vadnal, J., Houston, S., Freeman, E., and McDonough, J. (2009). Impaired regulation of electron transport chain subunit genes by nuclear respiratory factor 2 in multiple sclerosis. *J. Neurol. Sci.* 279, 14–20. doi: 10.1016/j.jns.2009.01.009

Patel, M. (2016). Targeting oxidative stress in central nervous system disorders. *Trends Pharm. Sci.* 37, 768–778. doi: 10.1016/j.tips.2016.06.007

Patel, N. V., Gordon, M. N., Connor, K. E., Good, R. A., Engelman, R. W., Mason, J., et al. (2005). Caloric restriction attenuates Abeta-deposition in Alzheimer transgenic models. *Neurobiol. Aging* 26, 995–1000. doi: 10.1016/j.neurobiolaging.2004.09.014

Pearson, K. J., Lewis, K. N., Price, N. L., Chang, J. W., Perez, E., Cascajo, M. V., et al. (2008). Nrf2 mediates cancer protection but not prolongevity induced by caloric restriction. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2325–2330. doi: 10.1073/pnas.0712162105

Peng, W., Robertson, L., Gallinetti, J., Mejia, P., Vose, S., Charlip, A., et al. (2012). Surgical stress resistance induced by single amino acid deprivation requires Gcn2 in mice. *Sci. Transl. Med.* 4:118ra11. doi: 10.1126/scitranslmed.3002629

Perluigi, M., Sultana, R., Cenini, G., Di Domenico, F., Memo, M., Pierce, W. M., et al. (2009). Redox proteomics identification of 4-hydroxynonenal-modified brain proteins in Alzheimer's disease: role of lipid peroxidation in Alzheimer's disease pathogenesis. *Proteomics Clin. Appl.* 3, 682–693. doi: 10.1002/prca.200800161

Piccio, L., Stark, J. L., and Cross, A. H. (2008). Chronic calorie restriction attenuates experimental autoimmune encephalomyelitis. *J. Leukoc. Biol.* 84, 940–948. doi: 10.1189/jlb.0208133

Pipatpiboon, N., Pratchayasakul, W., Chattipakorn, N., and Chattipakorn, S. C. (2012). PPARgamma agonist improves neuronal insulin receptor function in hippocampus and brain mitochondria function in rats with insulin resistance induced by long term high-fat diets. *Endocrinology* 153, 329–338. doi: 10.1210/en.2011-1502

Pistell, P. J., Morrison, C. D., Gupta, S., Knight, A. G., Keller, J. N., Ingram, D. K., et al. (2010). Cognitive impairment following high fat diet consumption is associated with brain inflammation. *J. Neuroimmunol.* 219, 25–32. doi: 10.1016/j.jneuroim.2009.11.010

Procaccini, C., Santopaolo, M., Faicchia, D., Colamatteo, A., Formisano, L., de Candia, P., et al. (2016). Role of metabolism in neurodegenerative disorders. *Metabolism* 65, 1376–1390. doi: 10.1016/j.metabol.2016.05.018

Qin, W., Chachich, M., Lane, M., Roth, G., Bryant, M., de Cabo, R., et al. (2006). Calorie restriction attenuates Alzheimer's disease type brain amyloidosis in Squirrel monkeys (*Saimiri sciureus*). *J. Alzheimers Dis.* 10, 417–422. doi: 10.3233/JAD-2006-10411

Qiu, G., Spangler, E. L., Wan, R., Miller, M., Mattson, M. P., So, K. F., et al. (2012). Neuroprotection provided by dietary restriction in rats is further enhanced by reducing glucocortocoids. *Neurobiol. Aging* 33, 2398–2410. doi: 10.1016/j.neurobiolaging.2011.11.025

Raina, A. K., Templeton, D. J., Deak, J. C., Perry, G., and Smith, M. A. (1999). Quinone reductase (NQO1), a sensitive redox indicator, is increased in Alzheimer's disease. *Redox Rep.* 4, 23–27. doi: 10.1179/135100099101534701

Ramsey, C. P., Glass, C. A., Montgomery, M. B., Lindl, K. A., Ritson, G. P., Chia, L. A., et al. (2007). Expression of Nrf2 in neurodegenerative diseases. *J. Neuropathol. Exp. Neurol.* 66, 75–85. doi: 10.1097/nen.0b013e31802d6da9

Rao, G., Xia, E., and Richardson, A. (1990). Effect of age on the expression of antioxidant enzymes in male Fischer F344 rats. *Mech. Ageing Dev.* 53, 49–60. doi: 10.1016/0047-6374(90)90033-C

Razay, G., Vreugdenhil, A., and Wilcock, G. (2006). Obesity, abdominal obesity and Alzheimer disease. *Dement. Geriatr. Cogn. Disord.* 22, 173–176. doi: 10.1159/000094586

Reed, T. T. (2011). Lipid peroxidation and neurodegenerative disease. *Free Radic. Biol. Med.* 51, 1302–1319. doi: 10.1016/j.freeradbiomed.2011.06.027

Rizzi, N., Brunialti, E., Cerri, S., Cermisoni, G., Levandis, G., Cesari, N., et al. (2018). In vivo imaging of early signs of dopaminergic neuronal death in an animal model of Parkinson's disease. *Neurobiol. Dis.* 114, 74–84. doi: 10.1016/j.nbd.2018.02.005

Roberge, M. C., Messier, C., Staines, W. A., and Plamondon, H. (2008). Food restriction induces long-lasting recovery of spatial memory deficits following global ischemia in delayed matching and non-matching-to-sample radial arm maze tasks. *Neuroscience* 156, 11–29. doi: 10.1016/j.neuroscience.2008.05.062

Safdar, A., deBeer, J., and Tarnopolsky, M. A. (2010). Dysfunctional Nrf2-Keap1 redox signaling in skeletal muscle of the sedentary old. *Free Radic. Biol. Med.* 49, 1487–1493. doi: 10.1016/j.freeradbiomed.2010.08.010

Sah, S. K., Lee, C., Jang, J. H., and Park, G. H. (2017). Effect of high-fat diet on cognitive impairment in triple-transgenic mice model of Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 493, 731–736. doi: 10.1016/j.bbrc.2017.08.122

Saleh, M. C., Connell, B. J., and Saleh, T. M. (2010). Resveratrol preconditioning induces cellular stress proteins and is mediated via NMDA and estrogen receptors. *Neuroscience* 166, 445–454. doi: 10.1016/j.neuroscience.2009.12.060

Samiec, P. S., Drews-Botsch, C., Flagg, E. W., Kurtz, J. C., Sternberg, P., Reed, R. L., et al. (1998). Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic. Biol. Med.* 24, 699–704. doi: 10.1016/S0891-5849(97)00286-4

Sandberg, M., Patil, J., D'Angelo, B., Weber, S. G., and Mallard, C. (2014). NRF2-regulation in brain health and disease: implication of cerebral inflammation. *Neuropharmacology* 79, 298–306. doi: 10.1016/j.neuropharm.2013.11.004

Satoh, T., Harada, N., Hosoya, T., Tohyama, K., Yamamoto, M., and Itoh, K. (2009). Keap1/Nrf2 system regulates neuronal survival as revealed through study of keap1 gene-knockout mice. *Biochem. Biophys. Res. Commun.* 380, 298–302. doi: 10.1016/j.bbrc.2009.01.063

Satoh, T., Okamoto, S. I., Cui, J., Watanabe, Y., Furuta, K., Suzuki, M., et al. (2006). Activation of the Keap1/Nrf2 pathway for neuroprotection by electrophilic [correction of electrophillic] phase II inducers. *Proc. Natl. Acad. Sci. U.S.A.* 103, 768–773. doi: 10.1073/pnas.0505723102

Schernhammer, E., Hansen, J., Rugbjerg, K., Wermuth, L., and Ritz, B. (2011). Diabetes and the risk of developing Parkinson's disease in Denmark. *Diabetes Care* 34, 1102–1108. doi: 10.2337/dc10-1333

Schreibelt, G., Musters, R. J., Reijerkerk, A., de Groot, L. R., van der Pol, S. M., Hendrikx, E. M., et al. (2006). Lipoic acid affects cellular migration into the central nervous system and stabilizes blood-brain barrier integrity. *J. Immunol.* 177, 2630–2637. doi: 10.4049/jimmunol.177.4.2630

Schwarz, S., and Leweling, H. (2005). Multiple sclerosis and nutrition. *Mult. Scler.* 11, 24–32. doi: 10.1191/1352458505ms1119oa

Selkoe, D. J. (1994). Alzheimer's disease: a central role for amyloid. *J. Neuropathol. Exp. Neurol.* 53, 438–447. doi: 10.1097/00005072-199409000-00003

Senanayake, V. K., Jin, W., Mochizuki, A., Chitou, B., and Goodenowe, D. B. (2015). Metabolic dysfunctions in multiple sclerosis: implications as to causation, early detection, and treatment, a case control study. *BMC Neurol.* 15:154. doi: 10.1186/s12883-015-0411-4

Sepcic, J., Mesaros, E., Materljan, E., and Sepic-Grahovac, D. (1993). Nutritional factors and multiple sclerosis in Gorski Kotar, Croatia. *Neuroepidemiology* 12, 234–240. doi: 10.1159/000110322

Shah, Z. A., Li, R. C., Thimmulappa, R. K., Kensler, T. W., Yamamoto, M., Biswal, S., et al. (2007). Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. *Neuroscience* 147, 53–59. doi: 10.1016/j.neuroscience.2007.02.066

Shih, A. Y., Li, P., and Murphy, T. H. (2005). A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J. Neurosci.* 25, 10321–10335. doi: 10.1523/JNEUROSCI.4014-05.2005

Shih, P. H., and Yen, G. C. (2007). Differential expressions of antioxidant status in aging rats: the role of transcriptional factor Nrf2 and MAPK signaling pathway. *Biogerontology* 8, 71–80. doi: 10.1007/s10522-006-9033-y

Shin, S., Wakabayashi, J., Yates, M. S., Wakabayashi, N., Dolan, P. M., Aja, S., et al. (2009). Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide. *Eur. J. Pharmacol.* 620, 138–144. doi: 10.1016/j.ejphar.2009.08.022

Simmons, S. B., Pierson, E. R., Lee, S. Y., and Goverman, J. M. (2013). Modeling the heterogeneity of multiple sclerosis in animals. *Trends Immunol.* 34, 410–422. doi: 10.1016/j.it.2013.04.006

Son, T. G., Camandola, S., Arumugam, T. V., Cutler, R. G., Telljohann, R. S., Mughal, M. R., et al. (2010). Plumbagin, a novel Nrf2/ARE activator, protects against cerebral ischemia. *J. Neurochem.* 112, 1316–1326. doi: 10.1111/j.1471-4159.2009.06552.x

Sporn, M. B., and Liby, K. T. (2012). NRF2 and cancer: the good, the bad and the importance of context. *Nat. Rev. Cancer* 12, 564–571. doi: 10.1038/nrc3278

Stanek, K. M., Grieve, S. M., Brickman, A. M., Korgaonkar, M. S., Paul, R. H., Cohen, R. A., et al. (2011). Obesity is associated with reduced white matter integrity in otherwise healthy adults. *Obesity* 19, 500–504. doi: 10.1038/oby.2010.312

Strong, K., Mathers, C., and Bonita, R. (2007). Preventing stroke: saving lives around the world. *Lancet Neurol.* 6, 182–187. doi: 10.1016/S1474-4422(07)70031-5

Suh, J. H., Shenvi, S. V., Dixon, B. M., Liu, H., Jaiswal, A. K., Liu, R. M., et al. (2004). Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3381–3386. doi: 10.1073/pnas.0400282101

Sun, Y., Yang, T., Leak, R. K., Chen, J., and Zhang, F. (2017). Preventive and protective roles of dietary Nrf2 activators against central nervous system diseases. *CNS Neurol. Disord. Drug Targets* 16, 326–338. doi: 10.2174/1871527316666170102120211

Takeda, S., Sato, N., Uchio-Yamada, K., Sawada, K., Kunieda, T., Takeuchi, D., et al. (2010). Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Abeta deposition in an Alzheimer mouse model with diabetes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7036–7041. doi: 10.1073/pnas.1000645107

Talbot, K., Wang, H. Y., Kazi, H., Han, L. Y., Bakshi, K. P., Stucky, A., et al. (2012). Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J. Clin. Investig.* 122, 1316–1338. doi: 10.1172/JCI59903

Tanigawa, S., Fujii, M., and Hou, D. X. (2007). Action of Nrf2 and keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radic. Biol. Med.* 42, 1690–1703. doi: 10.1016/j.freeradbiomed.2007.02.017

Tanito, M., Agbaga, M. P., and Anderson, R. E. (2007). Upregulation of thioredoxin system via Nrf2-antioxidant responsive element pathway in adaptive-retinal neuroprotection in vivo and in vitro. *Free Radic. Biol. Med.* 42, 1838–1850. doi: 10.1016/j.freeradbiomed.2007.03.018

Tanji, K., Maruyama, A., Odagiri, S., Mori, F., Itoh, K., Kakita, A., et al. (2013). Keap1 is localized in neuronal and glial cytoplasmic inclusions in various neurodegenerative diseases. *J. Neuropathol. Exp. Neurol.* 72, 18–28. doi: 10.1097/NEN.0b013e31827b5713

Tarantini, S., Valcarcel-Ares, M. N., Yabluchanskiy, A., Tucsek, Z., Hertelendy, P., Kiss, T., et al. (2018). Nrf2 deficiency exacerbates obesity-induced oxidative stress, neurovascular dysfunction, blood-brain barrier disruption, neuroinflammation, amyloidogenic gene expression, and cognitive decline in mice, mimicking the aging phenotype. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 73, 853–863. doi: 10.1093/gerona/glx177

Tebay, L. E., Robertson, H., Durant, S. T., Vitale, S. R., Penning, T. M., Dinkova-Kostova, A. T., et al. (2015). Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radic. Biol. Med.* 88(Pt B), 108–146. doi: 10.1016/j.freeradbiomed.2015.06.021

Thompson, A. J. (2008). Multiple sclerosis–a global disorder and still poorly managed. *Lancet Neurol.* 7, 1078–1079. doi: 10.1016/S1474-4422(08)70246-1

Tola, M. R., Granieri, E., Malagu, S., Caniatti, L., Casetta, I., Govoni, V., et al. (1994). Dietary habits and multiple sclerosis. A retrospective study in Ferrara, Italy. *Acta Neurol.* 16, 189–197.

Tucsek, Z., Toth, P., Sosnowska, D., Gautam, T., Mitschelen, M., Koller, A., et al. (2014). Obesity in aging exacerbates blood-brain barrier disruption, neuroinflammation, and oxidative stress in the mouse hippocampus: effects on expression of genes involved in beta-amyloid generation and Alzheimer's disease. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 69, 1212–1226. doi: 10.1093/gerona/glt177

Tufekci, K. U., Civi Bayin, E., Genc, S., and Genc, K. (2011). The Nrf2/ARE pathway: a promising target to counteract mitochondrial dysfunction in Parkinson's disease. *Parkinsons Dis.* 2011:314082. doi: 10.4061/2011/314082

Tzima, S., Victoratos, P., Kranidioti, K., Alexiou, M., and Kollias, G. (2009). Myeloid heme oxygenase-1 regulates innate immunity and autoimmunity by modulating IFN-beta production. *J. Exp. Med.* 206, 1167–1179. doi: 10.1084/jem.20081582

Ungvari, Z., Bailey-Downs, L., Gautam, T., Jimenez, R., Losonczy, G., Zhang, C., et al. (2011a). Adaptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. *Am. J. Physiol. Heart Circ. Physiol.* 300, H1133–H1140. doi: 10.1152/ajpheart.00402.2010

Ungvari, Z., Bailey-Downs, L., Sosnowska, D., Gautam, T., Koncz, P., Losonczy, G., et al. (2011b). Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of NRF2-mediated antioxidant response. *Am. J. Physiol. Heart Circ. Physiol.* 301, H363–H372. doi: 10.1152/ajpheart.01134.2010

Ungvari, Z., Bailey-Downs, L., Gautam, T., Sosnowska, D., Wang, M., Monticone, R. E., et al. (2011c). Age-associated vascular oxidative stress, Nrf2 dysfunction, and NF-{kappa}B activation in the nonhuman primate *Macaca mulatta*. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 66, 866–875. doi: 10.1093/gerona/glr092

Uttara, B., Singh, A. V., Zamboni, P., and Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* 7, 65–74. doi: 10.2174/157015909787602823

Van der Goes, A., Wouters, D., Van Der Pol, S. M., Huizinga, R., Ronken, E., Adamson, P., et al. (2001). Reactive oxygen species enhance the migration of monocytes across the blood-brain barrier *in vitro*. *FASEB J.* 15, 1852–1854. doi: 10.1096/fj.00-0881fje

Vasconcelos, A. R., Orellana, A. M. M., Paixão, A. G., Scavone, C., and Kawamoto, E. M. (2018). "Intermittent fasting and caloric restriction: neuroplasticity and neurodegeneration," in *Handbook of Famine, Starvation, and Nutrient Deprivation*, eds V. Preedy and V. Patel (Cham: Springer).

Vazquez-Medina, J. P., Zenteno-Savin, T., Forman, H. J., Crocker, D. E., and Ortiz, R. M. (2011). Prolonged fasting increases glutathione biosynthesis in postweaned northern elephant seals. *J. Exp. Biol.* 214(Pt 8), 1294–1299. doi: 10.1242/jeb.054320

Verweij, M., van Ginhoven, T. M., Mitchell, J. R., Sluiter, W., van den Engel, S., Roest, H. P., et al. (2011). Preoperative fasting protects mice against hepatic ischemia/reperfusion injury: mechanisms and effects on liver regeneration. *Liver Transpl.* 17, 695–704. doi: 10.1002/lt.22243

Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., et al. (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* 35, 238–245. doi: 10.1038/ng1248

Wang, J., Ho, L., Qin, W., Rocher, A. B., Seror, I., Humala, N., et al. (2005). Caloric restriction attenuates beta-amyloid neuropathology in a mouse model of Alzheimer's disease. *FASEB J.* 19, 659–661. doi: 10.1096/fj.04-3182fje

Wang, X., Svedin, P., Nie, C., Lapatto, R., Zhu, C., Gustavsson, M., et al. (2007). N-acetylcysteine reduces lipopolysaccharide-sensitized hypoxic-ischemic brain injury. *Ann. Neurol.* 61, 263–271. doi: 10.1002/ana.21066

Wang, Y., Miao, Y., Mir, A. Z., Cheng, L., Wang, L., Zhao, L., et al. (2016). Inhibition of beta-amyloid-induced neurotoxicity by pinocembrin through Nrf2/HO-1 pathway in SH-SY5Y cells. *J. Neurol. Sci.* 368, 223–230. doi: 10.1016/j.jns.2016.07.010

Willeumier, K. C., Taylor, D. V., and Amen, D. G. (2011). Elevated BMI is associated with decreased blood flow in the prefrontal cortex using SPECT imaging in healthy adults. *Obesity* 19, 1095–1097. doi: 10.1038/oby.2011.16

Winer, S., Paltser, G., Chan, Y., Tsui, H., Engleman, E., Winer, D., et al. (2009). Obesity predisposes to Th17 bias. *Eur. J. Immunol.* 39, 2629–2635. doi: 10.1002/eji.200838893

Witte, M. E., Mahad, D. J., Lassmann, H., and van Horssen, J. (2014). Mitochondrial dysfunction contributes to neurodegeneration in multiple sclerosis. *Trends Mol. Med.* 20, 179–187. doi: 10.1016/j.molmed.2013.11.007

World Health Organization [WHO] (2011). *World Health Statistics*. Geneva: WHO, 162.

Wruck, C. J., Claussen, M., Fuhrmann, G., Romer, L., Schulz, A., Pufe, T., et al. (2007). Luteolin protects rat PC12 and C6 cells against MPP+ induced toxicity via an ERK dependent Keap1-Nrf2-ARE pathway. *J. Neural Transm. Suppl.* 72, 57–67. doi: 10.1007/978-3-211-73574-9_9

Xu, Q., Park, Y., Huang, X., Hollenbeck, A., Blair, A., Schatzkin, A., et al. (2011). Diabetes and risk of Parkinson's disease. *Diabetes Care* 34, 910–915. doi: 10.2337/dc10-1922

Xu, X., Zhang, L., Ye, X., Hao, Q., Zhang, T., Cui, G., et al. (2018). Nrf2/ARE pathway inhibits ROS-induced NLRP3 inflammasome activation in BV2 cells after cerebral ischemia reperfusion. *Inflamm. Res.* 67, 57–65. doi: 10.1007/s00011-017-1095-6

Yang, T., Sun, Y., Mao, L., Zhang, M., Li, Q., Zhang, L., et al. (2018). Brain ischemic preconditioning protects against ischemic injury and preserves the blood-brain barrier via oxidative signaling and Nrf2 activation. *Redox Biol.* 17, 323–337. doi: 10.1016/j.redox.2018.05.001

Zagorski, J. W., Turley, A. E., Freeborn, R. A., VanDenBerg, K. R., Dover, H. E., Kardell, B. R., et al. (2018). Differential effects of the Nrf2 activators tBHQ and CDDO-Im on the early events of T cell activation. *Biochem. Pharm.* 147, 67–76. doi: 10.1016/j.bcp.2017.11.005

Zhang, P., Singh, A., Yegnasubramanian, S., Esopi, D., Kombairaju, P., Bodas, M., et al. (2010). Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol. Cancer Ther.* 9, 336–346. doi: 10.1158/1535-7163.MCT-09-0589

Zhu, H., Itoh, K., Yamamoto, M., Zweier, J. L., and Li, Y. (2005). Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in

cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS Lett.* 579, 3029–3036. doi: 10.1016/j.febslet.2005.04.058

Zlokovic, B. V. (2011). Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat. Rev. Neurosci.* 12, 723–738. doi: 10.1038/nrn3114

Zou, Y., Hong, B., Fan, L., Zhou, L., Liu, Y., Wu, Q., et al. (2013). Protective effect of puerarin against beta-amyloid-induced oxidative stress in neuronal cultures from rat hippocampus: involvement of the GSK-3beta/Nrf2 signaling pathway. *Free Radic. Res.* 47, 55–63. doi: 10.3109/10715762.2012.742518

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REVIEW
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Critical Role of Nrf2 in Experimental Ischemic Stroke

Lei Liu[1], *Logan M. Locascio*[1] *and Sylvain Doré*[1,2]*

[1] *Department of Anesthesiology, Center for Translational Research in Neurodegenerative Disease and McKnight Brain Institute, University of Florida, Gainesville, FL, United States,* [2] *Departments of Neurology, Psychiatry, Pharmaceutics, and Neuroscience, University of Florida, Gainesville, FL, United States*

Ischemic stroke is one of the leading causes of death and long-term disability worldwide; however, effective clinical approaches are still limited. The transcriptional factor Nrf2 is a master regulator in cellular and organismal defense against endogenous and exogenous stressors by coordinating basal and stress-inducible activation of multiple cytoprotective genes. The Nrf2 network not only tightly controls redox homeostasis but also regulates multiple intermediary metabolic processes. Therefore, targeting Nrf2 has emerged as an attractive therapeutic strategy for the prevention and treatment of CNS diseases including stroke. Here, the current understanding of the Nrf2 regulatory network is critically examined to present evidence for the contribution of Nrf2 pathway in rodent ischemic stroke models. This review outlines the literature for Nrf2 studies in preclinical stroke and focuses on the *in vivo* evidence for the role of Nrf2 in primary and secondary brain injuries. The dynamic change and functional importance of Nrf2 signaling, as well as Nrf2 targeted intervention, are revealed in permanent, transient, and global cerebral ischemia models. In addition, key considerations, pitfalls, and future potentials for Nrf2 studies in preclinical stroke investigation are discussed.

Keywords: antioxidant response element, global cerebral ischemia, middle cerebral artery occlusion, permanent MCAO, preclinical models, reperfusion, stroke, transient MCAO

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Javier Egea,
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Alexander A. Mongin,
Albany Medical College, United States
Siew Hua Gan,
Monash University Malaysia, Malaysia

***Correspondence:**
Sylvain Doré
sdore@ufl.edu

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INTRODUCTION

Ischemic stroke is one of the leading causes of death and long-term disability worldwide (Benjamin et al., 2018), an event which is more disabling than it is fatal. In the U.S. alone, ∼795,000 people each year suffer a new or recurrent stroke, of which 87% are ischemic (Dirnagl et al., 2003; Feigin et al., 2017; Benjamin et al., 2018). However, effective clinical approaches are still limited. A successful therapeutic strategy for salvaging ischemic brain tissue and promoting functional outcomes is to quickly restore blood flow during acute ischemic stroke by introducing intravenous recombinant tissue plasminogen activator (rtPA), which has been available since 1996 (Prabhakaran et al., 2015; Romano and Sacco, 2015), in combination with thrombectomy. However, more than 95% of patients do not benefit from rtPA due to the narrow therapeutic window (4.5 h) and limited indications (Hacke et al., 2008; Fonarow et al., 2011; Sandercock et al., 2012; Emberson et al., 2014). In addition, there is no efficacious treatment that exhibits long-term recovery improvement. Therefore, development of new therapeutic strategies by targeting vital cellular components of the ischemic cascade is urgently needed.

The transcriptional factor Nrf2 is a major regulator of cellular and organismal defense mechanism against endogenous and exogenous stresses by coordinating basal and stress-inducible activation of multiple cytoprotective genes (Leonardo and Doré, 2011; Suzuki et al., 2013; Hayes and Dinkova-Kostova, 2014; Tonelli et al., 2017; Cuadrado et al., 2018; Yamamoto et al., 2018).

Since its discovery in 1994 (Moi et al., 1994), Nrf2 biology has been extensively studied in understanding the structure, molecular mechanism, function, regulation of Nrf2 activity, downstream pathways and implications as a therapeutic target of diseases (Ma, 2013; Suzuki et al., 2013; Kumar et al., 2014; Cuadrado et al., 2018; Yamamoto et al., 2018). It is now recognized as a master regulator of redox homeostasis through the control of a wide array of target genes that share a common DNA sequence called the antioxidant response element (ARE) in the promoter region ensuring that its activity increases in response to redox perturbation, energy or nutrient fluxes, inflammation, toxicity, and disease conditions (Ma, 2013; Cuadrado et al., 2018; Yamamoto et al., 2018). The equivalent response element in mice and rats is called the electrophile response element (EpRE) (Friling et al., 1990; Wasserman and Fahl, 1997; Itoh et al., 2010; Yamamoto et al., 2018). Besides mediating antioxidant responses, Nrf2 contributes to the regulation of many primary and secondary metabolic processes (Hayes and Dinkova-Kostova, 2014; Tonelli et al., 2017). Consequently, targeting Nrf2 has emerged as an attractive therapeutic strategy for stroke prevention and reversal (Calkins et al., 2009; Leonardo and Doré, 2011; Wang Y. C. et al., 2011; Ma, 2013; Kumar et al., 2014; Tonelli et al., 2017).

This paper outlines the current understanding of the Nrf2 regulatory network and critically examined the recent evidence for the contribution of Nrf2 pathway in experimental ischemic stroke models. It includes extensive literature review for Nrf2 studies in experimental ischemic stroke mouse and rat models published by June 30, 2018 with a special focus on *in vivo* evidence for the role of Nrf2 in ischemic brain injury. We summarized the dynamic regulation of Nrf2 signaling, functional importance, and its targeted intervention in permanent, transient, and global cerebral ischemia preclinical models. Finally, we also assessed key considerations, pitfalls, and the potential for future Nrf2 studies in stroke investigation.

OVERVIEW OF THE NRF2 REGULATORY NETWORK

A Brief History

Nrf2 is widely expressed in mammalian cells. In 1991, a study in the field of toxicology revealed that oxidative stress activates antioxidant genes through the antioxidant-response element (ARE), a cis-acting regulatory element that contributes to cellular defense in eukaryotes (Rushmore et al., 1991; Hayes and Dinkova-Kostova, 2014). In 1994, Nrf2 was firstly reported in the molecular biology field as an NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region (Moi et al., 1994; Raghunath et al., 2018). However, its biological function was unclear. In 1996, the first study on Nrf2 knockout mice showed that no overt abnormal phenotype was detected although the mice were susceptible to stresses (Chan et al., 1996). In 1997, a landmark study illustrated that the induction of two ARE-driven genes, glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase-1 (NQO1), was abolished in the Nrf2 knockout mice by the phenolic antioxidant butylated hydroxyanisole (BHA), revealing that Nrf2 controls drug-metabolizing enzymes *in vivo* (Itoh et al., 1997). Recently, series of breakthrough studies have revealed that Nrf2 coordinately regulates a wide array of antioxidant response element/electrophile responsive element (ARE/EpRE)-driven genes that play critical roles in controlling endogenous resistance to various intrinsic and extrinsic stressors (Ma, 2013; Suzuki et al., 2013; Hayes and Dinkova-Kostova, 2014; Tonelli et al., 2017; Yamamoto et al., 2018).

Structure Domain of Nrf2

Nrf2 belongs to the cap'n'collar subfamily of the basic-region leucine zipper (CNC-bZIP) transcription factors. It is a modular protein composed of seven functional domains, Nrf2-ECH homology (Neh) domains 1–7, which have distinct functions (**Figure 1A**) (Chan et al., 1993; Katoh et al., 2005; Hayes and Dinkova-Kostova, 2014; Tonelli et al., 2017). The Neh1 domain contains the well-conserved CNC-bZIP region that heterodimerizes with small musculoaponeurotic fibrosarcoma oncogene (sMaf) proteins and binds ARE/EpRE sequence in DNA. This CNC-bZIP region, which can be found in several species, is vital for Nrf2 function. The N-terminal Neh2 is the domain by which Nrf2 binds to its primary negative regulator Keap1 through its low-affinity DLG and the high-affinity ETGE motifs. The C-terminal Neh3 region represents a transactivation domain that binds to chromo-ATPase/ helicase DNA-binding protein (CHD) 6 and activates transcription of Nrf2 target genes in concert with the Neh4 and Neh5 domains. The Neh4 and Neh5 regions of Nrf2 are transactivation domains that recruit the cAMP response element-binding protein (CREB)-binding protein (CBP) and/or receptor-associated coactivator (RAC) 3. The Neh6 domain, independent of Keap1, mediates the repression of Nrf2 stability with another negative regulator the dimeric β-transducin repeat-containing protein (β-TrCP), a substrate adaptor for the S-phase kinase-associated protein 1 (Skp1)–Cul1–Rbx1 core E3 complex, through DSGIS and DSAPGS motifs. The Neh7 domain is involved in the negative control of Nrf2 by physically binding to the retinoid X receptor (RXR).

Overview of the Nrf2 Pathway

Organisms are equipped with a defense system to maintain homeostasis against constant intrinsic and extrinsic insults that result in the damage of nucleic acids, proteins, and membrane lipids. Nrf2 is a master regulator of the inducible cell defense system by controlling a broad range of cytoprotective genes (Hayes and Dinkova-Kostova, 2014; Suzuki and Yamamoto, 2015). Under basal homeostatic conditions, Nrf2 in the cytoplasm is predominately bound to the Kelch-like ECH-associating protein 1 (Keap1) through the Keap1–Cullin3 (Cul3)-Rbx1 ubiquitin E3 ligase complex and is constitutively degraded by the proteasome. Cul3 serves as a scaffolding protein that is bound to both Keap1 and Rbx1. As a result, Nrf2 abundance and activity are maintained at low levels (**Figure 1B**). Under stress conditions, Nrf2 protein is liberated from Keap1-mediated repression. The stabilized and accumulated Nrf2

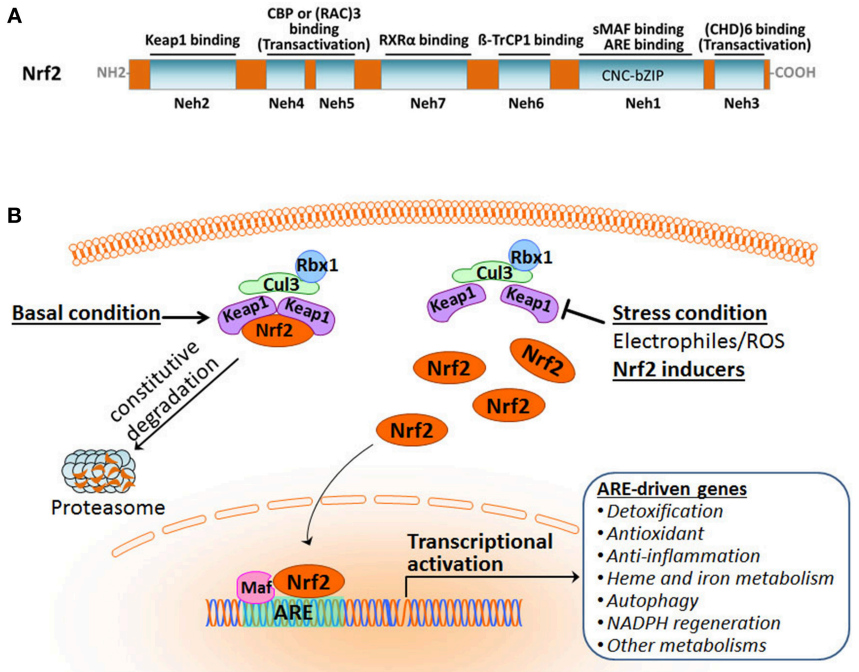

FIGURE 1 | Overview of the Nrf2 pathway activation. **(A)** Functional domains of human Nrf2 protein. **(B)** The Keap1-dependent Nrf2 activation and response.

translocates into the nucleus and, as a heterodimer with one of the small Maf proteins, binds to the ARE/EpRE in the promoter region of Nrf2 target genes, thus activating a wide array of cytoprotective genes (Ma, 2013; Kumar et al., 2014; Suzuki and Yamamoto, 2015; Taguchi and Yamamoto, 2017; Bellezza et al., 2018; Yamamoto et al., 2018).

It is becoming increasingly clear that Keap1 plays a central role in the regulatory mechanism of Nrf2. (1) Keap1 acts as a sensor of oxidative and electrophilic stresses for Nrf2 with a subcellular localization in the perinuclear cytoplasm (Suzuki and Yamamoto, 2017; Yamamoto et al., 2018). (2) Keap1 is a major repressor of Nrf2 through the activity of the Keap1–Cul3 complex. It is an adaptor protein for Cul3-dependent ubiquitination and specifically targets Nrf2 (Itoh et al., 1999, 2003; Kobayashi et al., 2004), thereby marking Nrf2 protein for rapid degradation by the ubiquitin–proteasome system. Under normal conditions, Nrf2 undergoes constitutive ubiquitination by the Keap1-Cul3 complex and resultant proteasomal degradation, leading to its low level of activity. Exposure to cellular insults like electrophiles or reactive oxygen species (ROS) brings activity of the E3 ubiquitin ligase Keap1-Cul3 complex to a halt, inhibiting Nrf2 ubiquitination and enabling Nrf2 protein stabilization and transcriptional activation. (3) Keap1 serves as a "floodgate" for Nrf2: Normally it functions to suppress Nrf2 nuclear translocation in response to cellular insults, the gene promotes translocation of Nrf2 into the nucleus to activate cytoprotective target gene expression. Keap1 has a relatively long half-life of 12 h compared to Nrf2's half-life of 20 min, contributing to its importance in the Nrf2 regulatory network.

Target Genes and Functions of Nrf2

The Nrf2-Maf complex binds to the specific promoter of its target genes. In the past decade, more than 200 target genes of Nrf2 have been identified through the analyses of gene expression profiling and chromatin immunoprecipitation (ChIP) (Suzuki et al., 2013; Kumar et al., 2014; Suzuki and Yamamoto, 2017; Rojo de la Vega et al., 2018). The Nrf2 target ARE-driven genes have been defined in encoding proteins for functional regulation of a wide range of biological processes including detoxification, antioxidation, anti-inflammation, NADPH regeneration, and intermediary metabolisms (**Figure 1B**). Nrf2 exerts multiple defense processes counteracting various stresses by the induction of these genes. Consequently, Nrf2 plays a fundamental role in maintaining the redox homeostasis of the cell.

Target Genes and Functions of Nrf2—*Detoxification of Xenobiotics*

Xenobiotics, including toxic chemicals and drugs, are detoxified by various drug-metabolizing enzymes and transporters. This detoxification mechanism is divided into three phases (Croom, 2012; Raghunath et al., 2018). Phase I enzymes mainly consist of cytochrome P450 oxidases that modify the xenobiotics through oxidation, reduction, or hydrolysis. Phase II conjugating enzymes include glutathione S-transferases, which catalyze the conjugation of reactive electrophile species with glutathione (GSH), attenuating the toxic potential of xenobiotics. Phase III transporters eliminate the GSH conjugates and offer protection against deleterious chemicals. It has been demonstrated that Nrf2 controls the expression of many drug-metabolizing enzymes [like distinct GST subunits and [NAD(P)H:quinone

oxidoreductase 1) NQO1] and transporters [(drug-resistance-associated proteins (MRP)].

Target Genes and Functions of Nrf2—*Antioxidant*

A major function of Nrf2 is to resist oxidative stress, thereby maintaining redox homeostasis between reactive oxidants and endogenous antioxidant systems. Groups of Nrf2 targeted genes that regulate antioxidant defense and oxidant signaling have been defined. (1) Glutathione (GSH)-based: glutamate–cysteine ligase catalytic (GCLC) subunit and glutamate-cysteine ligase modifier (GCLM) control the entry of cystine into cells; glutathione peroxidase (GPX) 2 produces oxidized glutathione (GSSG) during the reduction of peroxides; and glutathione reductase (GSR) 1 reduces GSSG for maintenance of reduced intracellular GSH levels. (2) Thioredoxin (TXN)-based: Thioredoxin (TXN) 1, thioredoxin reductase (TXNRD) 1, and sulfiredoxin (SRXN) 1 reduce oxidized protein thiols. (3) Others: G6PDH and 6PGD reduce synthesis of NADPH, antioxidant protein Trx, stress response protein heme oxygenase 1 (HO1), and other proteins. Nrf2 coordinately regulates key components in the antioxidant system that precisely controls antioxidant defense at multiple levels, thus ensuring an adequate response to oxidants in time and space (Lin and Beal, 2006; Ma, 2013).

Target Genes and Functions of Nrf2—*Anti-inflammation*

Besides protecting against xenobiotic and oxidative insults, Nrf2 plays a role in anti-inflammation, which is supported by several recent findings (Kobayashi et al., 2016; Suzuki and Yamamoto, 2017). Several findings support the anti-inflammatory effect. The absence of Nrf2 leads to exacerbated inflammation in different murine models. Attenuated inflammation by Nrf2 is related to the inhibition of the nuclear factor-κB (NF-κB) pathway and proinflammatory cytokine level (Wardyn et al., 2015). Most recently, it was demonstrated that Nrf2 suppressed lipopolysaccharide (LPS)-induced inflammation by blocking the transcriptional upregulation of proinflammatory cytokines, including IL-6 and IL-1β. ChIP-seq and ChIP-qPCR analyses revealed that Nrf2 binds near the promoter region of these genes, and this Nrf2-mediated inhibition is independent of the Nrf2-binding motif and ROS level (Ahmed et al., 2017).

Target Genes and Functions of Nrf2—*Heme and Iron Metabolism*

Nrf2 also modulates the key components of heme and iron metabolism. HO1 catalyzes the cleavage of heme (which cannot be recycled) to form iron, carbon monoxide (CO), and biliverdin, which is immediately reduced to bilirubin (Doré and Snyder, 1999; Doré et al., 1999). HO1 is considered to be a stress protein that can be highly induced in a cell-specific manner by numberous conditions (Ewing et al., 1992). Iron is intimately linked to oxygen by acting as an essential nutrient cofactor in enzymes for oxygen transport, oxidative phosphorylation, and metabolite oxidation. However, excess labile iron—not bound to ferritin—would facilitate the formation of oxygen-derived free radicals capable of damaging biomolecules (Rouault, 2013; Kerins and Ooi, 2017). The biological utilization of iron is a tightly regulated process. Constitutive Nrf2 activation and subsequent deregulation of iron metabolism have been implicated in cancer development. Nrf2-mediated upregulation of HO1 or the iron storage protein ferritin can lead to enhanced proliferation and therapy resistance.

Target Genes and Functions of Nrf2—NADPH Regeneration and Other Metabolic Processes

NADPH is an essential cofactor for many drug-metabolizing enzymes and antioxidants. Nrf2 controls NADPH production by regulating key NADPH-generating enzymes, such as glucose-6-phosphate dehydrogenase (G6pd), 6-phosphogluconate dehydrogenase (Pgd), isocitrate dehydrogenase 1 (Idh1), and malic enzyme 1 (Leonardo and Doré, 2011; Dinkova-Kostova and Abramov, 2015). In addition, Nrf2 controls the expression of multiple metabolic enzymes, indicating its unique role between redox and intermediary metabolism. Nrf2 also regulates fatty acid oxidation and lipases that influence lipid metabolism and transcription factors (Hayes and Dinkova-Kostova, 2014).

Regulatory Mechanisms of Nrf2

Besides the predominant view that Nrf2 activity is mainly regulated at the protein stability level by Keap-mediated repression, accumulated evidence show that Nrf2 activity is tightly controlled at the transcriptional and post-translational levels during both basal and stressful conditions (Hayes and Dinkova-Kostova, 2014; Tonelli et al., 2017; Yamamoto et al., 2018). Given the central role of Nrf2 in cellular defense, the regulatory mechanisms of Nrf2 activity are intricate and multifaceted.

Regulatory Mechanisms of Nrf2–Transcriptional Regulation

Nrf2 mRNA is broadly expressed across different species (Ma, 2013; Tonelli et al., 2017; Yamamoto et al., 2018), and its basal levels vary within different organs. Nrf2 activity is at least partially regulated at the transcriptional level. (1) The rodent Nrf2 gene comprises two ARE-like sequences in its promoter region and, in response to electrophilic stimuli, appropriately autoregulates its own expression level in concert with sustaining the induction of ARE-driven genes, offering a positive feedback mechanism to augment the Nrf2 signal. In contrast, the ARE-like sequences in the human Nrf2 gene promoter region are reportedly associated with reduced expression of Nrf2, which enhances vulnerability to lung cancer. (2) The Nrf2 gene promoter comprises a binding site for NF-κB, which can be induced by environmental stress, injury or inflammation. Indeed, Nfe2l2 transcription level in human monocytes is indeed activated by LPS-induced inflammation. (3) It was reported that Nfe2l2 transcription in tumor cells can be amplified by the Notch

signaling pathway and the phosphoinositide 3-kinase (PI3K)-Akt pathway, ultimately contributing to the increased expression of Nrf2.

Regulatory Mechanisms of Nrf2—Post-transcriptional Regulation

A microRNA (miRNA) is a small non-coding RNA molecule that functions in RNA silencing and post-transcriptional regulation of gene expression. At least eight miRNAs have been identified as direct modulators of Nrf2 expression at the transcriptomic level (Ayers et al., 2015). The miR-144 was identified to negatively regulate Nrf2 level in reticulocytes of homozygous sickle cell disease (HbSS) patients (Bryan et al., 2013; Kurinna and Werner, 2015) (140). Increased miR-144 is associated with the reduction of both Nrf2 level and GSH regeneration as well as impeded oxidative stress defense.

Regulatory Mechanisms of Nrf2—Post-translational Regulation in Nrf2 Protein Stability

Keap1-Dependent Repression Mechanism

(1) The prevailing view is that Keap1 is a primary repressor of Nrf2. Under basal conditions, Keap1 constantly targets Nrf2 for ubiquitination and proteasomal degradation, resulting in the disruption of Nrf2 protein stability and maintenance of Nrf2 signaling capacity at a very low level. In response to electrophiles or stressors, Nrf2 is liberated from the Keap1 repression (i.e., derepression) by inhibiting ubiquitylation and proteasomal degradation that allows newly-synthesized Nrf2 to be rapidly stabilized and activates transcriptional activation of cytoprotective genes. This view is supported by many reports of Keap1 knockout mice and Keap1 knockdown human cells that exhibited sufficient Nrf2 activity (Kensler et al., 2007; Yates et al., 2009; Bellezza et al., 2018; Yamamoto et al., 2018). Blockage of Keap1 expression by miR-141 or miR-200a triggers increased Nrf2 activity at the protein stabilization level. Transgenic complementation rescue assay is a comprehensive and powerful approach to delineate the *in vivo* functions of proteins (Motohashi et al., 2011; Katsuoka and Yamamoto, 2016). The esophagus lesions and resultant mortality in Keap1 knockout mice were fully retrieved by concurrent Nrf2 disruption, and aberrant phenotypes caused by Keap1 deficiency can also be rescued by transgene-derived Keap1. Epigenetic silencing of the Keap1 gene by hypermethylation of its promoter results in upregulation of Nrf2 in patients with gliomas and different cancers of breast, lung, prostate and colorectal (Tonelli et al., 2017). (2) The function of Keap1 within various electrophiles is related primarily to three critical cysteine residues: Cys151, Cys273, and Cys288 (Yamamoto et al., 2018). Keap1 is a thiol-rich protein with many cysteine residues and thus is sensitive to electrophiles (McMahon et al., 2010). Therefore, covalent modifications of the cysteine residues had been proposed to influence the function of Keap1, which releases Nrf2 (McMahon et al., 2010). Affirmatively, covalent binding of electrophiles to critical cysteine residues has been observed. Meanwhile, various lines of studies in cultured cells, zebrafish, and mice demonstrate that Keap1 functions as a sensor due to the prominent contribution of the cysteine residues. Multiple studies involving substitution of Cys151 strongly support its role as a main cysteine sensor by modulating the activity of the ubiquitin E3 ligase Keap1-Cul3 complex to respond to different electrophiles. (3) To activate Nrf2 by inhibiting Keap1 activity: Many ARE activity inducing agents are soft electrophiles that inhibit Keap1 by modifying Cys151, Cys226/Cys613, Cys-273/Cys-288, or Cys-434. Thus, the presence of multiple Cys-based sensors in Keap1 allows Nrf2 to be de-repressed in response to many xenobiotic stressors. (4) To activate Nrf2 by competitive inhibition of Keap1: p62, the first mammalian selective autophagy cargo receptor (Chu, 2018), is able to directly interact with the Nrf2- binding site on Keap1, a component of Cullin-3-type ubiquitin ligase for Nrf2 (Komatsu et al., 2010; Lau et al., 2010). Thus, an overproduction of p62 or a deficiency in autophagy competes with the interaction between Nrf2 and Keap1, resulting in Nrf2 stabilization and transcriptional activation of Nrf2 target genes. Additionally, p62 is a target gene of Nrf2 and creates a positive feedback loop by inducing ARE-driven gene transcription (Jain et al., 2010). Oxidative stress-triggered Nrf2 results in the induction and accumulation of p62 that in turn activates Nrf2 activation (Komatsu et al., 2010).

Keap1-Nondependent Repression Mechanism

(1) The Nrf2 is has been described to be suppressed by b-TrCP and GSK-3; in addition to the well-studied Keap1, E3 ubiquitin ligase adaptor β-TrCP is another negative repressor of Nrf2 stabilization (Rada et al., 2012). Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity (Chowdhry et al., 2013). Intraperitoneal injection of the GSK-3 inhibitor through the GSK-3/β-TrCP axis led to increased Nrf2 and HO1 levels in liver and hippocampus (Rada et al., 2012). Activation of the GSK3β/β-TrCP axis by gene knockdown of PHLPP1 in NRK52E cells enhanced Nrf2-responsive antioxidant enzymes HO1 and NQO1 (Mathur et al., 2018). (2) The Nrf2 is suppressed by CRIF1 and RNF4: CR6-interacting factor 1(CRIF1) physically interacts with both N- and C-terminal regions of Nrf2 and promotes Nrf2 ubiquitination and subsequent proteasome-mediated Nrf2 protein degradation (Kang et al., 2010). *Crif1*-knockdown BMMSCs caused increased oxidative stress and apoptosis after irradiation injury, partially due to a suppressed antioxidant response mediated by decreased Nrf2 nuclear translocation (Chen L. et al., 2017). RING finger protein 4 (Rnf4) mediates polyubiquitylation of polysumoylated Nrf2, leading to its subsequent degradation in promyelocytic leukemia-nuclear bodies (Malloy et al., 2013). Respiratory syncytial virus-induced Nrf2 degradation occurs in a SUMO-specific E3 ubiquitin ligase—RING finger protein 4 (Rnf4)-dependent manner.

CEREBRAL ISCHEMIA AND RELEVANT PRECLINICAL ANIMAL MODELS

Ischemic stroke is caused by cerebral arterial occlusion that leads to a critical reduction or loss of regional cerebral blood flow,

and it occurs as a consequence of multiple vascular diseases, including cardioembolism, atherosclerosis, small vessel disease, and cryptogenic diseases (Adams and Biller, 2015; Mehndiratta et al., 2015). Interruption of the blood supply initiates complex spatial and temporal events involving hemodynamic, biochemical, and neurophysiologic alterations that ultimately lead to a pathological disturbance and a wide range of clinical symptoms (Lo et al., 2003; Iadecola and Anrather, 2011). The severity and temporal evolution of ischemic injury depend on the extent of cerebral blood flow (CBF), localization, duration of ischemia and coexisting systemic diseases among individual patients (Martini and Kent, 2007; Bang et al., 2008; Shen and Duong, 2008; Liebeskind, 2010).

Experimental ischemic stroke models are indispensable to our understanding of the events occurring in the ischemic and reperfused brains, enabling us to elucidate the pathophysiological mechanisms of ischemic brain injury and develop novel therapeutic treatments (Bosetti et al., 2017; Quinn et al., 2018). The vast majority of stroke models are carried in rats or mice because they present clear advantages of lower cost, resemblance to human cerebrovascular anatomy and physiology, and reproducibility of studies (Durukan and Tatlisumak, 2007; Durukan et al., 2008). In addition, genetic modification is usually applied in mice to illuminate molecular pathophysiological conditions like stroke. Cerebral ischemia in humans is divided into two categories: focal and global. Focal cerebral ischemia occurs when CBF is disrupted within a specific brain region, whereas global cerebral ischemia occurs when CBF is blocked throughout most or all of the brain. Given that ischemic stroke in humans occurs mostly in the territory of the middle cerebral artery (MCA), experimental focal cerebral ischemia models, including permanent and transient types, have served as the most widely used tool in the stroke research field (Dorr et al., 2007; Mehta et al., 2007). In focal cerebral ischemia, there is no blood flow at the infarct core, but there is usually a gradient of blood flow from the inner core to the neighboring ischemic area due to the collateral circulation. In global cerebral ischemia, global blood flow is completely stopped or remarkably reduced, depleting the energy supply and hindering cerebral metabolism and function. The reperfusion of blood flow effectively treats acute stroke; however, it can also exacerbate tissue damage and limit the recovery of function. Oxidative stress plays a key role in the pathogenesis of cerebral ischemia-reperfusion injury.

(1) Permanent cerebral ischemia (pdMCAO and pMCAO): Technically, the MCA can be selectively occluded at a distal or proximal site, referred to as permanent distal MCA occlusion (pdMCAO) or permanent (proximal) MCA occlusion (pMCAO) cerebral ischemia model, respectively. The pdMCAO model produces highly reproducible ischemic cortical lesions that are predominantly restricted to the barrel regions of cortex, inducing definable sensorimotor deficits that closely mimics ischemic stroke in humans. Thus, it is believed to be one of the most predictable and useful stroke models, allowing researchers to look at long-term recovery with high survival rates (Doyle and Buckwalter, 2014). Permanent cerebral ischemia can also be generated by the intraluminal suture method used in MCAO (pMCAO).

(2) Transient focal cerebral ischemia (tMCAO): This model is easy to perform in a controlled manner. The intraluminal suture MCAO model in rats and mice is the most frequently used model. This model exhibits reproducible MCA region infarctions that depend on the shape, size, and insertion length of the thread, allowing reperfusion by retracting the suture. MCAO generates ischemic cell death in the striatum and overlying the frontal, parietal, temporal, and portions of the occipital cortex. MCAO also precipitates variable damage in the thalamus, cervicomedullary junction, substantia nigra, and hypothalamus. Ischemic brain injury widely affects diverse brain regions and leads to complex motor, sensory, autonomic, and cognitive deficits (Carmichael, 2005). Usually ischemia must be induced for 60–120 min to obtain reproducible infarct volumes in transient focal ischemia models. In contrast, focal ischemia for more than 3 h precludes reversibility (Kasner and Grotta, 1998; Sicard and Fisher, 2009).

(3) Global cerebral ischemia (GCI): global cerebral ischemia (GCI) during cardiac arrest results in selective and delayed neuronal death of pyramidal neurons in the hippocampal CA1 region, similar to the situation in humans, and consequent cognitive decline (Traystman, 2003; Ostrowski et al., 2016).

ROLE PLAYED BY NRF2 DURING CEREBRAL ISCHEMIA

In recent years, studies reported findings concerning the dynamic change of Nrf2 signaling, its functional importance, and its targeted intervention in cerebral ischemia. These findings provide insights into whether, when and how Nrf2 functions during brain injury. Accordingly, we mainly focused on the following questions. (1) What is the dynamic regulation of the Nrf2 signaling following cerebral ischemia? (2) Does the evidence from $Nrf2^{-/-}$ mice support the functional importance of Nrf2 during ischemic injury? (3) Whether Nrf2 induction is protective against ischemic injury and is facilitative for recovery? Specific focus is given to the *in vivo* evidence in different rodent cerebral ischemia models. Then, the pitfalls and concerns of current Nrf2 experimental ischemic stroke studies are discussed, which would be valuable for future studies.

THE DYNAMIC REGULATION OF THE NRF2 SIGNALING DURING CEREBRAL ISCHEMIA

Normally, Nrf2 is largely localized in the cytoplasm and is maintained at a low basal level due to its binding affinity to Keap1. However, when cells are exposed to excessive oxidative stimuli during cerebral ischemia, Nrf2 is liberated from Keap1, translocates into the nucleus, and binds to the ARE sequence, thereby upregulating the expression of its target genes, which code cytoprotective proteins like anti-oxidative enzymes. In recent years, studies have provided substantial *in vivo* evidence of dynamic alternation of Nrf2 expression, as well as its target genes, and cellular and subcellular distribution of Nrf2 during different stages of cerebral ischemia. These findings, utilizing

focal ischemia models with or without reperfusion in addition to global ischemia models, help us to identify the role of the Nrf2 regulatory network in the context of cerebral ischemia.

A number of studies with permanent cerebral ischemia models (pdMCAO and pMCAO) investigated the Nrf2/ARE pathway in response to ischemic insults (**Table 1**). Several permanent cerebral ischemia studies showed that, at 24 h after pMCAO, the protein expression levels of Nrf2 (total and nuclear) and its target antioxidant genes HO1 and SOD were upregulated in the ischemic cortex of mice or rats (Chang et al., 2013; Zhang J. et al., 2014; Zhao et al., 2014). The obvious increase of Nrf2 downstream antioxidant proteins can be sustained for at least 3 days following ischemia, revealed by the 1.8- to 3.6-fold increase in HO1, NQO1, SOD2, and GPx proteins after pdMCAO (Liu et al., 2018). The inflammatory factors IL-1β and IL-6 were dramatically increased in mouse brains for at least 48 h after pdMCAO (Clausen et al., 2017). These findings are supported by immunohistochemical analyses (Chen et al., 2012; Kao et al., 2013; Meng et al., 2014). Minimal Nrf2 and HO1 positive cells were detected in the sham group, indicating low baseline levels of Nrf2 and HO1 in the non-ischemic cortex areas, levels which increased significantly after pMCAO. Such signals were detected in neurons, astrocytes, and microglia, indicating concurrent expression of Nrf2 and HO1 in most cortical cells. On the contrary to above data, Nrf2 was reported to be significantly decreased at the mRNA level 6 h after pdMCAO, whereas the overall protein level was comparable to that under basal conditions (Clausen et al., 2017). A study reported a significantly decreased level of Nrf2 protein along with an increased level of antioxidant protein HO1 in rat cortex after pMCAO (Wang et al., 2018).

It appears that the majority of tMCAO studies support the activation of the Nrf2/ARE pathway in response to focal cerebral ischemia and perfusion (**Tables 2**, **3**). Because the ischemia-reperfusion injury measured by infarct volume is widely accepted to be most severe at 24 h following tMCAO, most studies investigated the Nrf2 pathway by Western blot (WB) or immunohistological analyses at that time point, with some extending to earlier (2–8 h) or later stages (3–14 d). In these studies, the Nrf2 pathway was examined in different ischemic brain tissues including the cortex, hemisphere, hippocampus, striatum, and cerebellum. The researchers selected 1–2 h ischemia when designing these tMCAO ischemia-reperfusion animal models. We summarized these studies together in order to provide an overview of this field. Most studies measured total Nrf2 level in the ischemic cortex or brain at 24 h after tMCAO (1–2 h); studies subjects showed Nrf2 upregulation at the mRNA level (Li et al., 2013; Guo et al., 2014) and protein level (up to 2–3 fold higher) (Han et al., 2014; Li et al., 2015; Peng et al., 2015; Shi et al., 2015; Cai et al., 2017; Miao et al., 2018; Shang et al., 2018). Such an increase in Nrf2 protein might begin at 8 h (Cai et al., 2017) and be sustained over 3–14 days (Ding et al., 2015; Lin et al., 2016; Bai et al., 2017; Shang et al., 2018). Following nuclear translocation, Nrf2 protein accumulated in the nucleus (Li et al., 2013; Lv et al., 2017) and increased the Nrf2-binding activity to the ARE. Studies presented that the high nuclear Nrf2 protein level (Ding et al., 2015; An et al., 2018) and increased DNA binding activity of Nrf2 with the ARE can be observed, at 7 days after tMCAO (Li et al., 2014). The Nrf2 target antioxidant protein HO1 (up to 3 fold) (Li et al., 2013; Zhang M. et al., 2014; Hua et al., 2015; Peng et al., 2015; Lv et al., 2017; Yang et al., 2017), NQO1(Miao et al., 2018), GCLC and LCLM (Han et al., 2014; Shi et al., 2015) were also upregulated. Several reports showed that the higher expression level of HO1 can continue over 2–14 days (Zhang M. et al., 2014; Ding et al., 2015; Lin et al., 2016). At 24 h after tMCAO, the total and nuclear Nrf2 proteins levels in the ischemic hippocampus also implied significantly higher expression levels (Shi et al., 2015; Lou et al., 2016), which may continue for at least 2 days. In addition, it was reported by immunohistochemistry that, Nrf2 levels in the peri-infarct regions began to show a significant increase at 2 h with a peak at 8 h of reperfusion after 1 h tMCAO, and its target antioxidative proteins such as thioredoxin, glutathione, and HO1 showed significant increases at 24–72 h (Tanaka et al., 2011). Another study presented that Nrf2 immunoreactivity was detectable in the neurons, endothelial cells, astrocytes, and microglia in the peri-infarct area at 7 days after 1 h tMCAO (Shang et al., 2018). A luciferase mouse model, a Keap1-dependent oxidative stress detector, was employed to visualize the time-dependent Nrf2 expression from brain ischemia onset through 7 days after tMCAO (Takagi et al., 2014; Nakano et al., 2017). The *in vivo* optical signals of Nrf2 expression were not detected in the earliest stages but peaked at 24 h after ischemia. Such Nrf2 expression was mainly detected in in the penumbra area, largely localizing inside neurons and astrocytes (Srivastava et al., 2013; Takagi et al., 2014). By quantifying Nrf2 labeled with green fluorescent protein (GFP), researchers have found that Nrf2 and HO1 exhibited higher protein levels in the ischemic cortex at 4–48 h and striatum at 24 h after ischemia, which are consistent with the findings above. In contrast, some studies present the results that indicate suppression of the Nrf2/ARE pathway during tMCAO. At 24 h after tMCAO, the total (Wang L. et al., 2012; Pang et al., 2016; Wang et al., 2016, 2018; Chumboatong et al., 2017; Janyou et al., 2017) and nuclear (Wicha et al., 2017) Nrf2 proteins were found to be downregulated, and the same was observed with HO1 (Wang L. et al., 2012; Pang et al., 2016; Wang et al., 2016, 2018; Chumboatong et al., 2017; Wicha et al., 2017) and NQO1 (Janyou et al., 2017). On the contrary to above findings, several reports found that no change was detected in the markers above (Wu L. et al., 2013; Zhao et al., 2016; Wu G. et al., 2017; Zhang W. et al., 2018).

The activation of the Nrf2/ARE pathway following global ischemia damage remains controversial (**Table 4**). This might be because of the various experimental factors like animal background, age, model, observation time points after ischemia, presence or absence of reperfusion, and site of the sample. At 24 h after GCI (2 vessel occlusion, 2VO), hippocampal Nrf2 content increased nearly 2-fold as determined by biochemical assay (Atef et al., 2018); striatal nuclear Nrf2 protein level and DNA binding activity of Nrf2 increased about 1.5-fold following reperfusion compared with the sham group; and there was notable upregulation in the detoxification and antioxidant proteins HO1, NQO1, GCLC, and GCLM (Ya et al., 2017). At 3 days after GCI (2VO), the total, cytoplasmic, and nuclear

TABLE 1 | Preclinical studies of Nrf2 in permanent cerebral ischemia models of mice and rats.

Species	Genetic background; sex; age or weight	Treatment	Dosage/Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism *(in vivo)*	References
pdMCAO							
Findings supporting the role of Nrf2 pathway (with Nrf2$^{-/-}$ mice)							
Mouse	C57BL/6 (WT and ***Nrf2***$^{-/-}$); M; 10–18 wks	Korean Red Ginsen	• Pre;100 mg/kg; gavage; daily • For 7 d	• Infarct volume (3 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Open field • Cylinder (3, 7, 28 d) ↓ • Corner (3, 7, 14, 21, 28 d) ↓ • Above changes are absent in Nrf2$^{-/-}$ mice	• HO1, NQO1, Gpx1 and SOD2 protein • Above changes are absent in Nrf2$^{-/-}$ mice	Liu et al., 2018
Mouse	C57BL/6 (WT and ***Nrf2***$^{-/-}$); M; 12 mo	-Epicatechin (EC)	• Pre; 15 mg/kg; gavage; 90 min before ischemia • Once	• Infarct volume (cortex; 7 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Adhesive removal (1 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice • DigiGait	• NA	Leonardo et al., 2015
Mouse	C57BL/6 (WT and ***Nrf2***$^{-/-}$); M; 2-3 mo	-Epicatechin (EC)	• Pre; 5, 10, or 15 mg/kg; gavage; 90 min before ischemia • Once	• Infarct volume (7 d; for 5 and 15 mg/kg) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Adhesive removal (1 d; for 5 and 10 mg/kg) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• NA	Leonardo et al., 2013
Mouse	C57BL/6 (WT and ***Nrf2***$^{-/-}$); 8-10wks	Carbon monoxide (CO)	• Post; 250 ppm at 1 L/min; inhalant; immediately after onset of ischemia • For 18 h	Infarct volume (cortex; 7 d) ↓	• Neurological deficits • Adhesive removal • Gross locomotor • Above change are decreased in Nrf2$^{-/-}$ mice	• Nrf2 (nuclear ↑, cytoplasmic; at 75 KDa) and HO1 proteins	Wang B. et al., 2011
Mouse	C57BL/6 (WT and ***Nrf2***$^{-/-}$); M; 6-8wks	Trichostatin A (TSA)	• Post; 1 mg/kg; i.p.; immediately and 6 h after onset of ischemia • Twice	• Infarct volume (cortex; 2 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Neurological deficits (2 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Nrf2 (nuclear ↑) and HO1, NQO1,GCLC proteins (*in vitro*) • Nrf2-ARE binding	Wang B. et al., 2011
Mouse/Rat	C57B/SV129 background ***Nrf2***$^{-/-}$ mice, M, 10-16wks	• Tert-butylhydroquinone (tBHQ)	• Mouse: 1% tBHQ (w/w) food pellets	• Infarct volume (cortex; between WT and Nrf2$^{-/-}$ mice; 1 d,7 d↓) • Above change is absent in Nrf2$^{-/-}$ mice	• NA	• NQO1 ↓ in Nrf2$^{-/-}$ mice	Shih et al., 2005
Findings supporting the role of Nrf2 pathway (without Nrf2$^{-/-}$ mice, indicated by Nrf2 protein nuclear translocation)							
NA							
Other findings involving the role of Nrf2 pathway							
Mouse	C57BL/6; M; 8–10weeks	Monomethyl-fumarate (MMF)	• Post; 20 mg/kg; i.v.; 30 min after ischemia • Once	• Infarct size • Brain edema (1, 2 d) ↓	• Open field • Grip strength (1, 2 d) ↓ • Rotarod	• Nrf2 (total ↑) protein	Clausen et al., 2017

(Continued)

TABLE 1 | Continued

Species	Genetic background; sex; age or weight	Treatment	Dosage/Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism *(in vivo)*	References
pMCAO							
Findings supporting the role of Nrf2 pathway (with Nrf2$^{-/-}$ mice)							
NA							
Findings supporting the role of Nrf2 pathway (without Nrf2$^{-/-}$ mice, with Nrf2 protein nuclear translocation)							
Mouse	CD-1 (ICR); M; 25–30 g	Paeonol (PN)	• Pre; 60 mg/kg; gavage; daily • For 3 d	• Infarct volume (24 h) ↓ • Brain edema (24 h) ↓	• Neurological deficits (24 h) ↓	• Nrf2 (nuclear ↑) and HO1 proteins • Nrf2 and HO1 mRNA	Zhao et al., 2014
Rat	SD; M; 250–300 g	Bicyclol	• Pre; 100 mg/kg; gavage; daily • For 3 d	• Infarct volume (24 h) ↓ • Brain edema (24 h) ↓	• Neurological deficits (24 h) ↓	• Nrf2 (nuclear ↑) and HO1, SOD proteins	Zhang J. et al., 2014
Rat	SD; M; 230–270 g	Recombinant human erythropoietin (rhEPO)	• Post; 5,000 IU/kg; i.p.; 2 h after onset of ischemia • Once	• Infarct volume (24 h) ↓ • Brain edema (24 h) ↓	• NA	• Nrf2 (nuclear ↑; at 70KDa) and HO1 proteins	Meng et al., 2014
Rat	SD; M; 250–280 g	Nobiletin	• Pre and Post; 25 mg/kg; i.p.; 3 d before AND once immediately after onset of ischemia; daily • For 4 d	• Infarct volume (cortex and striatum; 24 h) ↓ • Brain edema (24 h) ↓	• Neurological deficits (24 h) ↓	• Nrf2 (nuclear ↑) and HO1 proteins • Nrf2 and HO1 (IHC)	Zhang L. et al., 2016
Other findings involving the role of Nrf2 pathway							
Rat	SD; M; 300-350 g	Docosahexaenoic acid (DHA)	• Pre; 500 nmol/kg; i.p.; daily • For 3 d	• Infarct volume (3 d) ↓ • Brain edema (3 d) ↓	• Neurological deficits(3 d) ↓	• Nrf2 (total ↑) and HO1 proteins	Chang et al., 2013
Rat	SD; M/F; 250–280 g	HP-1c	• Post; 1 mg/kg; i.v.; 4 h after MCAO and the next 2 d; daily • 3 times	• Infarct volume (2 d) ↓	• Neurological deficits (3 d) ↓ • Rotarod (3, 7, 14, 21 d) • Corner (3, 7 d) ↓ • Open field (1 d) ↓	• Nrf2 (total ↑; at 100 kDa) and HO1 proteins (*in vitro*)	Wang et al., 2018
Rat	SD; M; 230–280 g	Octreotide (OCT)	• Post; 100 mg/kg; i.p.; immediately after onset of ischemia • Once	• Infarct volume (24 h) ↓ • Brain edema (24 h) ↓	• Neurological deficits (24 h) ↓	• Nrf2 (total ↑) and HO1 proteins • Nrf2 ↑ and HO1 (IHC) • SOD activity	Chen et al., 2012
Rat	SD; M; 250–300 g	Tetramethylpyrazine (TMP)	• Pre and Post; 20 mg/kg; i.p.; 30 min before and 60 min after onset of ischemia • Twice	• NA	• NA	• Nrf2 (total ↑) and HO1 proteins	Chang et al., 2015
Rat	SD; M; 300-350 g	Tetramethylpyrazine (TMP)	• Pre and Post; 20 mg/kg; i.p.; 30 min before and 60 min after onset of ischemia • Twice	• Infarct volume (3 d) ↓ • Brain edema (3 d) ↓	• Neurological deficits (3 d) ↓	• Nrf2 (total ↑) and HO1 proteins • Nrf2 and HO1 (IHC)	Kao et al., 2013

(Continued)

TABLE 1 | Continued

Species	Genetic background; sex; age or weight	Treatment	Dosage/Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism *(in vivo)*	References
Rat	SD; M; 8 wks; 220-260 g	Isoquercetin	• Post; 50 mg/kg; i.v.; daily • For 7 d	• Infarct volume (24 h) ↓	Neurological deficits (24 h) ↓	• Nrf2 mRNA/protein (total ↑)	Chen M. et al., 2017
Mouse	C57BL/6J; M; 3-4 mo; 25–30 g	Tert-butylhydroquinone (tBHQ)	• Pre; 0.582, 3.34, or 33.4 mg/kg; i.p.; started 24 h before ischemia; once every 12 h • 3 times	• Infarct volume (cortex; striatum; hemisphere)	• Neurological deficits (1 d) ↑ • Mortality ↑	• NA	Sun et al., 2016

MCAO, middle cerebral artery; pdMCAO, permanent distal middle cerebral artery occlusion; pMCAO, permanent (proximal end of) middle cerebral artery occlusion that is generated by the intraluminal suture MCAO; GCI, global cerebral ischemia; i.p., intraperitoneal; i.v., intravenous; i.c.v., intracerebroventricular; Pre, pretreatment; Post, posttreatment; KDa, kilodalton that indicates Nrf2 protein molecular weight by Western blot; the changes in brain lesion, edema, neurological deficits, and mRNA/protein expression level (↑ or ↓, increase or decrease at indicated time point; no label at indicated time point, no significant difference); h, hour; d, day; wk, week.

protein levels of Nrf2 were upregulated in the hippocampal CA1 region of rats although the increase in nuclear Nrf2 was not significant (Chen B. et al., 2016), a finding which is supported by another report (Lei et al., 2016). No change in HO1 protein level was detected at 3 days following brain injury (Chen B. et al., 2016). An immunofluorescence-based study showed that the Nrf2 level was slightly (but not significant) higher in the rats hippocampal CA1 area along with a decline of HO1 expression at 7 days after GCI (2VO) (Tulsulkar and Shah, 2013). In a rat GCI (4VO) model, activation of Nrf2 in the hippocampal CA1 region was examined over 3 days by four metrics including nuclear translocation of Nrf2, total Nrf2 protein level, DNA-binding of Nrf2, and induction of Nrf2 regulated proteins (Tu et al., 2015). The cytosolic but not nuclear Nrf2 protein was slightly upregulated from 12 to 72 h as evidenced by WB and immunostaining analyses, whereas the total Nrf2 protein level remained unchanged at 24 h. No change in the Nrf2 DNA binding activity was detected at 24 h and 72 h. The Nrf2-regulated antioxidant proteins HO1, NQO1, SOD2, and GPx1 were slightly altered within 72 h. These findings are supported by another report of GCI with less time of ischemia (Wang et al., 2013) that presented the upregulation of HO1 protein at 3 days. Lastly, it should be pointed out that the reports are inconclusive concerning the protein expression levels of Nrf2 with its target genes and subcellular distribution of Nrf2 following GCI. For example, hippocampal Nrf2 protein was reported to have not changed (Yang Y. et al., 2015), significantly decreased (Liu H. et al., 2015), or increased (Ashabi et al., 2015; Lee et al., 2015) following ischemia.

FUNCTIONAL BENEFIT OF NRF2 IN CEREBRAL ISCHEMIA—*IN VIVO* EVIDENCE FROM NRF2$^{-/-}$ MICE

Multiple lines of evidence demonstrate the beneficial contribution of Nrf2 in various pathological conditions, and most come from studies using Nrf2 knockout (Nrf2$^{-/-}$) or knockdown animals. Reports showed that Nrf2$^{-/-}$ mice do not exhibit any overt abnormal phenotype regarding size, body weight, food intake, mobility, fertility, or other characteristics at baseline (Itoh et al., 1997; Wang et al., 2007; Wang Y. C. et al., 2011; Wang B. et al., 2012; Leonardo et al., 2013, 2015; Doré, 2015; Liu et al., 2018). In addition, Nrf2$^{-/-}$ mice exhibit similar cerebrovascular architecture in anastomosis, vascular physiology, and blood pH compared to their WT counterparts (Leonardo et al., 2015). Basal GST and NQO1 activities, but not GSH content, modestly but significantly decreased in multiple brain regions of Nrf2$^{-/-}$ mice compared to their Nrf2$^{+/+}$ littermates, suggesting that the constitutive synthesis of brain GSH does not rely on Nrf2 function, at least in young adult mice (Shih et al., 2005).

However, in response to the various ischemic insults, it is becoming clear that Nrf2 plays a critical role in protection against ischemic brain damage (**Tables 1**–**3**). Indeed, older and recent studies with different cerebral ischemia models have revealed the functional benefit of Nrf2 on infarct volume, brain edema, and

TABLE 2 | Preclinical studies of Nrf2 in transient cerebral ischemia models of mice and rats (MCAO, 40 min-1.5 h).

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
MCAO (40 min-1.5 h)								
Findings supporting the role of Nrf2 pathway (with Nrf2$^{-/-}$ mice)								
MCAO (40 min)	Mouse	C57BL/6, ***Nrf2***$^{-/-}$, and Cx3cr1GFP/+; M; 23–30 g	3H-1,2-Dithiole-3-thione (D3T)	• Post; 50 mg/kg; i.p.; at 3 h after MCAO • Once	• Infarct volume (48 h) • Brain edema (48 h) • Above changes are decreased in Nrf2$^{-/-}$ mice	• Neurological deficits (48 h) ↓ • Above changes are absent in Nrf2$^{-/-}$ mice	• Nrf2 (total ↑) and HO1 proteins (*in vitro*) • Above changes are absent in Nrf2$^{-/-}$ mice • Nrf2 (IHC)	Kuo et al., 2017
MCAO (1 h)	Mouse	C57BL/6J WT, ***Nrf2***$^{-/-}$; M; 7–11wks;	Resveratrol	• Pre: 10 mg/kg; i.p.; 48 h before MCAO • Once	• Infarct volume (24 h) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• NA	• NQO1, SOD2 proteins • Above changes are decreased in Nrf2$^{-/-}$ mice	Narayanan et al., 2015
MCAO (1 h)	Mouse	C57BL/6J: ***Nrf2***$^{-/-}$ and WT; M; 8–10wks	Lentiviral transfection (for SIRT6 overexpression)	• Pre; 2.5 μl (10^9 infectious units/ml); i.c.v.; 2 wks before MCAO • Once	• Infarct volume (24 h) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Neurological deficits (24 h) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Nrf2 (total ↑) and HO1 proteins	Zhang et al., 2017
MCAO (1 h)	Mouse	C57BL/6: WT and ***Nrf2***$^{-/-}$; 20–25 g, 8 to 10 wks	Dimethyl fumarate (DMF), Monomethyl fumarate (MMF)	• Post: 30, 45 mg/kg (better); i.p.; 15 min before reperfusion twice a day • For 7 d	• Infarct volume (3,7 d) ↓ • Brain edema (3 d) ↓ • Above changes are absent in Nrf2$^{-/-}$ mice	• Neurological deficits (3,7 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Nrf2 (total ↑) and HO1 proteins	Yao et al., 2016
MCAO (1 h)	Mouse	WT and ***Nrf2***$^{-/-}$; M; 8–10 wks	Tanshinone IIA (TSA)	• Post; 25 mg/kg; i.p.; 10 min after MCAO • Once	• Infarct volume (72 h) • Above change is reduced in Nrf2$^{-/-}$ mice	• Neurological deficits (72 h) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Nrf2 (nuclear ↑) protein • Nrf2 mRNA	Cai et al., 2017
MCAO (1 h)	Mouse	ICR background ***Nrf2***$^{-/-}$ and WT; 25–28 g	Ursolic acid (UA)	• 130 mg/kg; i.p.; immediately after MCAO; once	• Infarct volume (24 h) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Neurological deficits (24 h) • Above change is reduced in Nrf2$^{-/-}$ mice	• Nrf2 (nuclear ↑, cytoplasmic ↓) and HO1 proteins • Nrf2 and HO1 mRNA	Li et al., 2013
MCAO (1.5 h)	Mouse	HO1$^{-/-}$, ***Nrf2***$^{-/-}$ and WT; M; 7–8 wks; 20–25 g	Epicatechin (EC)	• Pre: 2.5 (no effect), 5, 15, 30 mg/kg (best); oral; 90 min before; once • Post: 30 mg/kg EC was administered at 3.5 h (better) or 6 h (no effect) after MCAO	• Infarct volume (pre; 24 h) ↓ • Above change is absent in Nrf2$^{-/-}$ mice • Infarct volume (post; 72 h) ↓	• Neurological deficits (pre; 24 h) • Above change is reduced in Nrf2$^{-/-}$ mice • Neurological deficits (post; 72 h)	• Nrf2 (nuclear ↑, cytoplasmic) and HO1 proteins	Shah et al., 2010

(Continued)

TABLE 2 | Continued

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
MCAO (1.5 h)	Mouse	CD1 background ***Nrf2***$^{-/-}$ and WT; F; 20–25 g	Tert-butylhydroquinone (t-BHQ)	• NA	• Infarct volume (between Nrf2$^{-/-}$ and WT, 24 h) • Above change is reduced in Nrf2$^{-/-}$ mice	• Neurological deficits (between Nrf2$^{-/-}$ and WT; 24 h) • Above change is reduced in Nrf2$^{-/-}$ mice	• NA	Shah et al., 2007
Findings supporting the role of Nrf2 pathway (without Nrf2$^{-/-}$ mice, indicated by Nrf2 protein nuclear translocation)								
MCAO (1 h)	Mouse	ICR; M; 24–27 g	Isorhamnetin (Iso)	• Post; 5 mg/kg; i.p.; immediately at the onset of reperfusion; daily; • Twice	• Infarct volume (48 h) ↓ • Brain edema (48 h)	• Neurological deficits (48 h) ↓ • Rotarod (48 h)	• Nrf2 (nuclear ↑, cytoplasmic) and HO1 proteins	Zhao et al., 2016
MCAO (1.5 h)	Mouse	C57BL/6; M; 25–30 g; 10–12 wks	Epigallocatechin-3-gallate (EGCG)	• Post; 50 mg/kg; i.p.; immediately after; daily • For 7 d	• Infarct volume (7 d) ↓	• Neurological deficits (3, 7 and 14 d) ↓	• Nrf2 (nuclear ↑) protein • Nrf2 (IHC)	Bai et al., 2017
MCAO (1.5 h)	Rat	SD; M; 280–300 g	Diterpene ginkgolides meglumine injection (DGMI)	• Post; 1, 3 and 10 mg/kg, iv, at the onset of reperfusion and 12 h after reperfusion	• Infarct volume (3 and 10 mg/kg, 24 h) ↓	• Neurological deficits (1, 3 and 10 mg/kg dose-dependent, 24 h) ↓	• Nrf2 (nuclear ↑) and HO1	Zhang W. et al., 2018
MCAO (1 h)	Rat	SD; M; 3 mo	5-methoxyindole-2-carboxylic acid (MICA)	• Pre; diet supplemented with 0.33% MICA (200 mg/kg/d) for 4 wks before MCAO; i.p. injection (200 mg/kg body weight) once per day • For seven days until 24 h before MCAO	Infarct volume (24 h) ↓	• NA	• Nrf2 (nuclear ↑) and NQO1 proteins	Wu et al., 2017b
Other findings involving the role of Nrf2 pathway								
MCAO (1 h)	Mouse	C57BL/6J background WT and SHPS-1 mutant (MT); M; 10–12 wks	Src homology 2 domain–containing protein tyrosine phosphatase substrate−1 (SHPS-1)	• NA	• Infarct volume (24 h) ↓	• Neurological deficits (72 h) ↓	• Nrf2 (total ↑; at 98 KDa) and HO1 proteins	Wang B. et al., 2011

(Continued)

TABLE 2 | Continued

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion /Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
MCAO (1 h)	Mouse	C57BL/6 J; M; 22–25 g	MiR-93 antagomir	• Pre; 7 μl (at 100 μm); i.c.v.; 10 min before MCAO • Once	• Infarct volume (24 h) ↓	• Neurological deficits (24 h) ↓	• Nrf2 (total ↑) and HO1 proteins	Wang et al., 2016
MCAO (1 h)	Mouse	ICR; M; 6 wks old, 23–25 g	Tocovid	• 200 mg/kg/d orally once a day for 1 mo before MCAO	• Infarct volume (1, 3 d) ↓	• Bederson score (pre,1,3,7 d), • Rotarod (pre,1,3,7 d) • Corner (pre,1,3,7 d)	• Nrf2 (total ↑) protein • Nrf2 (IHC)	Shang et al., 2018
MCAO (1 h)	Mouse	C57BL/6 J	Gastrodin (GAS)	• Post: 10, 50, 100 mg/kg; i.p.; onset of cerebral reperfusion; • Once daily for 7 d	• Infarct volume (medium or high-dose, 24 h and 7 d) ↓	• Neurobehavioral scores (1, 7 d) ↓	• Nrf2 (total ↑; at 68 KDa) and HO1 proteins	Peng et al., 2015
MCAO (1 h)	Mouse	C57BL/6; F; 12–15 wks	Estradiol (EST)	• 0.05 mg; pellets; subcutaneous implantation; before; for 21 d	• Brain edema (24 h) ↓	• Neurological deficits (mNSS, 24 h) ↓	• Nrf2 (total ↑) and NQO1 proteins	Li et al., 2017
MCAO (1 h)	Rat	SD; M; 60–80 d old, 260–300 g	Tert-butylhydroquinone (tBHQ)	• Pre; 16.7 mg/kg; i.p. injection at intervals of 8 h before MCAO • Three times	• Infarct volume (24 h)	• Neurological deficits (24 h) ↓	• Nrf2 (total ↑) protein	Hou et al., 2018
MCAO (70 min)	Rat	SD; M; 250–330 g	Sulforaphane	• 5 mg/kg; i.p.; 1 h before; once	• Infarct volume (24 h, 72 h)	• Neurological deficits (24 h) ↓	• Nrf2 (total content ↑) and NQO1 proteins • HO1 (IHC)	Alfieri et al., 2013
MCAO (1 h)	Rat	SD; M; 3 mo	5-methoxyindole-2-carboxylic acid (MICA)	• Post; 100 mg/kg;i.p.; at the onset of reperfusion • Once	• Infarct volume (24 h) ↓	• NA	• Nrf2 (total ↑) and NQO1 proteins	Wu et al., 2018
MCAO (1.5 h)	Rat	SD; F; 250–300 g	Genistein	• Pre; 10 mg/kg, i.p., once daily • For 2 wks	• Infarct volume (72 h) ↓	• Neurological deficits (72 h) ↓	• Nrf2 (total ↑) and NQO1 proteins	Miao et al., 2018
MCAO (1 h)	Rat	SD; M; 60–80 d old, 240–300 g	Glycogen synthase kinase 3β (GSK-3β)	• Pre: 7 μl (2 μg/μl); i.c.v.; 48 h before MCAO • Once	• NA	• NA	• Nrf2 (total) and NQO1, HO1 proteins • NQO1, HO1 mRNA	Chen X. et al., 2016
MCAO (1 h)	Rat	SD; M; 250–280 g	siRNA targeting sulfiredoxin1 (Srxn1)	• Pre; i.c.v.; 24 h before MCAO • Once	Infarct volume (24 h)	• Neurological deficits (24 h) ↑	• Nrf2 (total ↓) and NQO1 proteins	Wu et al., 2017a
MCAO (1 h)	Rat	SD; M; 270–310 g	Thioredoxin-1 siRNA	• Pre: 10 μl (2 μg/μl); i.c.v.; 24 h before MCAO • Once	• Infarct volume (24 h) • Brain edema (24 h)	• Neurological deficits (24 h) ↓	• Nrf2 (total ↓) protein	Li et al., 2015

(Continued)

TABLE 2 | Continued

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
MCAO (1 h)	Rat	SD; M; 280–310 g	Sevoflurane	• Post: 2.6% for 1 h; inhalation; immediately at onset of reperfusion • Once	• Infarct volume (72 h)	• Neurological deficits (12, 24, 48, and 72 h) n	• Nrf2 (total ↓) and NQO1 • Nrf2-DNA binding activities	Li et al., 2014
MCAO (70 min)	Rat	SD; M; 250–300 g	Nrf2 inducer D, L-sulforaphane	• 5 mg/kg; i.p.; 1 h before MCAO • Once	• NA	• NA	• Nrf2 (IHC)	Srivastava et al., 2013
MCAO 1 h	Rat	SD; M; aged 8–9 wks; 300–350 g	• MicroRNA (miR-142-5p)	• NA	• NA	• NA	• Nrf2 mRNA	Wang et al., 2017
MCAO (1 h)	Rat	Wistar; M; 250–280 g	Danhong	• 0.9, 1.8 ml/kg; i.p.; 30 min before ischemia, with reperfusion and 24, 48, 72 h after ischemia	• Infarct volume (72 h) ↓ • Brain edema (72 h) ↓	• Neurological deficits (72 h)	• Nrf2 and HO1, NQO1 mRNA	Guo et al., 2014
MCAO (1.5 h)	Rat	SD; M; 180–220 g	Lactulose	• Pre; 0.25 g/kg; gavage; at start of ischemia • Once	• Infarct volume (24 h) ↓	• Neurological deficits (24 h) ↓ • Morris water maze ↓	• Nrf2 mRNA and activity • SOD activity	Zhai et al., 2013
MCAO (1.5 h)	Rat	SD; M; 260–290 g	β-caryophyllene (BCP)	• Pre; 34, 102, 306 mg/kg (best); gavage; once a day • For 7 d	• Infarct volume (24 h) ↓	Neurological deficits (24 h) ↓	• Nrf2 (total ↑) and HO1 mRNA	Lou et al., 2016
MCAO (1.5 h)	Rat	SD; M; 260–280 g	Neural stem cells (NSCs)	• Post; four 1.0 μl deposits of single-cell suspension in Dulbecco's PBS (10^5 cells per ul); along the anterior-posterior axis into the cortex; 6 h after stroke	• Infarct volume (cortex, 28 d) ↓	• Rotarod (1–28 d) • Beam-balance (28 d) ↓	Nrf2 mRNA	Sakata et al., 2012
MCAO (1.5 h)	Rat	Wistar; M; 250–280 g	Xueshuantong injection (Lyophilized, XST)	• Post; 25, 50,100 mg/kg; i.p.; 1 h after reperfusion, once a day • For 3 or 7 d	• NA	• Modified neurological severity (Mnss, 1, 3, and 7 d) ↓	• Nrf2 and HO1, NQO1 mRNA	Guo et al., 2018
MCAO (1 h)	Rat	SD; F; 300–350 g	p-hydro-xybenzyl alcohol (HBA)	• Pre; 25 mg/kg BW; i.m. with sesame oil; 3 d before • Once	• Infarct volume (cortex and striatum, 24 h) ↓	• Modified neurological severity score (mNSS) at 1, 7, 14, 21, and 28 d • Functional deficits from 7 d ↓	• Nrf2 DNA (PCR)	Kam et al., 2011

(Continued)

TABLE 2 | Continued

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion /Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
MCAO (1 h)	Rat	SD; M; 230–270 g	Curcumin	• Post; 300 mg/kg; i.p.; 1 h after MCAO • Once	• Infarct volume (24 h) ↓	• NA	• Nrf2-DNA binding activity	Wu J. et al., 2013
MCAO (45 min)	Mouse	OKD48 transgenic mice; M/F; 23–28 g	NA	• NA	• Infarct volume (12 h, 1, 3, 7 d)	• NA	• Nrf2 (IF)	Nakano et al., 2017
MCAO (1 h)	Mouse	ICR; M; 34–38 g; 8 wks	NA	• NA	• NA	• NA	• Nrf2 (IHC)	Tanaka et al., 2011
MCAO (1 h)	Rat	Hannover-Wistar; M; 250–350 g	Recombinant human erythropoietin (rhEpo)	• Post; 5000 IU/kg; i.p. immediately or 3 h after MCAO • Once	• Infarct volume (3, 24 h)	• NA	• Nrf2 (IHC)	Mrsic-Pelcic et al., 2017
MCAO (1 h)	Rat	Wistar; M; 10 wks; 250–300 g	Curcumin	• Post; 300 mg/kg; i.p.;Post; 300 mg/kg; i.p.; 30 min after MCAO • Once	• Infarct volume (d1) ↓ • Brain edema (d1) ↓	• Neurological deficits (d1) ↓	• Nrf2 (IHC)	Li et al., 2016
MCAO (1 h)	Mouse	Transgenic fatty acid metabolism-1 (*fat-1*) gene mice; C57BL/6; M	Omega-3 fatty acids (n-3 PUFAs) by fish oil (FO) diet	• Pre: 5% (w/w) was added to the regular diet, which increased the n-3 PUFA from 0.34 to 1.5%, and decreased the n-6:n-3 PUFA ratio from 5:1 to 1:1; oral; before; daily; for 6 wks	• Infarct volume (48 h) ↓	• Neurological deficits (48 h) ↓ • Dietary supplementation in Corner, Rotarod (7 d) d supplementation in Corner, Rotarod	HO1 protein	Zhang M. et al., 2014
MCAO (1 h)	Mouse	C57BL/6; M; 8-–0 wks	Dimethyl fumarate (DMF)	• Pre: 15 mg/kg; i.p.; twice a day for 3 d before stroke	• Infarct volume (4 h, 24 h) • Brain edema (4 h, 24 h) ↓	• NA	• HO1, NQO1,GCLC and GCLM mRNA	Kunze et al., 2015
MCAO (1.5 h)	Rat/ Mouse	Wistar (Osmotic pump studies) and SD, 250–350 g;	Tert-butylhydroquinone (tBHQ)	• Rat: 1 mM, i.c.v., osmotic mini-pump delivery (1 μl/h for 4 d), MCAO after 3 • d; i.p. in later experiments, 3.33 or 16.7 mg/kg, before; three times by 8 h intervals	• Rat: Infarct volume (cortex, 24 h) ↓	• Rat: tBHQ Neurological deficits (24 h to 1 mo) • Sensorimotor deficits (since 4 d) ↓	NA	Lou et al., 2016

MCAO, middle cerebral artery; pdMCAO, permanent distal middle cerebral artery occlusion; pMCAO, permanent (proximal end of) middle cerebral artery occlusion that is generated by the intraluminal suture MCAO; GCI, global cerebral ischemia; i.p., intraperitoneal; i.v., intravenous; i.c.v., intracerebroventricular; Pre, pretreatment; Post, posttreatment; the changes in brain lesion/edema and neurobehavioral deficits/Nrf2 protein expression level rf2 protein expression leveledema and neurobehavioral deficitseatmest, posttreatmeatme; IF, Immunofluorescence; KDa, kilodalton that indicates Nrf2 protein molecular weight by Western blot; the changes in brain lesion, edema, neurological deficits, and mRNA/protein expression level (↑ or ↓, increase or decrease at indicated time point; no label at indicated time point, no significant difference); h, hour; d, day; wk, week.

TABLE 3 | Preclinical studies of Nrf2 in transient cerebral ischemia models of mice and rats (MCAO, 2 h).

Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
MCAO (2 h)							
Findings supporting the role of Nrf2 pathway (with Nrf2$^{-/-}$ mice)							
Mouse	WT and **Nrf2**$^{-/-}$; 12 wks	S-allyl cysteine (SAC)	• Pre; 300 mg/kg; i.p.; 30 min before MCAO • Once	• Infarct volume (1 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Neurological deficits (1 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice	• Nrf2 (nuclear ↑) and HO1, GCLC,LCLM proteins	Shi et al., 2015
Mouse	WT and **Nrf2**$^{-/-}$; M; 30-35 g	Hydrogen sulfide (H2S)	• Pre; 40 ppm; inhalant; 7 d before MCAO; daily	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓ • Above change is absent in Nrf2$^{-/-}$ mice • Morris water maze ↓	• Nrf2 (nuclear ↑) protein	Ji et al., 2016
Findings supporting the role of Nrf2 pathway (without Nrf2$^{-/-}$ mice, indicated by Nrf2 protein nuclear translocation)							
Rat	SD; M; 220-240 g	Protocatechualdehyde (PCA)	• Pre; 40 mg/kg; i.v.; 1 h before reperfusion • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (nuclear ↑) and cytoplasmic HO1	Guo et al., 2017
Rat	SD; M; 200-250 g	Procyanidin B2 (PB)	• Post; 40 mg/kg; gavage; 3 h after MCAO then daily • For 14 d	• Brain edema (2 d) Infarct volume (2 d) ↓	• Neurological deficits (7, 11, 14 d) ↓ • Rotarod (7, 11, 14 d)	• Nrf2 (nuclear ↑) and HO1, NQO1, GSTa proteins	Wu et al., 2015
Rat	Wistar; M; 250-300 g	Hexahydrocurcumin (HHC)	• Post; 40 mg/kg; i.p.; immediately after MCAO • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (nuclear ↑; at 100 KDa) and HO1, NQO1 proteins • SOD activity	Wicha et al., 2017
Rat	SD; M; 220-250 g	Alpha-lipoic acid (α-LA)	• Post; 40 mg/kg; i.v.; immediately after reperfusion • Once	• Infarct volume (1 d) ↓ • Brain edema (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic ↓; at 68 KDa) and HO1 proteins	Lv et al., 2017
Rat	Wistar; M; 6 mo; 270–290 g	Hispidulin	• Post; 50 mg/kg; i.p.; onset of MCAO then daily • For 7 d	• Infarct volume (7 d) ↓ • Brain edema (7 d) ↓	• Neurological deficits (2, 3, 5, 7 d) ↓ • Beam-walking (2, 3, 5 7 d) ↓ • Morris water maze (1, 2, 3, 5, 7 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic ↑) protein • Nrf2 mRNA	An et al., 2018
Rat	SD; 280–320 g	Corilagin	• Post; 30 mg/kg; i.p.; 3 h after MCAO then daily • For 7 d	• Infarct volume (7 d) ↓	• Neurological deficits (7 d) ↓	• Nrf2 (nuclear ↑) protein, Nrf2 phosphorylation	Ding et al., 2017
Rat	SD; M; 240-280 g	Gualou Guizhi granule (GLGZG)	• Post; 3 g/kg; gavage; daily • For 7 d	• NA	• NA	• Nrf2 (nuclear ↑) and HO1, NQO1 proteins	Zhang Y. et al., 2018
Rat	SD; M; 3 mo; 210-230 g	Isoquercetin (Iso)	• Post; 20 mg/kg; gavage; after MCAO; daily for 3 d	• Infarct volume (3 d) ↓ • Brain edema (3 d) ↓	• Neurological deficits (3 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic ↓) protein	Dai et al., 2018

(Continued)

TABLE 3 | Continued

Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
Rat	SD; M; 10 mo; 350-400 g	Myricetin	• Pre and Post; 20 mg/kg; gavage; 2 h before MCAO then daily • For 2 d	• Infarct volume (1 d) ↓	• Neurological deficits (5, 7, 9, 11, 14 d) ↓ • Foot-fault (7, 9, 11, 14 d) ↓ • Modified balance beam (5, 7, 9, 11, 14 d) ↓ • Adhesive-removal somatosensory (7, 9, 11, 14 d) ↓ • Morris water maze (10 d) ↓ • Probe test (14 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic) and HO1 proteins	Wu et al., 2016
Rat	SD; M; 250-280 g	Lipoxin A4 (LXA4)	• Post; 1 nmol; i.c.v.; immediately after MCAO • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (nuclear ↑, total ↑) protein	Wu L. et al., 2013
Rat	SD; M; 230-270 g	Huang-Lian-Jie-Du-Decoction (HLJDD)	• Pre; 20 mg/kg; gavage; daily • For 7 d	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓ • Mortality ↓	• Nrf2 (nuclear ↑, cytoplasmic ↓) and HO1 proteins	Zhang Q. et al., 2016
Rat	Wistar; M; 220-250 g	Mangiferin	• Pre and Post; 100 mg/kg; gavage; 3 times before and once at 2 h after MCAO onset; daily • For 4 d	• Infarct volume (1 d) ↓ • Brain edema (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic ↓) protein	Yang et al., 2016
Other findings involving the role of Nrf2 pathway							
Mouse	ddY WT and C57BL/6 OKD-V, OKD-LUC; M; 8–12 wks	Bardoxolone methyl (BARD)	• Pre; 0.6 or 2 mg/kg; i.v.; immediately before reperfusion • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓ • Grid walk (1 d) ↓	• Nrf2 (total ↑) and HO1 • Nrf2 (IF)	Takagi et al., 2014
Mice	NA	Artesunate	10–40 mg/kg	• infarct volume (22 h) ↓	• NA	• Nrf2 (total ↑) protein	Lu et al., 2018
Mouse	ddY; M; 5–8 wks; 22–28 g	RS9	• Post; 0.2 mg/kg; i.p.; immediately after reperfusion • Once	• Infarct volume (1 d)	• Neurological deficits (1, 3, 5, 7 d) • Grid walk (1, 3, 5, 7 d) 1, • Mortality ↓	• Nrf2 (total ↑) protein	Yamauchi et al., 2016
Rat	SD; M/F; 250–280 g	HP-1c	• Post; 1 mg/kg; i.v.; 4 h after MCAO then daily • For 2 d	• Infarct volume (2 d) ↓	• Neurological deficits (3 d) ↓ • Rotarod (3, 7, 14, 21 d) c • Corner (3, 7 d) ↓ • Open-field ↓	• Nrf2 (total ↑; at 100 KDa) and HO1 proteins	Wang et al., 2018

(Continued)

TABLE 3 | Continued

Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
Rat	SD; M; 230–280 g	Resveratrol	• Pre; 15 or 30 mg/kg; i.p.; daily • For 7 d	• Infarct volume (1 d) ↓ • Brain edema (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) protein	Ren et al., 2011
Rat	SD; M; 220–280 g	Phloretin	• Pre; 80 mg/kg; i.p.; daily • For 14 d	• Infarct volume(1 d) ↓ • Brain edema (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑, at 61 KDa) protein and mRNA	Liu Y. et al., 2015
Rat	Wistar; M; 280–300 g	Dihydrocapsaicin (DHC)	• Pre; 5 or 10 mg/kg; i.p.; 15 min before reperfusion • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑; at 68 KDa) and NQO1 proteins • SOD and GPx activity	Janyou et al., 2017
Rat	Wistar; M; 220–250 g	Agomelatine	• Pre; 40 mg/kg; i.p.; 1 h before MCAO • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑; at 57 KDa) and NQO1 proteins • SOD and GPx activity	Chumboatong et al., 2017
Rat	SD; M; 270–320 g	(–)-Epigallocatechin gallate (EGCG)	• Pre; 40 mg/kg; i.p.; daily • For 3 d	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) and HO1, GCLC,GCLM proteins	Han et al., 2014
Rat	SD; M; 275–300 g	Dimethyl fumarate (DMF)	• Post; 50 mg/kg; gavage; 2–3 h after MCAO until d14; twice daily	• Infarct volume (14 d) ↓	• Neurological deficits (3, 7, 14 d) ↓	• Nrf2 (total ↑, at 110 KDa) and HO1 proteins	Lin et al., 2016
Rat	SD; M; 8–10 wks; 250–300 g	Tissue kallikrein (TK)	• Post; 8.75×10^{-3} PNAU/kg; i.v.; immediately after reperfusion; • once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) and HO1 proteins	Yang et al., 2017
Rat	SD; M; 57–61 d; 250–280 g	Compound 10 b	• Post; 140 mg/kg; gavage; immediately after MCAO • Once	• Infarct volume (1 d) ↓ • Brain edema (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑; at 68 KDa) and HO1 proteins	Hua et al., 2015
Rat	SD; M; 260–280 g	YQ138	• Post; 10 mg/kg; i.v.; 2, 4, and 6 h after MCAO onset • Three times	• Infarct volume (1 d) ↓ • Brain edema (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) and HO1 proteins	Pang et al., 2016
Rat	SD; M; 220–240 g	Protocatechualdehyde (PCA)	• Post; 40 mg/kg; i.v.; 1 h before reperfusion • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) and HO1 proteins	Guo et al., 2017
Rat	SD; M; 280–300 g	11-Keto-β-boswellic acid (KBA)	• Post; 25 mg/kg; i.p.; 1 h after reperfusion • Once	• Infarct volume (2 d) ↓	• Neurological deficits (2 d) ↓	• Nrf2 (total ↑) and HO1 proteins • Nrf2 and HO1 (IF)	Ding et al., 2015

(Continued)

TABLE 3 | Continued

Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion/ Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
Rat	SD; M; 230–260 g	Z-ligustilide (LIG)	• Post; 32 mg/kg; i.v.; immediately after MCAO • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) protein	Peng et al., 2013
Rat	Wistar; M; 280–300 g	Dihydrocapsaicin (DHC)	• Post; 10 mg/kg; i.p.; 15 min before reperfusion • Once	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑; at 68 KDa) and NQO1 proteins • SOD, GPx activity	Janyou et al., 2017
Rat	SD	Britanin	• Pre or Post; 50 mg/kg; gavage; 2 h before MCAO to 2 h after MCAO • Once	Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (total ↑) and HO1, NQO1 proteins	Wu G. et al., 2017
Rat	SD; M; 250–300 g	4-Hydroxybenzyl alcohol (4-HBA)	• Pre; 50 mg/kg; i.p.; daily • For 3 d	• Infarct volume (1 d) ↓	• Neurological deficits (0, 1, 2 d) ↓	• Nrf2 (IF)	Yu et al., 2013
Rat	SD; M; 260–280 g	Water extract (GUW) of *Gastrodia elata* and *Uncaria rhynchophylla*	• Post; 288.6 mg/kg; gavage; daily • For 7 d	• Infarct volume (7 d) ↓	• Neurological deficits (3, 5, 7 d) ↓ • Beam-walking (3, 5, 7 d) ↓	• Nrf2 (IHC)	Xian et al., 2016
Rat	SD; M; 7–8 wks; 250–280 g	Salidroside	• Pre and Post; 30 mg/kg; i.p.; immediately prior to MCAO and immediately after reperfusion • Twice	• Infarct volume (1 d) ↓	• Neurological deficits (1 d) ↓	• Nrf2 (IHC)	Han et al., 2015

MCAO, middle cerebral artery; pdMCAo, permanent distal middle cerebral artery occlusion; pMCAO, permanent (proximal end of) middle cerebral artery occlusion that is generated by the intraluminal suture MCAO; GCI, global cerebral ischemia; i.p., intraperitoneal; i.v., intravenous; i.c.v., intracerebroventricular; Pre, pretreatment; Post, posttreatment; the changes in brain lesion/edema and neurobehavioral deficits/Nrf2 protein expression level rf2 protein expression leveledema and neurobehavioral deficitseatmest, posttreatmeatme; IF, Immunofluorescence; KDa, kilodalton that indicates Nrf2 protein molecular weight by Western blot; the changes in brain lesion, edema, neurological deficits, and mRNA/protein expression level (↑ or ↓, increase or decrease at indicated time point; no label at indicated time point, no significant difference); h, hour; d, day; wk, week.

TABLE 4 | Preclinical studies of Nrf2 in global cerebral ischemia models of mice and rats.

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion /Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
Findings supporting the role of Nrf2 pathway (with Nrf2$^{-/-}$ mice)								**NA**
Findings supporting the role of Nrf2 pathway (without Nrf2$^{-/-}$ mice, indicated by Nrf2 protein nuclear translocation)								
2VO-20 min	Mouse	C57BL/6; M; 8 wks; 20-22 g	5-Hydroxymethyl-2-furfural (5-HMF)	• Pre and post;12 mg/kg; i.p.; 30 min before AND 5 min after the onset of reperfusion • Twice	• Neuronal injury (striatum; via cresyl violet, TUNEL; 1 d) • Brain edema (both hemispheres; 1 d) ↓	• Neurological deficits (24 h) ↓ • Locomotor activity (24 h) ↓ • Inclined beam walking (24 h) ↓	• Nrf2 (nuclear ↑) and HO1, GCLC, GCLM, NQO1 proteins • DNA binding activity of Nrf2	Ya et al., 2017
2VO-20 min	Rat	SD; M; 250–300 g	Rifampicin	• Post; 20 mg/kg; i.p.; 30 min after onset of reperfusion; daily; • For 7 d	• Neuronal death (CA1; via HE, TUNEL; 7 d) ↓	• Morris water maze (8, 9, 10 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic ↓, total) and HO1 proteins	Chen B. et al., 2016
4VO-10 min	Rat	SD; 320–360 g	Sevoflurane	• Post; inhalation of 2% sevoflurane for 10 min; inhalant; twice after ischemia	• Neuronal necrosis (CA1; via HE, TUNEL; 1, 7 d) ↓	• Neurological deficits (1, 7 d) L;	• Nrf2 (nuclear ↑) and HO1 proteins	Lee et al., 2015
4VO-10 min	Rat	SD; M; 250–300 g	Genistein	• Post; 1 mg/kg; i.c.v.; 5 min after onset of reperfusion • Once	• Neuronal death (CA1; via NeuN, TUNEL; 5 d) ↓	• Morris water maze (7, 8, 9 d) ↓	• Nrf2 (nuclear ↑; cytoplasmic ↓) protein • DNA Binding Activity of Nrf2 • HO1 protein (WB, staining)	Wang et al., 2013
4VO-15 min	Rat	SD; M; 250–300 g	DEETGE-CAL-Tat peptide	• Pre; 50 μg; i.c.v.; 30 min before ischemia; once • Post; 240 μg/d; subcutaneously; 1 d after ischemia until d9; daily	• For pretreatment: • Neuronal Injury (CA1; via NeuN, TUNEL; 7 d) ↓ For posttreatment: • Neuronal Injury (CA1; via NeuN, TUNEL; 9 d) ↓	• Morris water maze (7, 8, 9 d) ↓	• Nrf2 (nuclear ↑, cytoplasmic ↓) protein • Nrf2 DNA binding HO1, NQO1, GPx1, and SOD2	Tu et al., 2015
2VO-20 min	Mouse	C57BL/6; M; 12 wks; 20–24 g	Lycopene	• Pre; 20 mg/kg; i.p.; daily • For 7 d	• Neuronal degeneration (CA1; via HE, TUNEL; 3 d) ↓	• Neurological deficits (1, 2, 3 d) ↓	• Nrf2 (nuclear ↑, total ↑) and HO1 proteins	Lei et al., 2016
Other findings involving the role of Nrf2 pathway								
2VO-permanent	Rat	SD; M; 180–250 g	Sodium butyrate (SB)	• Post; 840 mg/kg; i.p.; 29–56 d after ischemia; daily; • For 28 d	• Immunoreactivity of neuronal/ synaptic proteins (HC; via NeuN; 56 d)	• Morris water maze (50-56 d) ↓ • Novel object recognition (49–56 d)	• Nrf2 (total ↑) protein Nrf2 • down-stream genes mRNA levels (GCLc, HO1, NQO1 and GCLm)	Liu H. et al., 2015

(Continued)

TABLE 4 | Continued

Model	Species	Genetic Background; Sex; Age or Weight	Treatment	Dosage/ Administration route	Brain lesion /Edema	Neurobehavioral deficits	Nrf2 Mechanism (*in vivo*)	References
2VO-permanent	Rat	Wistar; M; 7 wks; 220–250 g	Environment enrichment (EE)	• Post; 6 h/d; daily • For 28 d	• Oxidative neuronal damage (CA1, CA2, CA3; via 4-HNE; 56 d) ↓	• Morris water maze (3-5 d) ↓	• Nrf2 (total ↑) protein	Yang Y. et al., 2015
4VO-30 min	Rat	Wistar; M; 6 mo; 270–290 g	Metformin (MF)	• Pre; 200 mg/kg; gavage; daily • For 14 d	• NA	• NA	• Nrf2 (total ↑; at 68 KDa) and HO1 proteins	Ashabi et al., 2015
2VO-45 min	Rat	Wistar; M; 250–270 g	N6-cyclohexyl adenosine (CHA)	• Post; 6.25 nM in 1 μl; unilateral intrahippocampal injection; immediately after onset of reperfusion; • Once	• Neuronal degeneration (hippocampus; via HE; 1 d)	• Morris water maze (1 d) ↓ • Rotarod (1 d) r • Open field (1 d) ↓	• Nrf2 content (Elisa)	Atef et al., 2018
2VO-8 min	Mouse	C57BL/6; M; 8–10 wks; 20–25 g	Ginkgo biloba/EGb 761 (EGb 761)	• Pre; 100 mg/kg; gavage; daily; • For 7 d	• Neuronal injury (CA1; TUNEL; 7 d) ↓	• NA	• Nrf2 and HO1 (IF)	Tulsulkar and Shah, 2013

MCAO, middle cerebral artery; pdMCAO, permanent distal middle cerebral artery occlusion; pMCAO, permanent (proximal end of) middle cerebral artery occlusion that is generated by the intraluminal suture MCAO; GCI, global cerebral ischemia; i.p., intraperitoneal; i.v., intravenous; i.c.v., intracerebroventricular; Pre, pretreatment; Post, posttreatment; the changes in brain lesion/edema and neurobehavioral deficits/Nrf2 protein expression level rf2 protein expression leveledema and neurobehavioral deficitsntt is generated by the; IF, Immunofluorescence; KDa, kilodalton that indicates Nrf2 protein molecular weight by Western blot; the changes in brain lesion, edema, neurological deficits, and mRNA/protein expression level (↑ or ↓, increase or decrease at indicated time point; no label at indicated time point, no significant difference); h, hour; d, day; wk, week.

neurobehavioral deficits after ischemia. Following permanent ischemia injury without reperfusion, Nrf2 deficiency exacerbated the acute development of brain lesions, revealed by severe infarct volume at 3 days but not at 1 day after pdMCAO, and these mice exhibited relatively poor sensorimotor function over 28 days (Liu et al., 2018). At 2 days after pdMCAO, $Nrf2^{-/-}$ mice had significantly larger cortical infarct volume and more severe neurological deficits than WT controls (Wang B. et al., 2012). Another group showed that Nrf2 deficiency slightly (but not significantly) enlarged the cortical infarct size at 1 day and sustained considerably more damage at 7 day after pdMCAO ($P < 0.01$), which further supports the findings above (Shih et al., 2005). Moreover, it is well-known that reperfusion after a long period of ischemia can exacerbate brain damage. Following cerebral ischemia injury with reperfusion, several studies showed that $Nrf2^{-/-}$ mice exhibited aggravated acute brain damage in infarct volume and neurological deficits scores at 1 day after tMCAO with 1 h (Li et al., 2013; Yao et al., 2016; Zhang et al., 2017), 1.5 h (Shah et al., 2007), and 2 h (Shi et al., 2015) of ischemia. In these studies, no significant difference in blood pH, PaO_2, $PaCO_2$, or cerebral blood fluid (CBF) was observed before, during, or after ischemia-reperfusion between WT and $Nrf2^{-/-}$ at indicated time points as monitored by laser-Doppler flowmetry (Shah et al., 2010). In contrast, another contradictory report showed that no significant difference in infarct volume was observed between $Nrf2^{-/-}$ and WT mice 2 days after 1 h tMCAO (Narayanan et al., 2015). This discrepancy might be related to the different genetic background or age of mice or the experimental setting.

Nrf2 disruption depresses the capacity of the cellular antioxidant system and diminishes upregulation of its target cytoprotective proteins in response to ischemia (**Tables 1–3**). A study showed that, along with severe ischemic brain damage 3 days after pdMCAO, Nrf2 deficiency destroyed the ischemia-induced increase of antioxidant/detoxification proteins NQO1, HO1, SOD2, and Gpx1 (Liu et al., 2018). Oxidative stress and inflammation plays a key role in the pathological process of ischemia. Excessive production of ROS and reactive nitrogen species (RNS) contributes to the activation of inflammatory processes, further aggravating brain injury and functional deficits during or after stroke. Toll-like receptor 4 (TLR 4) initiates inflammatory processes and induces the expression of inflammatory elements IL-6, TNFα, and IL-1β through activation and nuclear translocation of NF-κB in cerebral ischemia/reperfusion injury (Arslan et al., 2010; Wang Y. C. et al., 2011). At 24 h after 1 h tMCAO, Nrf2 knockout upregulated the TLR4 and NF-κB proteins mediated by the inflammatory response in the ischemic cortex (Li et al., 2013).

Astrocytes interact closely with neurons to provide structural and functional support at multiple levels, including ion and water homeostasis, chemical signal transmission, blood flow regulation, immune and oxidative stress defense, and supply of metabolites (Liu et al., 2017). Given that the ARE-regulated genes are preferentially activated in glial cells, which have more effective detoxification and antioxidant capacities than neurons (Vargas and Johnson, 2009; Gan et al., 2012; Haskew-Layton et al., 2013), Nrf2 in glia cells can protect neurons from a wide array of insults like stroke (Vargas and Johnson, 2009). In response to ischemic insults, astrocytes undergo structural and functional changes, and interact with other glial cells to defend neurons (Magaki et al., 2018). Indeed, in the ischemic cortex, Nrf2 deficiency exacerbated reactive astrogliosis, dysfunction in glutamate metabolism, and water permeability within 3 days after pdMCAO. The spatiotemporal pattern of reactive astrogliosis correlated well with acute ischemic damage progression. Interestingly, the microglia activation was affected considerably under Nrf2 absence.

NRF2 TARGETED INTERVENTION IN ISCHEMIC STROKE—ADVANCEMENTS FROM EXPERIMENTAL ISCHEMIC STROKE MODEL

Given the fundamental role of Nrf2 in redox homeostasis, many studies have presented the unique contribution of Nrf2 in neuroprotection against various diseases (Ma, 2013; Kumar et al., 2014; Sun et al., 2017; Cuadrado et al., 2018). For ischemic stroke, Nrf2/ARE activation is expected to trigger a cytoprotective response counteracting deleterious ischemic events. The beneficial effects of some candidate Nrf2 inducers were directly proved by the findings in $Nrf2^{-/-}$ mice. The typical findings were listed below (just to name a few), and more are also depicted in **Tables 1–4**.

Dimethyl Fumarate and Monomethyl Fumarate

Dimethyl fumarate (DMF) and its primary metabolite monomethyl fumarate (MMF) are two typical Nrf2 inducers. Several studies support their neuroprotective efficacy against ischemic brain injury through the activation of the Nrf2/HO1 pathway. DMF and MMF dramatically reduced infarct volume, brain edema, and neurological deficits over 7 days after tMCAO, along with the suppressed acute glial activation (Yao et al., 2016). Importantly, such evident protection was abolished in $Nrf2^{-/-}$ mice, indicating that the Nrf2 pathway is required for the benefit of DMF and MMF. During the late phase (7–14 d), DMF also acted as a potent immunomodulator, reducing the infiltration of neutrophils and T cells and the number of activated microglia and macrophages in the infarct region (Lin et al., 2016). Interestingly, DMF also attenuated brain edema formation during ischemia and stabilized the blood brain barrier (BBB) by preventing disruption of interendothelial tight junctions (Kunze et al., 2015). DMF protected against acute brain damage in edema volume and sensorimotor deficits after pdMCAO through anti-inflammatory cytokines (IL-10 and IL-12p70), further supporting the neuroprotective role of MMF (Clausen et al., 2017).

Sulforaphane

Sulforaphane (SFN), a typical Nrf2 activator, is a naturally occurring isothiocyanate found in cruciferous vegetables. Upregulation of Nrf2 by sulforaphane pretreatment was associated with increased HO1 expression in perivascular astrocytes in the peri-infarct regions and cerebral endothelium of the infarct core. BBB disruption, lesion progression, and

neurological deficits were reduced after tMCAO. This indicates that the Nrf2 defense pathway in the cerebral microvasculature provides a novel therapeutic approach for preventing BBB breakdown and neurological dysfunction in stroke victims (Alfieri et al., 2013). There is a contradictory finding that sulforaphane treatment increased the overall mRNA levels of transcription of Nrf2, Hmox1, GCLC and GSTA4 but failed to reduce the infarct volume and motor deficits in a photothrombotic mouse model (Porritt et al., 2012).

Tert-Butylhydroquinone

Tert-butylhydroquinone (tBHQ) is a Nrf2 inducer widely used as a food additive. Nrf2 activation by tBHQ pretreatment reduced cortical damage and sensorimotor deficits from 24 h up to even 1 month after pdMCAO. Interestingly, larger infarcts were observed in $Nrf2^{-/-}$ mice 7 d after stroke, but not 24 h after stroke (Shih et al., 2005). This Nrf2-specific action of tBHQ *in vivo* was also validated in another study with tMCAO (Shah et al., 2007). A recent study showed that Nrf2 upregulation by tBHQ significantly reduced the expression of cytoplasmic thioredoxin interacting protein, nod-like receptor protein 3 (NLRP3) inflammasome, and downstream factors caspase-1, IL-1β, and IL-18, all of which contribute to the Nrf2-mediated neuroprotection against ischemic outcomes after tMCAO (Hou et al., 2018). Interestingly, there is a contradictory report that tBHQ-exacerbated stroke damage (Sun et al., 2016), revealed by increased post-stroke mortality and worsened outcomes after pMCAO.

MiR-93 Antagomir

MicroRNAs (miRNAs) play vital roles in regulating neuronal survival during cerebral ischemia/reperfusion injury. MiR-93, a direct negative modulator of Nrf2 expression at the transcriptomic level, serves as a potential therapeutic target for acute ischemic stroke. MiR-93 levels in the ischemic cortex of mice increased at 24 h and 48 h after tMCAO. MiR-93 antagomir treatment reduced infarction volume, neural apoptosis and neurological deficits through the Nrf2/HO1 antioxidant pathway (Wang et al., 2016).

Korean Red Ginseng

Korean Red Ginseng (Ginseng) is one of the most widely used herbal medicines with reported antioxidant and anti-inflammatory properties, displaying promising potential in neuroprotection. Ginseng pretreatment ameliorated short- and long-term sensorimotor deficits over 28 days, prevented the acute enlargement of lesion volume, attenuated reactive astroglial progression but not microglial activation, and enhanced the induction of Nrf2 target antioxidant proteins after pdMCAO in WT mice, an effect that was abolished in $Nrf2^{-/-}$ mice (Liu et al., 2018). Nrf2-dependent spatiotemporal reactive astrogliosis correlated well with acute ischemic damage progression. In contrast, Nrf2 deficiency exacerbated the ischemic conditions. This supported the conclusion that Ginseng pretreatment protects against acute sensorimotor deficits and promotes its long-term recovery after pdMCAO, at least partly, through Nrf2 activation. Attenuated reactive astrogliosis contributes to the Nrf2-depedent neuroprotection.

(–)-Epicatechin

The (–)-Epicatechin (EC) is especially abundant in cocoa, dark chocolate, and green tea, and it boosts antioxidant activity while supporting vascular function. EC-treated ischemic WT mice displayed a reduction of forelimb motor coordination impairments associated with reduced anatomical injury and microglia/macrophage activation in pMCAO and tMCAO models of adult WT mice, impairments which were abolished in tissues and neurons from $Nrf2^{-/-}$ mice (Shah et al., 2010). Interestingly, this neuroprotection was observed in 12 month-old WT and $Nrf2^{-/-}$ mice, indicating that age influences Nrf2 function (Leonardo et al., 2013, 2015).

Resveratrol

Resveratrol is a natural polyphenolic compound that is found in some dietary sources such as grapes, plums, and red wine. *Trans*-resveratrol pretreatment had neuroprotective effects on ischemic brain damage after tMCAO. This neuroprotective effect is likely exerted by upregulated expression of Nrf2, HO1 and SOD that ameliorates oxidative damage (Ren et al., 2011). Meanwhile, resveratrol preconditioning-mediated neuroprotection is reduced after tMCAO (Narayanan et al., 2015). WT and $Nrf2^{-/-}$ cortical mitochondria exhibited increased uncoupling and ROS production after resveratrol pre-treatments. $Nrf2^{-/-}$ astrocytes exhibited decreased mitochondrial antioxidant expression and were unable to upregulate cellular antioxidants.

Carbon Monoxide

Carbon monoxide (CO) is a gaseous second messenger produced when heme oxygenase enzymes catabolize heme. At low doses (Otterbein et al., 1999; Koehler and Traystman, 2002; Leffler et al., 2011), despite being traditionally viewed as a toxic agent (Cheng et al., 2012), CO displayed sustained neuroprotective efficacy against brain damage and progressive functional deficits via the Nrf2 pathway in tMCAO and pdMCAO models (Meffert et al., 1994; Maines, 1996; Alkadhi et al., 2001). Brain lesions in mice exposed to CO were 29.6 ± 12.6% smaller at 7 days after pdMCAO. Additionally, 18-h CO treatment led to Nrf2 dissociation from Keap1, nuclear translocation, increased activity of Nrf2 binding to the ARE sequence of the HO1 gene, and elevated HO1 expression from 6 to 48 h after CO exposure, the neuroprotection of which was abolished in $Nrf2^{-/-}$ mice (Wang B. et al., 2011).

Tetramethylpyrazine

Tetramethylpyrazine (TMP) is one of the main components of Ligusticum wallichii Franchat (Chuan Xiong) that has been used to treat neurovascular and cardiovascular diseases including stroke in traditional Chinese medicine. TMP has been found to protect against pdMCAO injury and reduce inflammation and ischemia-induced neutrophils through Nrf2/HO1 activation (Kao et al., 2013; Chang et al., 2015).

Curcumin

Curcumin is a phenolic pigment extracted from the rhizome of *Curuma longa* Linn, which has been widely used as medicine in many Asian countries. It has strong neuro-protective effects on neurological injuries, with virtually no toxicity, even at a high dose; though, its absorption-bioavailability is the main issue, and few groups are actively looking to test innovative formulation to circumvent this serious limitation. Curcumin significantly reduced infarct size, brain edema, neurological dysfunction, and oxidative stress levels in tMCAO rats, and this neuroprotective effect involves the Akt/Nrf2 and NF-κB pathways (Wu J. et al., 2013; Li et al., 2016).

Besides the candidate Nrf2 inducers above, many natural products, synthetic compounds and clinical drugs, such as ginkgo biloba (Tulsulkar and Shah, 2013), paeonol (Zhao et al., 2014), isoquercetin (Chen M. et al., 2017), tanshinone IIA (Cai et al., 2017), ursolic acid (Li et al., 2013), isorhamnetin (Zhao et al., 2016), docosahexaenoic acid (Chang et al., 2013), HP-1c (Wang et al., 2018), gastrodin (Peng et al., 2015), trichostatin A (Wang B. et al., 2012), bicyclol (Zhang J. et al., 2014), recombinant human erythropoietin (Meng et al., 2014), omega-3 fatty acids (Zhang M. et al., 2014), Tocovid (Shang et al., 2018), estradiol (Li et al., 2017), and more, have also been shown to benefit ischemic brain injury through mechanisms involving Nrf2.

PITFALLS AND CONSIDERATIONS OF CURRENT NRF2 EXPERIMENTAL ISCHEMIC STROKE STUDIES

Although most reports support the beneficial effect of Nrf2 activation by a multitude of variety of inducers against ischemic brain damage, many findings regarding the role of Nrf2 during ischemia or ischemic-reperfusion injury are contradictory. This could be attributed to a number of inconsistencies across studies, as briefly overview here. (1) Nrf2 antibody: Getting potent, selective and highly specific antibodies against the various species of Nrf2 has been an ongoing serious issue. Furthermore, what is the correct molecular weight of Nrf2 protein? The biologically relevant molecular weight of Nrf2 protein has been proven to be ∼95–110 KDa, but not ∼55–65 KDa, based on its 2-kb open reading frame or its tertiary structure (Lau et al., 2013; Kemmerer et al., 2015). Many reports presented the expression level of Nrf2 protein by Western blots at apparent different (or unspecified) molecular weights, which might be mainly responsible for the contradictory results among research groups, the lack of good negative controls to be run in parallel should be documented. Additionally, this issue may also contribute to the discrepancy regarding findings of Nrf2 in subcellular distribution and cell-type expression under normal and inducible conditions. (2) Questions remain as to which cell types would be most responsible for the neuroprotection associate with Nrf2. (3) Some reports also questioned the potential issues in the Nrf2 knockout lines and strains. (4) Furthermore, while many of these reported cytoprotective enzymes that are induced by Nrf2 activation, there are activate debates as to whether traditional freely available substrates are sufficiently high to explain the resulting beneficial properties of these metabolites/end-products. (5) While many of these compounds are assumed to be the bioactive entity, there are well-recognized issues on such drugs to be bioavailable and cross the blood brain barrier to then reach the target at a concentration that would be biologically relevant; thus, there is the possibility that indirect pathways are stimulated that would then indirectly activate the Nrf2 pathway. (6) Several of the studies have tested preventive medicine when give before ischemia while others have tested the therapeutic potential of the given drug concoction; though, one should not extrapolate conclusion of the universal protection until it is rigorously tested and independently validated. (7) Nrf2/ARE pathway: Is the Nrf2/ARE pathway activated or not? To validate that Nrf2 is activated, many reports based their conclusions on selected protein level measurements of total Nrf2 with or without its target markers like HO1 by WB. Based on the current understanding of Nrf2 activation, nucleus translocation of Nrf2 is essential to its activation, which is revealed by nuclear Nrf2 protein levels. Evidence that targets distinct components of the Nrf2/ARE pathway cascade will be expected. In addition, the application of pharmacological or genetic blockage of Nrf2 will be helpful to demonstrate whether Nrf2/ARE pathway activation is specific to the neuroprotection of the candidate Nrf2 inducer. (8) Ischemic brain tissue: Which part of the ischemic cerebral hemisphere was used for measurement? Despite variable ischemic damage to different brain regions (or together with reperfusion), various ischemic brain tissue like the cortex, ischemic region, and cerebral hemisphere was used for measurement, possibly leading to inconsistent results. These tissue samples such as the infarct core, peri-infarct, and healthy brain regions, could "dilute" and even mask the changes of markers interest during cerebral ischemia. The apparent alternation of Nrf2 expression happens inside the peri-infarct area. Accordingly, tissue from the same site of the peri-infarct area appears to be appropriate for the subsequent measurement that leads to the final conclusion. (9) Histological and neurobiological outcomes: Which marker and which time points are expected to have the functional benefit of a potent Nrf2 inducer? Most reports only selected infarct volume or neurological deficit score in the acute ischemic stage (1–3 days) without tracking long-term efficacy. As we know, ischemic insult induces severe motor, sensory, emotional, and cognitive deficits (Ferro et al., 2016), and long-term functional recovery is considered to be the goal of stroke intervention. More accurate and long-term histological and functional evaluations are expected for future studies. (10) Nrf2 activators: As mentioned above, a number of Nrf2 activators have been identified that appears to upregulate Nrf2/ARE pathway. However, thus far, only limited compounds including sulforaphane, CDDO-methyl ester, and DMF have consistently indicated sufficient pharmacokinetic and pharmacodynamic properties to act in brain (Lastres-Becker et al., 2016; Yamamoto et al., 2018). Future rigorous studies are needed to provide the necessary information concerning the clinical application prospect of Nrf2 targeted intervention.

This review focusses on Nrf2 activity in the context of ischemic stroke, though, over- or prolonged Nrf2 activation could potentially be problematic. For example, sustained activation of Nrf2 in drosophila would shorten its life

expectancy (Tsakiri et al., 2013), and it would also promote the malignant transformation of human cells (Yang X. et al., 2015). It has also been reported that mutations in Nrf2 and Keap1 would promote cancer cell survival (Shibata et al., 2008); furthermore, many cancer cells would overexpress Nrf2 and this would be associated with cell resistance to cancer therapies (Taguchi and Yamamoto, 2017).

CONCLUSIVE REMARKS AND PERSPECTIVE

Emerging evidence demonstrates that the Nrf2 network plays a crucial role in cellular adaption by controlling a wide range of cytoprotective proteins, counteracting distinct endogenous and exogenous insults while providing a promising optimal therapeutic target against various diseases from cancer to brain disorders. By using various ischemic stroke rodent models, recent preclinical studies provide direct *in vivo* evidence revealing the contribution of the Nrf2 pathway in ischemic stroke pathogenesis and neuroprotection. This review highlighted the promising potential of interventions targeting Nrf2, implying that the moderate activation of Nrf2 favors the attenuation of brain damage and long-term recovery from cerebral ischemia. Because the investigations of Nrf2 in stroke are still in the initial stages, future research is expected to elucidate the natural properties of Nrf2 in stroke leading to the development of novel drugs that target Nrf2.

AUTHOR CONTRIBUTIONS

LL and SD conceived the study and designed the databases analysis. LL prepared the manuscript with input from SD. LL and LML searched databases, collected data, performed analyses, and prepared the tables and figure. All authors reviewed, discussed and approved the final manuscript.

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REFERENCES

Adams, H. P. Jr., and Biller, J. (2015). Classification of subtypes of ischemic stroke: history of the trial of org 10172 in acute stroke treatment classification. *Stroke* 46, e114–117. doi: 10.1161/STROKEAHA.114.007773

Ahmed, S. M., Luo, L., Namani, A., Wang, X. J., and Tang, X. (2017). Nrf2 signaling pathway: pivotal roles in inflammation. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863, 585–597. doi: 10.1016/j.bbadis.2016.11.005

Alfieri, A., Srivastava, S., Siow, R. C., Cash, D., Modo, M., Duchen, M. R., et al. (2013). Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood-brain barrier disruption and neurological deficits in stroke. *Free Radic. Biol. Med.* 65, 1012–1022. doi: 10.1016/j.freeradbiomed.2013.08.190

Alkadhi, K. A., Al-Hijailan, R. S., Malik, K., and Hogan, Y. H. (2001). Retrograde carbon monoxide is required for induction of long-term potentiation in rat superior cervical ganglion. *J. Neurosci.* 21, 3515–3520. doi: 10.1523/JNEUROSCI.21-10-03515.2001

An, P., Wu, T., Yu, H., Fang, K., Ren, Z., and Tang, M. (2018). Hispidulin protects against focal cerebral ischemia reperfusion injury in rats. *J. Mol. Neurosci.* 65, 203–212. doi: 10.1007/s12031-018-1086-2

Arslan, F., Keogh, B., Mcguirk, P., and Parker, A. E. (2010). TLR2 and TLR4 in ischemia reperfusion injury. *Mediators Inflamm.* 2010:704202. doi: 10.1155/2010/704202

Ashabi, G., Khalaj, L., Khodagholi, F., Goudarzvand, M., and Sarkaki, A. (2015). Pre-treatment with metformin activates Nrf2 antioxidant pathways and inhibits inflammatory responses through induction of AMPK after transient global cerebral ischemia. *Metab. Brain Dis.* 30, 747–754. doi: 10.1007/s11011-014-9632-2

Atef, R. M., Agha, A. M., Abdel-Rhaman, A. A., and Nassar, N. N. (2018). The ying and yang of adenosine A1 and A2A receptors on ERK1/2 activation in a rat model of global cerebral ischemia reperfusion injury. *Mol. Neurobiol.* 55, 1284–1298. doi: 10.1007/s12035-017-0401-1

Ayers, D., Baron, B., and Hunter, T. (2015). miRNA Influences in NRF2 pathway interactions within cancer models. *J. Nucl. Acids* 2015:143636. doi: 10.1155/2015/143636

Bai, Q., Lyu, Z., Yang, X., Pan, Z., Lou, J., and Dong, T. (2017). Epigallocatechin-3-gallate promotes angiogenesis via up-regulation of Nfr2 signaling pathway in a mouse model of ischemic stroke. *Behav. Brain Res.* 321, 79–86. doi: 10.1016/j.bbr.2016.12.037

Bang, O. Y., Saver, J. L., Buck, B. H., Alger, J. R., Starkman, S., Ovbiagele, B., et al. (2008). Impact of collateral flow on tissue fate in acute ischaemic stroke. *J. Neurol Neuros. Psychiatry* 79, 625–629. doi: 10.1136/jnnp.2007.132100

Bellezza, I., Giambanco, I., Minelli, A., and Donato, R. (2018). Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim. Biophys. Acta* 1865, 721–733. doi: 10.1016/j.bbamcr.2018.02.010

Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., et al. (2018). Heart disease and stroke statistics-2018 update: a report from the american heart association. *Circulation* 137, e67–e492. doi: 10.1161/CIR.0000000000000558

Bosetti, F., Koenig, J. I., Ayata, C., Back, S. A., Becker, K., Broderick, J. P., et al. (2017). Translational stroke research: vision and opportunities. *Stroke* 48, 2632–2637. doi: 10.1161/STROKEAHA.117.017112

Bryan, H. K., Olayanju, A., Goldring, C. E., and Park, B. K. (2013). The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochem. Pharmacol.* 85, 705–717. doi: 10.1016/j.bcp.2012.11.016

Cai, M., Guo, Y., Wang, S., Wei, H., Sun, S., Zhao, G., et al. (2017). Tanshinone IIA elicits neuroprotective effect through activating the nuclear factor erythroid 2-related factor-dependent antioxidant response. *Rejuvenation Res.* 20, 286–297. doi: 10.1089/rej.2016.1912

Calkins, M. J., Johnson, D. A., Townsend, J. A., Vargas, M. R., Dowell, J. A., Williamson, T. P., et al. (2009). The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. *Antioxid. Redox Signal.* 11, 497–508. doi: 10.1089/ars.2008.2242

Carmichael, S. T. (2005). Rodent models of focal stroke: size, mechanism, and purpose. *NeuroRx* 2, 396–409. doi: 10.1602/neurorx.2.3.396

Chan, J. Y., Han, X. L., and Kan, Y. W. (1993). Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11371–11375. doi: 10.1073/pnas.90.23.11371

Chan, K., Lu, R., Chang, J. C., and Kan, Y. W. (1996). NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. U.S.A.* 93, 13943–13948. doi: 10.1073/pnas.93.24.13943

Chang, C. Y., Kao, T. K., Chen, W. Y., Ou, Y. C., Li, J. R., Liao, S. L., et al. (2015). Tetramethylpyrazine inhibits neutrophil activation following permanent cerebral ischemia in rats. *Biochem. Biophys. Res. Commun.* 463, 421–427. doi: 10.1016/j.bbrc.2015.05.088

Chang, C. Y., Kuan, Y. H., Li, J. R., Chen, W. Y., Ou, Y. C., Pan, H. C., et al. (2013). Docosahexaenoic acid reduces cellular inflammatory response following permanent focal cerebral ischemia in rats. *J. Nutr. Biochem.* 24, 2127–2137. doi: 10.1016/j.jnutbio.2013.08.004

Chen, B., Cao, H., Chen, L., Yang, X., Tian, X., Li, R., et al. (2016). Rifampicin attenuated global cerebral ischemia injury via activating the nuclear factor erythroid 2-related factor pathway. *Front. Cell Neurosci.* 10:273. doi: 10.3389/fncel.2016.00273

Chen, L., Ran, Q., Xiang, Y., Xiang, L., Li, F., Wu, J., et al. (2017). Co-Activation of PKC-delta by CRIF1 modulates oxidative stress in bone marrow multipotent mesenchymal stromal cells after irradiation by phosphorylating NRF2 Ser40. *Theranostics* 7, 2634–2648. doi: 10.7150/thno.17853

Chen, L., Wang, L., Zhang, X., Cui, L., Xing, Y., Dong, L., et al. (2012). The protection by octreotide against experimental ischemic stroke: up-regulated transcription factor Nrf2, HO-1 and down-regulated NF-kappaB expression. *Brain Res.* 1475, 80–87. doi: 10.1016/j.brainres.2012.07.052

Chen, M., Dai, L. H., Fei, A., Pan, S. M., and Wang, H. R. (2017). Isoquercetin activates the ERK1/2-Nrf2 pathway and protects against cerebral ischemia-reperfusion injury *in vivo* and *in vitro*. *Exp. Ther. Med.* 13, 1353–1359. doi: 10.3892/etm.2017.4093

Chen, X., Liu, Y., Zhu, J., Lei, S., Dong, Y., Li, L., et al. (2016). GSK-3beta downregulates Nrf2 in cultured cortical neurons and in a rat model of cerebral ischemia-reperfusion. *Sci. Rep.* 6:20196. doi: 10.1038/srep20196

Cheng, Y., Thomas, A., Mardini, F., Bianchi, S. L., Tang, J. X., Peng, J., et al. (2012). Neurodevelopmental consequences of sub-clinical carbon monoxide exposure in newborn mice. *PLoS ONE* 7:e32029. doi: 10.1371/journal.pone.0032029

Chowdhry, S., Zhang, Y., Mcmahon, M., Sutherland, C., Cuadrado, A., and Hayes, J. D. (2013). Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 32, 3765–3781. doi: 10.1038/onc.2012.388

Chu, C. T. (2018). Mechanisms of selective autophagy and mitophagy: Implications for neurodegenerative diseases. *Neurobiol Dis.* 122, 23–34. doi: 10.1016/j.nbd.2018.07.015

Chumboatong, W., Thummayot, S., Govitrapong, P., Tocharus, C., Jittiwat, J., and Tocharus, J. (2017). Neuroprotection of agomelatine against cerebral ischemia/reperfusion injury through an antiapoptotic pathway in rat. *Neurochem. Int.* 102, 114–122. doi: 10.1016/j.neuint.2016.12.011

Clausen, B. H., Lundberg, L., Yli-Karjanmaa, M., Martin, N. A., Svensson, M., Alfsen, M. Z., et al. (2017). Fumarate decreases edema volume and improves functional outcome after experimental stroke. *Exp. Neurol.* 295, 144–154. doi: 10.1016/j.expneurol.2017.06.011

Croom, E. (2012). Metabolism of xenobiotics of human environments. *Prog. Mol. Biol. Transl. Sci.* 112, 31–88. doi: 10.1016/B978-0-12-415813-9.00003-9

Cuadrado, A., Manda, G., Hassan, A., Alcaraz, M. J., Barbas, C., Daiber, A., et al. (2018). Transcription factor NRF2 as a therapeutic target for chronic diseases: a systems medicine approach. *Pharmacol. Rev.* 70, 348–383. doi: 10.1124/pr.117.014753

Dai, Y., Zhang, H., Zhang, J., and Yan, M. (2018). Isoquercetin attenuates oxidative stress and neuronal apoptosis after ischemia/reperfusion injury via Nrf2-mediated inhibition of the NOX4/ROS/NF-kappaB pathway. *Chem. Biol. Interact.* 284, 32–40. doi: 10.1016/j.cbi.2018.02.017

Ding, Y., Chen, M., Wang, M., Li, Y., and Wen, A. (2015). Posttreatment with 11-keto-beta-boswellic acid ameliorates cerebral ischemia-reperfusion injury: nrf2/ho-1 pathway as a potential mechanism. *Mol. Neurobiol.* 52, 1430–1439. doi: 10.1007/s12035-014-8929-9

Ding, Y., Ren, D., Xu, H., Liu, W., Liu, T., Li, L., et al. (2017). Antioxidant and pro-angiogenic effects of corilagin in rat cerebral ischemia via Nrf2 activation. *Oncotarget* 8, 114816–114828. doi: 10.18632/oncotarget.22023

Dinkova-Kostova, A. T., and Abramov, A. Y. (2015). The emerging role of Nrf2 in mitochondrial function. *Free Radic. Biol. Med.* 88, 179–188. doi: 10.1016/j.freeradbiomed.2015.04.036

Dirnagl, U., Simon, R. P., and Hallenbeck, J. M. (2003). Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci.* 26, 248–254. doi: 10.1016/S0166-2236(03)00071-7

Doré, S. (2015). Neuroprotective effert of carbon monoxide and Nrf2 in cerebral ischemia. *Springerplus* 4:L44. doi: 10.1186/2193-1801-4-S1-L44

Doré, S., and Snyder, S. H. (1999). Neuroprotective action of bilirubin against oxidative stress in primary hippocampal cultures. *Ann. N.Y. Acad. Sci.* 890, 167–172. doi: 10.1111/j.1749-6632.1999.tb07991.x

Doré, S., Takahashi, M., Ferris, C. D., Hester, L. D., Guastella, D., and Snyder, S. H. (1999). Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2445–2450. doi: 10.1073/pnas.96.5.2445

Dorr, A., Sled, J. G., and Kabani, N. (2007). Three-dimensional cerebral vasculature of the CBA mouse brain: a magnetic resonance imaging and micro computed tomography study. *Neuroimage* 35, 1409–1423. doi: 10.1016/j.neuroimage.2006.12.040

Doyle, K. P., and Buckwalter, M. S. (2014). A mouse model of permanent focal ischemia: distal middle cerebral artery occlusion. *Methods Mol. Biol.* 1135, 103–110. doi: 10.1007/978-1-4939-0320-7_9

Durukan, A., Strbian, D., and Tatlisumak, T. (2008). Rodent models of ischemic stroke: a useful tool for stroke drug development. *Curr. Pharm. Des.* 14, 359–370. doi: 10.2174/138161208783497688

Durukan, A., and Tatlisumak, T. (2007). Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. *Pharmacol. Biochem. Behav.* 87, 179–197. doi: 10.1016/j.pbb.2007.04.015

Emberson, J., Lees, K. R., Lyden, P., Blackwell, L., Albers, G., Bluhmki, E., et al. (2014). Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *Lancet* 384, 1929–1935. doi: 10.1016/S0140-6736(14)60584-5

Ewing, J. F., Haber, S. N., and Maines, M. D. (1992). Normal and heat-induced patterns of expression of heme oxygenase-1 (HSP32) in rat brain: hyperthermia causes rapid induction of mRNA and protein. *J. Neurochem.* 58, 1140–1149. doi: 10.1111/j.1471-4159.1992.tb09373.x

Feigin, V. L., Norrving, B., and Mensah, G. A. (2017). Global burden of stroke. *Circ. Res.* 120, 439–448. doi: 10.1161/CIRCRESAHA.116.308413

Ferro, J. M., Caeiro, L., and Figueira, M. L. (2016). Neuropsychiatric sequelae of stroke. *Nat. Rev. Neurol.* 12, 269–280. doi: 10.1038/nrneurol.2016.46

Fonarow, G. C., Smith, E. E., Saver, J. L., Reeves, M. J., Bhatt, D. L., Grau-Sepulveda, M. V., et al. (2011). Timeliness of tissue-type plasminogen activator therapy in acute ischemic stroke: patient characteristics, hospital factors, and outcomes associated with door-to-needle times within 60 minutes. *Circulation* 123, 750–758. doi: 10.1161/CIRCULATIONAHA.110.974675

Friling, R. S., Bensimon, A., Tichauer, Y., and Daniel, V. (1990). Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc. Natl. Acad. Sci. U.S.A.* 87, 6258–6262. doi: 10.1073/pnas.87.16.6258

Gan, L., Vargas, M. R., Johnson, D. A., and Johnson, J. A. (2012). Astrocyte-specific overexpression of Nrf2 delays motor pathology and synuclein aggregation throughout the CNS in the alpha-synuclein mutant (A53T) mouse model. *J. Neurosci.* 32, 17775–17787. doi: 10.1523/JNEUROSCI.3049-12.2012

Guo, C., Wang, S., Duan, J., Jia, N., Zhu, Y., Ding, Y., et al. (2017). Protocatechualdehyde protects against cerebral ischemia-reperfusion-induced oxidative injury via protein kinase cepsilon/Nrf2/HO-1 pathway. *Mol. Neurobiol.* 54, 833–845. doi: 10.1007/s12035-016-9690-z

Guo, H., Adah, D., James, P. B., Liu, Q., Li, G., Ahmadu, P., et al. (2018). Xueshuantong injection (Lyophilized) attenuates cerebral ischemia/reperfusion injury by the activation of Nrf2-VEGF pathway. *Neurochem. Res.* 43, 1096–1103. doi: 10.1007/s11064-018-2523-x

Guo, H., Li, M. J., Liu, Q. Q., Guo, L. L., Ma, M. M., Wang, S. X., et al. (2014). Danhong injection attenuates ischemia/reperfusion-induced brain damage which is associating with Nrf2 levels *in vivo* and *in vitro*. *Neurochem. Res.* 39, 1817–1824. doi: 10.1007/s11064-014-1384-1

Hacke, W., Kaste, M., Bluhmki, E., Brozman, M., Davalos, A., Guidetti, D., et al. (2008). Thrombolysis with alteplase 3 to 4.5 hours after acute

ischemic stroke. *N. Engl. J. Med.* 359, 1317–1329. doi: 10.1056/NEJMoa0804656

Han, J., Wang, M., Jing, X., Shi, H., Ren, M., and Lou, H. (2014). (-)-Epigallocatechin gallate protects against cerebral ischemia-induced oxidative stress via Nrf2/ARE signaling. *Neurochem. Res.* 39, 1292–1299. doi: 10.1007/s11064-014-1311-5

Han, J., Xiao, Q., Lin, Y. H., Zheng, Z. Z., He, Z. D., Hu, J., et al. (2015). Neuroprotective effects of salidroside on focal cerebral ischemia/reperfusion injury involve the nuclear erythroid 2-related factor 2 pathway. *Neural. Regen. Res.* 10, 1989–1996. doi: 10.4103/1673-5374.172317

Haskew-Layton, R. E., Payappilly, J. B., Xu, H., Bennett, S. A., and Ratan, R. R. (2013). 15-Deoxy-Delta12,14-prostaglandin J2 (15d-PGJ2) protects neurons from oxidative death via an Nrf2 astrocyte-specific mechanism independent of PPARgamma. *J. Neurochem.* 124, 536–547. doi: 10.1111/jnc.12107

Hayes, J. D., and Dinkova-Kostova, A. T. (2014). The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* 39, 199–218. doi: 10.1016/j.tibs.2014.02.002

Hou, Y., Wang, Y., He, Q., Li, L., Xie, H., Zhao, Y., et al. (2018). Nrf2 inhibits NLRP3 inflammasome activation through regulating Trx1/TXNIP complex in cerebral ischemia reperfusion injury. *Behav. Brain Res.* 336, 32–39. doi: 10.1016/j.bbr.2017.06.027

Hua, K., Sheng, X., Li, T. T., Wang, L. N., Zhang, Y. H., Huang, Z. J., et al. (2015). The edaravone and 3-n-butylphthalide ring-opening derivative 10b effectively attenuates cerebral ischemia injury in rats. *Acta Pharmacol. Sin.* 36, 917–927. doi: 10.1038/aps.2015.31

Iadecola, C., and Anrather, J. (2011). Stroke research at a crossroad: asking the brain for directions. *Nat. Neurosci.* 14, 1363–1368. doi: 10.1038/nn.2953

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* 236, 313–322. doi: 10.1006/bbrc.1997.6943

Itoh, K., Mimura, J., and Yamamoto, M. (2010). Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxid. Redox Signal.* 13, 1665–1678. doi: 10.1089/ars.2010.3222

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., et al. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes. Dev.* 13, 76–86. doi: 10.1101/gad.13.1.76

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'connor, T., and Yamamoto, M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 8, 379–391. doi: 10.1046/j.1365-2443.2003.00640.x

Jain, A., Lamark, T., Sjottem, E., Larsen, K. B., Awuh, J. A., Overvatn, A., et al. (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.* 285, 22576–22591. doi: 10.1074/jbc.M110.118976

Janyou, A., Wicha, P., Jittiwat, J., Suksamrarn, A., Tocharus, C., and Tocharus, J. (2017). Dihydrocapsaicin attenuates blood brain barrier and cerebral damage in focal cerebral ischemia/reperfusion via oxidative stress and inflammatory. *Sci. Rep.* 7:10556. doi: 10.1038/s41598-017-11181-5

Ji, K., Xue, L., Cheng, J., and Bai, Y. (2016). Preconditioning of H2S inhalation protects against cerebral ischemia/reperfusion injury by induction of HSP70 through PI3K/Akt/Nrf2 pathway. *Brain Res. Bull.* 121, 68–74. doi: 10.1016/j.brainresbull.2015.12.007

Kam, K. Y., Yu, S. J., Jeong, N., Hong, J. H., Jalin, A. M., Lee, S., et al. (2011). p-Hydroxybenzyl alcohol prevents brain injury and behavioral impairment by activating Nrf2, PDI, and neurotrophic factor genes in a rat model of brain ischemia. *Mol. Cells* 31, 209–215. doi: 10.1007/s10059-011-0028-4

Kang, H. J., Hong, Y. B., Kim, H. J., and Bae, I. (2010). CR6-interacting factor 1 (CRIF1) regulates NF-E2-related factor 2 (NRF2) protein stability by proteasome-mediated degradation. *J. Biol. Chem.* 285, 21258–21268. doi: 10.1074/jbc.M109.084590

Kao, T. K., Chang, C. Y., Ou, Y. C., Chen, W. Y., Kuan, Y. H., Pan, H. C., et al. (2013). Tetramethylpyrazine reduces cellular inflammatory response following permanent focal cerebral ischemia in rats. *Exp. Neurol.* 247, 188–201. doi: 10.1016/j.expneurol.2013.04.010

Kasner, S. E., and Grotta, J. C. (1998). Ischemic stroke. *Neurol. Clin.* 16, 355–372. doi: 10.1016/S0733-8619(05)70068-2

Katoh, Y., Iida, K., Kang, M. I., Kobayashi, A., Mizukami, M., Tong, K. I., et al. (2005). Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. *Arch. Biochem. Biophys.* 433, 342–350. doi: 10.1016/j.abb.2004.10.012

Katsuoka, F., and Yamamoto, M. (2016). Small Maf proteins (MafF, MafG, MafK): history, structure and function. *Gene* 586, 197–205. doi: 10.1016/j.gene.2016.03.058

Kemmerer, Z. A., Ader, N. R., Mulroy, S. S., and Eggler, A. L. (2015). Comparison of human Nrf2 antibodies: a tale of two proteins. *Toxicol. Lett.* 238, 83–89. doi: 10.1016/j.toxlet.2015.07.004

Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 47, 89–116. doi: 10.1146/annurev.pharmtox.46.120604.141046

Kerins, M. J., and Ooi, A. (2017). The Roles of NRF2 in Modulating Cellular Iron Homeostasis. *Antioxid Redox Signal* 29, 1756–1773. doi: 10.1089/ars.2017.7176

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., et al. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell. Biol.* 24, 7130–7139. doi: 10.1128/MCB.24.16.7130-7139.2004

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., et al. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat. Commun.* 7:11624. doi: 10.1038/ncomms11624

Koehler, R. C., and Traystman, R. J. (2002). Cerebrovascular effects of carbon monoxide. *Antioxid. Redox Signal.* 4, 279–290. doi: 10.1089/152308602753666334

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., et al. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat. Cell. Biol.* 12, 213–223. doi: 10.1038/ncb2021

Kumar, H., Kim, I. S., More, S. V., Kim, B. W., and Choi, D. K. (2014). Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases. *Nat. Prod. Rep.* 31, 109–139. doi: 10.1039/C3NP70065H

Kunze, R., Urrutia, A., Hoffmann, A., Liu, H., Helluy, X., Pham, M., et al. (2015). Dimethyl fumarate attenuates cerebral edema formation by protecting the blood-brain barrier integrity. *Exp. Neurol.* 266, 99–111. doi: 10.1016/j.expneurol.2015.02.022

Kuo, P. C., Yu, I. C., Scofield, B. A., Brown, D. A., Curfman, E. T., Paraiso, H. C., et al. (2017). 3H-1,2-Dithiole-3-thione as a novel therapeutic agent for the treatment of ischemic stroke through Nrf2 defense pathway. *Brain Behav. Immun.* 62, 180–192. doi: 10.1016/j.bbi.2017.01.018

Kurinna, S., and Werner, S. (2015). NRF2 and microRNAs: new but awaited relations. *Biochem. Soc. Trans.* 43, 595–601. doi: 10.1042/BST20140317

Lastres-Becker, I., Garcia-Yague, A. J., Scannevin, R. H., Casarejos, M. J., Kugler, S., Rabano, A., et al. (2016). Repurposing the NRF2 activator dimethyl fumarate as therapy against synucleinopathy in parkinson's disease. *Antioxid. Redox Signal.* 25, 61–77. doi: 10.1089/ars.2015.6549

Lau, A., Tian, W., Whitman, S. A., and Zhang, D. D. (2013). The predicted molecular weight of Nrf2: it is what it is not. *Antioxid. Redox Signal.* 18, 91–93. doi: 10.1089/ars.2012.4754

Lau, A., Wang, X. J., Zhao, F., Villeneuve, N. F., Wu, T., Jiang, T., et al. (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol. Cell. Biol.* 30, 3275–3285. doi: 10.1128/MCB.00248-10

Lee, H., Park, Y. H., Jeon, Y. T., Hwang, J. W., Lim, Y. J., Kim, E., et al. (2015). Sevoflurane post-conditioning increases nuclear factor erythroid 2-related factor and haemoxygenase-1 expression via protein kinase C pathway in a rat model of transient global cerebral ischaemia. *Br. J. Anaesth.* 114, 307–318. doi: 10.1093/bja/aeu268

Leffler, C. W., Parfenova, H., and Jaggar, J. H. (2011). Carbon monoxide as an endogenous vascular modulator. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1–H11. doi: 10.1152/ajpheart.00230.2011

Lei, X., Lei, L., Zhang, Z., and Cheng, Y. (2016). Neuroprotective effects of lycopene pretreatment on transient global cerebral ischemiareperfusion in rats:

the role of the Nrf2/HO1 signaling pathway. *Mol. Med. Rep.* 13, 412–418. doi: 10.3892/mmr.2015.4534

Leonardo, C. C., Agrawal, M., Singh, N., Moore, J. R., Biswal, S., and Dor,é, S. (2013). Oral administration of the flavanol (-)-epicatechin bolsters endogenous protection against focal ischemia through the Nrf2 cytoprotective pathway. *Eur. J. Neurosci.* 38, 3659–3668. doi: 10.1111/ejn.12362

Leonardo, C. C., and Doré, S. (2011). Dietary flavonoids are neuroprotective through Nrf2-coordinated induction of endogenous cytoprotective proteins. *Nutr. Neurosci.* 14, 226–236. doi: 10.1179/1476830511Y.0000000013

Leonardo, C. C., Mendes, M., Ahmad, A. S., and Doré, S. (2015). Efficacy of prophylactic flavan-3-ol in permanent focal ischemia in 12-mo-old mice. *Am. J. Physiol. Heart Circ. Physiol.* 308, H583–591. doi: 10.1152/ajpheart.00239.2014

Li, B., Sun, J., Lv, G., Yu, Y., Wang, G., Xie, K., et al. (2014). Sevoflurane postconditioning attenuates cerebral ischemia-reperfusion injury via protein kinase B/nuclear factor-erythroid 2-related factor 2 pathway activation. *Int. J. Dev. Neurosci.* 38, 79–86. doi: 10.1016/j.ijdevneu.2014.08.005

Li, L., Chen, J., Sun, S., Zhao, J., Dong, X., and Wang, J. (2017). Effects of estradiol on autophagy and Nrf-2/ARE signals after cerebral ischemia. *Cell. Physiol. Biochem.* 41, 2027–2036. doi: 10.1159/000475433

Li, L., Zhang, X., Cui, L., Wang, L., Liu, H., Ji, H., et al. (2013). Ursolic acid promotes the neuroprotection by activating Nrf2 pathway after cerebral ischemia in mice. *Brain Res.* 1497, 32–39. doi: 10.1016/j.brainres.2012.12.032

Li, L., Zhu, K., Liu, Y., Wu, X., Wu, J., Zhao, Y., et al. (2015). Targeting thioredoxin-1 with siRNA exacerbates oxidative stress injury after cerebral ischemia/reperfusion in rats. *Neuroscience* 284, 815–823. doi: 10.1016/j.neuroscience.2014.10.066

Li, W., Suwanwela, N. C., and Patumraj, S. (2016). Curcumin by down-regulating NF-kB and elevating Nrf2, reduces brain edema and neurological dysfunction after cerebral I/R. *Microvasc. Res.* 106, 117–127. doi: 10.1016/j.mvr.2015.12.008

Liebeskind, D. S. (2010). Reperfusion for acute ischemic stroke: arterial revascularization and collateral therapeutics. *Curr. Opin. Neurol.* 23, 36–45. doi: 10.1097/WCO.0b013e328334da32

Lin, M. T., and Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 443, 787–795. doi: 10.1038/nature05292

Lin, R., Cai, J., Kostuk, E. W., Rosenwasser, R., and Iacovitti, L. (2016). Fumarate modulates the immune/inflammatory response and rescues nerve cells and neurological function after stroke in rats. *J. Neuroinflammation* 13:269. doi: 10.1186/s12974-016-0733-1

Liu, B., Teschemacher, A. G., and Kasparov, S. (2017). Astroglia as a cellular target for neuroprotection and treatment of neuro-psychiatric disorders. *Glia* 65, 1205–1226. doi: 10.1002/glia.23136

Liu, H., Zhang, J. J., Li, X., Yang, Y., Xie, X. F., and Hu, K. (2015). Post-occlusion administration of sodium butyrate attenuates cognitive impairment in a rat model of chronic cerebral hypoperfusion. *Pharmacol. Biochem. Behav.* 135, 53–59. doi: 10.1016/j.pbb.2015.05.012

Liu, L., Vollmer, M. K., Fernandez, V. M., Dweik, Y., Kim, H., and Doré, S. (2018). Korean red ginseng pretreatment protects against long-term sensorimotor deficits after ischemic stroke likely through Nrf2. *Front. Cell. Neurosci.* 12:74.. doi: 10.3389/fncel.2018.00074

Liu, Y., Zhang, L., and Liang, J. (2015). Activation of the Nrf2 defense pathway contributes to neuroprotective effects of phloretin on oxidative stress injury after cerebral ischemia/reperfusion in rats. *J. Neurol. Sci.* 351, 88–92. doi: 10.1016/j.jns.2015.02.045

Lo, E. H., Dalkara, T., and Moskowitz, M. A. (2003). Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci.* 4, 399–415. doi: 10.1038/nrn1106

Lou, J., Cao, G., Li, R., Liu, J., Dong, Z., and Xu, L. (2016). beta-Caryophyllene attenuates focal cerebral ischemia-reperfusion injury by Nrf2/HO-1 pathway in rats. *Neurochem. Res.* 41, 1291–1304. doi: 10.1007/s11064-016-1826-z

Lu, H., Wang, B., Cui, N., and Zhang, Y. (2018). Artesunate suppresses oxidative and inflammatory processes by activating Nrf2 and ROSdependent p38 MAPK and protects against cerebral ischemiareperfusion injury. *Mol. Med. Rep.* 17, 6639–6646. doi: 10.3892/mmr.2018.8666

Lv, C., Maharjan, S., Wang, Q., Sun, Y., Han, X., Wang, S., et al. (2017). alpha-lipoic acid promotes neurological recovery after ischemic stroke by activating the Nrf2/HO-1 pathway to attenuate oxidative damage. *Cell. Physiol. Biochem.* 43, 1273–1287. doi: 10.1159/000481840

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Magaki, S. D., Williams, C. K., and Vinters, H. V. (2018). Glial function (and dysfunction) in the normal and ischemic brain. *Neuropharmacology* 134, 218–225. doi: 10.1016/j.neuropharm.2017.11.009

Maines, M. (1996). Carbon monoxide and nitric oxide homology: differential modulation of heme oxygenases in brain and detection of protein and activity. *Methods Enzymol.* 268, 473–488. doi: 10.1016/S0076-6879(96)68049-5

Malloy, M. T., Mcintosh, D. J., Walters, T. S., Flores, A., Goodwin, J. S., and Arinze, I. J. (2013). Trafficking of the transcription factor Nrf2 to promyelocytic leukemia-nuclear bodies: implications for degradation of NRF2 in the nucleus. *J. Biol. Chem.* 288, 14569–14583. doi: 10.1074/jbc.M112.437392

Martini, S. R., and Kent, T. A. (2007). Hyperglycemia in acute ischemic stroke: a vascular perspective. *J. Cereb. Blood Flow Metab.* 27, 435–451. doi: 10.1038/sj.jcbfm.9600355

Mathur, A., Pandey, V. K., and Kakkar, P. (2018). Activation of GSK3beta/beta-TrCP axis via PHLPP1 exacerbates Nrf2 degradation leading to impairment in cell survival pathway during diabetic nephropathy. *Free Radic. Biol. Med.* 120, 414–424. doi: 10.1016/j.freeradbiomed.2018.04.550

McMahon, M., Lamont, D. J., Beattie, K. A., and Hayes, J. D. (2010). Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18838–18843. doi: 10.1073/pnas.1007387107

Meffert, M. K., Haley, J. E., Schuman, E. M., Schulman, H., and Madison, D. V. (1994). Inhibition of hippocampal heme oxygenase, nitric oxide synthase, and long-term potentiation by metalloporphyrins. *Neuron* 13, 1225–1233. doi: 10.1016/0896-6273(94)90060-4

Mehndiratta, P., Chapman Smith, S., and Worrall, B. B. (2015). Etiologic stroke subtypes: updated definition and efficient workup strategies. *Curr. Treat. Options Cardiovasc. Med.* 17:357. doi: 10.1007/s11936-014-0357-7

Mehta, S. L., Manhas, N., and Raghubir, R. (2007). Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res. Rev.* 54, 34–66. doi: 10.1016/j.brainresrev.2006.11.003

Meng, H., Guo, J., Wang, H., Yan, P., Niu, X., and Zhang, J. (2014). Erythropoietin activates Keap1-Nrf2/ARE pathway in rat brain after ischemia. *Int. J. Neurosci.* 124, 362–368. doi: 10.3109/00207454.2013.848439

Miao, Z. Y., Xia, X., Che, L., and Song, Y. T. (2018). Genistein attenuates brain damage induced by transient cerebral ischemia through up-regulation of Nrf2 expression in ovariectomized rats. *Neurol. Res.* 40, 689–695. doi: 10.1080/01616412.2018.1462879

Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. U.S.A.* 91, 9926–9930. doi: 10.1073/pnas.91.21.9926

Motohashi, H., Fujita, R., Takayama, M., Inoue, A., Katsuoka, F., Bresnick, E. H., et al. (2011). Molecular determinants for small Maf protein control of platelet production. *Mol. Cell. Biol.* 31, 151–162. doi: 10.1128/MCB.00798-10

Mrsic-Pelcic, J., Pilipovic, K., Pelcic, G., Vitezic, D., and Zupan, G. (2017). Decrease in oxidative stress parameters after post-ischaemic recombinant human erythropoietin administration in the hippocampus of rats exposed to focal cerebral ischaemia. *Basic Clin. Pharmacol. Toxicol.* 121, 453–464. doi: 10.1111/bcpt.12833

Nakano, Y., Yamashita, T., Li, Q., Sato, K., Ohta, Y., Morihara, R., et al. (2017). Time-dependent change of *in vivo* optical imaging of oxidative stress in a mouse stroke model. *J. Neurosci. Res.* 95, 2030–2039. doi: 10.1002/jnr.24047

Narayanan, S. V., Dave, K. R., Saul, I., and Perez-Pinzon, M. A. (2015). Resveratrol preconditioning protects against cerebral ischemic injury via nuclear erythroid 2-related factor 2. *Stroke* 46, 1626–1632. doi: 10.1161/STROKEAHA.115.008921

Ostrowski, R. P., Stepien, K., Pucko, E., and Matyja, E. (2016). Hyperbaric oxygen modalities are differentially effective in distinct brain ischemia models. *Med. Gas. Res.* 6, 39–47. doi: 10.4103/2045-9912.179344

Otterbein, L. E., Mantell, L. L., and Choi, A. M. (1999). Carbon monoxide provides protection against hyperoxic lung injury. *Am. J. Physiol.* 276, L688–L694. doi: 10.1152/ajplung.1999.276.4.L688

Pang, T., Wang, Y. J., Gao, Y. X., Xu, Y., Li, Q., Zhou, Y. B., et al. (2016). A novel GSK-3beta inhibitor YQ138 prevents neuronal injury induced by glutamate and brain ischemia through activation of the Nrf2 signaling pathway. *Acta Pharmacol. Sin.* 37, 741–752. doi: 10.1038/aps.2016.3

Peng, B., Zhao, P., Lu, Y. P., Chen, M. M., Sun, H., Wu, X. M., et al. (2013). Z-ligustilide activates the Nrf2/HO-1 pathway and protects against cerebral ischemia-reperfusion injury *in vivo* and *in vitro*. *Brain Res.* 1520, 168–177. doi: 10.1016/j.brainres.2013.05.009

Peng, Z., Wang, S., Chen, G., Cai, M., Liu, R., Deng, J., et al. (2015). Gastrodin alleviates cerebral ischemic damage in mice by improving anti-oxidant and anti-inflammation activities and inhibiting apoptosis pathway. *Neurochem. Res.* 40, 661–673. doi: 10.1007/s11064-015-1513-5

Porritt, M. J., Andersson, H. C., Hou, L., Nilsson, A., Pekna, M., Pekny, M., et al. (2012). Photothrombosis-induced infarction of the mouse cerebral cortex is not affected by the Nrf2-activator sulforaphane. *PLoS ONE* 7:e41090. doi: 10.1371/journal.pone.0041090

Prabhakaran, S., Ruff, I., and Bernstein, R. A. (2015). Acute stroke intervention: a systematic review. *JAMA* 313, 1451–1462. doi: 10.1001/jama.2015.3058

Quinn, T. J., Elliott, E., and Langhorne, P. (2018). Cognitive and mood assessment tools for use in stroke. *Stroke* 49, 483–490. doi: 10.1161/STROKEAHA.117.016994

Rada, P., Rojo, A. I., Evrard-Todeschi, N., Innamorato, N. G., Cotte, A., Jaworski, T., et al. (2012). Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis. *Mol. Cell. Biol.* 32, 3486–3499. doi: 10.1128/MCB.00180-12

Raghunath, A., Sundarraj, K., Nagarajan, R., Arfuso, F., Bian, J., Kumar, A. P., et al. (2018). Antioxidant response elements: discovery, classes, regulation and potential applications. *Redox Biol.* 17, 297–314. doi: 10.1016/j.redox.2018.05.002

Ren, J., Fan, C., Chen, N., Huang, J., and Yang, Q. (2011). Resveratrol pretreatment attenuates cerebral ischemic injury by upregulating expression of transcription factor Nrf2 and HO-1 in rats. *Neurochem. Res.* 36, 2352–2362. doi: 10.1007/s11064-011-0561-8

Rojo de la Vega, M., Chapman, E., and Zhang, D. D. (2018). NRF2 and the hallmarks of cancer. *Cancer Cell* 34, 21–43. doi: 10.1016/j.ccell.2018.03.022

Romano, J. G., and Sacco, R. L. (2015). Decade in review-stroke: progress in acute ischaemic stroke treatment and prevention. *Nat. Rev. Neurol.* 11, 619–621. doi: 10.1038/nrneurol.2015.199

Rouault, T. A. (2013). Iron metabolism in the CNS: implications for neurodegenerative diseases. *Nat. Rev. Neurosci.* 14, 551–564. doi: 10.1038/nrn3453

Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991). The antioxidant responsive element. activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* 266, 11632–11639.

Sakata, H., Niizuma, K., Yoshioka, H., Kim, G. S., Jung, J. E., Katsu, M., et al. (2012). Minocycline-preconditioned neural stem cells enhance neuroprotection after ischemic stroke in rats. *J. Neurosci.* 32, 3462–3473. doi: 10.1523/JNEUROSCI.5686-11.2012

Sandercock, P., Wardlaw, J. M., Lindley, R. I., Dennis, M., Cohen, G., Murray, G., et al. (2012). The benefits and harms of intravenous thrombolysis with recombinant tissue plasminogen activator within 6 h of acute ischaemic stroke (the third international stroke trial [IST-3]): a randomised controlled trial. *Lancet* 379, 2352–2363. doi: 10.1016/S0140-6736(12)60768-5

Shah, Z. A., Li, R. C., Ahmad, A. S., Kensler, T. W., Yamamoto, M., Biswal, S., et al. (2010). The flavanol (-)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway. *J. Cereb. Blood Flow Metab.* 30, 1951–1961. doi: 10.1038/jcbfm.2010.53

Shah, Z. A., Li, R. C., Thimmulappa, R. K., Kensler, T. W., Yamamoto, M., Biswal, S., et al. (2007). Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. *Neuroscience* 147, 53–59. doi: 10.1016/j.neuroscience.2007.02.066

Shang, J., Yan, H., Jiao, Y., Ohta, Y., Liu, X., Li, X., et al. (2018). Therapeutic effects of pretreatment with tocovid on oxidative stress in postischemic mice brain. *J. Stroke Cerebrovasc. Dis.* 27, 2096–2105. doi: 10.1016/j.jstrokecerebrovasdis.2018.03.012

Shen, Q., and Duong, T. Q. (2008). Quantitative prediction of ischemic stroke tissue fate. *NMR Biomed.* 21, 839–848. doi: 10.1002/nbm.1264

Shi, H., Jing, X., Wei, X., Perez, R. G., Ren, M., Zhang, X., et al. (2015). S-allyl cysteine activates the Nrf2-dependent antioxidant response and protects neurons against ischemic injury *in vitro* and *in vivo*. *J. Neurochem.* 133, 298–308. doi: 10.1111/jnc.12986

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., et al. (2008). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13568–13573. doi: 10.1073/pnas.0806268105

Shih, A. Y., Li, P., and Murphy, T. H. (2005). A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia *in vivo*. *J. Neurosci.* 25, 10321–10335. doi: 10.1523/JNEUROSCI.4014-05.2005

Sicard, K. M., and Fisher, M. (2009). Animal models of focal brain ischemia. *Exp. Transl. Stroke Med.* 1:7. doi: 10.1186/2040-7378-1-7

Srivastava, S., Alfieri, A., Siow, R. C., Mann, G. E., and Fraser, P. A. (2013). Temporal and spatial distribution of Nrf2 in rat brain following stroke: quantification of nuclear to cytoplasmic Nrf2 content using a novel immunohistochemical technique. *J. Physiol.* 591, 3525–3538. doi: 10.1113/jphysiol.2013.257964

Sun, J., Hu, H., Ren, X., and Simpkins, J. W. (2016). Tert-butylhydroquinone compromises survival in murine experimental stroke. *Neurotoxicol. Teratol.* 54, 15–21. doi: 10.1016/j.ntt.2016.01.004

Sun, Y., Yang, T., Leak, R. K., Chen, J., and Zhang, F. (2017). Preventive and protective roles of dietary Nrf2 activators against central nervous system diseases. *CNS Neurol. Disord. Drug Targets* 16, 326–338. doi: 10.2174/1871527316666170102120211

Suzuki, T., Motohashi, H., and Yamamoto, M. (2013). Toward clinical application of the Keap1-Nrf2 pathway. *Trends Pharmacol. Sci.* 34, 340–346. doi: 10.1016/j.tips.2013.04.005

Suzuki, T., and Yamamoto, M. (2015). Molecular basis of the Keap1-Nrf2 system. *Free Radic. Biol. Med.* 88, 93–100. doi: 10.1016/j.freeradbiomed.2015.06.006

Suzuki, T., and Yamamoto, M. (2017). Stress-sensing mechanisms and the physiological roles of the Keap1-Nrf2 system during cellular stress. *J. Biol. Chem.* 292, 16817–16824. doi: 10.1074/jbc.R117.800169

Taguchi, K., and Yamamoto, M. (2017). The KEAP1-NRF2 system in cancer. *Front. Oncol.* 7:85. doi: 10.3389/fonc.2017.00085

Takagi, T., Kitashoji, A., Iwawaki, T., Tsuruma, K., Shimazawa, M., Yoshimura, S., et al. (2014). Temporal activation of Nrf2 in the penumbra and Nrf2 activator-mediated neuroprotection in ischemia-reperfusion injury. *Free Radic. Biol. Med.* 72, 124–133. doi: 10.1016/j.freeradbiomed.2014.04.009

Tanaka, N., Ikeda, Y., Ohta, Y., Deguchi, K., Tian, F., Shang, J., et al. (2011). Expression of Keap1-Nrf2 system and antioxidative proteins in mouse brain after transient middle cerebral artery occlusion. *Brain Res.* 1370, 246–253. doi: 10.1016/j.brainres.2010.11.010

Tonelli, C., Chio, I. I. C., and Tuveson, D. A. (2017). Transcriptional Regulation by Nrf2. *Antioxid Redox Signal.* doi: 10.1089/ars.2017.7342

Traystman, R. J. (2003). Animal models of focal and global cerebral ischemia. *ILAR J.* 44, 85–95. doi: 10.1093/ilar.44.2.85

Tsakiri, E. N., Sykiotis, G. P., Papassideri, I. S., Terpos, E., Dimopoulos, M. A., Gorgoulis, V. G., et al. (2013). Proteasome dysfunction in drosophila signals to an Nrf2-dependent regulatory circuit aiming to restore proteostasis and prevent premature aging. *Aging Cell* 12, 802–813. doi: 10.1111/acel.12111

Tu, J., Zhang, X., Zhu, Y., Dai, Y., Li, N., Yang, F., et al. (2015). Cell-permeable peptide targeting the Nrf2-Keap1 interaction: a potential novel therapy for global cerebral ischemia. *J. Neurosci.* 35, 14727–14739. doi: 10.1523/JNEUROSCI.1304-15.2015

Tulsulkar, J., and Shah, Z. A. (2013). Ginkgo biloba prevents transient global ischemia-induced delayed hippocampal neuronal death through antioxidant and anti-inflammatory mechanism. *Neurochem. Int.* 62, 189–197. doi: 10.1016/j.neuint.2012.11.017

Vargas, M. R., and Johnson, J. A. (2009). The Nrf2-ARE cytoprotective pathway in astrocytes. *Expert Rev. Mol. Med.* 11:e17. doi: 10.1017/S1462399409001094

Wang, B., Cao, W., Biswal, S., and Doré, S. (2011). Carbon monoxide-activated Nrf2 pathway leads to protection against permanent focal cerebral ischemia. *Stroke* 42, 2605–2610. doi: 10.1161/STROKEAHA.110.607101

Wang, B., Zhu, X., Kim, Y., Li, J., Huang, S., Saleem, S., et al. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Radic. Biol. Med.* 52, 928–936. doi: 10.1016/j.freeradbiomed.2011.12.006

Wang, J., Fields, J., Zhao, C., Langer, J., Thimmulappa, R. K., Kensler, T. W., et al. (2007). Role of Nrf2 in protection against intracerebral hemorrhage injury in mice. *Free Radic. Biol. Med.* 43, 408–414. doi: 10.1016/j.freeradbiomed.2007.04.020

Wang, L., Lu, Y., Deng, S., Zhang, Y., Yang, L., Guan, Y., et al. (2012). SHPS-1 deficiency induces robust neuroprotection against experimental stroke by attenuating oxidative stress. *J. Neurochem.* 122, 834–843. doi: 10.1111/j.1471-4159.2012.07818.x

Wang, N., Zhang, L., Lu, Y., Zhang, M., Zhang, Z., Wang, K., et al. (2017). Down-regulation of microRNA-142-5p attenuates oxygen-glucose deprivation and reoxygenation-induced neuron injury through up-regulating Nrf2/ARE signaling pathway. *Biomed. Pharmacother.* 89, 1187–1195. doi: 10.1016/j.biopha.2017.03.011

Wang, P., Liang, X., Lu, Y., Zhao, X., and Liang, J. (2016). MicroRNA-93 downregulation ameliorates cerebral ischemic injury through the Nrf2/HO-1 defense pathway. *Neurochem. Res.* 41, 2627–2635. doi: 10.1007/s11064-016-1975-0

Wang, R., Tu, J., Zhang, Q., Zhang, X., Zhu, Y., Ma, W., et al. (2013). Genistein attenuates ischemic oxidative damage and behavioral deficits via eNOS/Nrf2/HO-1 signaling. *Hippocampus* 23, 634–647. doi: 10.1002/hipo.22126

Wang, Y., Huang, Y., Xu, Y., Ruan, W., Wang, H., Zhang, Y., et al. (2018). A dual AMPK/Nrf2 activator reduces brain inflammation after stroke by enhancing microglia M2 polarization. *Antioxid. Redox Signal.* 28, 141–163. doi: 10.1089/ars.2017.7003

Wang, Y. C., Lin, S., and Yang, Q. W. (2011). Toll-like receptors in cerebral ischemic inflammatory injury. *J. Neuroinflammation* 8:134. doi: 10.1186/1742-2094-8-134

Wardyn, J. D., Ponsford, A. H., and Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-kappaB response pathways. *Biochem. Soc. Trans.* 43, 621–626. doi: 10.1042/BST20150014

Wasserman, W. W., and Fahl, W. E. (1997). Functional antioxidant responsive elements. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5361–5366. doi: 10.1073/pnas.94.10.5361

Wicha, P., Tocharus, J., Janyou, A., Jittiwat, J., Changtam, C., Suksamrarn, A., et al. (2017). Hexahydrocurcumin protects against cerebral ischemia/reperfusion injury, attenuates inflammation, and improves antioxidant defenses in a rat stroke model. *PLoS ONE* 12:e0189211. doi: 10.1371/journal.pone.0189211

Wu, G., Zhu, L., Yuan, X., Chen, H., Xiong, R., Zhang, S., et al. (2017). Britanin ameliorates cerebral ischemia-reperfusion injury by inducing the Nrf2 protective pathway. *Antioxid. Redox Signal.* 27, 754–768. doi: 10.1089/ars.2016.6885

Wu, J., Chen, Y., Yu, S., Li, L., Zhao, X., Li, Q., et al. (2017a). Neuroprotective effects of sulfiredoxin-1 during cerebral ischemia/reperfusion oxidative stress injury in rats. *Brain Res. Bull.* 132, 99–108. doi: 10.1016/j.brainresbull.2017.05.012

Wu, J., Jin, Z., Yang, X., and Yan, L. J. (2018). Post-ischemic administration of 5-methoxyindole-2-carboxylic acid at the onset of reperfusion affords neuroprotection against stroke injury by preserving mitochondrial function and attenuating oxidative stress. *Biochem. Biophys. Res. Commun.* 497, 444–450. doi: 10.1016/j.bbrc.2018.02.106

Wu, J., Li, Q., Wang, X., Yu, S., Li, L., Wu, X., et al. (2013). Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway. *PLoS ONE* 8:e59843. doi: 10.1371/journal.pone.0059843

Wu, J., Li, R., Li, W., Ren, M., Thangthaeng, N., Sumien, N., et al. (2017b). Administration of 5-methoxyindole-2-carboxylic acid that potentially targets mitochondrial dihydrolipoamide dehydrogenase confers cerebral preconditioning against ischemic stroke injury. *Free Radic. Biol. Med.* 113, 244–254. doi: 10.1016/j.freeradbiomed.2017.10.008

Wu, L., Liu, Z. J., Miao, S., Zou, L. B., Cai, L., Wu, P., et al. (2013). Lipoxin A4 ameliorates cerebral ischaemia/reperfusion injury through upregulation of nuclear factor erythroid 2-related factor 2. *Neurol. Res.* 35, 968–975. doi: 10.1179/1743132813Y.0000000242

Wu, S., Yue, Y., Li, J., Li, Z., Li, X., Niu, Y., et al. (2015). Procyanidin B2 attenuates neurological deficits and blood-brain barrier disruption in a rat model of cerebral ischemia. *Mol. Nutr. Food Res.* 59, 1930–1941. doi: 10.1002/mnfr.201500181

Wu, S., Yue, Y., Peng, A., Zhang, L., Xiang, J., Cao, X., et al. (2016). Myricetin ameliorates brain injury and neurological deficits via Nrf2 activation after experimental stroke in middle-aged rats. *Food Funct.* 7, 2624–2634. doi: 10.1039/C6FO00419A

Xian, J. W., Choi, A. Y., Lau, C. B., Leung, W. N., Ng, C. F., and Chan, C. W. (2016). Gastrodia and uncaria (tianma gouteng) water extract exerts antioxidative and antiapoptotic effects against cerebral ischemia *in vitro* and *in vivo*. *Chin. Med.* 11:27. doi: 10.1186/s13020-016-0097-6

Ya, B. L., Li, H. F., Wang, H. Y., Wu, F., Xin, Q., Cheng, H. J., et al. (2017). 5-HMF attenuates striatum oxidative damage via Nrf2/ARE signaling pathway following transient global cerebral ischemia. *Cell Stress Chaperones* 22, 55–65. doi: 10.1007/s12192-016-0742-0

Yamamoto, M., Kensler, T. W., and Motohashi, H. (2018). The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol. Rev.* 98, 1169–1203. doi: 10.1152/physrev.00023.2017

Yamauchi, K., Nakano, Y., Imai, T., Takagi, T., Tsuruma, K., Shimazawa, M., et al. (2016). A novel nuclear factor erythroid 2-related factor 2 (Nrf2) activator RS9 attenuates brain injury after ischemia reperfusion in mice. *Neuroscience* 333, 302–310. doi: 10.1016/j.neuroscience.2016.07.035

Yang, J., Su, J., Wan, F., Yang, N., Jiang, H., Fang, M., et al. (2017). Tissue kallikrein protects against ischemic stroke by suppressing TLR4/NF-kappaB and activating Nrf2 signaling pathway in rats. *Exp. Ther. Med.* 14, 1163–1170. doi: 10.3892/etm.2017.4614

Yang, X., Wang, D., Ma, Y., Xu, X., Zhu, Z., Wang, X., et al. (2015). Continuous activation of Nrf2 and its target antioxidant enzymes leads to arsenite-induced malignant transformation of human bronchial epithelial cells. *Toxicol. Appl. Pharmacol.* 289, 231–239. doi: 10.1016/j.taap.2015.09.020

Yang, Y., Zhang, J., Xiong, L., Deng, M., Wang, J., Xin, J., et al. (2015). Cognitive improvement induced by environment enrichment in chronic cerebral hypoperfusion rats: a result of upregulated endogenous neuroprotection? *J. Mol. Neurosci.* 56, 278–289. doi: 10.1007/s12031-015-0529-2

Yang, Z., Weian, C., Susu, H., and Hanmin, W. (2016). Protective effects of mangiferin on cerebral ischemia-reperfusion injury and its mechanisms. *Eur. J. Pharmacol.* 771, 145–151. doi: 10.1016/j.ejphar.2015.12.003

Yao, Y., Miao, W., Liu, Z., Han, W., Shi, K., Shen, Y., et al. (2016). Dimethyl fumarate and monomethyl fumarate promote post-ischemic recovery in mice. *Transl. Stroke Res.* 7, 535–547. doi: 10.1007/s12975-016-0496-0

Yates, M. S., Tran, Q. T., Dolan, P. M., Osburn, W. O., Shin, S., Mcculloch, C. C., et al. (2009). Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid-treated mice. *Carcinogenesis* 30, 1024–1031. doi: 10.1093/carcin/bgp100

Yu, S., Zhao, J., Wang, X., Lei, S., Wu, X., Chen, Y., et al. (2013). 4-Hydroxybenzyl alcohol confers neuroprotection through up-regulation of antioxidant protein expression. *Neurochem. Res.* 38, 1501–1516. doi: 10.1007/s11064-013-1052-x

Zhai, X., Chen, X., Shi, J., Shi, D., Ye, Z., Liu, W., et al. (2013). Lactulose ameliorates cerebral ischemia-reperfusion injury in rats by inducing hydrogen by activating Nrf2 expression. *Free Radic. Biol. Med.* 65, 731–741. doi: 10.1016/j.freeradbiomed.2013.08.004

Zhang, J., Fu, B., Zhang, X., Zhang, L., Bai, X., Zhao, X., et al. (2014). Bicyclol upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. *Brain Res. Bull.* 100, 38–43. doi: 10.1016/j.brainresbull.2013.11.001

Zhang, L., Zhang, X., Zhang, C., Bai, X., Zhang, J., Zhao, X., et al. (2016). Nobiletin promotes antioxidant and anti-inflammatory responses and elicits protection against ischemic stroke *in vivo*. *Brain Res.* 1636, 130–141. doi: 10.1016/j.brainres.2016.02.013

Zhang, M., Wang, S., Mao, L., Leak, R. K., Shi, Y., Zhang, W., et al. (2014). Omega-3 fatty acids protect the brain against ischemic injury by activating Nrf2 and upregulating heme oxygenase 1. *J. Neurosci.* 34, 1903–1915. doi: 10.1523/JNEUROSCI.4043-13.2014

Zhang, Q., Wang, J., Zhang, C., Liao, S., Li, P., Xu, D., et al. (2016). The components of Huang-Lian-Jie-Du-Decoction act synergistically to exert protective effects in a rat ischemic stroke model. *Oncotarget* 7, 80872–80887. doi: 10.18632/oncotarget.12645

Zhang, W., Song, J. K., Yan, R., Li, L., Xiao, Z. Y., Zhou, W. X., et al. (2018). Diterpene ginkgolides protect against cerebral ischemia/reperfusion damage in rats by activating Nrf2 and CREB through PI3K/Akt signaling. *Acta Pharmacol Sin*. 39, 1259–1272. doi: 10.1038/aps.2017.149

Zhang, W., Wei, R., Zhang, L., Tan, Y., and Qian, C. (2017). Sirtuin 6 protects the brain from cerebral ischemia/reperfusion injury through NRF2 activation. *Neuroscience* 366, 95–104. doi: 10.1016/j.neuroscience.2017.09.035

Zhang, Y., Fan, L., Li, H., Wang, X., Xu, W., Chu, K., et al. (2018). Gualou guizhi granule protects against oxidative injury by activating Nrf2/ARE pathway in rats and PC12 cells. *Neurochem. Res.* 43, 1003–1009. doi: 10.1007/s11064-018-2507-x

Zhao, J. J., Song, J. Q., Pan, S. Y., and Wang, K. (2016). Treatment with isorhamnetin protects the brain against ischemic injury in mice. *Neurochem. Res.* 41, 1939–1948. doi: 10.1007/s11064-016-1904-2

Zhao, Y., Fu, B., Zhang, X., Zhao, T., Chen, L., Zhang, J., et al. (2014). Paeonol pretreatment attenuates cerebral ischemic injury via upregulating expression of pAkt, Nrf2, HO-1 and ameliorating BBB permeability in mice. *Brain Res. Bull.* 109, 61–67. doi: 10.1016/j.brainresbull.2014.09.008

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Nrf2 as a Potential Mediator of Cardiovascular Risk in Metabolic Diseases

Rafael M. da Costa[1,2], Daniel Rodrigues[1], Camila A. Pereira[1], Josiane F. Silva[1], Juliano V. Alves[1], Núbia S. Lobato[2] and Rita C. Tostes[1]*

[1] Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil, [2] Special Academic Unit of Health Sciences, Federal University of Goiás, Jataí, Brazil

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Edited by:
Javier Egea, Institute of Health Research of the University Hospital of La Princesa, Spain

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Soon Yew Tang, University of Pennsylvania, United States
Carole L. Wilson, Medical University of South Carolina, United States

***Correspondence:**
Rafael M. da Costa
rafael.menezess@yahoo.com.br

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Free radicals act as secondary messengers, modulating a number of important biological processes, including gene expression, ion mobilization in transport systems, protein interactions and enzymatic functions, cell growth, cell cycle, redox homeostasis, among others. In the cardiovascular system, the physiological generation of free radicals ensures the integrity and function of cardiomyocytes, endothelial cells, and adjacent smooth muscle cells. In physiological conditions, there is a balance between free radicals generation and the activity of enzymatic and non-enzymatic antioxidant systems. Redox imbalance, caused by increased free radical's production and/or reduced antioxidant defense, plays an important role in the development of cardiovascular diseases, contributing to cardiac hypertrophy and heart failure, endothelial dysfunction, hypertrophy and hypercontractility of vascular smooth muscle. Excessive production of oxidizing agents in detriment of antioxidant defenses in the cardiovascular system has been described in obesity, diabetes mellitus, hypertension, and atherosclerosis. The transcription factor Nrf2 (nuclear factor erythroid 2–related factor 2), a major regulator of antioxidant and cellular protective genes, is primarily activated in response to oxidative stress. Under physiological conditions, Nrf2 is constitutively expressed in the cytoplasm of cells and is usually associated with Keap-1, a repressor protein. This association maintains low levels of free Nrf2. Stressors, such as free radicals, favor the translocation of Nrf2 to the cell nucleus. The accumulation of nuclear Nrf2 allows the binding of this protein to the antioxidant response element of genes that code antioxidant proteins. Although little information on the role of Nrf2 in the cardiovascular system is available, growing evidence indicates that decreased Nrf2 activity contributes to oxidative stress, favoring the pathophysiology of cardiovascular disorders found in obesity, diabetes mellitus, and atherosclerosis. The present mini-review will provide a comprehensive overview of the role of Nrf2 as a contributing factor to cardiovascular risk in metabolic diseases.

Keywords: Nrf2, oxidative stress, cardiovascular risk, metabolic diseases, therapeutic target

OVERVIEW OF REACTIVE OXYGEN SPECIES AND CARDIOVASCULAR FUNCTION

Although reactive oxygen species (ROS) were initially presumed to cause cell damage, they are now recognized as important molecules that regulate many cell signaling and biological processes, such as induction of defense genes, activation of transcription factors, phosphorylation of kinases, and mobilization of ions in transport systems (reviewed in Droge, 2002; Touyz and Briones, 2011; Brown and Griendling, 2015). In the cardiovascular system, ROS generation is important to maintain endothelial and vascular smooth muscle cells (VSMCs) function, including vascular tone control, inflammation-related responses, cell growth and proliferation, modulation of extracellular matrix production, apoptosis and angiogenesis (Siti et al., 2015; reviewed in Galley and Straub, 2017). Alterations of the balance between cellular ROS production and the capacity to rapidly detoxify reactive intermediates play an important role in the development of risk factors for cardiovascular diseases (reviewed in Harrison et al., 2003 and Touyz et al., 2018). Such events are observed in patients with essential hypertension (Redón et al., 2003) and various experimental models of arterial hypertension, such as spontaneously hypertensive rats (SHR) that exhibit increased levels of vascular superoxide anion and vascular smooth muscle hypercontractility (Paravicini et al., 2004). The same occurs in obesity and experimental models of diabetes, in which insulin resistance and hyperglycemia culminate in increased NAD(P)H oxidase enzyme activity and endothelial nitric oxide synthase (eNOS) uncoupling, thereby contributing to increased ROS generation and impaired vascular function (Youn et al., 2012; da Costa et al., 2017; Neves et al., 2018).

Vascular cells generate ROS in response to several stimuli, including cytokines, angiotensin II, endothelin-1, aldosterone and platelet-derived growth factor (PDGF) (reviewed in Thannickal and Fanburg, 2000). ROS signaling in endothelial cells and VSMCs involves alterations in the intracellular redox state and oxidative modification of regulatory and contractile proteins (da Costa et al., 2017). The oxidative modification of these redox-sensitive proteins alters their conformation, stability, activity and/or ability to interact with other proteins, resulting in modulation of vascular function. Redox-sensitive proteins include proteins involved in calcium handling as well as contractile proteins, proteins involved in various signaling and transcriptional activities. Redox modulation of calcium handling proteins directly affects cardiac and vascular contraction by altering intracellular calcium levels. Examples of redox-sensitive calcium handling proteins are calcium-calmodulin kinase II (CaMKII), the ryanodine receptor on the sarcoplasmic reticulum, sarcoplasmic reticulum ATPase (SERCA), and phospholamban (reviewed in Burgoyne et al., 2012 and Steinberg, 2013). Protein kinases and phosphatases are also affected by direct redox modification, resulting in modulation of various signal transduction pathways in cardiac and vascular cells, including altered modulation of calcium sensitivity, phosphorylation of the myofilaments and receptor tyrosine kinase signaling (reviewed in Knock and Ward, 2011 and Steinberg, 2013).

The long-term effects of ROS in cardiovascular function depend on the balance between signals promoting proliferation or growth inhibition and/or cell death. This dual role is evidenced by the findings that endothelial apoptosis initiated by tumor necrosis factor-alpha (TNF-α) is attenuated by ROS scavenging (Xia et al., 2006) and that, during ischemia-reperfusion injury, ROS trigger apoptosis, whereas ROS generated during ischemic preconditioning prevent apoptosis (Maulik et al., 1999; Hattori et al., 2001; reviewed in Becker, 2004). In vascular cells, ROS can alter this balance leading to either excessive angiogenesis or loss of endothelial cells (reviewed in Papaharalambus and Griendling, 2007).

To contain ROS generation, there are antioxidant defenses that prevent or neutralize the generation of highly reactive compounds in biological systems. Among the major intracellular antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase are major players. In cooperative action, these enzymes catalyze ROS in molecular oxygen and water (reviewed in Rodriguez and Redman, 2005). Other agents, such as ascorbic acid (reviewed in May and Harrison, 2013) and phenolic acids (reviewed in Santhakumar et al., 2014), which do not present enlightened mechanisms of action, also contribute to redox balance.

Although the exact mechanisms underlying cardiovascular oxidative stress during obesity, diabetes, and atherosclerosis still remain unclear, the discovery of mechanisms and subsequent new pharmacological interventions related to the disruption of the antioxidant system has become of great interest to identify key mediators that will potentially instigate and exacerbate these cardiovascular complications.

Nrf2 ACTIVITY AND REGULATION

Several cell types throughout evolution have developed adaptive programs to counteract oxidative stress, and the role of the nuclear factor erythroid 2-related factor-2 (Nrf2) in these programs has been a subject of recent interest. Nrf2 is a basic leucine zipper region (bZip) transcription factor of the Cap n'Collar family, with approximately 589 amino acid residues and seven conserved domains (Neh1 – Neh7) (reviewed in Kansanen et al., 2013). When the levels of ROS and electrophiles become greater than the cell's ability to detoxify them, a series of events results in Nrf2 activation. Activated Nrf2 heterodimerizes with Jun and small musculoaponeurotic fibrosarcoma (sMaf) proteins, translocates to the nucleus and binds to the antioxidant response element (ARE) or the electrophile-response element (EpRE) in the promoter region of Nrf2 target genes. This results in the coordinated expression and activation of antioxidant, antiapoptotic, metabolic, and detoxification proteins. Among the proteins with antioxidant activity regulated by Nrf2 are superoxide dismutase (SOD), catalase (CAT), heme-oxygenase 1 (HO-1), glutathione peroxidase 1 (GPx-1) and NAD(P)H: quinone oxidoreductase 1 (reviewed in Tebay et al., 2015).

The conserved Nrf2 domains are crucial for their self-regulation and activity. In this context, the Neh-1 domain is responsible for the binding of Nrf2 to DNA through dimerization with the low molecular weight sMaf proteins of type F, G, and K (sMafF, sMafG, and sMafK), favored by the bZip domain, and by recognition of the ARE (Mohler et al., 1991; Oyake et al., 1996; reviewed in Motohashi et al., 2002). The Neh-2 domain, rich in lysines, is responsible for binding of Nrf2 to its cytoplasmic repressor protein Keap-1. This domain also has high and low affinity regions for Keap-1 binding, the ETGE and DLG motifs, respectively (Itoh et al., 1999; Kobayashi et al., 2002). The Neh-3 domain, located in the carboxy-terminal portion of Nrf2, is sumoylated. This post-translational modification is important for Nrf2 transactivation. In addition to Neh-3, the Neh-4, and Neh-5 domains, when acetylated or oxidized, in oxidative stress conditions, are responsible for the transactivation of Nrf2 (Katoh et al., 2001; Nioi et al., 2005). The Neh-6 and Neh-7 domains are responsible for the degradation of Nrf2, independently of the regulation by Keap-1 (McMahon et al., 2004; Wang et al., 2013).

In physiological conditions, when there is a balance between oxidant species and the adequate activity of antioxidant proteins, Nrf2 is suppressed in the cytoplasm. This suppression occurs adjacent to the cellular cytoskeleton by the interaction of Nrf2 with Keap-1 and Cullin3, a protein of the E3 ligase family (reviewed in Canning et al., 2015). The formation of the Nrf2-Keap-1-Cullin3 complex becomes a signal for the ubiquitination and proteasome degradation of Nrf2, maintaining its basal levels along with the genes regulated by this transcription factor (Furukawa and Xiong, 2005; reviewed in Canning et al., 2015).

Under oxidative or electrophilic stress conditions, ROS promote the breakdown of the Nrf2-Keap1-Cullin3 complex, allowing Nrf2 to translocate to the nucleus and activate the expression of antioxidant proteins. The ARE is normally suppressed by a heterodimer formed by the sMaf and Bach-1 proteins, preventing Nrf2 heterodimerization and binding to the ARE (reviewed in Tebay et al., 2015 and Tong et al., 2015). However, under conditions of oxidative stress, there is an accumulation of heme groups in the cytosol, a frequent substrate for ROS formation. In this condition, Bach-1 undergoes dissociation of sMaf proteins and migrates to the cytosol to act on the heme group metabolism, allowing Nrf2 to dimerize with the sMaf protein and initiate gene transcription (reviewed in Ryter and Choi, 2005 and Tebay et al., 2015).

Among the proteins that participate in Nrf2 regulation and activity, Keap-1 and Bach-1 stand out from the others due to their direct role in redox balance and cardiovascular homeostasis (Lopes et al., 2015). Of importance, the negative regulation of Nrf2 by Keap-1 and Bach-1 raises the possibility of new therapeutic targets to prevent oxidative stress-associated cardiac hypertrophy and vascular dysfunction (Abed et al., 2015; Jiao et al., 2018).

Nrf2 AND OBESITY

The mechanisms accompanying the progression of obesity and its cardiovascular comorbidities have been the focus of considerable research over the last 30 years. As the mechanistic insights clarify, it is evident that the expansion of visceral adipose tissue plays a pivotal role in the pathophysiology of obesity. Increased ROS generation by the adipocytes, which, in turn, increases expression and secretion of inflammatory adipokines, causes dramatic consequences for the regulation of energy homeostasis and vascular function (Keaney et al., 2003; reviewed in DeMarco et al., 2010 and Otani, 2011). This is a common combination associated with individual cardiovascular disease risk (Furukawa et al., 2004; reviewed in Sowers et al., 2011).

Recent evidence shows that Nrf2 is involved in the control of energy metabolism and might be a promising therapeutic target for obesity. The underlying mechanism of Nrf2 in obesity has been investigated using various experimental approaches, including Nrf2 gene deletion, Nrf2 pharmacological activators and Nrf2 gene overexpression, but only few of these approaches were clinically tested. Specific pharmacological activators of Nrf2, including epigallocatechin 3-gallate, oltipraz, sulforaphane, curcumin and 1-[2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oyl] imidazole (CDDO-Im), induce the expression and activity of Nrf2 both *in vitro* and *in vivo* (Shin et al., 2009; Yu et al., 2011; Xu et al., 2012; Gao et al., 2013). The epigallocatechin 3-gallate-induced activation of Nrf2 in liver and adipose tissue of obese mice improves lipidemic control, decreases oxidative products generation, and reduces body mass, insulin and glucose levels (Sampath et al., 2017). Interestingly, knockout mice for the Keap-1 protein exhibit similar features, including suppression of high-fat diet-induced obesity and decreased deposition of lipids and cholesterol in the liver (Slocum et al., 2016). Of importance, improvement of metabolic profile is closely associated with improved cardiovascular function (da Costa et al., 2016; Neves et al., 2018).

A recent study demonstrated that the Nrf2 activator sulforaphane, during revascularization procedures in metabolically compromised individuals, has the potential to suppress the progression of intimal hyperplasia. In addition, Nrf2 activation attenuates leptin-induced proliferation of VSMCs in the diet-induced obesity scenario (Shawky et al., 2016).

Natural compounds are also promising elements in Nrf2 activation during obesity. As an example, Zeng et al. (2015) observed that curcumin, a natural Nrf2 activator, suppresses oxidative stress, inflammation and hypertrophy induced by treatment of cardiac cells with free fatty acids. Similarly, high-fat diet induced oxidative stress, inflammation, fibrosis, hypertrophy and tissue remodeling are attenuated by curcumin treatment. These benefits are closely associated with increased Nrf2 expression and activity, as well as reduced ROS generation (Zeng et al., 2015).

Deletion of the Nrf2 gene is expected to increase ROS generation and to aggravate the phenotypes of obesity. Nrf2 knockout mice show increased ROS generation, deposition of fatty acids in the liver and increased expression of genes related to the synthesis of lipids and cholesterol (Tanaka et al., 2008). Consistent with these findings, our group showed that obesity in mice favors vascular oxidative stress by increasing the expression of the downregulatory proteins of Nrf2, Keap-1 and Bach-1 (Costa et al., 2017).

Nrf2 in adipose tissue function and metabolic syndrome has also been examined. Xue et al. (2013) determined the role of Nrf2 in the development of obesity and associated metabolic disorders, using Nrf2-knockout mice on a leptin-deficient ob/ob background, a model with an extremely positive energy balance. In obese mice, ablation of Nrf2, globally or specifically in adipocytes, reduces white adipose tissue mass. However, Nrf2 deletion results in even more severe metabolic syndrome with aggravated insulin resistance, hyperglycemia, and hypertriglyceridemia. In addition, when compared to wild-type mice, the white adipose tissue of obese mice expresses substantially higher levels of many genes related to antioxidant response, inflammation, adipogenesis, lipogenesis, glucose uptake, and lipid transport (Xue et al., 2013). These findings support a role for Nrf2 in regulating adipose tissue development and function, insulin sensitivity, glucose and lipid homeostasis.

In contrast to the above-mentioned study, Nrf2 knockout mice treated with a high-fat diet tend to gain less body mass and display increased insulin sensitivity and glucose tolerance. In addition, they do not exhibit increased glucose, cholesterol, or plasma triglycerides. Of importance, the altered metabolic phenotype of Nrf2- knockout mice on high-fat diet is associated with higher expression and abundance of fibroblast growth factor 21 (FGF21), a novel hormone that regulates energy metabolism, glucose tolerance and adipose tissue expansion (Chartoumpekis et al., 2011).

The complex roles of Nrf2 in adipogenesis and adipose tissue functions were recently examined by adipose tissue-specific ablation of Nrf2 in mice. This condition is associated with a transient delayed increase of body weight in high-fat diet-fed mice. However, the benefit is eventually suppressed after prolonged feeding. The phenotypic changes induced by adipose tissue-specific ablation of Nrf2 also extend to the whole-body level, reducing blood glucose and altering the expression of genes involved in glucose, lipid and energy metabolism (Zhang et al., 2016). These findings are consistent with those of previous studies using Nrf2 knockout mice (Pi et al., 2010; Xu J. et al., 2015) and provide additional mechanistic insights into the role of Nrf2 as an important mediator of glucose, lipid and energy metabolism. To date there are no studies showing direct effects of Nrf2 deletion on cardiovascular function.

The apparent contradictory role of Nrf2 protecting mice from obesity and insulin resistance in conditions of Nrf2 deficiency in comparison to Nrf2 pharmacological activation, may be explained by the observations that Nrf2 deficiency leads to a mild increase in the levels of ROS, which stimulate the antioxidant system in a manner similar to the Nrf2 activators (reviewed in Bocci et al., 2014). Another possible explanation is that Nrf2 activators also regulate non-Nrf2 signaling pathways to modulate glucose and lipid metabolism. The same is true for Keap-1, the Nrf2 repressor protein, which may also have Nrf2-independent effects on transcription factors and, consequently, on metabolic homeostasis (Huang et al., 2012). Controversial results on the role of Nrf2 in obesity may be linked to differences in the pathophysiological characteristics of obesity (such as diet content or time under obesity conditions), as well as specific genetic characteristics. **Table 1** summarizes the contribution of Nrf2 signaling in different obesity conditions. Further studies are required to explore this apparent discrepancy on the role of Nrf2 in obesity.

The Nrf2/Keap1/ARE signaling pathway also represents a mechanism by which the gut microbiome activates a wide range of host signaling and homeostatic processes (reviewed in Murota et al., 2018). The intestinal microbiome and its metabolites display a pivotal role in host physiological processes including immune, metabolic, neurological, and nutritional homeostasis (reviewed in Lynch and Pedersen, 2016). Many of these physiological processes are under influence of ROS generation in the gut epithelia. Alterations in the gut microbiota have recently emerged as major triggers of abnormalities in the integrity of the intestinal barrier, facilitating blood translocation of bacteria and uremic toxins, systemic inflammation and adverse outcomes in obesity and diabetes (reviewed in Belizário et al., 2018). Nrf2 activation in intestinal barrier leads to upregulation of antioxidant enzymes, thereby strengthening the cell's ability to neutralize several types of free radicals (Zheng et al., 2018). In addition, the relationship between bacterial-dependent ROS generation and Nrf2 pathway activity was recently revealed by observations that lactobacilli-induced and Nox1-mediated generation of ROS evokes Nrf2-dependent activation of cytoprotective antioxidants genes (Jones et al., 2015). In fact, microbe-induced ROS generation and activation of Keap1/Nrf2/ARE signaling may contribute to our understanding on the mechanisms involved in the genesis of obesity and diabetes, comorbidities associated with increased cardiovascular risk.

Nrf2 AND TYPE 2 DIABETES MELLITUS

There is abundant evidence that oxidative damage caused by free radicals contributes to the pathogenesis and progression of type 2 diabetes mellitus and its complications (reviewed in Dandona et al., 1996; Brownlee, 2001 and Giacco and Brownlee, 2010). Only recently, however, has the role of the Nrf2/Keap-1/ARE pathway in the pathophysiology of this condition and the wide range of its complications, such as diabetic nephropathy and impaired cutaneous wound healing begun to be elucidated (Jiménez-Osorio et al., 2014; reviewed in Uruno et al., 2015; Long et al., 2016). Furthermore, as noted in an excellent and recent review on Nrf2 (David et al., 2017), this pathway is implicated in diabetic damage to the pancreas (Yagishita et al., 2014) and heart (Li et al., 2009). There are promising results provided by animal studies and clinical trials suggesting that activation of this pathway can delay or even reverse type 2 diabetes mellitus-associated dysfunctions (Ichikawa et al., 2009; Uruno et al., 2013).

A consistent alteration in diabetic patients is the presence of endothelial dysfunction, which precedes the development of diabetes-associated vascular complications and may explain, in part, the increased cardiovascular risk in this condition. Endothelial dysfunction in diabetes is associated with enhanced vascular contractility, oxidative stress and vascular inflammation (Zakkar et al., 2009; Tabit et al., 2010

TABLE 1 | Nrf2 signaling and actions in obesity and atherosclerosis animals models.

Genotype strain mice	Metabolic and body parameters	Atherosclerotic plaque	Diet / feeding time period	Effect on Nrf2 and target genes	References
Male C57BL/6J	HFD vs. LFD ↓ glycaemia and body weight. HFD + E-75 vs. LFD ↔ glycaemia and body weight.	Not assessed	LFD (10% calories from fat) or HFD (45% calories from fat), and HFD + E-25 or HFD + E-75 for 17 weeks.	HFD vs. LFD ↔ Nrf2 nuclear fraction in the liver. HFD + E-75 vs. LFD ↑ Nrf2 nuclear fraction and HO-1 protein expression.	Sampath et al. (2017)
Male C57BL/6J WT and Keap1-hypo	HFD Keap1-hypo vs. HFD WT ↓ glycaemia, hepatic triglyceride and body weight.	Not assessed	SD (10 kcal % fat) or HFD (60 kcal % fat) for 90 days.	Keap1-hypo vs. WT ↑ NQO-1 mRNA.	Slocum et al. (2016)
Male C57BL/6J	HFHS+SFN vs. HFHS ↓ glycaemia, weight gain, plasma leptin, plasma insulin, cLDL and triglycerides.	HFHS+SFN vs. HFHS ↓ neointima formation in the injured femoral artery.	SD (10% calories from fat and 72% from carbohydrate) or HFHS diet (40% calories from fat, 42% from carbohydrate and 0.15% w/w cholesterol), and HFHS + SFN for 8 weeks.	SFN induces Nrf2 activation.	Shawky et al. (2016)
Male C57BL/6J	HFD curcumin vs. HFD ↔ triglycerides, LDL, cholesterol total and body weight.	Not assessed	SD or HFD, and HFD + curcumin treatment for 8 weeks.	HFD curcumin vs. HFD ↑ Nrf2, HO-1 and NQO-1 gene and protein expression in the myocardium.	Zeng et al. (2015)
Male C57BL/6J WT; $Nrf2^{+/+}$:ob/ob; $Nrf2^{-/-}$:ob/ob and Adipocyte-specific Nrf2-KO	$Nrf2^{-/-}$:ob/ob vs. $Nrf2^{+/+}$:ob/ob ↓ weight gain and white adipose tissue; ↑ insulin resistance, triglycerides, glycaemia. Adipocyte-specific Nrf2-KO and $Nrf2^{-/-}$:ob/ob mice have a similar phenotype.	Not assessed	SD for 11 weeks.	WT vs. $Nrf2^{+/+}$:ob/ob ↑ HO-1 and NQO-1 mRNA.	Xue et al. (2013)
Male C57BL/6J WT and $Nrf2^{-/-}$	Nrf2-/- HFD vs. HFD WT ↓ weight gain, basal glucose, insulin resistance, leptin. ↑ triglycerides.	Not assessed	SD (10 kcal % fat) or HFD (60 kcal % fat) for 180 days.	HFD WT vs. SD WT ↑ Nrf2 mRNA.	Chartoumpekis et al. (2011)
Male adipose-specific Nrf2-KO (NK) and Nrf2 control (NC)	NK HFD vs. NC HFD ↓ weight gain, basal glucose. ↔ cholesterol, leptin, free fatty acid.	Not assessed	SD (5.55% kcal soybean oil and 4.44% kcal) or HFD (5.55% kcal soybean oil and 54.35% kcal) for 14 weeks.	NK mice has a reduction in adipose tissue Nrf2 expression	Zhang et al. (2016)
Male C57BL/6J WT and $Nrf2^{-/-}$	$Nrf2^{-/-}$ HFD vs. WT HFD, 12 weeks ↓ weight gain, adipose tissue.	Not assessed	Regular diet (11% fat) or HFD (41% fat) for 4, 8, and 12 weeks.	Not assessed	Pi et al. (2010)
Male $Lep^{ob/ob}$ (OB) and Nrf2/$Lep^{ob/ob}$ (OB-Nrf2 KO)	OB-Nrf2 KO vs. OB, 8 weeks ↓ body weight, adipose tissue, glucose tolerance. ↑ VLDL/triglycerides hepatic secretion, triglycerides, cholesterol. ↔ not-fasting glucose.	Not assessed	SD for 4, 8, and 12 weeks.	Not assessed	Xu J. et al., 2015

(Continued)

TABLE 1 | Continued

Genotype strain mice	Metabolic and body parameters	Atherosclerotic plaque	Diet / feeding time period	Effect on Nrf2 and target genes	References
Male C57BL/6J WT and $Nrf2^{-/-}$	Not assessed	Not assessed	SD and Carotid artery treatment with oxPAPC for 6 h and 24 h.	WT + oxPAPC vs. WT ↑ HO-1 and NQO-1 expression.	Jyrkkänen et al. (2008)
Female $HO\text{-}1^{-/-}$:$ApoE^{-/-}$ and $HO\text{-}1^{+/+}$:$ApoE^{+/+}$	↔ cholesterol.	↑ Atherosclerotic lesion formation.	Western Diet (1.25% cholesterol and 21% fat) for 8 weeks.	Not assessed	Yet et al. (2003)
Male $Nrf2^{-/-}$:$ApoE^{-/-}$ and $Nrf2^{+/+}$:$ApoE^{-/-}$	↓ cholesterol, VLDL in $Nrf2^{-/-}$:$ApoE^{-/-}$ irradiated.	↔ Atherosclerotic lesion at 3 and 5 weeks. ↓ atherosclerotic plaque at 12 weeks in $Nrf2^{-/-}$: $ApoE^{-/-}$.	HFD (1.25% cholesterol) for 3, 5, and 12 weeks.	↓ HO-1 expression in $Nrf2^{+/+}$: $ApoE^{-/-}$ with HFD at 12 weeks.	Harada et al. (2012)
Male $LDLr^{-/-}$ and $LDLr^{-/-}$ transplanted with $Nrf2^{-/-}$ BM ($Nrf2^{+/+}$ BM)	↔ cholesterol, triglycerides.	↑ Lesion, necrotic cord.	SD or HFD.	HFD-fed $Nrf2^{-/-}$ vs. $Nrf2^{+/+}$ BM mice ↓ NQO-1, catalase, Gpx-1.	Collins et al. (2012)
Male C57BL/6J WT and $Nrf2^{-/-}$	Not informed	$Nrf2^{-/-}$ ↑ neointimal formation.	SD for 4 weeks.	Nrf2 depletion.	Ashino et al. (2013)
Male C57BL/6J WT and $Nrf2^{-/-}$	Not informed	$Nrf2^{-/-}$ ↑ neointimal formation after femoral injury.	SD.	Femoral artery injury WT ↑ Nrf2. ↓ Keap1.	Ashino et al. (2016)

↔, not altered; ↓, decreased; ↑, increased; Adipocyte-specific Nrf2-KO, mice with Nrf2 deletion only in adipose tissue; $ApoE^{-/-}$, knockout mice to apolipoprotein E; BM, bone marrow; cLDL, cholesterol fraction of the Low density lipoprotein; E-25 and E-75, treatment with Epigallocatechin-3-gallate 25 and 75 mg/kg, respectively; Gpx-1, glutathione peroxidase 1; HFD, high fat diet; HFHS + SFN, HFHS diet-fed mice that receive SFN treatment; HFHS, high fat high sugar diet; SFN, sulforaphane; HO-1, Hemeoxygenase 1; Keap1-hypo, hypomorphic Keap1 allele mice; LDL, low density lipoprotein; $LDLr^{-/-}$, knockout mice to low density lipoprotein receptor; $Lep^{ob/ob}$ or ob/ob, leptin-deficient mice; LFD, low fat diet; NQO-1, NAD(P)H:Quinone Oxidoreductase 1; Nrf2, Nuclear factor-like 2; $Nrf2^{-/-}$:ob/ob mice, Nrf2 knockout and leptin-deficient mice; Nrf2-KO or $NRF2^{-/-}$, knockout mice to Nrf2; oxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-phosphocholine; SD, standard diet; WT, wild type mice.

and Hamilton and Watts, 2013). The importance of Nrf2 and its downstream elements in the control of vascular function in diabetes has become increasingly apparent and is reinforced by multiple studies using many of the same agents for protection from conditions other than diabetes. Bardoxolone methyl, a synthetic small molecule activator of the Nrf2/Keap-1 pathway, improves structural and functional changes in animal models of renal disease (Wu et al., 2011; Chin et al., 2013). Its therapeutic potential was extended to clinical trials in type 2 diabetic patients with chronic kidney disease (Pergola et al., 2011). The recent studies published by Tan and Sharma's group demonstrated that the bardoxolone analog, dh404, at low doses, attenuates atherosclerosis in diabetic Apolipoprotein E (ApoE) knockout mice (Tan et al., 2014) and protects against diabetes-induced endothelial dysfunction, both *in vivo* and *in vitro* (Sharma et al., 2017). Considering that endothelial dysfunction, which is the first step in the development of vascular complications in diabetes, is accompanied by pro-oxidative and pro-inflammatory processes, the atheroprotective effects of dh404 could be mediated by improvement of the endothelial function. In agreement with these observations, increased Nrf2 activity induced, for example, by bardoxolone or sulforaphane, abrogates augmented vascular contraction (Alves-Lopes et al., 2016; Sharma et al., 2017) and attenuates reduced vasodilation in diabetic mice (Lu et al., 2017).

One proposed mechanism for the improvement of endothelial function following Nrf2 activation is the increased expression of a subunit of the BK (big potassium or large conductance, Ca^{2+}-activated potassium) channel, the BK-β1, and its attenuated degradation (Lu et al., 2017), a process commonly accelerated by diabetes-induced oxidative stress (Li et al., 2017). Other mechanisms include reduced systemic and vascular oxidative stress as well as increased nitric oxide (NO) bioavailability (Liu et al., 2016; Pereira et al., 2017). Beyond this immediate homeostatic response, long-term consequences of Nrf2 activity have also been described as important culprits of micro- and macrovascular complications associated with diabetes. Accordingly, the literature describe functional connections between Nrf2 and signaling pathways involving nuclear factor-κB (NF-κB), p53, ERK5, mTOR46, heat shock proteins, activator protein-1 (AP-1) and Notch homolog 1, translocation-associated (Drosophila) (NOTCH1) (Hwang et al., 2017). This implies that Nrf2 modulates many cellular activities, beyond its immediate homeostatic and cytoprotective actions, influencing processes as diverse as inflammation, proliferation, apoptosis, cell differentiation, tissue regeneration and even metabolism. In fact, decreased activation of Nrf2 is observed in experimental diabetic cardiomyopathy, along with a decrease in the downstream activity of antioxidant enzymes and increased oxidative stress

(Wang et al., 2017). In this sense, activation of the Nrf2 system attenuates vascular remodeling by decreasing proliferation, migration, and fibrotic processes. These effects are mediated by reduced metalloproteinase activity and decreased protein expression of adhesion molecules and TNF-α (Wang et al., 2014; Choi et al., 2015).

Such widespread protective effects of Nrf2 might constitute the underlying mechanism involved in the progression of diabetes-associated complications. These results have also provided strong support for the development of new potent enhancers of Nrf2 activity for the prevention and treatment of many diseases in which both inflammatory and oxidative processes have a key pathogenic role.

Nrf2 AND ATHEROSCLEROSIS

Atherosclerosis, a progressing inflammatory disease produced by many risk factors, such as diabetes, hypertension, and hyperlipidemia, is one of the major cardiovascular diseases, which, together with myocardial infarction and coronary heart disease, will account for more than 20 million deaths in 2030 (reviewed in Yahagi et al., 2016). Even though much is known about the mechanisms that result in the formation of atherosclerotic plaque, the processes are not entirely understood.

During the atherogenesis process, the build-up of lipids in the arterial intima triggers several changes in the microenvironment of the arterial wall, such as the formation of fatty streaks, endothelial dysfunction, recruitment and activation of immune cells and VSMCs proliferation (reviewed in Raggi et al., 2018). Recruitment and retention of inflammatory cells lead to persistent production of cytokines and ROS that contribute to the progression of atherosclerotic lesion (reviewed in Koelwyn et al., 2018). Increased ROS induces the oxidation of low-density lipoprotein (LDL) to ox-LDL that contributes to oxidative stress and foam cell formation in the arterial wall, aggravating the atherosclerotic plaque formation (reviewed in Koelwyn et al., 2018). In this scenario, the transcription factor Nrf2 is considered a protective signaling molecule, since it induces the expression of many antioxidant genes that may attenuate atherosclerosis progression (Jyrkkänen et al., 2008; reviewed in Guerrero-Hue et al., 2017). For instance, deficiency of GPX-1, a Nrf2 target gene, in mice increases ox-LDL-induced foam cell formation and leads to amplified proliferative activity of peritoneal macrophages, indicating that this gene is atheroprotective (Cheng et al., 2013). Moreover, atherosclerotic lesion development and oxidative stress are accelerated in HO-1 deficient ApoE knockout mice (Yet et al., 2003). Deletion of HO-1 in macrophages increases lipid build-up and foam cell formation and, consequently, the production of ROS and pro-inflammatory cytokines (Orozco et al., 2007). Cheng et al. found that HO-1 expression is increased in vulnerable plaque from patients with symptomatic carotid artery disease and this increase correlates with unstable plaque phenotype. These findings indicate that HO-1 is a major regulator of advanced atherosclerotic lesion progression (Cheng et al., 2009). However, it is not clear whether the induction of HO-1 is a compensatory atheroprotective response, trying to reduce increased levels of ROS in the plaque, or if it contributes to increased plaque vulnerability. Therefore, more studies are necessary to understand the role of HO-1 in advanced atherosclerotic plaque stage.

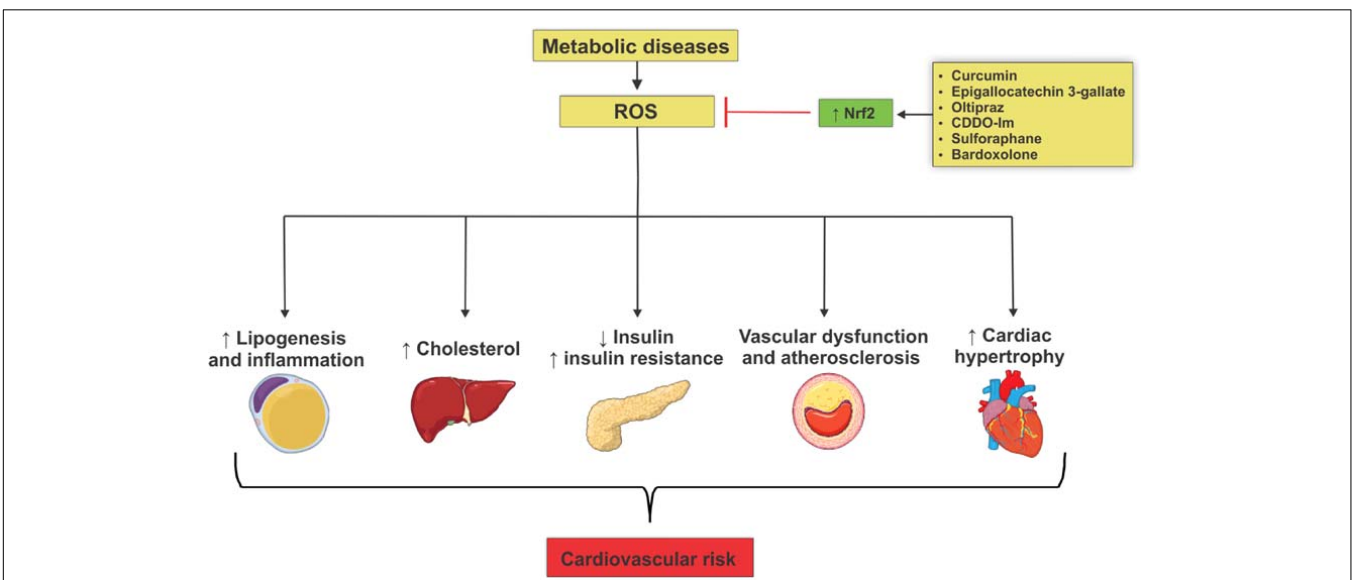

FIGURE 1 | Mechanisms involved in the actions of reactive oxygen species that lead to metabolic diseases and cardiovascular risk development. Metabolic diseases are closely associated with increased generation of reactive oxygen species (ROS) due to reduced Nrf2 antioxidant activity. This phenomenon culminates in target-organ damage and metabolism disorders, such as adipogenesis and adipose tissue inflammation, increased production of hepatic cholesterol, decreased insulin secretion, insulin resistance, loss of integrity of vascular tone control, endothelial dysfunction and atheroma formation, all contributing to increased cardiovascular risk. Nrf2 activation by several agents reduces ROS levels, decreasing metabolic damage and reducing cardiovascular risk.

On the other hand, Nrf2 gene deletion in ApoE knockout mice decreases atherosclerotic lesions at a late stage, whereas it does not affect atherosclerotic lesions in earlier stages (Harada et al., 2012). These observations suggest that Nrf2 inhibition may be atheroprotective in advanced plaques. Additionally, Ishii et al. observed that oxidized lipids induce Nrf2-dependent CD36 scavenger receptor expression in macrophages, resulting in intracellular accumulation of ox-LDL (Ishii et al., 2004). However, deletion of Nrf2 in myeloid cells of LDL receptor knockout mice ($LDLr^{-/-}$) exacerbates atherosclerotic lesions and increases pro-inflammatory genes expression (Collins et al., 2012), indicating that Nrf2 activity in myeloid cells and macrophages modulates the pro-inflammatory vascular milieu associated with atherosclerosis. These conflicting findings demonstrate that Nrf2 may also exhibit pro-atherogenic functions, depending on atherosclerotic lesion stage or animal model.

The accumulation of lipids into the vascular intima is related to oxidative and pro-inflammatory stress that result in endothelial cells dysfunction (reviewed in Sitia et al., 2010). Pro-inflammatory cytokines contribute to monocytes recruitment into the intima by inducing expression of endothelial adhesion molecules and chemokines. Nrf2 activity reduces the inflammatory response in endothelial cells. Ox-LDL-induced expression of vascular cellular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) is reduced by a HO-1 inducer (Zhang et al., 2013). Furthermore, Nrf2/ARE pathway suppresses TNF-α-induced expression of redox-sensitive inflammatory genes, including monocyte chemoattractant protein (MCP)-1 and VCAM-1 (Chen et al., 2006). These results indicate that Nrf2 activation is atheroprotective due to its antioxidant and anti-inflammatory actions that limit the deleterious effects imposed by hyperlipidemic and inflammatory processes to endothelial cells.

Nrf2 anti-atherogenic effects have also been linked to its modulatory effects on migration and proliferation of VSMCs. PDGF-induced VSMCs migration is increased by Nrf2 depletion, and Nrf2-deficient mice exhibit higher neointimal hyperplasia, as shown in a wire injury model (Ashino et al., 2013). Nrf2 control of neointima hyperplasia may be linked to the ability of Nrf2/Keap-1 system to regulate VSMCs apoptosis and, consequently, to inhibit neointimal hyperplasia after vascular injury (Ashino et al., 2016). Also, increased expression of Nrf2 target genes reduces VSMCs proliferation (Duckers et al., 2001; Kim et al., 2009). Moreover, Nrf2 activity is important to maintain VSMCs phenotype (Xu M. et al., 2015) and the activation of Keap-1/Nrf2/NQO1 pathway attenuates VSMCs calcification circulating calciprotein particles (CPP)-induced VSMCs calcification (Aghagolzadeh et al., 2017). Taken together, these studies indicate that Nrf2 may protect against atherogenesis by decreasing VSMCs migration, proliferation, calcification and vascular remodeling.

In recent years, microRNAs (non-coding small RNAs) were identified as key regulators in the cellular events and molecular signaling pathways involved in atherosclerosis (reviewed in Feinberg and Moore, 2016). Multiple microRNAs that participate in cholesterol homeostasis (miR-33), macrophage activation (miR-155), endothelium dysfunction (miR-146), VSMCs proliferation (miR-221), and other processes that lead to plaque progression have already been identified (reviewed in Feinberg and Moore, 2016). In this context, the Nrf2 system and microRNAs can establish regulatory loops and influence vascular responses to oxidative and inflammatory injury. Accordingly, Nrf2 and miR93 regulate endothelial glycolysis, proliferation, and quiescence by Krüppel-like Factor 2 (KLF2)- and vascular endothelial growth factor A (VEGFA)-dependent mechanisms (Kuosmanen et al., 2017). Moreover, oxidized palmitoyl-arachidonoyl-phosphatidylcholine (Ox-PAPC)- induced HO-1 expression is partially dependent on miR-320a in endothelial cells (Schrottmaier et al., 2014). However, the role of microRNAs in the Nrf2 system and their implications in atherogenesis need to be further explored.

In conclusion, anti-oxidant and anti-inflammatory effects of Nrf2 play an essential modulatory role in the formation and progression of atherosclerotic lesions, regulating functional and structural vascular responses. However, additional studies are necessary to explain the discrepant results related to the role of Nrf2 in the different stages of plaque progression. The interactions between microRNAs and Nrf2 target genes during atherosclerosis development also deserve further investigation.

CONCLUSION

Activation of the Nrf2-dependent antioxidant system plays an important role in cell defense against oxidative stress damage, whereas the insufficiency of the Nrf2 system is associated with multiple aspects of the genesis and progression of metabolic diseases, posing a great risk to the cardiovascular system (**Figure 1**). The systemic increase of Nrf2 activity by several activators may be beneficial in the treatment of metabolic diseases. In addition, selective upregulation of Nrf2 genes may represent a potential therapy in obesity, diabetes and atherosclerosis. Looking to the future, experimental research that elucidates the role of Nrf2 activation in specific tissues, such as adipose tissue, liver, pancreas and others, is important for better understanding of the multiple roles of Nrf2. Additional studies may also provide new redox balance-targeted therapy for the treatment of metabolic diseases and consequent mitigation of cardiovascular risk.

AUTHOR CONTRIBUTIONS

RdC, DR, CP, JS, JA, NL, and RT equally contributed to the conception and draft of manuscript and approved its final version.

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REFERENCES

Abed, D. A., Goldstein, M., Albanyan, H., Jin, H., and Hu, L. (2015). Discovery of direct inhibitors of Keap1–Nrf2 protein–protein interaction as potential therapeutic and preventive agents. *Acta Pharm. Sin. B* 5, 285–299. doi: 10.1016/j.apsb.2015.05.008

Aghagolzadeh, P., Radpour, R., Bachtler, M., van Goor, H., Smith, E. R., Lister, A., et al. (2017). Hydrogen sulfide attenuates calcification of vascular smooth muscle cells via KEAP1/NRF2/NQO1 activation. *Atherosclerosis* 265, 78–86. doi: 10.1016/j.atherosclerosis.2017.08.012

Alves-Lopes, R., Neves, K. B., Montezano, A. C., Harvey, A., Carneiro, F. S., Touyz, R. M., et al. (2016). Internal pudental artery dysfunction in diabetes mellitus is mediated by NOX1-derived ROS-, Nrf2-, and rho kinase-dependent mechanisms. *Hypertension* 68, 1056–1064. doi: 10.1161/HYPERTENSIONAHA.116.07518

Ashino, T., Yamamoto, M., and Numazawa, S. (2016). Nrf2/Keap1 system regulates vascular smooth muscle cell apoptosis for vascular homeostasis: role in neointimal formation after vascular injury. *Sci. Rep.* 6:26291. doi: 10.1038/srep26291

Ashino, T., Yamamoto, M., Yoshida, T., and Numazawa, S. (2013). Redox-sensitive transcription factor Nrf2 regulates vascular smooth muscle cell migration and neointimal hyperplasia significance. *Arterioscler. Thromb. Vasc. Biol.* 33, 760–768. doi: 10.1161/ATVBAHA.112.300614

Becker, L. B. (2004). New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc. Res.* 61, 461–470. doi: 10.1016/j.cardiores.2003.10.025

Belizário, J. E., Faintuch, J., and Garay-Malpartida, M. (2018). Gut Microbiome dysbiosis and immunometabolism: new frontiers for treatment of metabolic diseases. *Mediat. Inflamm.* 2018:2037838. doi: 10.1155/2018/2037838

Bocci, V., Zanardi, I., Huijberts, M. S., and Travagli, V. (2014). An integrated medical treatment for type-2 diabetes. *Diabetes Metab. Syndr.* 8, 57–61. doi: 10.1016/j.dsx.2013.10.004

Brown, D. I., and Griendling, K. K. (2015). Regulation of signal transduction by reactive oxygen species in the cardiovascular system. *Circ. Res.* 116, 531–549. doi: 10.1161/CIRCRESAHA.116.303584

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* 414, 813–820. doi: 10.1038/414813a

Burgoyne, J. R., Mongue-Din, H., Eaton, P., and Shah, A. M. (2012). Redox signaling in cardiac physiology and pathology. *Circ. Res.* 111, 1091–1106. doi: 10.1161/CIRCRESAHA.111.255216

Canning, P., Sorrell, F. J., and Bullock, A. N. (2015). Structural basis of Keap1 interactions with Nrf2. *Free Radic. Biol. Med.* 88, 101–107. doi: 10.1016/j.freeradbiomed.2015.05.034

Chartoumpekis, D. V., Ziros, P. G., Psyrogiannis, A. I., Papavassiliou, A. G., Kyriazopoulou, V. E., Sykiotis, G. P., et al. (2011). Nrf2 represses FGF21 during long-term high-fat diet–induced obesity in mice. *Diabetes* 60, 2465–2473. doi: 10.2337/db11-0112

Chen, X. L., Dodd, G., Thomas, S., Zhang, X., Wasserman, M. A., Rovin, B. H., et al. (2006). Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. *Am. J. Physiol. Heart Circ. Physiol.* 290, H1862–H1870. doi: 10.1152/ajpheart.00651.2005

Cheng, C., Noordeloos, A. M., Jeney, V., Soares, M. P., Moll, F., Pasterkamp, G., et al. (2009). Heme oxygenase 1 determines atherosclerotic lesion progression into a vulnerable plaque. *Circulation* 119, 3017–3027. doi: 10.1161/CIRCULATIONAHA.108.808618

Cheng, F., Torzewski, M., Degreif, A., Rossmann, H., Canisius, A., and Lackner, K. J. (2013). Impact of glutathione peroxidase-1 deficiency on macrophage foam cell formation and proliferation: implications for atherogenesis. *PLoS One* 8:e72063. doi: 10.1371/journal.pone.0072063

Chin, M., Lee, C. Y. I., Chuang, J. C., Bumeister, R., Wigley, W. C., Sonis, S. T., et al. (2013). Bardoxolone methyl analogs RTA 405 and dh404 are well tolerated and exhibit efficacy in rodent models of type 2 diabetes and obesity. *Am. J. Physiol. Renal Physiol.* 304, F1438–F1446. doi: 10.1152/ajprenal.00387.2012

Choi, S. H., Park, S., Oh, C. J., Leem, J., Park, K. G., and Lee, I. K. (2015). Dipeptidyl peptidase-4 inhibition by gemigliptin prevents abnormal vascular remodeling via NF-E2-related factor 2 activation. *Vascul. Pharmacol.* 73, 11–19. doi: 10.1016/j.vph.2015.07.005

Collins, A. R., Gupte, A. A., Ji, R., Ramirez, M. R., Minze, L. J., Liu, J. Z., et al. (2012). Myeloid deletion of nuclear factor erythroid 2-related factor 2 increases atherosclerosis and liver injury. *Arterioscler. Thromb. Vasc. Biol.* 32, 2839–2846. doi: 10.1161/ATVBAHA.112.300345

Costa, R. M., Alves-Lopes, R., Mestriner, F., and Tostes, R. (2017). Testosterone downregulates the Nrf2 system and promotes vascular dysfunction in HFD-fed mice. *FASEB J.* 31, (1_Suppl.), 837.18.

da Costa, R. M., Fais, R. S., Dechandt, C. R., Louzada-Junior, P., Alberici, L. C., Lobato, N. S., et al. (2017). Increased mitochondrial ROS generation mediates the loss of the anti-contractile effects of perivascular adipose tissue in high-fat diet obese mice. *Br. J. Pharmacol.* 174, 3527–3541. doi: 10.1111/bph.13687

da Costa, R. M., Neves, K. B., Mestriner, F. L., Louzada-Junior, P., Bruder-Nascimento, T., and Tostes, R. C. (2016). TNF-α induces vascular insulin resistance via positive modulation of PTEN and decreased Akt/eNOS/NO signaling in high fat diet-fed mice. *Cardiovasc. Diabetol.* 15:119. doi: 10.1186/s12933-016-0443-0

Dandona, P., Thusu, K., Cook, S., Snyder, B., Makowski, J., Armstrong, D., et al. (1996). Oxidative damage to DNA in diabetes mellitus. *Lancet* 347, 444–445. doi: 10.1016/S0140-6736(96)90013-6

David, J. A., Rifkin, W. J., Rabbani, P. S., and Ceradini, D. J. (2017). The Nrf2/Keap1/ARE pathway and oxidative stress as a therapeutic target in type II diabetes mellitus. *J. Diabetes Res.* 2017, 4826724. doi: 10.1155/2017/4826724

DeMarco, V. G., Johnson, M. S., Whaley-Connell, A. T., and Sowers, J. R. (2010). Cytokine abnormalities in the etiology of the cardiometabolic syndrome. *Curr. Hypertens. Rep.* 12, 93–98. doi: 10.1007/s11906-010-0095-5

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* 82, 47–95. doi: 10.1152/physrev.00018.2001

Duckers, H. J., Boehm, M., True, A. L., Yet, S. F., San, H., Park, J. L., et al. (2001). Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat. Med.* 7:693. doi: 10.1038/89068

Feinberg, M. W., and Moore, K. J. (2016). MicroRNA regulation of atherosclerosis. *Circ. Res.* 118, 703–720. doi: 10.1161/CIRCRESAHA.115.306300

Furukawa, M., and Xiong, Y. (2005). BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Mol. Cell. Biol.* 25, 162–171. doi: 10.1128/MCB.25.1.162-171.2005

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., et al. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *J. Clin. Invest.* 114, 1752–1761. doi: 10.1172/JCI21625

Galley, J. C., and Straub, A. C. (2017). Redox control of vascular function. *Arterioscler. Thromb Vasc. Biol.* 37, e178–e184. doi: 10.1161/ATVBAHA.117.309945

Gao, S., Duan, X., Wang, X., Dong, D., Liu, D., Li, X., et al. (2013). Curcumin attenuates arsenic-induced hepatic injuries and oxidative stress in experimental mice through activation of Nrf2 pathway, promotion of arsenic methylation and urinary excretion. *Food Chem. Toxicol.* 59, 739–747. doi: 10.1016/j.fct.2013.07.032

Giacco, F., and Brownlee, M. (2010). Oxidative stress and diabetic complications. *Circ. Res.* 107, 1058–1070. doi: 10.1161/CIRCRESAHA.110.223545

Guerrero-Hue, M., Farre-Alins, V., Palomino-Antolin, A., Parada, E., Rubio-Navarro, A., Egido, J., et al. (2017). Targeting Nrf2 in protection against renal disease. *Curr. Med. Chem.* 24, 3583–3605. doi: 10.2174/0929867324666170511120814

Hamilton, S. J., and Watts, G. F. (2013). Endothelial dysfunction in diabetes: pathogenesis, significance, and treatment. *Rev. Diabetes Stud.* 10, 133–156. doi: 10.1900/RDS.2013.10.133

Harada, N., Ito, K., Hosoya, T., Mimura, J., Maruyama, A., Noguchi, N., et al. (2012). Nrf2 in bone marrow-derived cells positively contributes to the advanced stage of atherosclerotic plaque formation. *Free Radic. Biol. Med.* 53, 2256–2262. doi: 10.1016/j.freeradbiomed.2012.10.001

Harrison, D., Griendling, K. K., Landmesser, U., Hornig, B., and Drexler, H. (2003). Role of oxidative stress in atherosclerosis. *Am. J. Cardiol.* 91, 7–11. doi: 10.1016/S0002-9149(02)03144-2

Hattori, R., Otani, H., Uchiyama, T., Imamura, H., Cui, J., Maulik, N., et al. (2001). Src tyrosine kinase is the trigger but not the mediator of ischemic preconditioning. *Am. J. Physiol. Heart Circ. Physiol.* 281, H1066–H1074. doi: 10.1152/ajpheart.2001.281.3.H1066

Huang, X., Ordemann, J., Müller, J. M., and Dubiel, W. (2012). The COP9 signalosome, cullin 3 and Keap1 supercomplex regulates CHOP stability and adipogenesis. *Biol. Open* 1, 705–710. doi: 10.1242/bio.20121875

Hwang, A. R., Han, J. H., Lim, J. H., Kang, Y. J., and Woo, C. H. (2017). Fluvastatin inhibits AGE-induced cell proliferation and migration via an ERK5-dependent Nrf2 pathway in vascular smooth muscle cells. *PLoS One* 12:e0178278. doi: 10.1371/journal.pone.0178278

Ichikawa, T., Li, J., Meyer, C. J., Janicki, J. S., Hannink, M., and Cui, T. (2009). Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes. *PLoS One* 4:e8391. doi: 10.1371/journal.pone.0008391

Ishii, T., Itoh, K., Ruiz, E., Leake, D. S., Unoki, H., Yamamoto, M., et al. (2004). Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ. Res.* 94, 609–616. doi: 10.1161/01.RES.0000119171.44657.45

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., et al. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76–86. doi: 10.1101/gad.13.1.76

Jiao, Y., Watts, T., Xue, J., Hannafon, B., and Ding, W. Q. (2018). Sorafenib and docosahexaenoic acid act in synergy to suppress cancer cell viability: a role of heme oxygenase 1. *BMC Cancer* 18:1042. doi: 10.1186/s12885-018-4946-9

Jiménez-Osorio, A. S., Picazo, A., González-Reyes, S., Barrera-Oviedo, D., Rodríguez-Arellano, M. E., and Pedraza-Chaverri, J. (2014). Nrf2 and redox status in prediabetic and diabetic patients. *Int. J. Mol. Sci.* 15, 20290–20305. doi: 10.3390/ijms151120290

Jones, R. M., Desai, C., Darby, T. M., Luo, L., Wolfarth, A. A., Scharer, C. D., et al. (2015). Lactobacilli modulate epithelial cytoprotection through the Nrf2 pathway. *Cell Rep.* 12, 1217–1225. doi: 10.1016/j.celrep.2015.07.042

Jyrkkänen, H. K., Kansanen, E., Inkala, M., Kivelä, A. M., Hurttila, H., Heinonen, S. E., et al. (2008). Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo. *Circ Res.* 103, e1–e9. doi: 10.1161/CIRCRESAHA.108.176883

Kansanen, E., Kuosmanen, S. M., Leinonen, H., and Levonen, A. L. (2013). The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. *Redox Biol.* 1, 45–49. doi: 10.1016/j.redox.2012.10.001

Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., and Yamamoto, M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells* 6, 857–868. doi: 10.1046/j.1365-2443.2001.00469.x

Keaney, J. F. Jr., Larson, M. G., Vasan, R. S., Wilson, P. W., Lipinska, I., Corey, D., et al. (2003). Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. *Arterioscler. Thromb. Vasc. Biol.* 23, 434–439. doi: 10.1161/01.ATV.0000058402.34138.11

Kim, S. Y., Jeoung, N. H., Oh, C. J., Choi, Y. K., Lee, H. J., Kim, H. J., et al. (2009). Activation of NAD (P) H: quinone oxidoreductase 1 prevents arterial restenosis by suppressing vascular smooth muscle cell proliferation. *Circ. Res.* 104, 842–850. doi: 10.1161/CIRCRESAHA.108.189837

Knock, G. A., and Ward, J. P. (2011). Redox regulation of protein kinases as a modulator of vascular function. *Antioxid. Redox Signal.* 15, 1531–1547. doi: 10.1089/ars.2010.3614

Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., et al. (2002). Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells* 7, 807–820. doi: 10.1046/j.1365-2443.2002.00561.x

Koelwyn, G. J., Corr, E. M., Erbay, E., and Moore, K. J. (2018). Regulation of macrophage immunometabolism in atherosclerosis. *Nat. Immunol.* 19, 526–537. doi: 10.1038/s41590-018-0113-3

Kuosmanen, S. M., Kansanen, E., Kaikkonen, M. U., Sihvola, V., Pulkkinen, K., Jyrkkänen, H. K., et al. (2017). NRF2 regulates endothelial glycolysis and proliferation with miR-93 and mediates the effects of oxidized phospholipids on endothelial activation. *Nucleic Acids Res.* 46, 1124–1138. doi: 10.1093/nar/gkx1155

Li, J., Ichikawa, T., Villacorta, L., Janicki, J. S., Brower, G. L., Yamamoto, M., et al. (2009). Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. *Arterioscler. Thromb Vasc. Biol.* 29, 1843–1850. doi: 10.1161/ATVBAHA.109.189480

Li, Y., Wang, X. L., Sun, X., Chai, Q., Li, J., Thompson, B., et al. (2017). Regulation of vascular large-conductance calcium-activated potassium channels by Nrf2 signalling. *Diabetes Vasc. Dis. Res.* 14, 353–362. doi: 10.1177/1479164117703903

Liu, J., Wu, J., Sun, A., Sun, Y., Yu, X., Liu, N., et al. (2016). Hydrogen sulfide decreases high glucose/palmitate-induced autophagy in endothelial cells by the Nrf2-ROS-AMPK signaling pathway. *Cell Biosci.* 6:33. doi: 10.1186/s13578-016-0099-1

Long, M., De La Vega, M. R., Wen, Q., Bharara, M., Jiang, T., Zhang, R., et al. (2016). An essential role of NRF2 in diabetic wound healing. *Diabetes* 65, 780–793. doi: 10.2337/db15-0564

Lopes, R. A., Neves, K. B., Tostes, R. C., Montezano, A. C., and Touyz, R. M. (2015). Downregulation of nuclear factor erythroid 2–related factor and associated antioxidant genes contributes to redox-sensitive vascular dysfunction in hypertension. *Hypertension* 66, 1240–1250. doi: 10.1161/HYPERTENSIONAHA.115.06163

Lu, T., Sun, X., Li, Y., Chai, Q., Wang, X. L., and Lee, H. C. (2017). Role of Nrf2 signaling in the regulation of vascular BK channel β1 subunit expression and BK channel function in high-fat diet-induced diabetic mice. *Diabetes* 66, 2681–2690. doi: 10.2337/db17-0181

Lynch, S. V., and Pedersen, O. (2016). The human intestinal microbiome in health and disease. *N. Engl. J. Med.* 375, 2369–2379. doi: 10.1056/NEJMra1600266

Maulik, N., Sasaki, H., and Galang, N. (1999). Differential regulation of apoptosis by Ischemia-reperfusion and ischemic adaptation a. *Ann. N. Y. Acad. Sci.* 874, 401–411. doi: 10.1111/j.1749-6632.1999.tb09254.x

May, J. M., and Harrison, F. E. (2013). Role of vitamin C in the function of the vascular endothelium. *Antioxid. Redox Signal.* 19, 2068–2083. doi: 10.1089/ars.2013.5205

McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J. D. (2004). Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *J. Biol. Chem.* 279, 31556–31567. doi: 10.1074/jbc.M403061200

Mohler, J., Vani, K., Leung, S., and Epstein, A. (1991). Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis. *Mech. Dev.* 34, 3–9. doi: 10.1016/0925-4773(91)90086-L

Motohashi, H., O'Connor, T., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2002). Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* 294, 1–12. doi: 10.1016/S0378-1119(02)00788-6

Murota, K., Nakamura, Y., and Uehara, M. (2018). Flavonoid metabolism: the interaction of metabolites and gut microbiota. *Biosci. Biotechnol. Biochem.* 82, 600–610. doi: 10.1080/09168451.2018.1444467

Neves, K. B., Nguyen Dinh Cat, A., Alves-Lopes, R., Harvey, K. Y., da Costa, R. M., Lobato, N. S., et al. (2018). Chemerin receptor blockade improves vascular function in diabetic obese mice via redox-sensitive-and Akt-dependent pathways. *Am. J. Physiol. Heart Circ. Physiol.* 315, H1851–H1860. doi: 10.1152/ajpheart.00285.2018

Nioi, P., Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2005). The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. *Mol. Cell. Biol.* 25, 10895–10906. doi: 0.1128/MCB.25.24.10895-10906.2005

Orozco, L. D., Kapturczak, M. H., Barajas, B., Wang, X., Weinstein, M. M., Wong, J., et al. (2007). Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. *Circ. Res.* 100, 1703–1711. doi: 10.1161/CIRCRESAHA.107.151720

Otani, H. (2011). Oxidative stress as pathogenesis of cardiovascular risk associated with metabolic syndrome. *Antioxid. Redox Signal.* 15, 1911–1926. doi: 10.1089/ars.2010.3739

Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., et al. (1996). Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol. Cell. Biol.* 16, 6083–6095. doi: 10.1128/MCB.16.11.6083

Papaharalambus, C. A., and Griendling, K. K. (2007). Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends Cardiovasc. Med.* 17, 48–54. doi: 10.1016/j.tcm.2006.11.005

Paravicini, T. M., Chrissobolis, S., Drummond, G. R., and Sobey, C. G. (2004). Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced cerebral vasodilatation to NADPH in vivo. *Stroke* 35, 584–589. doi: 10.1161/01.STR.0000112974.37028.58

Pereira, A., Fernandes, R., Crisóstomo, J., Seiça, R. M., and Sena, C. M. (2017). The Sulforaphane and pyridoxamine supplementation normalize endothelial dysfunction associated with type 2 diabetes. *Sci. Rep.* 7:14357. doi: 10.1038/s41598-017-14733-x

Pergola, P. E., Krauth, M., Huff, J. W., Ferguson, D. A., Ruiz, S., Meyer, C. J., et al. (2011). Effect of bardoxolone methyl on kidney function in patients with T2D and stage 3b–4 CKD. *Am. J. Nephrol.* 33, 469–476. doi: 10.1159/000327599

Pi, J., Leung, L., Xue, P., Wang, W., Hou, Y., Liu, D., et al. (2010). Deficiency in the nuclear factor E2-related factor 2 transcription factor results in impaired adipogenesis and protects against diet-induced obesity. *J. Biol. Chem.* 285, 9292–9300. doi: 10.1074/jbc.M109.093955

Raggi, P., Genest, J., Giles, J. T., Rayner, K. J., Dwivedi, G., Beanlands, R. S., et al. (2018). Role of inflammation in the pathogenesis of atherosclerosis and therapeutic interventions. *Atherosclerosis* 276, 98–108. doi: 10.1016/j.atherosclerosis.2018.07.014

Redón, J., Oliva, M. R., Tormos, C., Giner, V., Chaves, J., Iradi, A., et al. (2003). Antioxidant activities and oxidative stress byproducts in human hypertension. *Hypertension* 41, 1096–1101. doi: 10.1161/01.HYP.0000068370.21009.38

Rodriguez, R., and Redman, R. (2005). Balancing the generation and elimination of reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3175–3176. doi: 10.1073/pnas.0500367102

Ryter, S. W., and Choi, A. M. (2005). Heme oxygenase-1: redox regulation of a stress protein in lung and cell culture models. *Antioxid. Redox Signal.* 7, 80–91. doi: 10.1089/ars.2005.7.80

Sampath, C., Rashid, M. R., Sang, S., and Ahmedna, M. (2017). Green tea epigallocatechin 3-gallate alleviates hyperglycemia and reduces advanced glycation end products via nrf2 pathway in mice with high fat diet-induced obesity. *Biomed. Pharmacother.* 87, 73–81. doi: 10.1016/j.biopha.2016.12.082

Santhakumar, A. B., Bulmer, A. C., and Singh, I. (2014). A review of the mechanisms and effectiveness of dietary polyphenols in reducing oxidative stress and thrombotic risk. *J. Hum. Nutr. Diet.* 27, 1–21. doi: 10.1111/jhn.12177

Schrottmaier, W. C., Oskolkova, O. V., Schabbauer, G., and Afonyushkin, T. (2014). MicroRNA miR-320a modulates induction of HO-1, GCLM and OKL38 by oxidized phospholipids in endothelial cells. *Atherosclerosis* 235, 1–8. doi: 10.1016/j.atherosclerosis.2014.03.026

Sharma, A., Rizky, L., Stefanovic, N., Tate, M., Ritchie, R. H., Ward, K. W., et al. (2017). The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator dh404 protects against diabetes-induced endothelial dysfunction. *Cardiovasc. Diabetol.* 16:33. doi: 10.1186/s12933-017-0513-y

Shawky, N. M., Pichavaram, P., Shehatou, G. S., Suddek, G. M., Gameil, N. M., Jun, J. Y., et al. (2016). Sulforaphane improves dysregulated metabolic profile and inhibits leptin-induced VSMC proliferation: implications toward suppression of neointima formation after arterial injury in western diet-fed obese mice. *J. Nutr. Biochem.* 32, 73–84. doi: 10.1016/j.jnutbio.2016.01.009

Shin, S., Wakabayashi, J., Yates, M. S., Wakabayashi, N., Dolan, P. M., Aja, S., et al. (2009). Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide. *Eur. J. Pharmacol.* 620, 138–144. doi: 10.1016/j.ejphar.2009.08.022

Siti, H. N., Kamisah, Y., and Kamsiah, J. (2015). The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review). *Vascul. Pharmacol.* 71, 40–56. doi: 10.1016/j.vph.2015.03.005

Sitia, S., Tomasoni, L., Atzeni, F., Ambrosio, G., Cordiano, C., Catapano, A., et al. (2010). From endothelial dysfunction to atherosclerosis. *Autoimmun. Rev.* 9, 830–834. doi: 10.1016/j.autrev.2010.07.016

Slocum, S. L., Skoko, J. J., Wakabayashi, N., Aja, S., Yamamoto, M., Kensler, T. W., et al. (2016). Keap1/Nrf2 pathway activation leads to a repressed hepatic gluconeogenic and lipogenic program in mice on a high-fat diet. *Arch. Biochem. Biophys.* 591, 57–65. doi: 10.1016/j.abb.2015

Sowers, J. R., Whaley-Connell, A., and Hayden, M. R. (2011). The role of overweight and obesity in the cardiorenal syndrome. *Cardiorenal Med.* 1, 5–12. doi: 10.1159/000322822

Steinberg, S. F. (2013). Oxidative stress and sarcomeric proteins. *Circ. Res.* 112, 393–405. doi: 10.1161/CIRCRESAHA.111.300496

Tabit, C. E., Chung, W. B., Hamburg, N. M., and Vita, J. A. (2010). Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Rev. Endocr. Metab. Disord.* 11, 61–74. doi: 10.1007/s11154-010-9134-4

Tan, S. M., Sharma, A., Stefanovic, N., Yuen, D. Y., Karagiannis, T. C., Meyer, C., et al. (2014). A derivative of bardoxolone methyl, dh404, in an inverse dose-dependent manner, lessens diabetes-associated atherosclerosis and improves diabetic kidney disease. *Diabetes* 63, 3091–3103. doi: 10.2337/db13-1743

Tanaka, Y., Aleksunes, L. M., Yeager, R. L., Gyamfi, M. A., Esterly, N., Guo, G. L., et al. (2008). NF-E2-related factor 2 inhibits lipid accumulation and oxidative stress in mice fed a high-fat diet. *J. Pharmacol. Exp. Ther.* 325, 655–664. doi: 10.1124/jpet.107.135822

Tebay, L. E., Robertson, H., Durant, S. T., Vitale, S. R., Penning, T. M., Dinkova-Kostova, A. T., et al. (2015). Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radic. Biol. Med.* 88, 108–146. doi: 10.1016/j.freeradbiomed.2015.06.021

Thannickal, V. J., and Fanburg, B. L. (2000). Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 279, L1005–L1028. doi: 10.1152/ajplung.2000.279.6.L1005

Tong, Y. H., Zhang, B., Fan, Y., and Lin, N. M. (2015). Keap1–Nrf2 pathway: a promising target towards lung cancer prevention and therapeutics. *Chronic Dis. Transl. Med.* 1, 175–186. doi: 10.1016/j.cdtm.2015.09.002

Touyz, R. M., Anagnostopoulou, A., Camargo, L., Rios, F., and Montezano, A. (2018). Vascular biology of superoxide-generating NADPH oxidase 5 (Nox5)-implications in hypertension and cardiovascular disease. *Antioxid. Redox Signal.* 30, 1027–1040. doi: 10.1089/ars.2018.7583

Touyz, R. M., and Briones, A. M. (2011). Reactive oxygen species and vascular biology: implications in human hypertension. *Hypertens. Res.* 34, 5–14. doi: 10.1038/hr.2010.201

Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., et al. (2013). The Keap1-Nrf2 system prevents onset of diabetes mellitus. *Mol. Cell. Biol.* 33, 2996–3010. doi: 10.1128/MCB.00225-13

Uruno, A., Yagishita, Y., and Yamamoto, M. (2015). The Keap1–Nrf2 system and diabetes mellitus. *Arch. Biochem. Biophys.* 566, 76–84. doi: 10.1016/j.abb.2014.12.012

Wang, H., Liu, K., Geng, M., Gao, P., Wu, X., Hai, Y., et al. (2013). RXRα inhibits the NRF2-ARE signalling pathway through a direct interaction with the Neh7 domain of NRF2. *Cancer Res.* 73, 3097–3108. doi: 10.1158/0008-5472.CAN-12-3386

Wang, S., Wang, B., Wang, Y., Tong, Q., Liu, Q., Sun, J., et al. (2017). Zinc prevents the development of diabetic cardiomyopathy in db/db mice. *Int. J. Mol. Sci.* 18:E580. doi: 10.3390/ijms18030580

Wang, Y., Zhang, Z., Sun, W., Tan, Y., Liu, Y., Zheng, Y., et al. (2014). Sulforaphane attenuation of type 2 diabetes-induced aortic damage was associated with the upregulation of Nrf2 expression and function. *Oxid. Med. Cell. Longev.* 2014:123963. doi: 10.1155/2014/123963

Wu, Q. Q., Wang, Y., Senitko, M., Meyer, C., Wigley, W. C., Ferguson, D. A., et al. (2011). Bardoxolone methyl (BARD) ameliorates ischemic AKI and increases expression of protective genes Nrf2, PPARγ, and HO-1. *Am. J. Physiol. Renal Physiol.* 300, F1180–F1192. doi: 10.1152/ajprenal.00353.2010

Xia, Z., Liu, M., Wu, Y., Sharma, V., Luo, T., Ouyang, J., et al. (2006). N-acetylcysteine attenuates TNF-α-induced human vascular endothelial cell apoptosis and restores eNOS expression. *Eur. J. Pharmacol.* 550, 134–142. doi: 10.1016/j.ejphar.2006.08.044

Xu, J., Donepudi, A. C., More, V. R., Kulkarni, S. R., Li, L., Guo, L., et al. (2015). Deficiency in N rf2 transcription factor decreases adipose tissue mass and hepatic lipid accumulation in leptin-deficient mice. *Obesity* 23, 335–344. doi: 10.1002/oby.20929

Xu, M., Li, X. X., Wang, L., Wang, M., Zhang, Y., and Li, P. L. (2015). Contribution of Nrf2 to atherogenic phenotype switching of coronary arterial smooth muscle cells lacking CD38 gene. *Cell Physiol. Biochem.* 37, 432–444. doi: 10.1159/000430366

Xu, J., Kulkarni, S. R., Donepudi, A. C., More, V. R., and Slitt, A. L. (2012). Enhanced Nrf2 activity worsens insulin resistance, impairs lipid accumulation in adipose tissue, and increases hepatic steatosis in leptin-deficient mice. *Diabetes* 61, 3208–3218. doi: 10.2337/db11-1716

Xue, P., Hou, Y., Chen, Y., Yang, B., Fu, J., Zheng, H., et al. (2013). Adipose deficiency of Nrf2 in ob/ob mice results in severe metabolic syndrome. *Diabetes* 62, 845–854. doi: 10.2337/db12-0584

Yagishita, Y., Fukutomi, T., Sugawara, A., Kawamura, H., Takahashi, T., Pi, J., et al. (2014). Nrf2 protects pancreatic β-cells from oxidative and nitrosative stress in diabetic model mice. *Diabetes* 63, 605–618. doi: 10.2337/db13-0909

Yahagi, K., Kolodgie, F. D., Otsuka, F., Finn, A. V., Davis, H. R., Joner, M., et al. (2016). Pathophysiology of native coronary, vein graft, and in-stent atherosclerosis. *Nat. Rev. Cardiol.* 13, 79–98. doi: 10.1038/nrcardio.2015.164

Yet, S. F., Layne, M. D., Liu, X., Chen, Y. H., Ith, B., Sibinga, N. E., et al. (2003). Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J.* 17, 1759–1761. doi: 10.1096/fj.03-0187fje

Youn, J. Y., Gao, L., and Cai, H. (2012). The p47phox-and NADPH oxidase organiser 1 (NOXO1)-dependent activation of NADPH oxidase 1 (NOX1) mediates endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction in a streptozotocin-induced murine model of diabetes. *Diabetologia* 55, 2069–2079. doi: 10.1007/s00125-012-2557-6

Yu, Z., Shao, W., Chiang, Y., Foltz, W., Zhang, Z., Ling, W., et al. (2011). Erratum to: oltipraz upregulates the nuclear factor (erythroid-derived 2)-like 2 (NRF2) antioxidant system and prevents insulin resistance and obesity induced by a high-fat diet in C57BL/6J mice. *Diabetologia* 54, 989–989. doi: 10.1007/s00125-010-2001-8

Zakkar, M., Van der Heiden, K., Luong, L. A., Chaudhury, H., Cuhlmann, S., Hamdulay, S. S., et al. (2009). Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state. *Arterioscler. Thromb. Vasc. Biol.* 29, 1851–1857. doi: 10.1161/ATVBAHA.109.193375

Zeng, C., Zhong, P., Zhao, Y., Kanchana, K., Zhang, Y., Khan, Z. A., et al. (2015). Curcumin protects hearts from FFA-induced injury by activating Nrf2 and inactivating NF-κB both in vitro and in vivo. *J. Mol. Cell. Cardiol.* 79, 1–12. doi: 10.1016/j.yjmcc.2014.10.002

Zhang, H. P., Zheng, F. L., Zhao, J. H., Guo, D. X., and Chen, X. L. (2013). Genistein inhibits ox-LDL-induced VCAM-1, ICAM-1 and MCP-1 expression of HUVECs through heme oxygenase-1. *Arch. Med. Res.* 44, 13–20. doi: 10.1016/j.arcmed.2012.12.001

Zhang, L., Dasuri, K., Fernandez-Kim, S. O., Bruce-Keller, A. J., and Keller, J. N. (2016). Adipose-specific ablation of Nrf2 transiently delayed high-fat diet-induced obesity by altering glucose, lipid and energy metabolism of male mice. *Am. J. Transl. Res.* 8, 5309–5319.

Zheng, L., Wei, H., Yu, H., Xing, Q., Zou, Y., and Zhou, Y. (2018). Fish skin gelatin hydrolysate production by ginger powder induces glutathione synthesis to prevent hydrogen peroxide induced intestinal oxidative stress via the Pept1-p62-Nrf2 Cascade. *J. Agric. Food Chem.* 66, 11601–11611. doi: 10.1021/acs.jafc.8b02840

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Quinolinic Acid and Nuclear Factor Erythroid 2-Related Factor 2 in Depression: Role in Neuroprogression

*Yashika Bansal[1], Raghunath Singh[1], Ishwar Parhar[2], Anurag Kuhad[1] and Tomoko Soga[2]**

[1]*Pharmacology Research Lab, University Institute of Pharmaceutical Sciences UGC-Centre of Advanced Study, Panjab University, Chandigarh, India,* [2]*Brain Research Institute Monash Sunway, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway, Malaysia*

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***Correspondence:**
Tomoko Soga
tomoko.soga@monash.edu

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Depression is an incapacitating neuropsychiatric disorder. The serotonergic system in the brain plays an important role in the pathophysiology of depression. However, due to delayed and/or poor performance of selective serotonin reuptake inhibitors in treating depressive symptoms, the role of the serotonergic system in depression has been recently questioned further. Evidence from recent studies suggests that increased inflammation and oxidative stress may play significant roles in the pathophysiology of depression. The consequences of these factors can lead to the neuroprogression of depression, involving neurodegeneration, astrocytic apoptosis, reduced neurogenesis, reduced plasticity (neuronal and synaptic), and enhanced immunoreactivity. Specifically, increased proinflammatory cytokine levels have been shown to activate the kynurenine pathway, which causes increased production of quinolinic acid (QA, an N-Methyl-D-aspartate agonist) and decreases the synthesis of serotonin. QA exerts many deleterious effects on the brain *via* mechanisms including N-methyl-D-aspartate excitotoxicity, increased oxidative stress, astrocyte degeneration, and neuronal apoptosis. QA may also act directly as a pro-oxidant. Additionally, the nuclear translocation of antioxidant defense factors, such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), is downregulated in depression. Hence, in the present review, we discuss the role of QA in increasing oxidative stress in depression by modulating the nuclear translocation of nuclear factor (erythroid-derived 2)-like 2 and thus affecting the synthesis of antioxidant enzymes.

Keywords: oxidative stress, depression, serotonin, quinolinic acid, tryptophan, Nrf2

INTRODUCTION

Depression is a heterogeneous mood disorder characterized by mood alterations, anhedonia, low self-esteem, social withdrawal, feelings of guilt, idiopathic pain, loss of interest in enjoyable activities, and suicidal tendencies. According to the World Health Organization, more than 300 million people currently have depression and approximately 800,000 individuals with

depression commit suicide every year. Suicide due to depression is the second leading cause of death among individuals 16–25 years of age (WHO, 2018).

Various antidepressants are available for the clinical treatment of depression, including typical antidepressants, atypical antidepressants, monoamine oxidase inhibitors, and selective serotonin reuptake inhibitors. These drugs' mechanism of action aligns well with the monoamine theory of depression. This theory states that decreased levels of serotonin in the synaptic cleft in various brain regions correspond to increased activity of the monoamine degrading enzyme and decreased synthesis of serotonin by serotonergic neurons, ultimately leading to depression (Cowen and Browning, 2015).

Although advancements in medical research have contributed to increase depression treatment options, one-third of patients do not respond to conventional drug therapies. Given this, there is a pressing need to elucidate the pathophysiology of depression so that new pathways can be explored for the investigation of novel therapeutics. Emerging evidence from various clinical and preclinical studies has revealed that oxidative stress and increased activity of immune factor cascades play significant roles in the pathophysiology of depression (Liu et al., 2015; Kohler et al., 2016). Maes et al. were the first to report that abnormal activation of the immune system and hypothalamic-pituitary-adrenal axis hyperactivity occur in depression. Additionally, decreased serotonin has been implicated in depression as a consequence of cell-mediated immune activation leading to decrease availability of plasma L-tryptophan (a precursor for serotonin synthesis). Tryptophan is a common substrate for the kynurenine pathway (KP) and serotonin synthesis pathway (methoxyindole pathway). Activation of indoleamine 2,3-dioxygenase (IDO), a rate limiting enzyme of the KP in the brain and periphery, results in decreased tryptophan availability (Maes et al., 1991, 1993; Maes, 1993). Interferon (IFN)-γ is the major inducer of IDO while tumor necrosis factor-α, interleukin (IL)-6, IFN-β, and IFN-α also activate IDO to some extent. Many studies have found a positive link between neuroinflammation and IDO expression in neurodegenerative diseases and depression (Sapko et al., 2006; Kim et al., 2012; Dobos et al., 2015).

Tryptophan metabolism *via* the KP results in the production of a neurotoxin, quinolinic acid (QA), and a neuroprotective compound, kynurenic acid (KA). KA binds to the glutamate recognition site of the N-methyl-D-aspartate (NMDA) receptor and antagonizes it, while QA binds to the glycine site of the NMDA receptor with agonistic properties. Thus, KA prevents excitotoxicity induced by NMDA overstimulation. Kynurenine is the first metabolite of the KP and is catalyzed by IDO. IDO enhances the activity of the kynureninase enzyme, which catalyzes the breakdown of kynurenine to anthranilic acid. However, kynureninase also prevents the activity of kynurenine aminotransferase (KAT), which catalyzes the formation of neuroprotective KA from kynurenine. Eventually, the breakdown of kynurenine is linked to neurotoxic QA production and reduced KA production (Réus et al., 2015).

Imbalance in the levels of QA and KA has been reported in patients with major depressive disorder (MDD). Furthermore, increased levels of QA exert neurotoxic effects in the brain of patients with depression (explained in the next section) (Myint et al., 2012). Studies conducted by three different groups have shown that QA acts as a pro-oxidant and is associated with oxidative stress (Behan et al., 1999; Santamaría et al., 1999; Rossato et al., 2002). Although QA is an NMDA receptor agonist, QA-induced oxidative stress occurs in both NMDA-dependent and independent fashion and requires further exploration (Orlando et al., 2001; Behan and Stone, 2002; Guillemin, 2012).

A key factor crucial to combat increased oxidative stress is nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is a basic leucine zipper protein factor that acts as a master regulator of oxidative stress, maintains redox homeostasis, and provides protection against oxidative stress by transcribing various antioxidant enzymes. More specifically, studies have also shown downregulation of Nrf2 in depression and that Nrf2 activators, such as sulforaphane and its precursor glucoraphanin, exert antidepressive-like effects in depression (Martín-de-Saavedra et al., 2013; Yao et al., 2016).

In the present review, we discuss the role of QA, which might act as a pro-oxidant by impeding Nrf2 activity, an antioxidant protein implicated in clinical depression. Research on these two factors and their role in depression has led to emerging insight into the neuroprogression theory of depression and potential novel pharmacotherapeutics for its treatment.

THE KP AND GLIAL CELLS IN DEPRESSION

The KP is a metabolic pathway of tryptophan both in the periphery and central nervous system (CNS). In the periphery, 90% of tryptophan is found in the unbound form while 10% is bound to albumin. Only the free form of tryptophan can be transported through the blood-brain barrier (Jones et al., 2013). Tryptophan is metabolized to kynurenine by tryptophan 2,3-dioxygenase or IDO, a rate limiting enzyme of the KP. Tryptophan 2,3-dioxygenase catalyzes tryptophan catabolism in the liver and contributes to the peripheral levels of tryptophan, whereas IDO catalyzes tryptophan metabolism extrahepatically. In inflammatory conditions, IDO is induced by proinflammatory cytokines and shifts tryptophan metabolism to kynurenine. Kynurenine is further metabolized *via* three branches to KA, anthranilic acid, and QA by the enzymatic activity of KAT, kynureninase, and kynurenine monooxygenase, respectively. As shown in **Figure 1**, kynurenine is metabolized to KA through branch 1, to anthranilic acid through branch 2, and to 3-hydroxy kynurenine through branch 3. 3-Hydroxykynurenine is further metabolized to 3-hydroxyanthranilic acid in the presence of kynureninase. Finally, 3-hydroxyanthranilic acid is metabolized to QA in the presence of 3-hydroxyanthranilate 3,4-dioxygenase. Anthranilic acid formed *via* branch 2 is readily metabolized

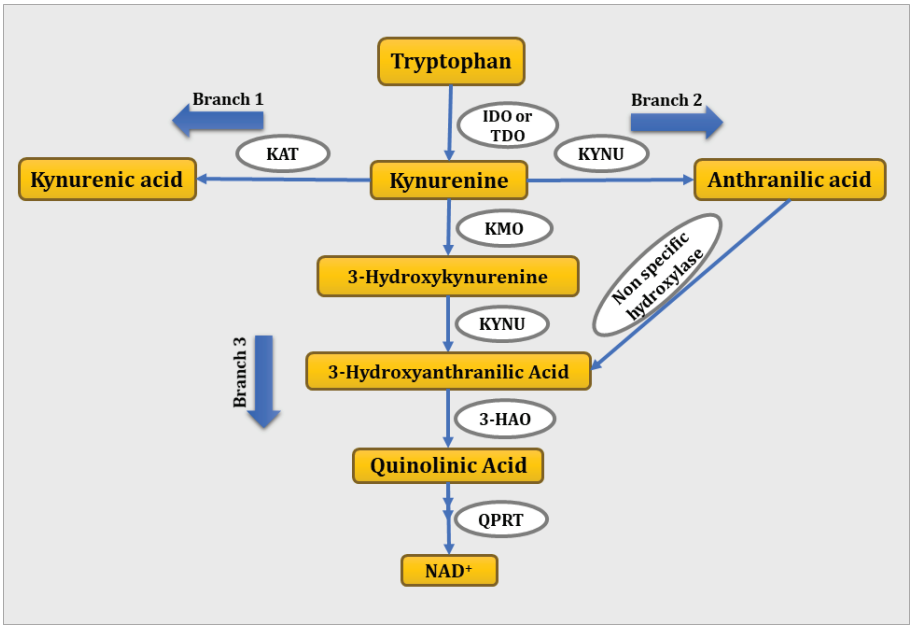

FIGURE 1 | Schematic representation of tryptophan-kynurenine pathway. IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KMO, kynurenine monooxygenase; KYNU, kynureninase; 3-HAO, 3-hydroxyanthranilate 3,4-dioxygenase; QPRT: quinolinate phosphoribosyl transferase.

to 3-hydrocyanthranilic acid through non-specific hydroxylase, which further contributes to the synthesis of QA (Lima, 1998).

Glial cells, i.e., astrocytes and microglia, play a significant role in the development and proper function of the adult brain. Astrocytes are crucial for the formation and maturation of synapses, receptor trafficking, control of the homeostasis of ions and energy metabolites, and clearance of neurotransmitters for maintenance of the neuronal microenvironment (Araque et al., 2014; Dallérac and Rouach, 2016). Astrocytes and microglia have been found to play a potential role in inflammatory and neurodegenerative diseases as they act as both source and target of various inflammatory cytokines. Increased astrogliosis and microgliosis have been observed in several neurological and neurodegenerative disorders such as CNS injury, brain tumors, Huntington's disease, stroke, epilepsy, Parkinson's disease, or Alzheimer's disease, whereas no astrogliosis takes place in depression. Postmortem studies have revealed considerably reduced number and packing density of astrocytes in subjects with depression compared to age-matched normal controls (Cotter et al., 2002; Gittins and Harrison, 2011). In addition, immunohistochemical analysis of glial fibrillary acidic protein, an astrocytic marker, revealed a significantly reduced area covered by glial fibrillary acidic protein-positive cell bodies and processes in various brain regions of young patients with depression compared to a control group (Müller et al., 2001; Fatemi et al., 2004; Miguel-Hidalgo et al., 2010; Gittins and Harrison, 2011; Chandley et al., 2013). In a recent preclinical study by Mendoza et al. (2018), reduced number and complexity of astrocytes in the dentate gyrus region of the hippocampus was seen in a restraint stress mouse model of depression. Contrary to this, animal studies have shown a clear increase in microglial activity in depressed rodents. Mice subjected to lipopolysaccharide (LPS)-induced microglial activation showed depressive-like behaviors (Henry et al., 2008). In a clinical study by Setiawan et al. (2015), significant elevation in the brain translocator protein density, a marker of microglial activation and neuroinflammation, was found in the prefrontal cortex, anterior angulate cortex, thalamus, hippocampus, dorsal putamen, and ventral striatum in patients with MDD compared to healthy controls. The mechanism by which activated microglia induce depression is through activation of microglial IDO and a further signaling pathway, i.e., the KP. Psychological stress, increased glucocorticoid levels, increased inflammatory cytokines, mainly IFN-γ but also tumor necrosis factor-α, IL-6, and their inducers such as LPS, activate microglial IDO (Kiank et al., 2010; Dantzer et al., 2011; Vaváková et al., 2015). Microglial KP-mediated depression is supported by clinical studies where IFN-α-induced immunotherapy increased peripheral and central KP metabolites. The severity of depressive-like symptoms is positively correlated with increased tryptophan metabolism (Capuron et al., 2003; Raison et al., 2010; Savitz, 2017). Additionally, postmortem studies of patients with unipolar depression have shown elevated number of QA-positive microglia in the subgenual anterior cingulate gyrus and anterior mid-cingulate cortex (Steiner et al., 2011). 1-Methlytryptophan (an inhibitor of IDO) and ketamine (an NMDA antagonist) significantly decreased depressive-like behavior by inhibiting QA-mediated NMDA signaling in rodents in an LPS-induced depression model (Aan Het Rot et al., 2012; Dobos et al., 2015). These findings revealed the prominent role of the KP in astrocytes and microglia in depression (**Figure 2**).

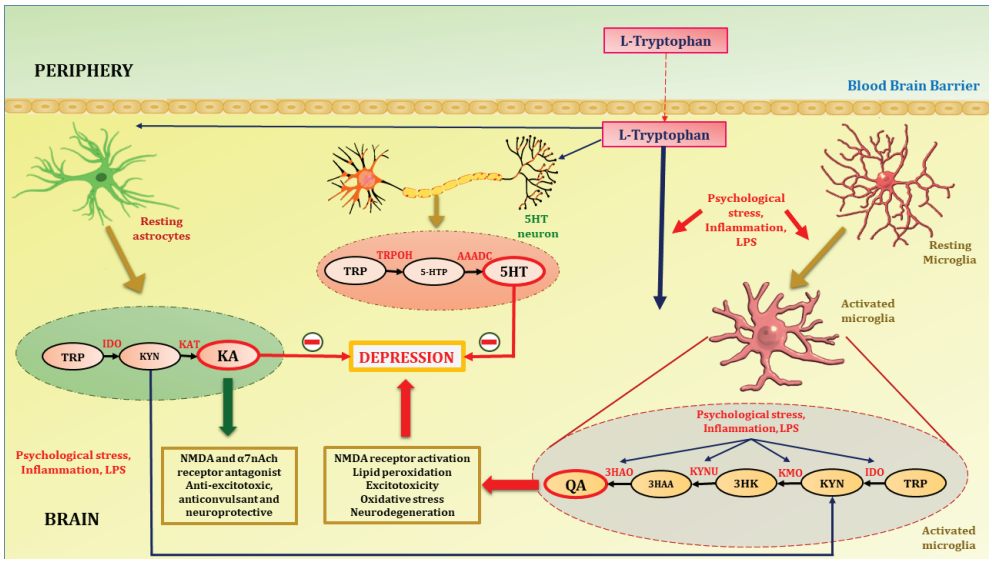

FIGURE 2 | Tryptophan metabolism in astrocytes, microglia and 5HT neuron. Tryptophan, precursor of serotonin is transported to brain with the aid of non-specific competitive L-type amino acid transporters. At homeostatic conditions tryptophan is metabolized to KA in astrocytes and 5HT in serotonergic neurons. KA is an NMDA and α7nAch receptor antagonist, hence acts as anti-excitotoxic and anticonvulsant, thus provide neuroprotection. Increased activation of inflammatory cascades either by psychological stress and LPS activates microglia (microgliosis). Increased inflammation due to psychological stress or LPS activates the enzymes of neurotoxic branch of kynurenine pathway in microglia. This leads to increased production of QA. Psychological stress and activation of inflammatory cascades diverts the metabolism of tryptophan towards QA. This shift hampers the neuroprotection provided by KA and decreases synthesis of 5HT in serotonergic neurons thus contribute to the depression pathophysiology. TRP, tryptophan; IDO, indoleamine-2,3-dioxygenase; KAT, kynurenine aminotransferase; TRPOH, tryptophan hydroxylase; AAADC, aromatic L-amino acid decarboxylase; 5HT, serotonin; 5HTP, 5-hydroxytryptophan; QA, quinolinic acid; KA, kynurenic acid; KYN, kynurenine; 3HK, 3-hydroxykynurenine; 3HAA, 3-hydroxyanthranilic acid; KMO, kynurenine monooxygenase; KYNU, kynureninase; 3HAO, 3-hydroxyanthranilate 3,4-dioxygenase.

QUINOLINIC ACID IN DEPRESSION

QA is a neurotoxic compound involved in the neuroprogression of depression. Neuroprogression is a term inclusive of the various stages of neurodegeneration including apoptosis, reduced neurogenesis, reduced plasticity (neuronal and synaptic), and increased immunoreactivity (Berk et al., 2011). Various dynamics are involved in the neuroprogression of depression, including disturbances in the serotonergic system, increased inflammation and cell-mediated immunity, oxidative and nitrosative stress, and neurotoxic compound production (Vaváková et al., 2015; Ruiz et al., 2018). QA is one such neurotoxic compound that acts as an NMDA receptor agonist and elicits excitotoxic damage *via* glutamatergic activation of neurons and astrocytes (Zhou et al., 2013).

The serotonin theory of depression asserts that "depression is a mood disorder characterized by decreased levels of monoamines, i.e., serotonin in the brain as a consequence of the increased activity of monoamine degrading enzyme, i.e., MAO or reduced serotonin synthesis" (Delgado, 2000). The reduced synthesis of serotonin accounts for the activation of the KP, as a consequence of increased inflammatory cascades in the brain (Ball et al., 2007). Critically, the fate of tryptophan depends on proinflammatory factor levels in the brain. During stress, the levels of circulating and brain proinflammatory cytokines (IL-6, IL-β, tumor necrosis factor-α, and IFN-γ) increase (Lanquillon et al., 2000; Kaestner et al., 2005; Thomas et al., 2005). This increase activates IDO and thus shifts tryptophan metabolism toward the tryptophan-KP (Leonard, 2010). Under basal conditions, kynurenine is metabolized predominantly to KA in astrocytes, the glia cells responsible for maintenance of homeostasis within the brain. KAT catalyzes the synthesis of KA from kynurenine and is not responsive to increased inflammation as are other enzymes of the KP. KAT is predominantly expressed in astrocytes (Rossi et al., 2008). During neuroinflammation, kynurenine metabolism shifts toward QA synthesis in microglia due to microglial activation (Delgado, 2000). Increased kynurenine levels and kynurenine monooxygenase expression are associated with neuroinflammation, characterized by activation of microglia and inflammatory cascades in the CNS. This shift from the methoxyindole pathway toward the KP increases the levels of the deleterious metabolite, QA, in the brain during increased neuroinflammation (Yan et al., 2015).

IDO-directed activity of pro-inflammatory cytokines (IL-6, IFN-α, and IFN-γ) can outpace KA synthesis by inhibiting KAT. A clinical study by Savitz et al. revealed reduced KA/QA levels in the cerebrospinal fluid (CSF) of patients with depression, MDD, and remitted patients with MDD compared to healthy individuals (Savitz et al., 2015). Additional studies have also shown a negative correlation between hippocampal and amygdala volume and increased QA levels in patients with bipolar disorder (Savitz et al., 2015). Another study reported that QA and IL-6 levels in the CSF are positively correlated

with suicide attempts in patients with MDD. Clinical and postmortem studies with individuals who had attempted to commit suicide have shown clear increase in the levels of QA in the brain (Erhardt et al., 2013; Brundin et al., 2016). In a preclinical study by Laugeray et al. (2011), tryptophan was found to be metabolized to QA in the amygdala and striatum but not in the cingulate cortex. Furthermore, peripheral catabolism of tryptophan to kynurenine was found to increase in a murine model of depression, resulting in lower availability of plasma tryptophan to the brain (Laugeray et al., 2011).

Given the relationship between QA and depression, the next question that arises is how QA serves as an oxidative stress modulator. QA is not only excitotoxic but also a modulator of oxidative stress (Kubicova et al., 2013). Specifically, QA increases reactive oxygen species (ROS) by increasing the excitotoxicity of the NMDA receptor (Guillemin, 2012; Schwarcz et al., 2012). QA-induced toxicity can be mediated through both NMDA-dependent and independent mechanisms. In line with this, studies have shown that at pathological concentrations (i.e., 10–40 μM), QA forms complexes with iron (Fe) *via* the Fenton reaction and thus increases ROS levels (Stipek et al., 1997; Iwahashi et al., 1999; Müller et al., 2007; Kubicova et al., 2013). In a study by Goda et al. (1996), it was found that the QA-Fe^{2+} complex causes cell death *via* hydroxyl ion-induced DNA chain breakage and lipid peroxidation. QA also enhances free radical production by inducing nitric oxide synthase activity in neurons and astrocytes and also increases poly(ADP-ribose) polymerase and extracellular lactate dehydrogenase activities and impairs mitochondrial function (Braidy et al., 2010; Pérez-De La Cruz et al., 2010). QA has also been found to impair the activity of endogenous antioxidant enzymes. In a study by Rodríguez-Martínez et al. (2000), intrastriatal injection of QA reduced the activity of reduced glutathione and cytosolic copper/zinc superoxide dismutase, whereas it increased the levels of oxidized glutathione. Melatonin and deprenyl, potent free radical scavengers, prevented QA-induced oxidative stress *via* NMDA receptor-independent action and also elevated antioxidant enzyme levels (Behan et al., 1999; Antunes Wilhelm et al., 2013). Increased brain microglial density was reported by a postmortem study with individuals who had attempted to commit suicide (Steiner et al., 2008). In addition, microglial QA levels have also been shown to be upregulated in the brain of patients with depression (Steiner et al., 2011). In their study with patients with depression, Meier et al. found a positive correlation between a decreased KA/QA ratio in the CSF and reductions in gray matter volume in the rostral and subgenual anterior cingulate cortex in the medial prefrontal cortex (Meier et al., 2016). Patients with bipolar disorder were found to have decreased KA levels in the hippocampus and amygdala compared with healthy controls (Savitz et al., 2015). A recent meta-analysis by Ogyu et al. (2018) revealed increased QA levels and decreased KA and kynurenine levels in patients with depression. Additionally, at pathological concentrations (i.e., 10–40 μM), QA reduces metabolic and physical buffering of neurons by instigating astrocytic apoptosis and thus hampering protection to neurons from ROS and inflammatory processes that lead to neurodegeneration (Grant and Kapoor, 1998). Given this, constraining the activity of QA may reduce the neuroprogression of depression and thus serve as a potential target for its pharmacotherapeutic treatment.

Nrf2 IN DEPRESSION

Nrf2, also known as NFE2L2, is a redox-sensitive transcription factor. It is a basic leucine zipper protein factor that belongs to the cap "n" collar subfamily. It plays an important role in the body's endogenous antioxidant defense system by maintaining the intracellular redox homeostasis that when dysregulated, triggers oxidative stress (Moi et al., 1994). Nrf2 transcribes various antioxidant enzymes to protect against oxidative stress induced by injury and/or inflammation. Under physiological conditions, Nrf2 is retained in the cytosol in a dormant form *via* tethering to Kelch-like ECH associated protein-1 (Keap-1), an adaptor for the cullin-3/Rbx complex. Keap-1, in association with cullin-3/Rbx, causes ubiquitination and finally causes proteasomal degradation of Nrf2. Given this, Nrf2 is highly unstable at physiological conditions due to its negative regulation by Keap-1 (Dhakshinamoorthy and Jaiswal, 2001). In inflammatory and oxidative stress conditions, Nrf2 translocates to the nucleus after dissociating from Keap-1 and binds to antioxidant response element, also referred to as electrophile responsive element. Transcription of electrophile responsive element target genes that encode phase-II antioxidant proteins such as GCLC, NOQ1, and HO-1; detoxifying enzymes, antiapoptotic proteins, and proteasomes are then enhanced (**Figure 3**; Niture et al., 2014).

Of special relevance here, studies have shown that downregulation of Nrf2 occurs in depression. In a recent study by Bouvier et al. (2016), rats that were exposed to persistently stressful conditions became progressively more vulnerable to depression due to persistent oxidative stress. This oxidative stress was found to be caused by diminished nuclear translocation of Nrf2, which prevented the transcription of antioxidants and detoxifying enzymes. In an *in vitro* study by Rojo et al. (2008), GSK-3β downregulated Nrf2 in neuronal cultures, providing a possible mechanism of inability to combat oxidative stress under persistent oxidant exposure (**Figure 3**).

The neurotrophic hypothesis of depression proposes that depression is also accompanied by decreased neurotrophic support, which is primarily linked to the brain-derived neurotrophic factor (BDNF) protein. A recent study reported that BDNF-deficient mice were more susceptible to stress-induced oxidative damage, indicating a direct link between oxidative stress and BDNF levels (Hacioglu et al., 2016). Bouvier et al. (2016) reported a positive correlation between BDNF and Nrf2 levels in rodents vulnerable to depression. In a study by Mendez-David et al. (2015), a positive correlation between BDNF and Nrf2 in the hippocampus of corticosterone-treated depressed rats was established. Besides depression, reduced levels of Nrf2 have further been noted in hippocampal astrocytes in patients with Alzheimer's disease (Ramsey et al., 2007). Furthermore, when Nrf2 knockout mice were treated with corticosterone, they exhibited significant changes in prefrontal cortex neurotransmitter

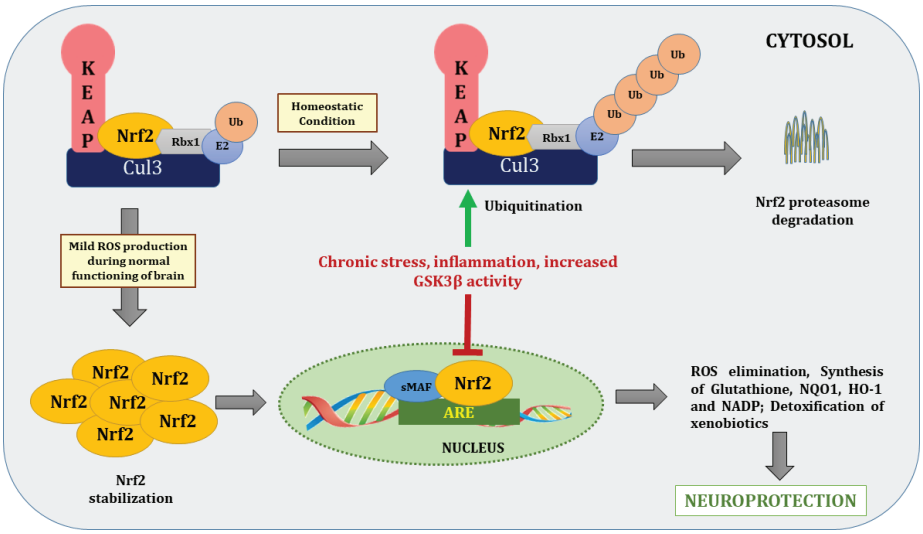

FIGURE 3 | Nrf2/ARE pathway during basal and stressed conditions. During homeostatic conditions Nrf2 remains in tethering with Keap1 and Cul3-Rbx1-E2 ligase and undergoes proteasome degradation through ubiquitination. In normal brain functioning, mild ROS production dissociates Nrf2 from Keap1 and translocate it to the nucleus. After nuclear translocation, it binds with ARE to transcribe genes of various antioxidants enzymes to combat deleterious effects of ROS. On contrary during chronic stress, increased activity of inflammatory cascades and GSK-3β increased Nrf2 proteasome degradation via ubiquitination. Nrf2, nuclear factor (erythroid-derived 2)-like 2; Keap1, Kelch-like ECH associated protein-1; Cul-3, cullin 3; ROS, reactive oxygen species; ARE, antioxidant response element; Rbx1, ring box-1; E2, E2 ubiquitin-conjugating enzyme; Ub, ubiquitin.

levels, including serotonin, glutamate, and dopamine, compared to wild-type controls (Mendez-David et al., 2015).

In addition to its role in neurotransmission and depression, Nrf2 is also linked to neuroprotection. Nrf2 protects neurons from the deleterious effects of oxidative and nitrosative stress and inflammatory cytokines by regulating astrocytic enzymes integral to glutathione redox signaling (Habas et al., 2013; Baxter and Hardingham, 2016). In a recent study by Yao et al. (2016), mice exhibiting depression-like behavior were found to have decreased levels of Keap-1 and Nrf2 protein in the prefrontal cortex, CA3, and dentate gyrus compared to controls. The authors also found that pre-treatment with dietary sulforaphane and 0.1% glucoraphanin (Nrf2 modulators) during the juvenile and adolescent stages prevented emergence of depression-like behavioral phenotypes in animals exposed to repeated social defeat stress (Yao et al., 2016). Additionally, Freitas et al. (2016) found that agmatine, an endogenous metabolite of L-arginine, protects corticosterone-induced apoptotic cell death and ROS production in hippocampal neuronal cells *via* induction of Nrf2. This group further evaluated the antidepressant potential of agmatine in a corticosterone mouse model of depression. Agmatine showed antidepressant properties in $Nrf2^{(+/+)}$ mice *via* induction of Nrf2 and BDNF and was unable to reverse the depression-like effect in Nrf2 knockout ($Nrf2^{(-/-)}$) mice. Agmatine also prevented morphological changes in astrocytes and microglia in the CA1 region of the hippocampus of $Nrf2^{(-/-)}$ mice (Freitas et al., 2016).

Nrf2 is predominantly expressed in astrocytes. In studies by Calkins et al. (2004, 2010), specific Nrf2 activation in astrocytes provided protection against malonate and 3-nitropropionic acid induced mitochondrial complex II inhibition-mediated neurotoxicity both *in vitro* and *in vivo*. Additionally, the presence of *tert*-butyl hydroquinone (tBHQ; an Nrf2 activator) was unable to buffer against oxidative stress in pure neuronal cultures, whereas in a mixed culture of astrocytes and neurons, tBHQ caused Nrf2 activation and provided protection from oxidative stress (Bell et al., 2015). In another complimentary study conducted by (Kraft, 2004), similar *in vitro* results were reported wherein the pharmacological activation of mixed cultures by tBHQ protected against oxidative stress, whereas specific inhibition of Nrf2 in astrocytes reduced the protection. Although Nrf2 is predominantly expressed in astrocytes, overactivation of Nrf2 in astrocytes is protective in various neurogenerative diseases such as Parkinson's disease (Chen et al., 2009), amyotrophic lateral sclerosis (Vargas et al., 2008), Huntington's disease (Shih et al., 2005), and multiple sclerosis (Draheim et al., 2016). In another study, overactivation of neuronal Nrf2 was found to be protective in Alzheimer's disease (Vargas et al., 2013), suggesting potential neural involvement. Collectively, these findings indicate that astrocytic activation of Nrf2 may be a potent pharmacotherapeutic target in various CNS diseases.

New findings on the role of Nrf2 in various CNS diseases have shown that Nrf2 is also important in the regulation of neuroinflammation that arises as a consequence of oxidative stress in the brain. In a study by Martín-de-Saavedra et al. (2013), Nrf2 was shown to reverse depression symptoms *via* an anti-inflammatory mechanism. This study also found that depletion of Nrf2 caused inflammation-induced depressive symptoms that were reversed by treatment with an anti-inflammatory drug, rofecoxib. Furthermore, this study also found that sulforaphane treatment in an LPS-induced inflammatory mouse model of depression increased nuclear translocation of

Nrf2 and led to antidepressant-like effects (Martín-de-Saavedra et al., 2013). Also in the same study, Nrf2$^{(-/-)}$ animals displayed a reduction in BDNF expression in the hippocampus, demonstrating a prospective relationship between the neurotropic factor and Nrf2. Suggesting a potential mechanism for these effects of Nrf2, in a recent study by Kobayashi et al. (2016), Nrf2 was found to be an upstream regulator of cytokine production *via* inhibiting transcriptional upregulation of proinflammatory cytokine genes and thus decreased the levels of proinflammatory cytokines. Thus, Nrf2 exerts anti-inflammatory and antioxidant effects on biological systems. Collectively, the studies discussed here strongly suggest an essential role for Nrf2 in depression, pointing at the induction of Nrf2 as a potential target in the investigation of novel antidepressant drugs.

INTERACTIONS BETWEEN QA AND Nrf2 IN DEPRESSION

As suggested by the studies outlined previously, both QA and Nrf2 play substantial, and probably opposite, roles in the etiology of depression. While QA has been shown to contribute to depression, Nrf2 likely protects against depression. Given this, it is important to understand whether QA and Nrf2 are directly associated. To date, only two known *in vitro* studies have examined the direct relationship between QA and Nrf2 (Tasset et al., 2010; Colín-González et al., 2014). Suggesting a potential mechanism for this, Tasset et al. (2010) reported that exposure to QA abolished nuclear translocation of Nrf2 in rat striatal tissues. Nuclear translocation of Nrf2 in QA-treated striatal slices was noted after tBHQ (a Nrf2 modulator) treatment (Tasset et al., 2010). This indicates that QA abolishes Nrf2 translocation in the nucleus.

An additional study by Colín-González et al. (2014) reported that striatal slices from Nrf2$^{(-/-)}$ animals were more vulnerable to the oxidative damage caused by QA than were wild-type tissues. In mouse striatum slices, QA treatment after 1 h was found to stimulate Nrf2 nuclear translocation, while after 3 h, it was found to decrease Nrf2 translocation and phase II enzymatic activity and increase lipid peroxidation. Enhanced Nrf2 nuclear translocation may thus represent a compensatory mechanism for QA-induced oxidative stress, while downregulation of Nrf2 might contribute to cellular oxidative damage. Further, Nrf2$^{(-/-)}$ animals were found to be less responsive to QA-induced toxicity compared to wild-type animals (Colín-González et al., 2014). In a recent study by Ferreira et al. (2018), KA prevented QA-induced oxidative imbalance, mitochondrial dysfunction, and decreased Nrf2 levels in striatal slices.

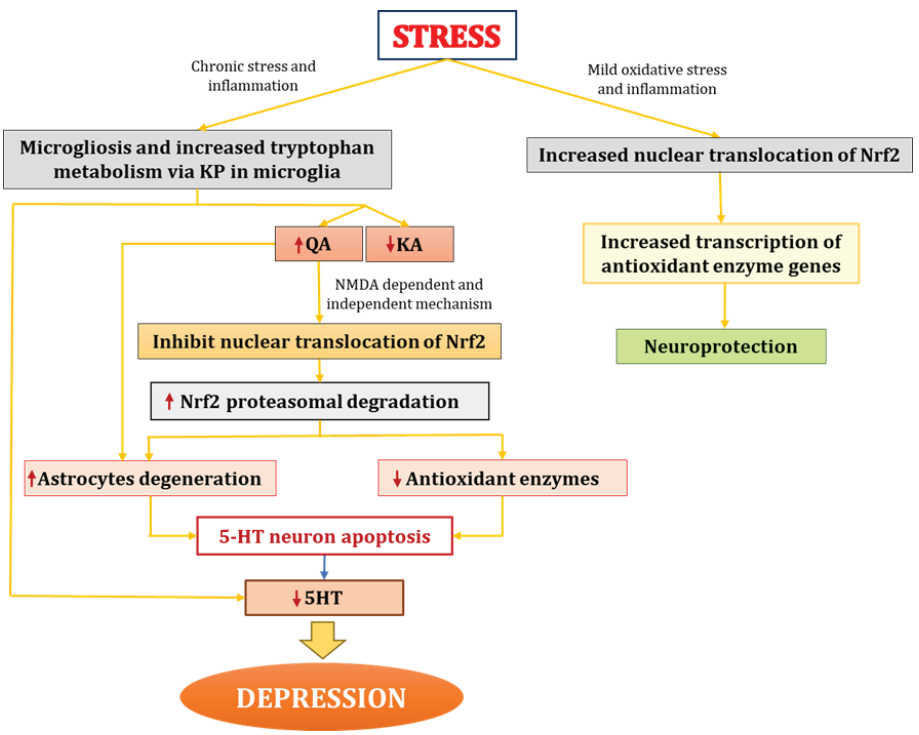

FIGURE 4 | Interaction between QA and Nrf2 in depression. In chronic stress conditions, increased proinflammatory cytokines lead to microgliosis. Increased proinflammatory cytokines activate IDO (enzyme catalyzing first rate-limiting step of KP) and shift tryptophan metabolism from serotonin synthesis to KP in microglia. Elevated levels of QA further increases oxidative stress through NMDA agonistic activity and secondly might be through directly inhibiting nuclear translocation of Nrf2 and hence causes increased proteasome degradation of Nrf2 which ultimately led to decreased antioxidant levels and increased oxidative stress. QA also causes degeneration of astrocytes thus hampering protection and nutritional support to neurons. All this together lead to degeneration of 5HT neurons and decreased 5HT synthesis which results in depression. On the other hand mild oxidative stress stabilizes Nrf2 and increases Nrf2 transcribed antioxidant enzymes. IDO, indoleamine-2,3-dioxygenase; KP, kynurenine pathway; QA, quinolinic acid; KA, kynurenic acid; Nrf2, nuclear factor (erythroid-derived 2)-like 2; 5HT, serotonin.

The NADPH inhibitor, apocynin, prevented QA-induced decrease in antioxidant levels such as glutathione, γ-GCL, and GPx activities and Nrf2 mRNA levels which is involved in the maintenance of antioxidant levels (Cruz-Álvarez et al., 2017).

Apart from NMDA-induced excitotoxicity by QA, studies have also shown that QA exerts toxic effects by increasing the oxidant-to-antioxidant ratio. Specifically, QA alters the ratio of reduced glutathione to oxidized glutathione and hinders the activity of other antioxidants, such as Cu and zinc-superoxide dismutase (Rodríguez-Martínez et al., 2000). However, Nrf2 protects against these pro-oxidant effects by increasing the transcription of various cytoprotective antioxidant genes such as *GSH*. For example, Harvey et al. (2009) demonstrated that Nrf2 is protective against oxidative stress by maintaining the glutathione redox state *via* transcriptional regulation of glutathione reductase. As noted previously, pathological concentrations of QA can cause astrocytic apoptosis, limiting the role of these cells in protecting neurons from ROS and other pro-inflammatory processes, thus causing neurodegeneration (Grant and Kapoor, 1998). In sum, Nrf2 protects against the deleterious effects of oxidative and nitrosative stress and inflammatory cytokines on neurons by cytoprotection of astrocytes *via* regulation of enzymes belonging to the glutathione redox system (Habas et al., 2013; Baxter and Hardingham, 2016). Based on the aforementioned studies, there may also be a direct link between QA and Nrf2, wherein QA constrains Nrf2 nuclear translocation. Further examining this process may serve as a viable potential target for the development of pharmacological treatments for depression (**Figure 4**).

CONCLUDING REMARKS

A growing body of preclinical and postmortem evidence has revealed that depression is often accompanied by hippocampal and prefrontal cortex neuronal atrophy. Decreased neuronal activity and plasticity across multiple brain regions have also been noted in clinical and modeled depression. Collectively, these morphological changes and altered neuronal functions may be due to increased oxidative stress, which contributes to neuronal and glial atrophy in depression. Specifically, the role of QA in mediating these changes and the broader pathophysiology of depression has become a subject of clinical and preclinical studies. A decreased KA/QA ratio has been noted in the CSF of remittent patients with depression. Moreover, QA is an endogenous toxin that also acts as a pro-oxidant and leads to the neuroprogression of depression. Furthermore, QA is linked to the activity of Nrf2, an endogenous antioxidant transcription factor. While the contributions of Nrf2 to CNS diseases have been understudied, it has been reported that Nrf2 is both a potent antioxidant transcription factor and also has anti-inflammatory effects that may be responsible for its antidepressant properties. Hence, further study of the associations among QA, Nrf2, and oxidative stress may provide novel insight into the neuroprogression and etiology of depression.

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REFERENCES

Aan Het Rot, M., Zarate, C. A., Charney, D. S., and Mathew, S. J. (2012). Ketamine for depression: where do we go from here? *Biol. Psychiatry* 72, 537–547. doi: 10.1016/j.biopsych.2012.05.003

Antunes Wilhelm, E., Ricardo Jesse, C., Folharini Bortolatto, C., and Wayne Nogueira, C. (2013). Correlations between behavioural and oxidative parameters in a rat quinolinic acid model of Huntington's disease: protective effect of melatonin. *Eur. J. Pharmacol.* 701, 65–72. doi: 10.1016/j.ejphar.2013.01.007

Araque, A., Carmignoto, G., Haydon, P. G., Oliet, S. H. R., Robitaille, R., and Volterra, A. (2014). Gliotransmitters travel in time and space. *Neuron* 81, 728–739. doi: 10.1016/j.neuron.2014.02.007

Ball, H. J., Sanchez-Perez, A., Weiser, S., Austin, C. J. D., Astelbauer, F., Miu, J., et al. (2007). Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. *Gene* 396, 203–213. doi: 10.1016/j.gene.2007.04.010

Baxter, P. S., and Hardingham, G. E. (2016). Adaptive regulation of the brain's antioxidant defences by neurons and astrocytes. *Free Radic. Biol. Med.* 100, 147–152. doi: 10.1016/j.freeradbiomed.2016.06.027

Behan, W. M., McDonald, M., Darlington, L. G., and Stone, T. W. (1999). Oxidative stress as a mechanism for quinolinic acid-induced hippocampal damage: protection by melatonin and deprenyl. *Br. J. Pharmacol.* 128, 1754–1760. doi: 10.1038/sj.bjp.0702940

Behan, W. M. H., and Stone, T. W. (2002). Enhanced neuronal damage by co-administration of quinolinic acid and free radicals, and protection by adenosine A2A receptor antagonists. *Br. J. Pharmacol.* 135, 1435–1442. doi: 10.1038/sj.bjp.0704613

Bell, K. F. S., Al-Mubarak, B., Martel, M. A., McKay, S., Wheelan, N., Hasel, P., et al. (2015). Neuronal development is promoted by weakened intrinsic antioxidant defences due to epigenetic repression of Nrf2. *Nat. Commun.* 6, 1–15. doi: 10.1038/ncomms8066

Berk, M., Kapczinski, F., Andreazza, A. C., Dean, O. M., Giorlando, F., Maes, M., et al. (2011). Pathways underlying neuroprogression in bipolar disorder: focus on inflammation, oxidative stress and neurotrophic factors. *Neurosci. Biobehav. Rev.* 35, 804–817. doi: 10.1016/j.neubiorev.2010.10.001

Bouvier, E., Brouillard, F., Molet, J., Claverie, D., Cabungcal, J. H., Cresto, N., et al. (2016). Nrf2-dependent persistent oxidative stress results in stress-induced vulnerability to depression. *Mol. Psychiatry.* 22, 1701–1713. doi: 10.1038/mp.2016.144

Braidy, N., Grant, R., Adams, S., and Guillemin, G. J. (2010). Neuroprotective effects of naturally occurring polyphenols on quinolinic acid-induced excitotoxicity in human neurons. *FEBS J.* 277, 368–382. doi: 10.1111/j.1742-4658.2009.07487.x

Brundin, L., Sellgren, C. M., Lim, C. K., Grit, J., Pålsson, E., Landén, M., et al. (2016). An enzyme in the kynurenine pathway that governs vulnerability to suicidal behavior by regulating excitotoxicity and neuroinflammation. *Transl. Psychiatry* 6, 1–9. doi: 10.1038/tp.2016.133

Calkins, M. J., Jakel, R. J., Johnson, D. A., Chan, K., Kan, Y. W., and Johnson, J. A. (2004). Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. *Proc. Natl. Acad. Sci. USA* 102, 244–249. doi: 10.1073/pnas.0408487101

Calkins, M. J., Vargas, M. R., Johnson, D. A., and Johnson, J. A. (2010). Astrocyte-specific overexpression of Nrf2 protects striatal neurons from mitochondrial complex II inhibition. *Toxicol. Sci.* 115, 557–568. doi: 10.1093/toxsci/kfq072

Capuron, L., Neurauter, G., Musselman, D. L., Lawson, D. H., Nemeroff, C. B., Fuchs, D., et al. (2003). Interferon-alpha-induced changes in tryptophan metabolism: relationship to depression and paroxetine treatment. *Biol. Psychiatry* 54, 906–914. doi: 10.1016/S0006-3223(03)00173-2

Chandley, M. J., Szebeni, K., Szebeni, A., Crawford, J., Stockmeier, C. A., Turecki, G., et al. (2013). Gene expression deficits in pontine locus coeruleus astrocytes in men with major depressive disorder. *J. Psychiatry Neurosci.* 38, 276–284. doi: 10.1503/jpn.120110

Chen, P.-C., Vargas, M. R., Pani, A. K., Smeyne, R. J., Johnson, D. A., Kan, Y. W., et al. (2009). Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: critical role for the astrocyte. *Proc. Natl. Acad. Sci. USA* 106, 2933–2938. doi: 10.1073/pnas.0813361106

Colín-González, A. L., Luna-López, A., Königsberg, M., Ali, S. F., Pedraza-Chaverrí, J., and Santamaría, A. (2014). Early modulation of the transcription factor Nrf2 in rodent striatal slices by quinolinic acid, a toxic metabolite of the kynurenine pathway. *Neuroscience* 260, 130–139. doi: 10.1016/j.neuroscience.2013.12.025

Cotter, D., Mackay, D., Chana, G., Beasley, C., Landau, S., and Everall, I. P. (2002). Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. *Cereb. Cortex* 12, 386–394. doi: 10.1093/cercor/12.4.386

Cowen, P. J., and Browning, M. (2015). What has serotonin to do with depression? *World Psychiatry* 14, 158–160. doi: 10.1002/wps.20229

Cruz-Álvarez, S., Santana-Martínez, R., Avila-Chávez, E., Barrera-Oviedo, D., Hernández-Pando, R., Pedraza-Chaverri, J., et al. (2017). Apocynin protects against neurological damage induced by quinolinic acid by an increase in glutathione synthesis and Nrf2 levels. *Neuroscience* 350, 65–74. doi: 10.1016/j.neuroscience.2017.03.011

Dallérac, G., and Rouach, N. (2016). Astrocytes as new targets to improve cognitive functions. *Prog. Neurobiol.* 144, 48–67. doi: 10.1016/j.pneurobio.2016.01.003

Dantzer, R., O'Connor, J. C., Lawson, M. A., and Kelley, K. W. (2011). Inflammation-associated depression: from serotonin to kynurenine. *Psychoneuroendocrinology* 36, 426–436. doi: 10.1016/j.psyneuen.2010.09.012

Delgado, P. L. (2000). Depression: the case for a monoamine deficiency. *J. Clin. Psychiatry* 61(Suppl. 6), 7–11.

Dhakshinamoorthy, S., and Jaiswal, A. K. (2001). Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* 20, 3906–3917. doi: 10.1038/sj.onc.1204506

Dobos, N., de Vries, E. F. J., Kema, I. P., Patas, K., Prins, M., Nijholt, I. M., et al. (2015). "The role of indoleamine 2,3-dioxygenase in a mouse model of neuroinflammation-induced depression" in *Handbook of depression in Alzheimer's disease* (IOS Press), 163–174. doi: 10.3233/978-1-61499-542-5-163

Draheim, T., Liessem, A., Scheld, M., Wilms, F., Weißflog, M., Denecke, B., et al. (2016). Activation of the astrocytic Nrf2/ARE system ameliorates the formation of demyelinating lesions in a multiple sclerosis animal model. *Glia* 64, 2219–2230. doi: 10.1002/glia.23058

Erhardt, S., Lim, C. K., Linderholm, K. R., Janelidze, S., Lindqvist, D., Samuelsson, M., et al. (2013). Connecting inflammation with glutamate agonism in suicidality. *Neuropsychopharmacology* 38, 743–752. doi: 10.1038/npp.2012.248

Fatemi, S. H., Laurence, J. A., Araghi-Niknam, M., Stary, J. M., Schulz, S. C., Lee, S., et al. (2004). Glial fibrillary acidic protein is reduced in cerebellum of subjects with major depression, but not schizophrenia. *Schizophr. Res.* 69, 317–323. doi: 10.1016/j.schres.2003.08.014

Ferreira, F. S., Biasibetti-Brendler, H., Pierozan, P., Schmitz, F., Bertó, C. G., Prezzi, C. A., et al. (2018). Kynurenic acid restores Nrf2 levels and prevents quinolinic acid-induced toxicity in rat striatal slices. *Mol. Neurobiol.* 55, 1–12. doi: 10.1007/s12035-018-1003-2

Freitas, A. E., Egea, J., Buendia, I., Gómez-Rangel, V., Parada, E., Navarro, E., et al. (2016). Agmatine, by improving neuroplasticity markers and inducing Nrf2, prevents corticosterone-induced depressive-like behavior in mice. *Mol. Neurobiol.* 53, 3030–3045. doi: 10.1007/s12035-015-9182-6

Gittins, R. A., and Harrison, P. J. (2011). A morphometric study of glia and neurons in the anterior cingulate cortex in mood disorder. *J. Affect. Disord.* 133, 328–332. doi: 10.1016/j.jad.2011.03.042

Goda, K., Kishimoto, R., Shimizu, S., Hamane, Y., and Ueda, M. (1996). Quinolinic acid and active oxygens. Possible contribution of active oxygens during cell death in the brain. *Adv. Exp. Med. Biol.* 398, 247–254.

Grant, R. S., and Kapoor, V. (1998). Murine glial cells regenerate NAD, after peroxide-induced depletion, using either nicotinic acid, nicotinamide, or quinolinic acid as substrates. *J. Neurochem.* 70, 1759–1763. doi: 10.1046/j.1471-4159.1998.70041759.x

Guillemin, G. J. (2012). Quinolinic acid, the inescapable neurotoxin. *FEBS J.* 279, 1356–1365. doi: 10.1111/j.1742-4658.2012.08485.x

Habas, A., Hahn, J., Wang, X., and Margeta, M. (2013). Neuronal activity regulates astrocytic Nrf2 signaling. *Proc. Natl. Acad. Sci. USA* 110, 18291–18296. doi: 10.1073/pnas.1208764110

Hacioglu, G., Senturk, A., Ince, I., and Alver, A. (2016). Assessment of oxidative stress parameters of brain-derived neurotrophic factor heterozygous mice in acute stress model. *Iran. J. Basic Med. Sci.* 19, 388–393. doi: 10.22038/ijbms.2016.6810

Harvey, C. J., Thimmulappa, R. K., Singh, A., Blake, D. J., Ling, G., Wakabayashi, N., et al. (2009). Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free Radic. Biol. Med.* 46, 443–453. doi: 10.1016/j.freeradbiomed.2008.10.040

Henry, C. J., Huang, Y., Wynne, A., Hanke, M., Himler, J., Bailey, M. T., et al. (2008). Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. *J. Neuroinflammation* 5, 1–14. doi: 10.1186/1742-2094-5-15

Iwahashi, H., Kawamori, H., and Fukushima, K. (1999). Quinolinic acid, α-picolinic acid, fusaric acid, and 2,6-pyridinedicarboxylic acid enhance the Fenton reaction in phosphate buffer. *Chem. Biol. Interact.* 118, 201–215. doi: 10.1016/S0009-2797(99)00080-0

Jones, S. P., Guillemin, G. J., and Brew, B. J. (2013). The Kynurenine pathway in stem cell biology. *Int. J. Tryptophan Res.* 6, 57–66. doi: 10.4137/IJTR.S12626

Kaestner, F., Hettich, M., Peters, M., Sibrowski, W., Hetzel, G., Ponath, G., et al. (2005). Different activation patterns of proinflammatory cytokines in melancholic and non-melancholic major depression are associated with HPA axis activity. *J. Affect. Disord.* 87, 305–311. doi: 10.1016/j.jad.2005.03.012

Kiank, C., Zeden, J. P., Drude, S., Domanska, G., Fusch, G., Otten, W., et al. (2010). Psychological stress-induced, IDO1-dependent tryptophan catabolism: implications on immunosuppression in mice and humans. *PLoS One* 5, 1–15. doi: 10.1371/journal.pone.0011825

Kim, H., Chen, L., Lim, G., Sung, B., Wang, S., McCabe, M. F., et al. (2012). Brain indoleamine 2,3-dioxygenase contributes to the comorbidity of pain and depression. *J. Clin. Invest.* 122, 2940–2954. doi: 10.1172/JCI61884

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., et al. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat. Commun.* 7, 1–14. doi: 10.1038/ncomms11624

Kohler, O., Krogh, J., Mors, O., and Eriksen Benros, M. (2016). Inflammation in depression and the potential for anti-inflammatory treatment. *Curr. Neuropharmacol.* 14, 732–742. doi: 10.2174/1570159X14666151208113700

Kraft, A. D. (2004). Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. *J. Neurosci.* 24, 1101–1112. doi: 10.1523/JNEUROSCI.3817-03.2004

Kubicova, L., Hadacek, F., and Chobot, V. (2013). Quinolinic acid: neurotoxin or oxidative stress modulator? *Int. J. Mol. Sci.* 14, 21328–21338. doi: 10.3390/ijms141121328

Lanquillon, S. (2000). Cytokine Production and Treatment Response in Major Depressive Disorder. *Neuropsychopharmacology* 22, 370–379. doi: 10.1016/S0893-133X(99)00134-7

Laugeray, A., Launay, J. M., Callebert, J., Surget, A., Belzung, C., and Barone, P. R. (2011). Evidence for a key role of the peripheral kynurenine pathway in the modulation of anxiety- and depression-like behaviours in mice: focus on individual differences. *Pharmacol. Biochem. Behav.* 98, 161–168. doi: 10.1016/j.pbb.2010.12.008

Leonard, B. E. (2010). "The concept of depression as a dysfunction of the immune system" in *Depression: From Psychopathology to Pharmacotherapy* 53–71. doi: 10.1159/000319504

Lima, W. S. (1998). Seasonal infection pattern of gastrointestinal nematodes of beef cattle in Minas Gerais State–Brazil. *Vet. Parasitol.* 74, 203–214. doi: 10.1016/S0304-4017(97)00164-7

Liu, T., Zhong, S., Liao, X., Chen, J., He, T., Lai, S., et al. (2015). A meta-analysis of oxidative stress markers in depression. *PLoS One* 10. doi: 10.1371/journal.pone.0138904

Maes, M. (1993). A review on the acute phase response in major depression. *Rev. Neurosci.* 4, 407–416. doi: 10.1515/REVNEURO.1993.4.4.407

Maes, M., Bosmans, E., Suy, E., Vandervorst, C., DeJonckheere, C., and Raus, J. (1991). Depression-related disturbances in mitogen-induced lymphocyte responses and interleukin-1β and soluble interleukin-2 receptor production. *Acta Psychiatr. Scand.* 84, 379–386. doi: 10.1111/j.1600-0447.1991.tb03163.x

Maes, M., Scharpé, S., Meltzer, H. Y., Bosmans, E., Suy, E., Calabrese, J., et al. (1993). Relationships between interleukin-6 activity, acute phase proteins, and function of the hypothalamic-pituitary-adrenal axis in severe depression. *Psychiatry Res.* 49, 11–27. doi: 10.1016/0165-1781(93)90027-E

Martín-de-Saavedra, M. D., Budni, J., Cunha, M. P., Gómez-Rangel, V., Lorrio, S., del Barrio, L., et al. (2013). Nrf2 participates in depressive disorders through an anti-inflammatory mechanism. *Psychoneuroendocrinology* 38, 2010–2022. doi: 10.1016/j.psyneuen.2013.03.020

Meier, T. B., Drevets, W. C., Wurfel, B. E., Ford, B. N., Morris, H. M., Victor, T. A., et al. (2016). Relationship between neurotoxic kynurenine metabolites and reductions in right medial prefrontal cortical thickness in major depressive disorder. *Brain Behav. Immun.* 53, 39–48. doi: 10.1016/j.bbi.2015.11.003

Mendez-David, I., Tritschler, L., El Ali, Z., Damiens, M. H., Pallardy, M., David, D. J., et al. (2015). Nrf2-signaling and BDNF: a new target for the antidepressant-like activity of chronic fluoxetine treatment in a mouse model of anxiety/depression. *Neurosci. Lett.* 597, 121–126. doi: 10.1016/j.neulet.2015.04.036

Mendoza, C., Perez-Urrutia, N., Alvarez-Ricartes, N., Barreto, G. E., Pérez-Ordás, R., Iarkov, A., et al. (2018). Cotinine plus krill oil decreased depressive behavior, and increased astrocytes survival in the hippocampus of mice subjected to restraint stress. *Front. Neurosci.* 12, 1–11. doi: 10.3389/fnins.2018.00952

Miguel-Hidalgo, J. J., Waltzer, R., Whittom, A. A., Austin, M. C., Rajkowska, G., and Stockmeier, C. A. (2010). Glial and glutamatergic markers in depression, alcoholism, and their comorbidity. *J. Affect. Disord.* 127, 230–240. doi: 10.1016/j.jad.2010.06.003

Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. USA* 91, 9926–9930. doi: 10.1073/pnas.91.21.9926

Müller, A. C., Dairam, A., Limson, J. L., and Daya, S. (2007). Mechanisms by which acyclovir reduces the oxidative neurotoxicity and biosynthesis of quinolinic acid. *Life Sci.* 80, 918–925. doi: 10.1016/j.lfs.2006.11.031

Müller, M. B., Lucassen, P. J., Yassouridis, A., Hoogendijk, W. J. G., Holsboer, F., and Swaab, D. F. (2001). Neither major depression nor glucocorticoid treatment affects the cellular integrity of the human hippocampus. *Eur. J. Neurosci.* 14, 1603–1612. doi: 10.1046/j.0953-816X.2001.01784.x

Myint, A. M., Schwarz, M. J., and Müller, N. (2012). The role of the kynurenine metabolism in major depression. *J. Neural Transm.* 119, 245–251. doi: 10.1007/s00702-011-0741-3

Niture, S. K., Khatri, R., and Jaiswal, A. K. (2014). Regulation of Nrf2–an update. *Free Radic. Biol. Med.* 66, 36–44. doi: 10.1016/j.freeradbiomed.2013.02.008

Ogyu, K., Kubo, K., Noda, Y., Iwata, Y., Tsugawa, S., Omura, Y., et al. (2018). Kynurenine pathway in depression: A systematic review and meta-analysis. *Neurosci. Biobehav. Rev.* doi: 10.1016/j.neubiorev.2018.03.023

Orlando, L. R., Alsdorf, S. A., Penney, J. B., and Young, A. B. (2001). The role of group I and group II metabotropic glutamate receptors in modulation of striatal NMDA and quinolinic acid toxicity. *Exp. Neurol.* 167, 196–204. doi: 10.1006/exnr.2000.7542

Pérez-De La Cruz, V., Elinos-Calderón, D., Carrillo-Mora, P., Silva-Adaya, D., Konigsberg, M., Morán, J., et al. (2010). Time-course correlation of early toxic events in three models of striatal damage: modulation by proteases inhibition. *Neurochem. Int.* 56, 834–842. doi: 10.1016/j.neuint.2010.03.008

Raison, C. L., Dantzer, R., Kelley, K. W., Lawson, M. A., Woolwine, B. J., Vogt, G., et al. (2010). CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-α: relationship to CNS immune responses and depression. *Mol. Psychiatry* 15, 393–403. doi: 10.1038/mp.2009.116

Ramsey, C. P., Glass, C. A., Montgomery, M. B., Lindl, K. A., Ritson, G. P., Chia, L. A., et al. (2007). Expression of Nrf2 in neurodegenerative diseases. *J. Neuropathol. Exp. Neurol.* 66, 75–85. doi: 10.1097/nen.0b013e31802d6da9

Réus, G. Z., Jansen, K., Titus, S., Carvalho, A. F., Gabbay, V., and Quevedo, J. (2015). Kynurenine pathway dysfunction in the pathophysiology and treatment of depression: evidences from animal and human studies. *J. Psychiatr. Res.* 68, 316–328. doi: 10.1016/j.jpsychires.2015.05.007

Rodríguez-Martínez, E., Camacho, A., Maldonado, P. D., Pedraza-Chaverrí, J., Santamaría, D., Galván-Arzate, S., et al. (2000). Effect of quinolinic acid on endogenous antioxidants in rat corpus striatum. *Brain Res.* 858, 436–439. doi: 10.1016/S0006-8993(99)02474-9

Rojo, A. I., De Sagarra, M. R., and Cuadrado, A. (2008). GSK-3β down-regulates the transcription factor Nrf2 after oxidant damage: relevance to exposure of neuronal cells to oxidative stress. *J. Neurochem.* 105, 192–202. doi: 10.1111/j.1471-4159.2007.05124.x

Rossato, J. I., Ketzer, L. A., Centurião, F. B., Silva, S. J. N., Lüdtke, D. S., Zeni, G., et al. (2002). Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. *Neurochem. Res.* 27, 297–303. doi: 10.1023/A:1014907228580

Rossi, F., Schwarcz, R., and Rizzi, M. (2008). Curiosity to kill the KAT (kynurenine aminotransferase): structural insights into brain kynurenic acid synthesis. *Curr. Opin. Struct. Biol.* 18, 748–755. doi: 10.1016/j.sbi.2008.09.009

Ruiz, N. A. L., Del Angel, D. S., Olguin, H. J., and Silva, M. L. (2018). Neuroprogression: the hidden mechanism of depression. *Neuropsychiatr. Dis. Treat.* 14, 2837–2845. doi: 10.2147/NDT.S177973

Santamaría, D., Espinoza-González, V., Ríos, C., and Santamaría, A. (1999). Nomega-nitro-L-arginine, a nitric oxide synthase inhibitor, antagonizes quinolinic acid-induced neurotoxicity and oxidative stress in rat striatal slices. *Neurochem. Res.* 24, 843–848. doi: 10.1023/A:1020949812581

Sapko, M. T., Guidetti, P., Yu, P., Tagle, D. A., Pellicciari, R., and Schwarcz, R. (2006). Endogenous kynurenate controls the vulnerability of striatal neurons to quinolinate: implications for Huntington's disease. *Exp. Neurol.* 197, 31–40. doi: 10.1016/j.expneurol.2005.07.004

Savitz, J. (2017). "Role of kynurenine metabolism pathway activation in major depressive disorders" in *Current topics in behavioral neurosciences*, 249–268. doi: 10.1007/7854_2016_12

Savitz, J., Dantzer, R., Wurfel, B. E., Victor, T. A., Ford, B. N., Bodurka, J., et al. (2015). Neuroprotective kynurenine metabolite indices are abnormally reduced and positively associated with hippocampal and amygdalar volume in bipolar disorder. *Psychoneuroendocrinology* 52, 200–211. doi: 10.1016/j.psyneuen.2014.11.015

Savitz, J., Drevets, W. C., Wurfel, B. E., Ford, B. N., Bellgowan, P. S. F., Victor, T. A., et al. (2015). Reduction of kynurenic acid to quinolinic acid ratio in both the depressed and remitted phases of major depressive disorder. *Brain Behav. Immun.* 46, 55–59. doi: 10.1016/j.bbi.2015.02.007

Schwarcz, R., Bruno, J. P., Muchowski, P. J., and Wu, H. Q. (2012). Kynurenines in the mammalian brain: when physiology meets pathology. *Nat. Rev. Neurosci.* 13, 465–477. doi: 10.1038/nrn3257

Setiawan, E., Wilson, A. A., Mizrahi, R., Rusjan, P. M., Miler, L., Rajkowska, G., et al. (2015). Role of translocator protein density, a marker of neuroinflammation, in the brain during major depressive episodes. *JAMA Psychiatry* 72, 268–275. doi: 10.1001/jamapsychiatry.2014.2427

Shih, A. Y., Imbeault, S., Barakauskas, V., Erb, H., Jiang, L., Li, P., et al. (2005). Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *J. Biol. Chem.* 280, 22925–22936. doi: 10.1074/jbc.M414635200

Steiner, J., Bielau, H., Brisch, R., Danos, P., Ullrich, O., Mawrin, C., et al. (2008). Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide. *J. Psychiatr. Res.* 42, 151–157. doi: 10.1016/j.jpsychires.2006.10.013

Steiner, J., Walter, M., Gos, T., Guillemin, G. J., Bernstein, H. G., Sarnyai, Z., et al. (2011). Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission? *J. Neuroinflammation* 8, 1–9. doi: 10.1186/1742-2094-8-94

Stipek, S., Stastny, F., Platenik, J., Crkovska, J., and Zima, T. (1997). The effect of quinolinate on rat brain lipid peroxidation is dependent on iron. *Neurochem. Int.* 30, 233–237. doi: 10.1016/S0197-0186(97)90002-4

Tasset, I., Pérez-De La Cruz, V., Elinos-Calderón, D., Carrillo-Mora, P., González-Herrera, I. G., Luna-López, A., et al. (2010). Protective effect of

tert-butylhydroquinone on the quinolinic-acid-induced toxicity in rat striatal slices: role of the Nrf2-antioxidant response element pathway. *Neurosignals* 18, 24–31. doi: 10.1159/000243650

Thomas, A. J., Davis, S., Morris, C., Jackson, E., Harrison, R., and O'Brien, J. T. (2005). Increase in interleukin-1beta in late-life depression. *Am. J. Psychiatry* 162, 175–177. doi: 10.1176/appi.ajp.162.1.175

Vargas, M. R., Burton, N. C., Gan, L., Johnson, D. A., Schäfer, M., Werner, S., et al. (2013). Absence of Nrf2 or its selective overexpression in neurons and muscle does not affect survival in ALS-linked mutant hSOD1 mouse models. *PLoS One* 8. doi: 10.1371/journal.pone.0056625

Vargas, M. R., Johnson, D. A., Sirkis, D. W., Messing, A., and Johnson, J. A. (2008). Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. *J. Neurosci.* 28, 13574–13581. doi: 10.1523/JNEUROSCI.4099-08.2008

Vaváková, M., Ďuračková, Z., and Trebatická, J. (2015). Markers of oxidative stress and neuroprogression in depression disorder. *Oxidative Med. Cell. Longev.* 2015, 1–12. doi: 10.1155/2015/898393

WHO (2018). Depression. Available at: http://www.who.int/news-room/fact-sheets/detail/depression [Accessed March 8, 2019].

Yan, E. B., Frugier, T., Lim, C. K., Heng, B., Sundaram, G., Tan, M., et al. (2015). Activation of the kynurenine pathway and increased production of the excitotoxin quinolinic acid following traumatic brain injury in humans. *J. Neuroinflammation* 12, 1–17. doi: 10.1186/s12974-015-0328-2

Yao, W., Zhang, J. C., Ishima, T., Dong, C., Yang, C., Ren, Q., et al. (2016). Role of Keap1-Nrf2 signaling in depression and dietary intake of glucoraphanin confers stress resilience in mice. *Sci. Rep.* 6, 1–13. doi: 10.1038/srep30659

Zhou, X., Hollern, D., Liao, J., Andrechek, E., and Wang, H. (2013). NMDA receptor-mediated excitotoxicity depends on the coactivation of synaptic and extrasynaptic receptors. *Cell Death Dis.* 4, 1–11. doi: 10.1038/cddis.2013.82

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Nrf2 Plays a Protective Role Against Intravascular Hemolysis-Mediated Acute Kidney Injury

Alfonso Rubio-Navarro[1†‡], Cristina Vázquez-Carballo[1†], Melania Guerrero-Hue[1†], Cristina García-Caballero[1], Carmen Herencia[1], Eduardo Gutiérrez[2], Claudia Yuste[2], Ángel Sevillano[2], Manuel Praga[2], Javier Egea[3,4,5], Pablo Cannata[6], Isabel Cortegano[7], Belén de Andrés[7], María Luisa Gaspar[7], Susana Cadenas[8], Patrycja Michalska[3,4], Rafael León[3,4], Alberto Ortiz[1], Jesús Egido[1] and Juan Antonio Moreno[1,9*]*

[1] Renal, Vascular and Diabetes Research Lab, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz, Autónoma University, Madrid, Spain, [2] Department of Nephrology, Hospital 12 de Octubre, Madrid, Spain, [3] Instituto de Investigación Sanitaria-Hospital Universitario de la Princesa, Madrid, Spain, [4] Instituto Teófilo Hernando, Departamento de Farmacología y Terapéutica, Facultad de Medicina, UAM, Madrid, Spain, [5] Hospital Santa Cristina, Madrid, Spain, [6] Pathology Department, Fundación Instituto de Investigaciones Sanitarias-Fundación Jiménez Díaz, Autónoma University, Madrid, Spain, [7] Immunology Department, Centro Nacional de Microbiologìa, Instituto de Salud Carlos III (ISCIII), Madrid, Spain, [8] Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain, [9] Department of Cell Biology, Physiology and Immunology, Maimonides Biomedical Research Institute of Cordoba (IMIBIC), University of Cordoba, Cordoba, Spain

Massive intravascular hemolysis is associated with acute kidney injury (AKI). Nuclear factor erythroid-2-related factor 2 (Nrf2) plays a central role in the defense against oxidative stress by activating the expression of antioxidant proteins. We investigated the role of Nrf2 in intravascular hemolysis and whether Nrf2 activation protected against hemoglobin (Hb)/heme-mediated renal damage *in vivo* and *in vitro*. We observed renal Nrf2 activation in human hemolysis and in an experimental model of intravascular hemolysis promoted by phenylhydrazine intraperitoneal injection. In wild-type mice, Hb/heme released from intravascular hemolysis promoted AKI, resulting in decreased renal function, enhanced expression of tubular injury markers (KIM-1 and NGAL), oxidative and endoplasmic reticulum stress (ER), and cell death. These features were more severe in Nrf2-deficient mice, which showed decreased expression of Nrf2-related antioxidant enzymes, including heme oxygenase 1 (HO-1) and ferritin. Nrf2 activation with sulforaphane protected against Hb toxicity in mice and cultured tubular epithelial cells, ameliorating renal function and kidney injury and reducing cell stress and death. Nrf2 genotype or sulforaphane treatment did not influence the severity of hemolysis. In conclusion, our study identifies Nrf2 as a key molecule involved in protection against renal damage associated with hemolysis and opens novel therapeutic approaches to prevent renal damage in patients with severe hemolytic crisis. These findings provide new insights into novel aspects of Hb-mediated renal toxicity and may have important therapeutic implications for intravascular hemolysis-related diseases.

Keywords: intravascular hemolysis, hemoglobin, heme, Nrf2, oxidative stress, tubular injury, sulforaphane

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***Correspondence:**
Alfonso Rubio-Navarro
arr2015@med.cornell.edu
Juan Antonio Moreno
juan.moreno@uco.es

†These authors have contributed equally to this work.

‡Present Address:
Alfonso Rubio-Navarro
Department of Medicine, Weill Cornell Medicine, Cornell University, New York City, NY, United States

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INTRODUCTION

Recurrent and massive intravascular hemolysis induces renal toxicity, leading to acute kidney injury (AKI) and increasing the risk to develop chronic kidney disease (CKD) (Moreno et al., 2012; Naik et al., 2014). Intravascular hemolysis is a common characteristic of diseases such as sickle cell disease (SCD), paroxysmal nocturnal hemoglobinuria, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, and surgical procedures, including cardiopulmonary bypass or percutaneous mechanical thrombectomy (Ballarin et al., 2011; Baddam et al., 2017; Escobar et al., 2017; Mammen et al., 2017; Wetz et al., 2017; Guerrero-Hue et al., 2018).

Erythrocyte destruction leads to the release of hemoglobin (Hb) and heme-derived products to plasma, which are then filtered by kidney glomeruli and reabsorbed by the proximal tubular epithelium. Renal cell exposure to Hb and metabolites promotes side effects, such as inflammation, cell death, and oxidative stress characterized by lipid peroxidation and mitochondrial dysfunction (Balla et al., 1993; McFaul et al., 1994; Reiter et al., 2002; Liu and Spolarics 2003; Gonzalez-Michaca et al., 2004). Thus, in an oxidant milieu, heme-derived iron group may be oxidized, producing hydroxyl and hydroperoxyl radicals (Gutteridge, 1986). These radicals are highly reactive and promote the formation of reactive oxygen species and subsequent lipid peroxidation and DNA damage (Jia et al., 2007; Buehler et al., 2010). Endoplasmic reticulum (ER) stress, a common form of cell stress, has recently emerged as a pathophysiologic mechanism underlying the renal damage associated with Hb and heme accumulation (Deuel et al., 2016; Feng et al., 2018). ER stress is generated by the accumulation of misfolded proteins in the ER, initiating the unfolded protein response (UPR) that also can be triggered by several insults such as inhibition of proteasome, hypoxia, and oxidative stress (Kim et al., 2008; Liu et al., 2019). UPR compensates stress maintaining cellular homeostasis and avoiding cell death. Heme induces ER stress by expression of activating transcription factor-4 (ATF4) as well as splicing of X-box binding protein-1 (XBP1), promoting upregulation of targets such as CHOP (C/EBP homology protein) (Gall et al., 2018). Administration of transferrin, hemopexin, and haptoglobin resulted in beneficial extravascular/intravascular hemolytic anemias (Buehler and Karnaukhova, 2018). However, it is necessary to unravel the pathogenesis of Hb-mediated renal damage to identify novel therapeutic targets to protect against AKI and subsequent progression to CKD.

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor that drives the expression of target genes involved in cellular defense against xenobiotics and cellular stress such as oxidative and ER stress (Itoh et al., 1997; Cullinan and Diehl, 2004; Chang et al., 2018). In these harmful conditions, Nrf2 is translocated into the nucleus and interacts with antioxidant response elements (AREs) to upregulate a battery of phase II and antioxidant enzymes, such as HO-1 (the enzyme that catalyzes heme degradation) and ferritin (involved in iron storage and with ferroxidase activity) (Tsuji et al., 2000; Kobayashi and Yamamoto, 2006; Liu et al., 2009). Both proteins have been shown to be protective in heme-associated pathologies (Balla et al., 1992; Nath et al., 1992; Zarjou et al., 2013). Several studies have shown that Nrf2 induction protects against oxidative stress and inflammation in AKI (Shelton et al., 2013; Zarjou et al., 2013). Interestingly, pharmacological activation of Nrf2/HO-1 pathway was found to significantly protect against rhabdomyolysis-induced AKI (Shelton et al., 2013; Wang et al., 2014). Although Nrf2 activation has been postulated as a possible therapeutic target to treat diseases associated with intravascular hemolysis (Keleku-Lukwete et al., 2015; Doss et al., 2016; Ghosh et al., 2016; Vasconcellos et al., 2016; Belcher et al., 2017), additional studies are needed to unravel the specific effect of Nrf2 activation in kidney in this pathological setting. In the present study, we investigate the role of Nrf2 in Hb-induced AKI during intravascular hemolysis episodes in humans and in an experimental animal model resembling massive intravascular hemolysis. Nrf2 knockout mice showed more severe AKI. To further clarify the role of Nrf2 in AKI induced by intravascular hemolysis, we analyzed the expression of several Nrf2-related proteins. Finally, we also demonstrate that Nrf2 activation ameliorates AKI induced by intravascular hemolysis both *in vivo* and in cultured tubular epithelial cells, indicating that Nrf2 may be a therapeutic target for the treatment of these diseases.

MATERIAL AND METHODS

Human Renal Biopsy

We identified a renal biopsy from a 28-year-old patient with massive intravascular hemolysis secondary to percutaneous mechanical thrombectomy. At time of biopsy, the patient showed characteristics of AKI (sCr 9.78 mg/dl) and intravascular hemolysis (Hb 11 g/dl, platelets 180,000/μl, LDH 1,030 IU/L, and haptoglobin 5 mg/dl). Healthy kidney samples were obtained from non-tumor renal sections obtained after surgery in patients with kidney cancer and stored at the Instituto de Investigaciones Sanitarias-Fundacion Jimenez Diaz (IIS-FJD) biobank. Patients provided informed consent, and the biobank was approved by the IIS-FJD ethics committee.

Animal Model

Intravascular hemolysis was induced by the intraperitoneal administration of a freshly prepared phenylhydrazine solution (2 mg/10 g of body weight) in 12-week-old wild-type C57BL/6 mice (Jackson Laboratory) or Nrf2-deficient mice (Nrf2−/−) (obtained from Dr. Susana Cadenas, CBMSO, Spain). Mice were housed in a pathogen-free, temperature-controlled environment with a 12-h/12-h light/dark photocycle and had free access to food and water. Phenylhydrazine hydrochloride (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml, and the pH was adjusted to pH 7.4 with NaOH. For Nrf2 activation, sulforaphane (12.5 mg/kg of body weight, Cayman Chemical) was administrated intraperitoneally 48, 24, and 2 h before phenylhydrazine injection. At 24 h after phenylhydrazine injection, mice were

anesthetized (100 mg/kg of ketamine and 15 mg/kg of xylazine), saline perfused, and euthanized. Blood samples were collected for biochemistry analysis (ADVIA® 2400 Clinical Chemistry System, Siemens Healthcare) and hematological analysis (Scil Vet ABC hematology analyser; Scil). Urine samples were collected for measuring urinary creatinine (creatinine assay kit, Abcam). The presence of heme in tissue, blood, and urine was quantified with a commercial kit (MAK316, Sigma). Dissected kidneys were fixed in 4% paraformaldehyde and embedded in paraffin for histological studies or snap frozen for RNA and protein studies, as previously described (Moreno et al., 2011; Sastre et al., 2013). All reported experiments were conducted in accordance with the Directive 2010/63/EU of the European Parliament and were approved by a local Institutional Animal Care and Use Committee (IIS-FJD).

Immunohistochemistry/ Immunofluorescence

Paraffin-embedded kidneys were cross-sectioned into 3-µm-thick pieces, and immunohistochemistry/immunofluorescence was performed as previously described (Rubio-Navarro et al., 2016). Specific primary antibodies were rabbit anti-Hb (1:100 dilution, ab92492, Abcam), rabbit anti-HO-1 (1:200 dilution, ADI-OSA-150-DEnzo Life technologies), rabbit anti-ferritin light chain (1:500 dilution, ab69090, Abcam), rabbit anti-phospho Nrf2 (1:50 dilution, bs-2013R, Bioss), Nrf2 (1:100 sc-722, Santa Cruz), rabbit anti-mouse 4-hydroxynonenal (4-HNE) (1:100, ab46545, Abcam), mouse anti-calnexin (1:100, 610523 BD Biosciences), and mouse anti-BiP (1:100, sc376768, Santa Cruz). The biotinylated secondary antibodies were applied for 1 h. Avidin–biotin peroxidase complex (Vectastain ABC kit, PK-7200, Vector Laboratories) was added for 30 min. Sections were stained with 3,3′-diaminobenzidine or 3-amino-9-ethyl carbazol (S1967, DAKO) and counterstained with hematoxylin. Images were taken with a Nikon Eclipse E400 microscope (Japan) and Nikon ACT-1 software (Japan). In immunofluorescence studies, slides were incubated with Alexa 488 as secondary antibodies (A11090, Invitrogen) and analyzed in an inverted confocal microscope (Leica TCS SP5). Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Negative controls using the corresponding IgG were included to check for non-specific staining. Quantification of protein expression was performed using the Image-J software (National Institutes of Health, Bethesda, MD, USA) with similar acquisition settings in all tissues. Samples were examined in a blinded manner.

TUNEL Assay

The degree of apoptosis was assessed using a terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay. Detection of DNA fragmentation was performed using a kit from Roche Applied Sciences (06432344001, Indianapolis, IN, USA). A semi-quantitative analysis was performed by counting the number of TUNEL-positive cells per field in the renal tissue at ×400 magnification. At least 10 areas in the cortex per slide were randomly selected. The mean number of green colored cells in these selected fields was expressed as the number of TUNEL-positive cells.

Cell Culture

Proximal murine tubular epithelial (MCT) cells were cultured in RPMI 1640 (R0883, Sigma, MO, USA) supplemented with 10% decomplemented fetal bovine serum (FBS, F7524, Sigma, MO, USA), glutamine (2 mmol/L, G7513, Sigma, MO, USA), and penicillin/streptomycin (100 U/ml; P0781, Sigma, MO, USA) in 5% CO_2 at 37°C. MCT cells were stimulated with Hb (0–500 µg/ml, corresponding to 0–30 µM of heme molar equivalents) (H0267, Sigma) and hemin/heme (0–30 µM) (H9039, Sigma). Some cells were pre-treated with sulforaphane (SFN, 2 µM, Cayman Chemical) or *tert*-butyl hydroquinone (tBHQ, 1 µM) (112941, Sigma) for 16 h before Hb/heme stimulation.

Luciferase Assay in AREc32 Cell Cultures

Transformed MCF-7 breast cancer cells, stably expressing luciferase under the control of ARE sequences, AREc32 [kindly provided by Prof. Roland Wolf (University of Dundee, U.K.)], were cultured in DMEM with GlutaMAX and high glucose, supplemented with 1% penicillin-streptomycin (10,000 units), Geneticin (0.8 mg/ml), and 10% FBS at 37°C in a 5% CO_2-supplemented air atmosphere. Nrf2 was induced in AREc32 cells as previously described (Buendia et al., 2015). In brief, AREc32 cells were seeded in 96-well white plates (2×10^4 cells/well). Twenty-four hours later, cells were treated with Hb (0–500 µg/ml, 0–30 µM of heme molar equivalents) or heme (10 µM) for another 24 h. Thereafter, luciferase content was determined using a Luciferase Assay System (Promega E1500) and quantified in an Orion II microplate luminometer (Berthold, Germany).

RNA Extraction and Real-Time PCR

Total RNA from kidneys or cultured cells was isolated with TriPure reagent (Roche) and reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed on ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA, USA) using the DeltaDelta Ct method.

Expression of target genes was analyzed by real-time quantitative PCR using Taqman® gene expression assays for murine NGAL (Mm01324470_m1), KIM-1 (Mm00506686_m1), HO-1 (Mm00516005_m1), ferritin light chain (Mm03030144_g1), Nrf2 (Mm 00477784_m1), catalase (Mm00437992_m1), and NQO1 (Mm01253561_m1) (Applied Biosystems, Foster City, CA, USA) and designed probes for Atf4 (forward-5′-GGGTTCTGTCTTCCACTCCA-3′, reverse-5′-AAGCAGCAGAGTCAGGCTTTC-3′), CHOP/Ddit3 (forward-5′-CCACCACACCTGAAAGCAGAA-3′, reverse-5′-AGGTGAAAGGCAGGGACTCA-3′), spliced XBP1 (sXBP1) (forward-5′-CTGAGTCCGAATCAGGTGCAG-3′, reverse-5′-GTCCATGGGAAGATGTTCTGG-3′), ferritin heavy chain (forward-5′-AGACCGTGATGACTGGGAGA-3′, reverse-5′-TGAAGTCACATAAGTGGGGATCA-3′), and GAPDH (forward-5′-TGCACCACCAACTGCTTAGC-3′, reverse-5′-GGCATGGACTGTGGTCATGAG-3′) (Fisher Scientific, Spain). Expression levels are given as ratios to eukaryotic 18S rRNA (VIC, 4310893E).

Western Blot

Tissue samples were homogenized in lysis buffer (50 mM of Tris–HCl, 150 mM of NaCl, 2 mM of EDTA, 2 mM of EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mM of PMSF, and 1 μg/ml of pepstatin A) and then separated by 10% SDS-PAGE under reducing conditions. After electrophoresis, samples were transferred to PVDF membranes (IPVH00010, Millipore, Bedford, MA, USA), blocked with 5% skimmed milk in TBS/0.5% v/v Tween 20 for 1 h, washed with TBS/Tween, and incubated with anti-HO-1 (1:2000 dilution, ADI-OSA-150-D Enzo Life Technologies), rabbit anti-ferritin light chain (1:500 dilution, ab69090, Abcam), rabbit anti-ferritin heavy chain (1:1000 dilution, Thermo Fisher 701934), and anti-phospho Nrf2 (1:1000 dilution, bs-2013R, Bioss). Antibodies were diluted in 5% milk TBS/Tween. Blots were washed with TBS/Tween and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham, Aylesbury, UK). After being washed with TBS/Tween, blots were developed with the chemiluminescence method (ECL Luminata Crescendo, WBLUR0500, Millipore) and scanned using the ImageQuant LAS-4000 (GE Healthcare). Blots were then probed with mouse monoclonal anti-α-tubulin antibody (1:5000, T6199, Sigma, MO, USA), and levels of expression were corrected for minor differences in loading. Quantification was expressed as arbitrary densitometric units (AU).

Assessment of Oxidative Stress

The molecular probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (C6827; Invitrogen) was used to measure intracellular reactive oxygen species (ROS). Cells were incubated with H_2DCFDA (5 μM) for 30 min, and ROS production was estimated using flow cytometry as previously reported (Rubio-Navarro et al., 2018). To determine cell superoxide anion production, cells were assayed with dihydroethidium (DHE) (Invitrogen) (Sastre et al., 2013). To quantify mitochondrial superoxide production, cells were incubated with MitoSOX Red (0.5 μM) for 30 min in the dark. Nuclei were counterstained with DAPI. Cells were then analyzed using confocal microscope (Leica TCS SP5).

Cell Viability Assay

Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma) colorimetric assay. After treatment, cells were incubated with 1 mg/ml of MTT in PBS for 1 h at 37°C. The resulting formazan crystals were dried and dissolved in dimethylsulfoxide. Absorbance (indicative of cell viability) was measured at 570 nm in a microplate reader (BMG Labtech Offenburg, Germany).

GSH Measurement

GSH quantification was performed as previously described (Kamencic et al., 2000). In brief, cells were incubated with monochlorobimane (100 μM) in free serum media for 45 min. Then, cells were washed twice with RPMI and treated for 6 h. Fluorescence intensity was measured in a Fluostar optima microplate reader (BMG Labtech Offenburg, Germany) at excitation and emission wavelengths of 410 and 485 nm, respectively.

Statistical Analysis

Data were expressed as mean ± SEM. Differences between groups were analyzed with the Kruskal–Wallis test and the Mann–Whitney *U*-test. p values <0.05 were considered significant. Statistical analysis was performed using SPSS 11.0 statistical software.

RESULTS

Nrf2 Is Activated in Human Kidney After Massive Intravascular Hemolysis

To determine whether Nrf2 is activated in kidneys as consequence of intravascular hemolysis, we performed histological studies in a patient with AKI-associated with intravascular hemolysis. Perls' Prussian blue staining revealed iron accumulation in tubular cells compared with renal tissue from healthy donor (**Figure 1A**). Immunofluorescence studies confirmed the presence of Hb within tubular cells, as well as Nrf2 phosphorylation and translocation to nuclei and induction of HO-1 expression as compared with those of healthy control (**Figure 1B**). In this patient, we also observed increased tubular cell death, determined by TUNEL staining (**Figure 1B**), and exacerbated oxidative stress, as determined by 4-hydroxynonenal (4-HNE) staining (**Figure 1C**) and presence of the endoplasmic reticulum stress markers BiP (binding immunoglobulin protein) (**Figure 1D**) and calnexin (**Figure 1E**). These results suggest that kidney Hb/iron accumulation as a consequence of intravascular hemolysis promotes oxidative stress and tubular cell injury and further activation of Nrf2 in human disease.

Hb/Heme Induces Nrf2 Activation in Both AREc32 Cells and Cultured Tubular Epithelial Cells

To further explore how intravascular hemolysis modulates Nrf2 activity, we studied whether Hb and heme directly induce Nrf2 transcriptional activity in AREc32 cells. In these experiments, we observed that Hb or heme increased Nrf2-dependent luciferase reporter activity in a dose-dependent manner (**Figure 2A** and **B**). Moreover, we found that both Hb and heme induced Nrf2 mRNA expression and Nrf2 nuclear translocation and reduced the intracellular levels of the Nrf2 repressor Keap1 in a time-dependent manner in MCT (**Figure 2C–H**). Stimulation of MCT cells with Hb and heme increased mRNA and protein expression of two Nrf2-regulated proteins, HO-1 and ferritin subunits, heavy (FtH) (**Supplemental Figure 3A–C**) and light ferritin chain (FtL) (**Figure 2I–L**).These results suggest that intravascular hemolysis activates Nrf2 in the kidney *via* Hb and heme accumulation.

Nrf2 Is a Protective Factor Against Kidney Injury Induced by Intravascular Hemolysis

To determine whether Nrf2 plays a protective role against hemolysis-induced kidney injury, we set up a mouse model of

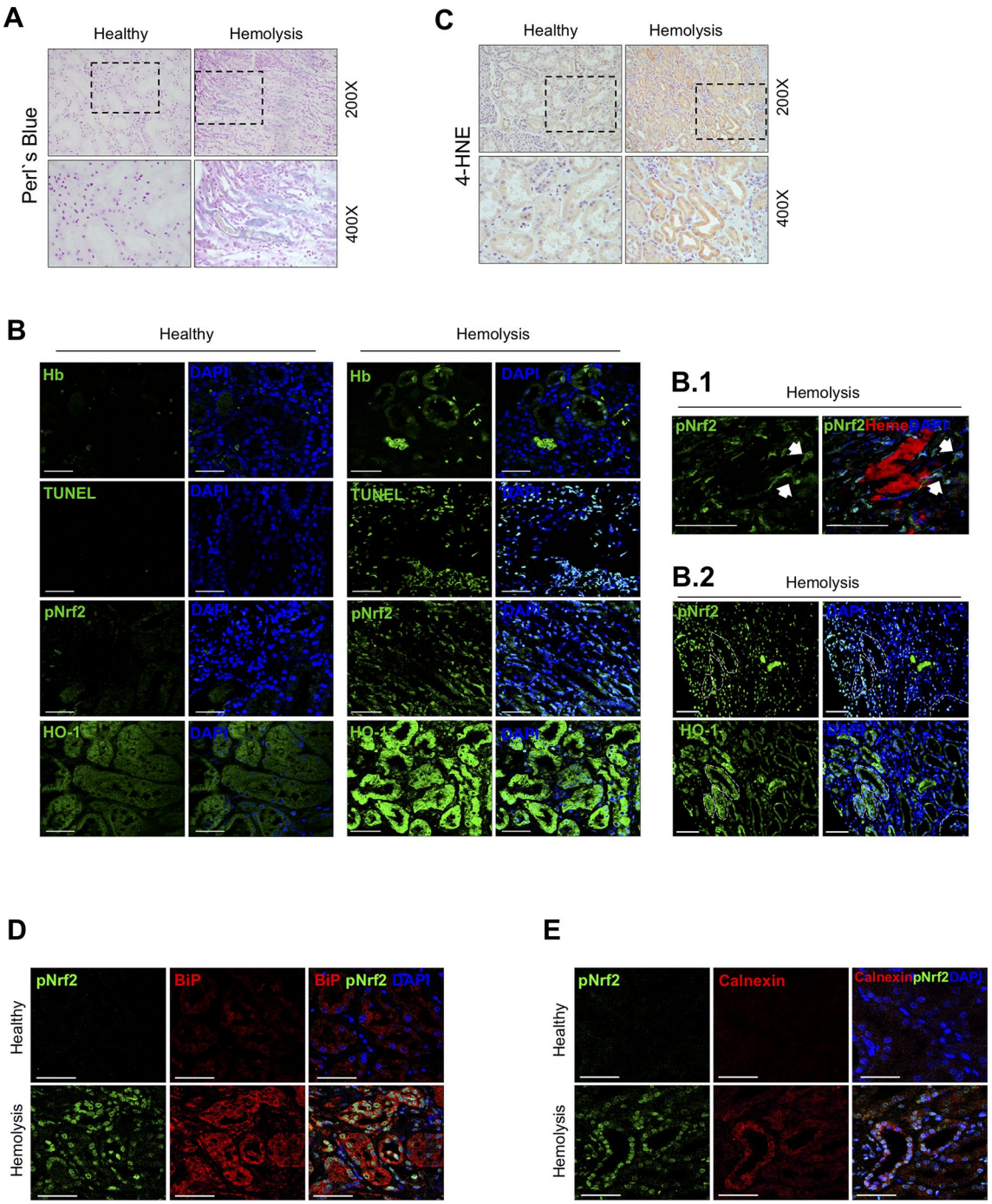

FIGURE 1 | Nuclear factor erythroid-2-related factor 2 (Nrf2) activation in a patient with massive intravascular hemolysis-associated acute kidney injury (AKI). **(A)** Representative image of Perls' Prussian blue staining showing iron accumulation in the renal biopsy of a patient with massive intravascular hemolysis as compared with healthy control (200× upper panel, 400× lower panel). **(B)** Representative confocal microscopy images showing Hb accumulation (green, first row), terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL)-positive cells (green, second row), phospho-Nrf2 (green, third row), and HO-1 (green, fourth row). Nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) (blue); scale bar, 100 μm. **B.1.** Representative confocal microscopy images showing the presence of heme cast (red) and nuclear phospho-Nrf2 (green); scale bar, 50 μm. **B.2.** Representative serial immunofluorescence images showing nuclear Nrf2 translocation (phospho-Nrf2, green) in HO-1-rich areas. White circles indicate similar regions in serial immunostained sections. Scale bar, 100 μm. **(C)** Representative image of 4-hydroxynonenal (4-HNE) staining (200× upper panel, 400× lower panel). Representative confocal microscopy images showing the presence of nuclear Nrf2 translocation (phospho-Nrf2, green) and the endoplasmic reticulum stress markers BiP **(**red, **D)** and calnexin **(**red, **E)**. Scale bar, 50 μm.

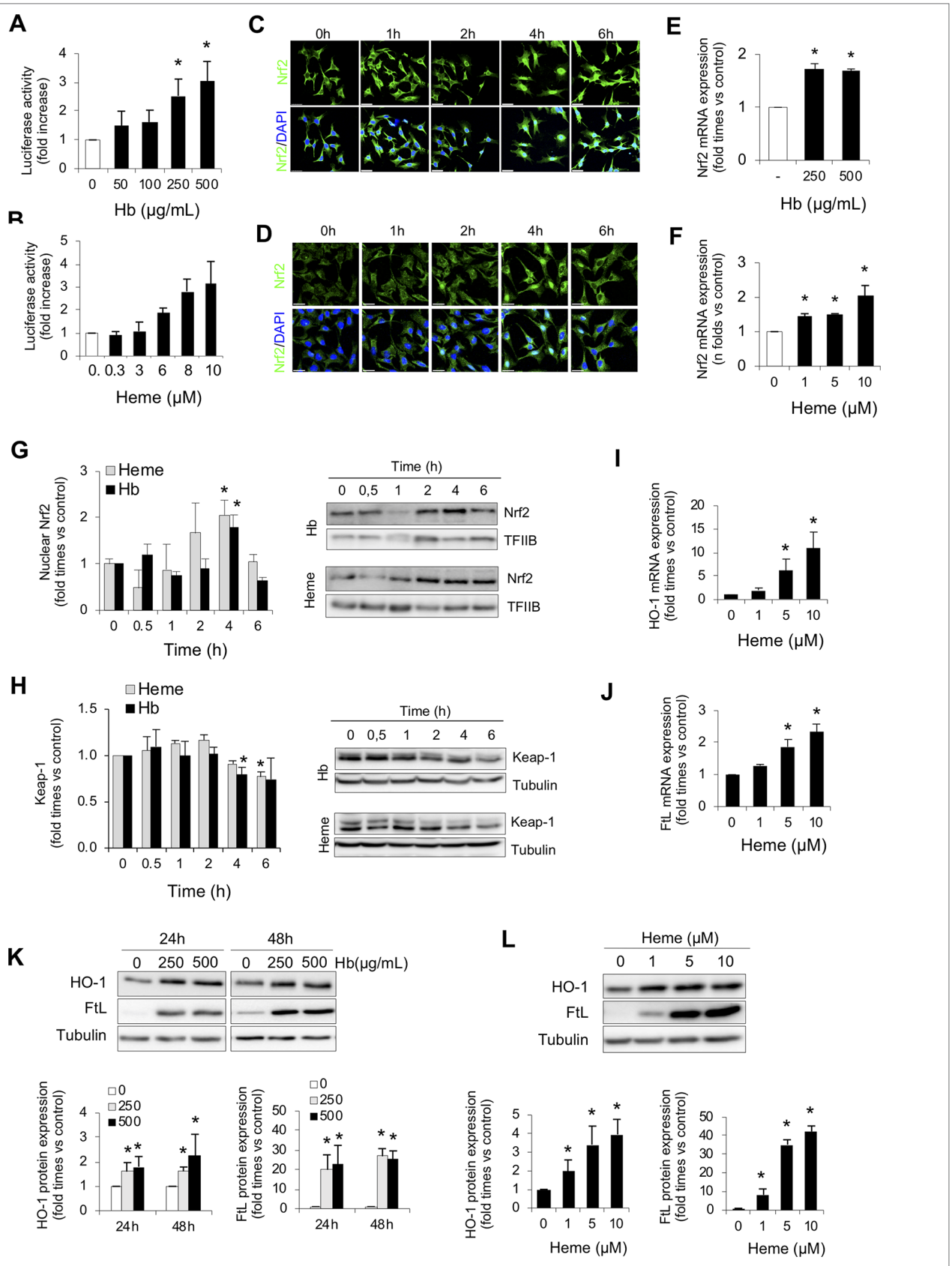

FIGURE 2 | Continued

FIGURE 2 | Hemoglobin and heme promote Nrf2 activation in cultured renal cells. Nrf2 transcriptional activity was measured by a luciferase reporter assay in AREc32 cells treated with increasing concentrations of Hb (0–500 μg/ml, 0–30 μM heme equivalents) **(A)** or heme (0–10 μM) **(B)** for 24 h. Representative confocal microscopy images showing Nrf2 nuclear translocation (green) in murine tubular (MCT) cells after exposure to Hb (500 μg/ml, 30 μM heme equivalents) **(C)** or heme (1 μM) **(D)** at different times (0–6 h). Nuclei were stained with DAPI (blue). Nrf2 mRNA expression measured by real-time-quatitative polymerase chain reaction (RT-qPCR) in MCT cells treated with Hb **(E)** or heme **(F)** for 6 h. **(G)** Nuclear Nrf2 levels in MCT cells treated with Hb (500 μg/ml, 30 μM heme equivalents) and heme (1 μM) at different times (0–6 h). Representative western blot image from nuclear protein fraction. TFIIB (transcription factor II B) was used as loading control. **(H)** Keap1 protein content in MCT cells treated with Hb (500 μg/ml, 30 μM heme equivalents) and heme (1 μM) at different times (0–6 h). HO-1 **(I)** and FtL **(J)** mRNA expression measured by RT-qPCR in MCT cells treated with heme for 6 h. **(K–L)** Representative western blot image showing HO-1 and FtL expression in MCT cells treated with Hb (0–500 μg/ml, 0–30 μM heme equivalents) and heme (0–10 μM) for up to 48 h (upper panel). Quantification of HO-1 and FtL by western blot (lower panel). Results are expressed as mean ± SE. $^*p < 0.05$ vs non-treated cells.

intravascular hemolysis induced by the intraperitoneal injection of phenylhydrazine in wild-type (Nrf2+/+) and Nrf2 knockout mice (Nrf2−/−) (**Figure 3A**). This is a well-established mouse model of AKI-associated with massive intravascular hemolysis promoted by phenylhydrazine-mediated lipid peroxidation of erythrocytes membranes, leading to the extracellular release of Hb and heme (Merle et al., 2018). In line with the human findings, we observed nuclear staining for phospho-Nrf2 (Ser40) (pNrf2) in kidneys from Nrf2+/+ mice with intravascular hemolysis (**Supplemental Figure 1**). Interestingly, our results show a decline of renal function (increase of serum creatinine and BUN levels) after induction of intravascular hemolysis in WT mice; and this was more severe in Nrf2−/− mice (**Figure 3B** and **C**). Similarly, we observed more severe histological changes (presence of intratubular debris, pyknotic nuclei from apoptotic cells, tubular epithelial cells into the lumen, and loss of nuclei in the tubular epithelium) and enhanced mRNA expression of the tubular injury biomarkers NGAL and KIM-1 in Nrf2−/− mice with intravascular hemolysis than in wild-type mice (**Figure 3D–F**). These data suggest that Nrf2 could be a protective factor against kidney injury promoted by intravascular hemolysis.

We next examined whether Nrf2 deficiency determines degree of systemic intravascular hemolysis. As expected, phenylhydrazine-injected mice showed a reduction in hematocrit and erythrocyte number, as well as an increased concentration of serum free Hb and heme, but no significant differences were observed between Nrf2+/+ and Nrf2−/− mice (**Figure 3G–K**). Hemolysis also promoted a similar renal accumulation of Hb and heme between mice strains, mainly in tubular cells (**Figure 3L–N**). Altogether, these data reveal that Nrf2 did not prevent erythrocyte lysis or Hb accumulation after the intravascular hemolysis episode.

Nrf2 Protects From Kidney Oxidative Stress and Cell Death Promoted by Intravascular Hemolysis

To fully address the mechanism involved in Nrf2-mediated renal protection against intravascular hemolysis, we characterized oxidative stress and cell death, pathogenic processes characteristic of this scenario (Gutteridge, 1986; Gonzalez-Michaca et al., 2004). Intraperitoneal phenylhydrazine injection increased renal levels of 4-HNE, a marker of lipid peroxidation (**Figure 4A**), and decreased kidney GSH contents (**Figure 4B**). These effects were more severe in Nrf2−/− than in wild-type mice. Recently, ER stress has been shown as a new mechanism underlying the renal toxicity induced by Hb and heme accumulation (Deuel et al., 2016; Feng et al., 2018). We observed an increase in expression of UPR-related proteins such as CHOP, ATF4, and sXBP1 in kidney from WT mice injected with Phe. However, Nrf2−/− mice injected with phenylhydrazine showed a higher induction of these UPR-related genes (**Figure 4C**). Renal tubule cell death is an important consequence of Hb accumulation, leading to AKI and renal failure in patients with intravascular hemolysis (Guerrero-Hue et al., 2017). Therefore, we explored the potential beneficial effect of the Nrf2 transcription factor on cell death prevention. Intraperitoneal injection of phenylhydrazine induced tubular cell death, as determined by TUNEL-positive staining, in wild-type mice, an effect that was significantly increased in Nrf2−/− mice (**Figure 4D**).

Next, to unravel the mechanism involved in Nrf2 protection, we analyzed the expression of some Nrf2-regulated genes, such as HO-1 and ferritin, which are implicated in the intracellular degradation of toxic heme (Stocker, 1990). Intravascular hemolysis upregulated HO-1 and both ferritin subunits, light ferritin (FtL) (**Figure 5A–F**) and heavy ferritin (FtH) (**Supplemental Figure 3D**). However, this compensatory response, consisting of heme-related and anti-oxidant proteins, was attenuated in Nrf2−/− as compared with wild-type mice, despite the more severe injury in Nrf2−/− mice. Similar effects were observed for other antioxidant Nrf2-dependent genes, such as catalase and NQO1 (**Figure 5G** and **H**). These results could explain why intravascular hemolysis induced more severe kidney injury in mice lacking Nrf2.

Nrf2 Activation Protects Against Hb/Heme-Mediated Oxidative Stress and Cell Death *In Vitro*

We further investigated whether the Nrf2 inducers sulforaphane or *tert*-butylhydroquinone (tBHQ) protected MCT cells from Hb/heme-mediated damage. Thus, we performed *in vitro* experiments pre-treating MCT cells with sulforaphane or tBHQ for 16 h before adding Hb/heme. As expected, Nrf2 activators increased mRNA and protein HO-1 expression in time- and dose-dependent manner (**Figure 6A–C**). Importantly, we observed that sulforaphane and tBHQ reduced Hb- and heme-mediated ROS production, i.e., hydrogen peroxide (**Figure 6D** and **E**), mitochondrial superoxide (**Figure 6F**), and total superoxide (**Figure 6G**). Similarly, we found that pre-treatment with Nrf2 inducers abolished the Hb- and heme-mediated intracellular GSH reduction (**Figure 6H**). We next tested whether Nrf2 activation could protect tubular cells from cell death *in vitro*.

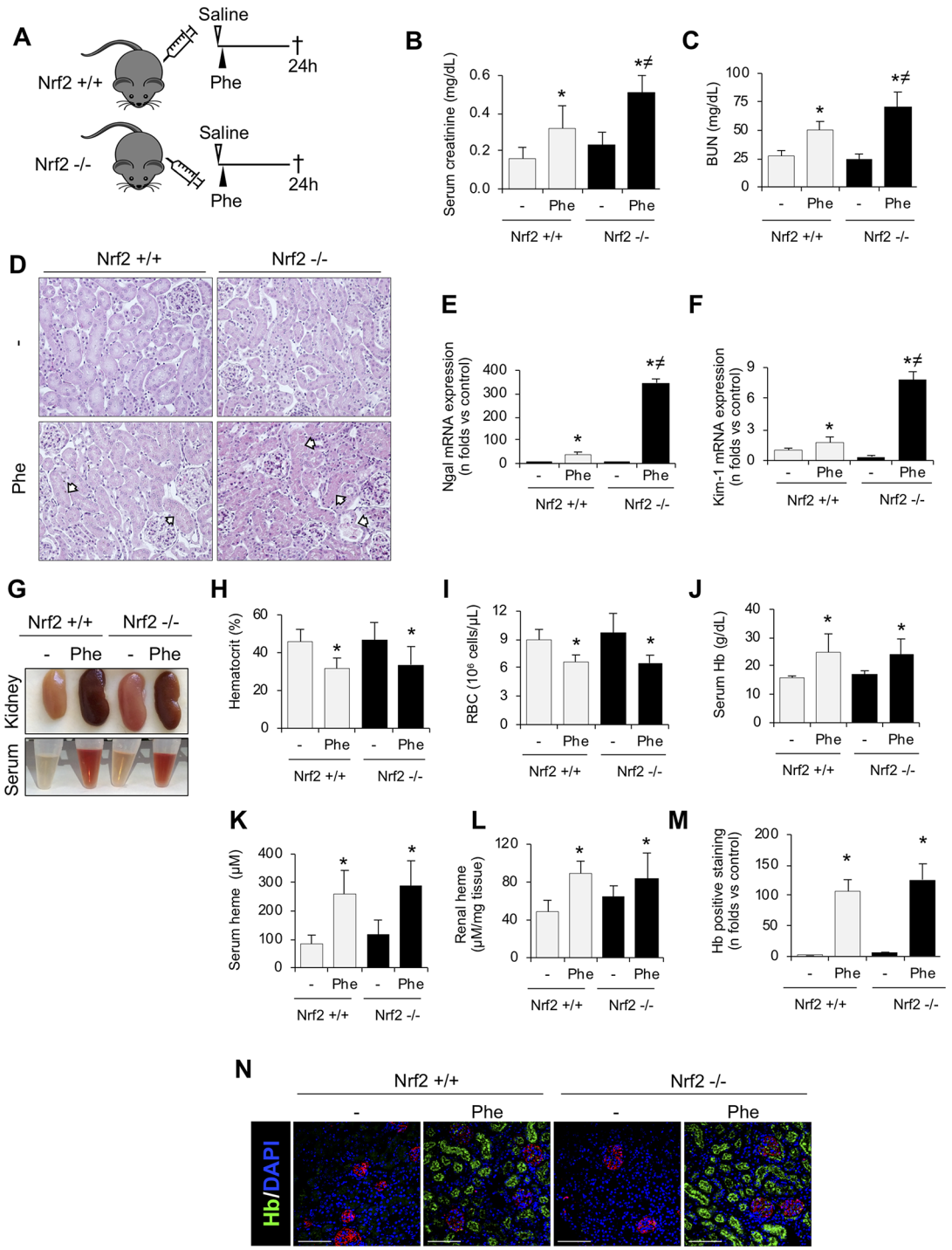

FIGURE 3 | Nrf2 plays a protective role against kidney injury associated with intravascular hemolysis. C57BL/6 (Nrf2+/+) or Nrf2–/– mice (12 weeks old) were i.p. injected with saline (vehicle) or phenylhydrazine (Phe, 2 mg/10 g of body weight) to induce intravascular hemolysis (n = 8/group). **(A)** Schematic representation of intravascular hemolysis mouse model. Serum measurement of creatinine **(B)** and blood urea nitrogen (BUN) **(C)**. **(D)** Representative images showing hematoxylin and eosin staining in kidneys from mice with intravascular hemolysis. Arrows indicate signs of acute tubular injury: presence of intratubular debris, pyknotic nuclei from apoptotic cells, tubular epithelial cells into the lumen, and loss of nuclei in the tubular epithelium. Expression of tubular injury biomarkers NGAL **(E)** and KIM-1 **(F)**, as determined by real-time RT-qPCR, in kidneys from mice with intravascular hemolysis. **(G)** Representatives images showing kidneys (upper row) and serum (lower row) after intravascular hemolysis in both Nrf2+/+ and Nrf2–/– mice. Hematocrit **(H)**, total red blood cell (RBC) counts **(I)**, serum levels of Hb **(J)** and heme **(K)**, and heme concentrations in renal tissue **(L)**. **(M)** Semi-quantification of hemoglobin (Hb)-positive staining per renal cross section. **(N)** Representative images showing Hb (green) accumulation obtained by confocal microscopy. The podocyte marker nephrin (red) was used to delimitate the glomerular area. Nuclei were stained with DAPI (blue); scale bar, 100 μm. Results are expressed as mean ± SE. $^{*}p < 0.05$ vs Nrf2+/+ control mice, $^{\neq}p < 0.05$ vs Nrf2+/+ Phe-injected mice.

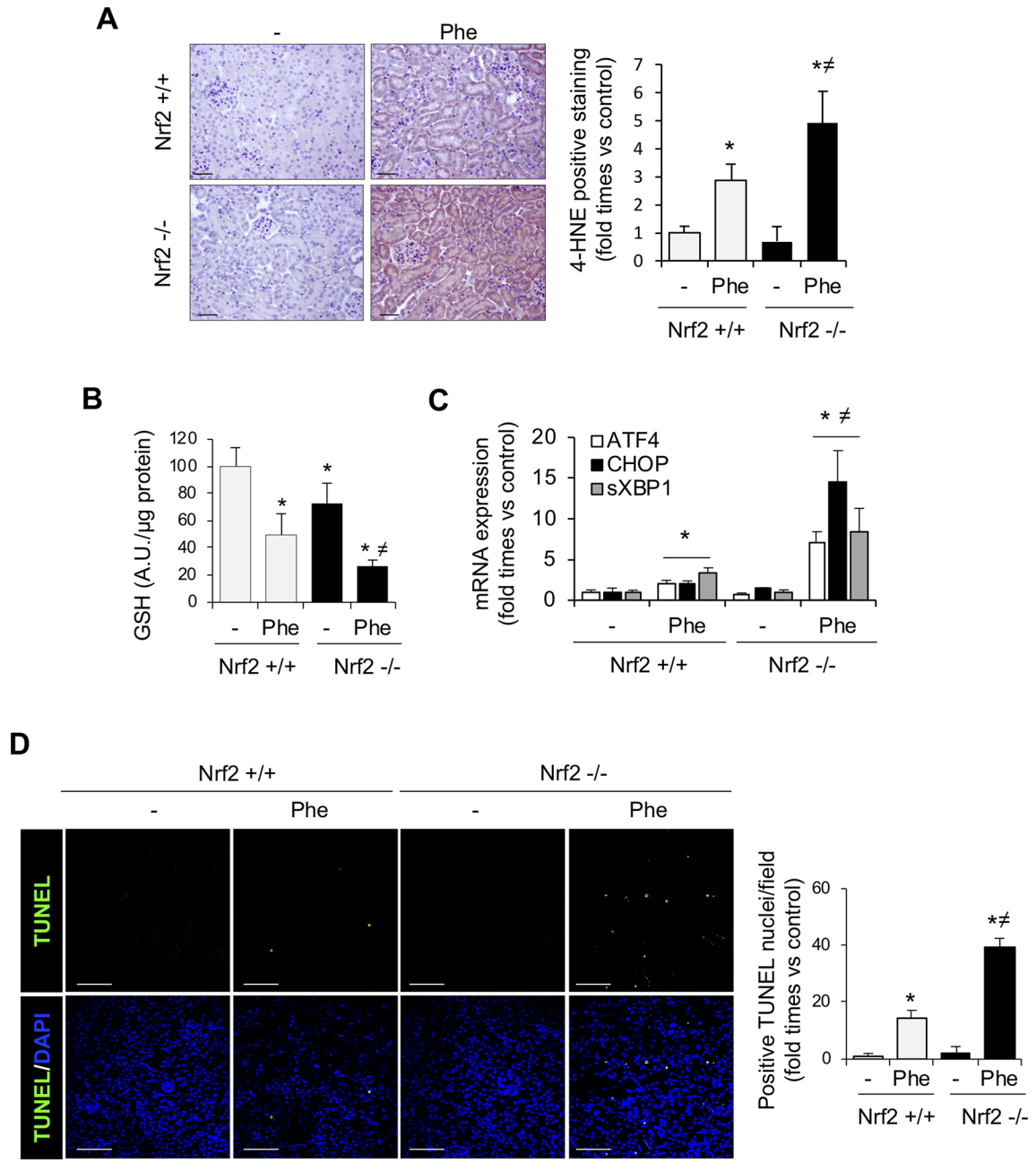

FIGURE 4 | Nrf2 protects against oxidative stress and cell death associated with intravascular hemolysis. C57BL/6 (Nrf2+/+) or Nrf2–/– mice (12 weeks old) were i.p. injected with saline (vehicle) or phenylhydrazine (Phe, 2 mg/10 g of body weight) to induce intravascular hemolysis (n = 8/group). **(A)** Representative image of 4-hydroxynonenal (4-HNE) staining. Semi-quantification of 4-HNE-positive staining per renal cross section. **(B)** GSH content in renal tissue. **(C)** ATF4, CHOP, and sXBP1 mRNA levels determined in kidney by real-time RT-qPCR. **(D)** Representative confocal microscopy images showing nuclear staining of TUNEL (green) (left panel). Nuclei were stained with DAPI (blue); scale bar, 100 µm. Semi-quantitative analysis of TUNEL-positive cells (right panel). Results are expressed as mean ± SE. $^{*}p < 0.05$ vs Nrf2+/+ control mice, $^{\neq}p < 0.05$ vs Nrf2+/+ Phe-injected mice.

Treatment with sulforaphane and tBHQ improved cell viability after exposure to heme (**Figure 6I** and **J**). Altogether, our results show that pharmacological activation of Nrf2 *in vitro* avoided Hb- and heme-mediated oxidative stress and cell death, indicating that Nrf2 could be a therapeutic target against kidney injury induced by intravascular hemolysis.

In Vivo Nrf2 Activation Protects Against Intravascular Hemolysis-Associated AKI

Once we demonstrated the beneficial effects of Nrf2 activation *in vitro*, we tested whether this therapeutic approach may prove effective *in vivo*. Thus, in a first set of experiments, we administrated sulforaphane once a day for 3 days to analyze

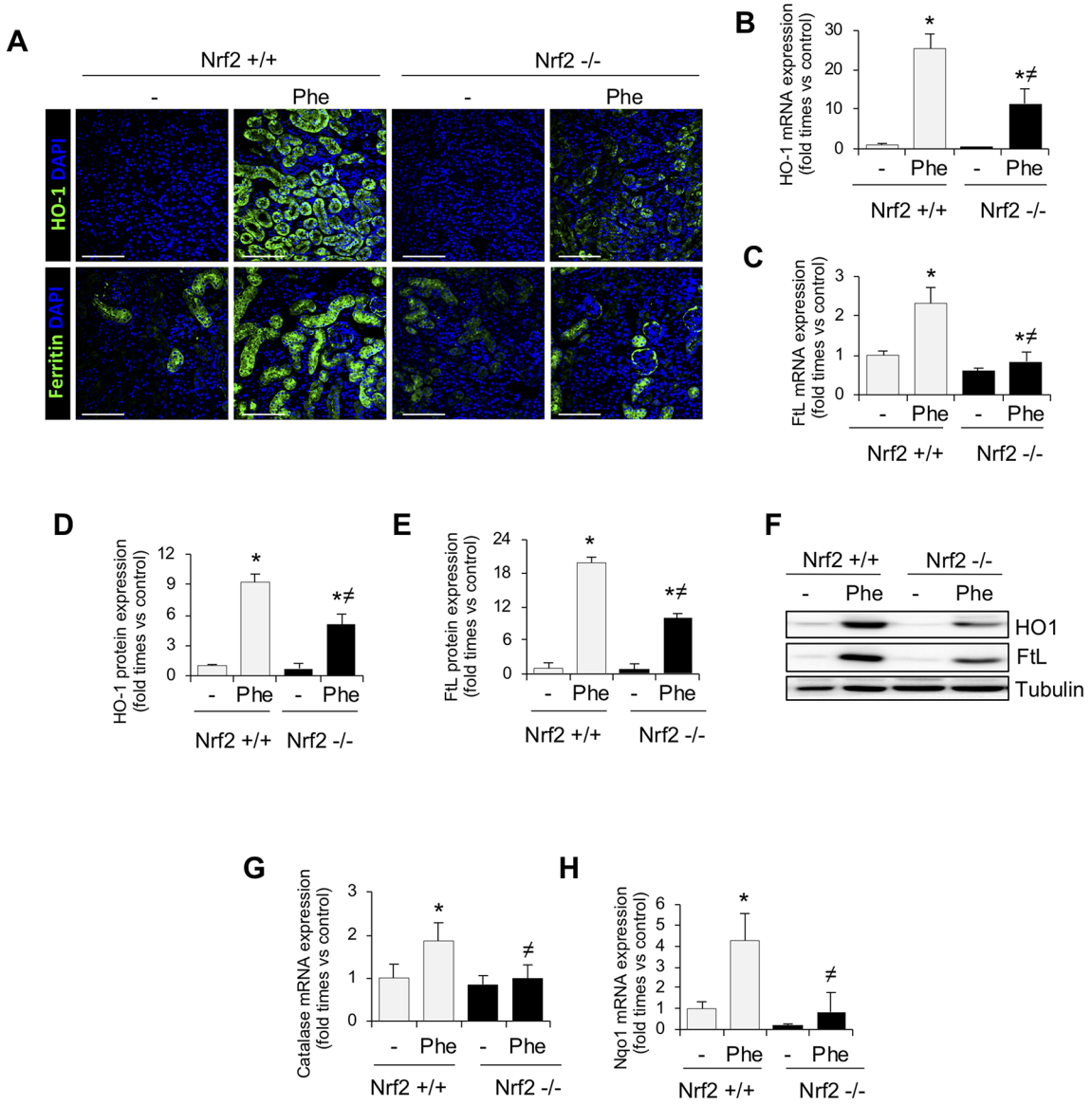

FIGURE 5 | Nrf2-related proteins in kidney from mice with intravascular hemolysis. C57BL/6 (Nrf2+/+) or Nrf2–/– mice (12 weeks old) were i.p. injected with saline (vehicle) or phenylhydrazine (Phe, 2 mg/10 g of body weight) to induce intravascular hemolysis (n = 8/group). **(A)** Representative immunofluorescence images obtained by confocal microscopy showing expression of HO-1 (green, upper panel) and FtL (green, lower panel). Nuclei were stained with DAPI (blue); scale bar, 100 μm. HO-1 **(B)** and FtL **(C)** mRNA expression measured by RT-qPCR. Semi-quantification of HO-1 **(D)** and FtL **(E)** protein expression determined by western blot. **(F)** Representative western blot image of HO-1 amd FtL expression in kidneys from mice of the experimental model. Catalase **(G)** and NQO1 **(H)** mRNA expression measured by RT-qPCR. Results are expressed as mean ± SEM. *$p < 0.05$ vs Nrf2+/+ control mice, ≠$p < 0.05$ vs Nrf2+/+ Phe-injected mice.

whether this treatment produced Nrf2 activation in kidney. As reported in **Figure 7A**, sulforaphane treatment induced Nrf2 phosphorylation and increased HO-1 and FtL levels in the kidney. We also ruled out any sulforaphane-derived adverse effect in the kidney (**Supplemental Figure 2**). We next conducted new experiments to determine whether sulforaphane administration protects from phenylhydrazine-mediated renal injury in mice (**Figure 7B**). Consistent with our previous findings, sulforaphane treatment improved renal function in mice with hemolysis, as assessed by serum creatinine and BUN (**Figure 7C** and **D**). Moreover, sulforaphane reduced histological injury (**Figure 7E**) and decreased the gene expression of the tubular injury markers KIM-1 and NGAL (**Figure 7F** and **G**). In line with our previous results, we did not observe any differences in the severity of hemolysis (hematocrit, erythrocyte number, and serum free Hb/heme concentration) between sulforaphane-treated and non-treated mice (**Figure 7H–L**). Moreover, kidney Hb/heme accumulation did not change according to sulforaphane treatment (**Figure 7M** and **N**).

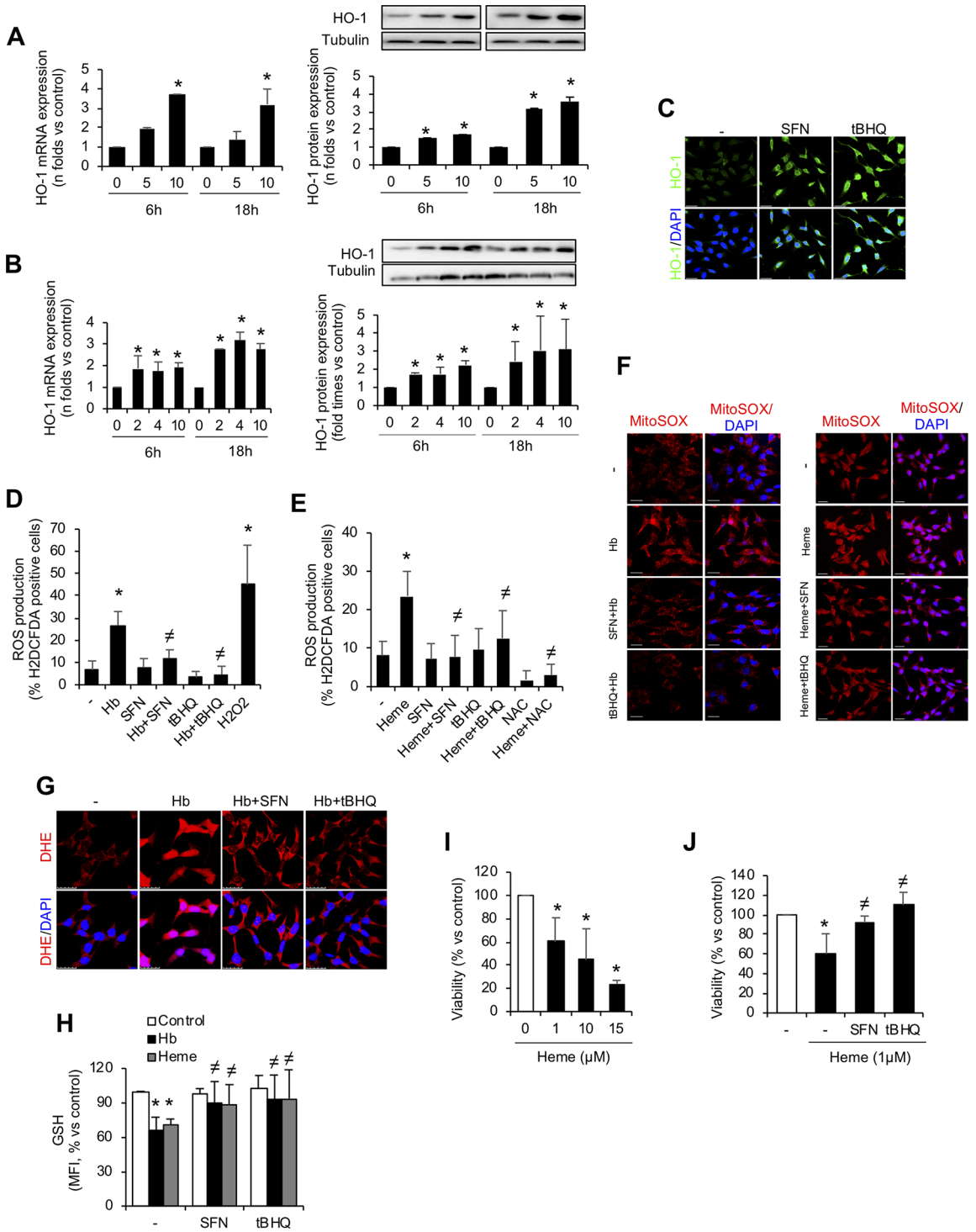

FIGURE 6 | *In vitro* Nrf2 induction ameliorates oxidative stress and cell death. HO-1 mRNA and protein expression in MCT cells treated with the Nrf2 inducers *tert*-butylhydroquinone (tBHQ) **(A)** or sulforaphane (SFN) **(B)** at different concentrations (μM) for up to 18 h. **(C)** Representative confocal microscopy images showing HO-1 (green) staining in MCT cells pre-treated with Nrf2 inducers SFN and tBHQ with or without Hb. Nuclei were stained with DAPI (blue); scale bar, 20 μm. Quantification of ROS production (hydrogen peroxide) by flow cytometry with the fluorescent dye H_2DCFDA in MCT cells stimulated with Hb **(D)** or heme **(E)** for 6 h. **(F)** Representative image of MitoSOX showing mitochondrial superoxide production by Hb (left panel) and heme (right panel) in cells pre-treated with SFN (2 μM) and tBHQ (1 μM) for 16 h. Nuclei were stained with DAPI (blue); scale bar, 20 μm. **(G)** Representative image of superoxide anion production determined by confocal microscopy using dihydroethidium (DHE) assay. Nuclei were stained with DAPI (blue); scale bar, 20 μm. **(H)** Intracellular GSH content in MCT cells treated with heme (1 μM) for 6 h with or without SFN (2 μM) or tBHQ (1 μM). **(I–J)** Cell viability determined in MCT cells stimulated with heme (1 μM) for 24 h and pre-treated with SFN (2 μM) or tBHQ (1 μM). Results are expressed as mean ± SEM. $^*p < 0.05$ vs non-treated cells. $^{\neq}p < 0.05$ vs Hb or heme-treated cells.

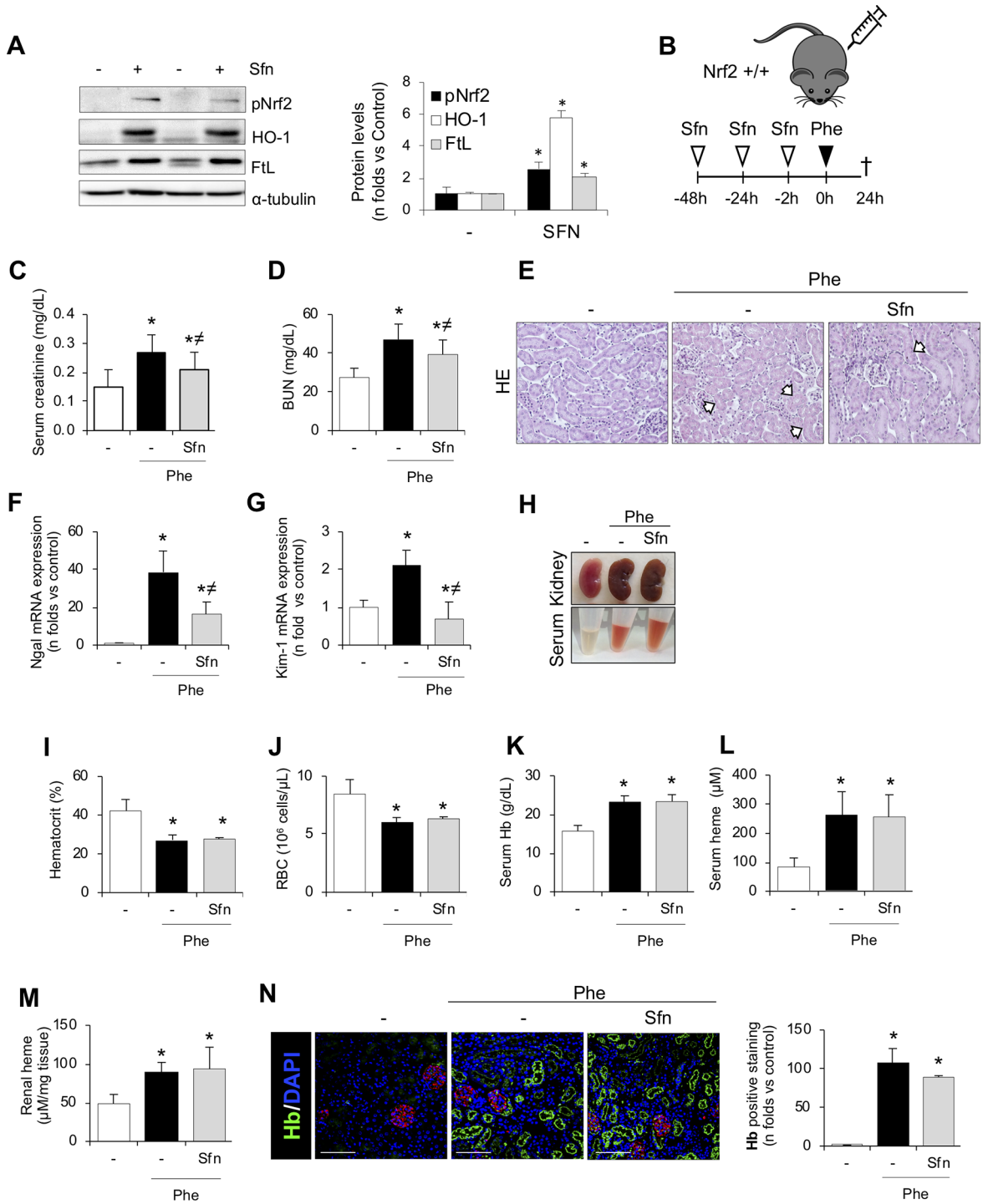

FIGURE 7 | SFN treatment ameliorates acute kidney injury and tubular damage induced by intravascular hemolysis. **(A)** C57Bl/6 mice (males, 12 weeks old) were i.p. treated with saline or sulforaphane (SFN, 12.5 mg/kg) for 3 days (n = 5/group). Representative western blot image showing phospho-Nrf2 (Ser40), HO-1, and FtL protein expression. **(B)** C57Bl/6 mice (males, 12 weeks old) were i.p. treated with saline or sulforaphane (SFN, 12.5 mg/kg) for 48, 24, and 2 h before phenylhydrazine (n = 5/group). Mice were sacrificed 24 h after phenylhydrazine administration. Schematic representation of intravascular hemolysis mouse model. Serum measurement of creatinine **(C)** and blood urea nitrogen (BUN) **(D)**. Representative images showing hematoxylin and eosin staining **(E)**. Expression of tubular injury biomarkers NGAL **(F)** and KIM-1**(G)**, as determined by real-time RT-qPCR, in kidneys from mice with intravascular hemolysis. **(H)** Representatives images showing kidney and serum after intravascular hemolysis in mice treated with SFN. Hematocrit **(I)** and total red blood cell (RBC) counts **(J)**. Serum levels of Hb **(K)** and heme **(L)**. **(M)** Heme concentrations in renal tissue. **(N)** Representative images showing hemoglobin (Hb, green) accumulation obtained by confocal microscopy (left panel). The podocyte marker nephrin (red) was used to delimitate the glomerular area. Nuclei were stained with DAPI (blue); scale bar, 100 μm. Semi-quantification Hb-positive staining per cross section (right panel). Results are expressed as mean ± SEM. *$p < 0.05$ vs control mice, ≠$p < 0.05$ vs Phe-injected mice.

Interestingly, 4-HNE staining revealed lower oxidative stress in sulforaphane-treated than non-treated mice (**Figure 8A**). In addition, sulforaphane decreased the renal expression of UPR-related molecules induced by intravascular hemolysis (**Figure 8B**). Moreover, the decrease in renal GSH levels was milder in sulforaphane-treated mice with hemolysis and was not significantly different from that in controls as opposed to the significant GSH decrease observed in non-treated mice with hemolysis (**Figure 8C**).

Finally, we observed that sulforaphane significantly decreased renal tubular epithelial cell death, evaluated by TUNEL staining, after intravascular hemolysis, as compared with that in non-treated mice (**Figure 8D** and **E**). Collectively, these results suggest that administration of the pharmacologic inducer of Nrf2, sulforaphane, relieves the kidney adverse effects observed in experimental hemolysis. We conclude that Nrf2 might be a potential therapeutic target for kidney injury induced by intravascular hemolysis.

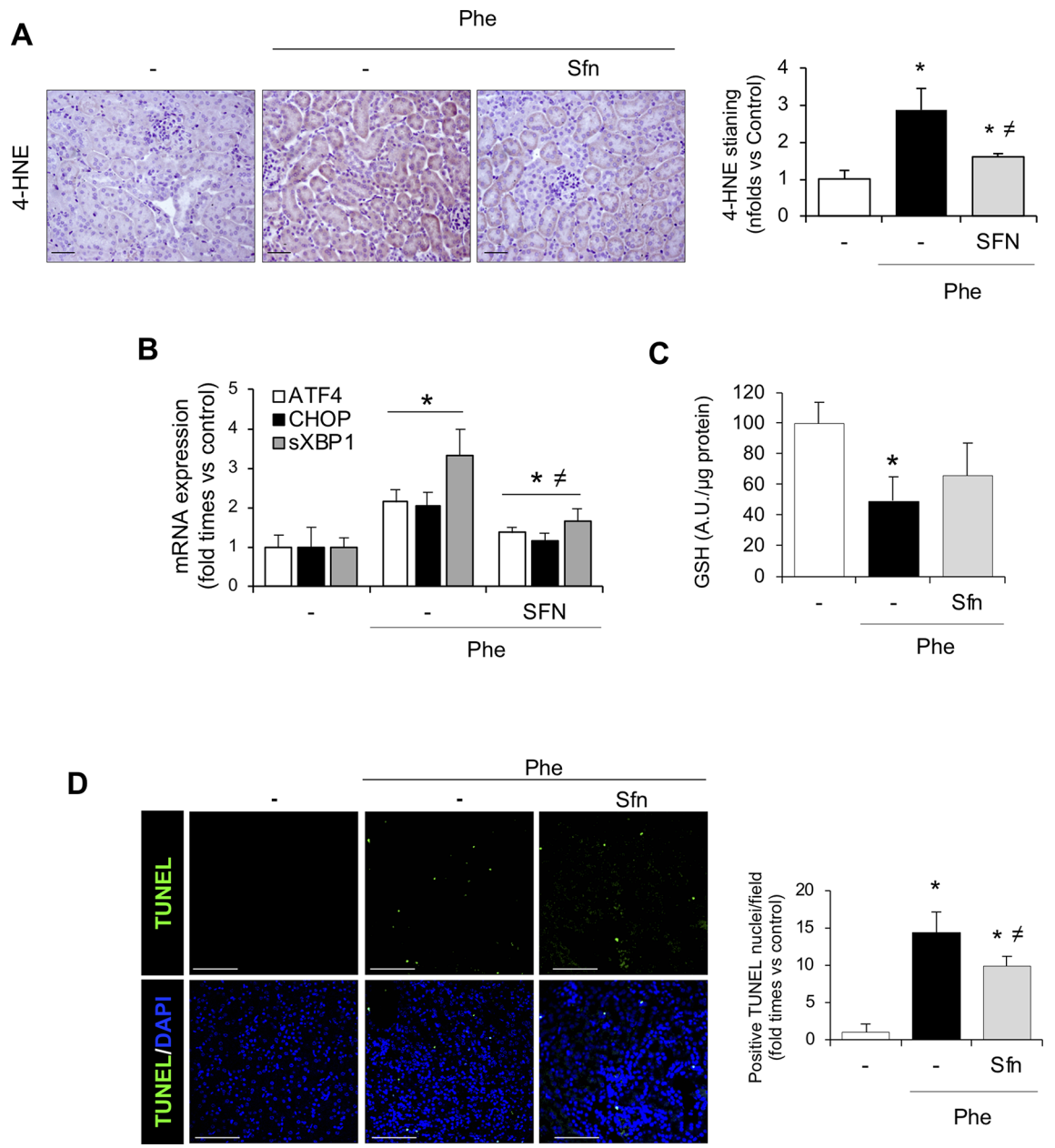

FIGURE 8 | Nrf2 activation pathway in kidney from SFN-treated mice. **(A)** Representative image of 4-HNE staining in the experimental model (left). Semi-quantification 4-HNE-positive staining per cross section (right). **(B)** ATF4, CHOP, and sXBP1 mRNA levels determined in kidney by real-time RT-qPCR. **(C)** GSH content in renal tissue. **(D)** Representative confocal microscopy images showing nuclear staining of TUNEL (green) (left) and semi-quantitative analysis of TUNEL-positive cells (right). Nuclei were stained with DAPI (blue); scale bar, 100 µm. Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs control mice, $^{\neq}p < 0.05$ vs Phe-injected mice.

DISCUSSION

In the present study, we demonstrate that Hb/heme accumulation in the kidney after massive intravascular hemolysis induces Nrf2 activation. We also found that massive intravascular hemolysis promotes AKI, resulting in increased serum creatinine concentration, tubular injury, oxidative and ER stress, and subsequent kidney cell death. These harmful effects were more severe in Nrf2 knockout mice, which showed a decreased expression of the Nrf2-dependent antioxidant proteins HO-1, FtL, and FtH and reduced levels of GSH, suggesting a critical protective role against Hb-mediated ROS production and cell toxicity in the kidney. Moreover, therapeutic Nrf2 activation reduced Hb/heme-mediated cellular stress and cytotoxicity in experimental hemolysis and cultured renal cells. Therefore, enhancing Nrf2 activity may be a potential therapeutic strategy to decrease renal injury associated with hemolytic diseases.

Recurrent or massive erythrocyte lysis increases plasma free Hb concentration and leads to renal Hb overload and toxicity, contributing to acute and chronic kidney injury (Tracz et al., 2007; Moreno et al., 2012; Naik et al., 2014). In our study, we administrated phenylhydrazine to induce severe hemolysis, thus promoting accumulation of Hb and its heme derivatives as well as a rapid decrease of renal function. It is well known that Hb triggers ROS production (Jia et al., 2007), similar to our observations in renal proximal tubular epithelial cells after exposure with Hb or heme. Recently, ER stress has been shown as a novel mechanism underlying the renal toxicity of Hb and heme (Deuel et al., 2016; Feng et al., 2018). Interestingly, Nrf2−/− mice showed an exacerbated renal expression of UPR-related genes, suggesting a protective role of this transcription factor against ER stress in kidney. Mitochondria are susceptible to Hb-mediated toxicity, resulting in decreased oxygen consumption and exacerbating mitochondrial ROS production (Nath et al., 1998). In this line, our results show an increased mitochondrial ROS production in renal cells exposed to Hb and heme. We also found that Hb/heme-mediated oxidative stress was related not only to production of superoxide anion and hydrogen peroxide but also to a reduction in GSH levels, the main cellular antioxidant. There is accumulating evidence supporting a direct link between mitochondria, oxidative stress, and cell death (Ott et al., 2007). Previous studies from our group showed that Hb induced apoptosis in renal podocytes by mitochondrial-related intrinsic pathway (Rubio-Navarro et al., 2018). Moreover, it has been shown that oxidative stress may contribute to ER stress and subsequent cell death (Liu et al., 2019). Specifically, rhabdomyolysis-induced ER stress promoted apoptosis, playing a crucial role in progression of AKI (Feng et al., 2018). According to these observations, the oxidative and ER stress as well as mitochondrial oxidation induced by Hb/heme may explain the increased cell death rate reported in our study, both *in vitro* and in mice with intravascular hemolysis (Nath et al., 1995; Gonzalez-Michaca et al., 2004). Moreover, this could explain the exacerbated cell death observed in kidney from Nrf2−/−, suggesting a protective role against renal toxicity subsequent to intravascular hemolysis.

There is no specific treatment to avoid the toxic effects caused by free Hb in the kidney. Therefore, understanding molecular mechanisms involved in this pathological setting is crucial to design new therapeutic strategies. In this line, Nrf2 has emerged as a master regulator of cellular resistance to oxidation (Guerrero-Hue et al., 2017). In basal conditions, Nrf2 is bound to Keap1, a cytosolic repressor that targets Nrf2 to the proteasome for degradation (Jaiswal, 2004). However, oxidative stress modifies cysteine residues in Keap1 and allows the nuclear translocation of Nrf2, such as what we observed in tubular cells stimulated with Hb and in mice with hemolysis. Once in the nucleus, Nrf2 transcriptionally induces the expression of cytoprotective enzymes and proteins involved in heme-degradation, including HO-1 and both ferritin subunits, FtH with ferroxidase activity and FtL that induces iron nucleation. Thus, we surmised that Nrf2 might improve intravascular hemolysis-induced kidney injury. To test this hypothesis, we induced intravascular hemolysis in both Nrf2+/+ and Nrf2−/− mice. Here, we show that mice lacking Nrf2 gene had more severe kidney injury, characterized by loss of renal function and increased cell stress and death. In agreement with our data, several studies have demonstrated the protective role of Nrf2 in a number of renal diseases associated with oxidative stress, such as cisplatin-induced nephropathy (Li et al., 2016), ischemia–reperfusion (Bayrak et al., 2008; Yoon et al., 2008; Trujillo et al., 2013), and rhabdomyolysis (Zhao et al., 2016). Oxidative stress drives ER stress and UPR activation in order to attenuate cellular stress through Nrf2 activation (Cullinan and Diehl, 2004; Liu et al., 2019). Interestingly, our data showed an increased expression of UPR-related proteins in kidneys from phenylhydrazine-injected Nrf2−/− mice as compared with wild-type mice, suggesting that the Nrf2 pathway may regulate kidney ER stress induced by intravascular hemolysis (Mukaigasa et al., 2018). In our study, the Nrf2-regulated molecules HO-1, FtL, and FtH were found upregulated in kidneys from wild-type mice after hemolysis or in cultured tubular epithelial cells stimulated with Hb/heme. Supporting the translational relevance of our findings, we observed increased Nrf2 and HO-1 expression, lipid peroxidation, and tubular cell death in a patient suffering from massive Hb renal accumulation after hemolysis-associated AKI. Similarly, patients with warm antibody hemolytic anemia, SCD, or thrombotic microangiopathy also showed increased expression and activity of HO-1 in renal tubules (Nath et al., 2001; Maroti et al., 2004; Fervenza et al., 2008). However, it is important to note that in our study, kidneys from Nrf2−/− mice with hemolysis showed an attenuated adaptive response characterized by lower expression of NQO-1, catalase, HO-1, FtH, and FtL than did wild-type mice with hemolysis. These Nrf2-regulated proteins play a key protective role against heme-mediated oxidative stress. HO-1 is the principal enzyme involved in heme degradation and, therefore, plays a cytoprotective function during heme-mediated tissue injury (Vogt et al., 1995). Interestingly, a known human case of HO-1 deficiency showed persistent hemolytic anemia with kidney iron deposits as well as loss of renal function (Yachie et al., 1999). Altogether, these data suggest the key role of the Nrf2/HO-1 axis in renal diseases associated with Hb accumulation. Recent studies have demonstrated the relevance of Nrf2 pathway in the regulation of hemolysis

(Keleku-Lukwete et al., 2015; Krishnamoorthy et al., 2017). Thus, targeted disruption of Nrf2 induced hemolytic anemia in old mice by increasing erythrocyte susceptibility to oxidative stress (Lee et al., 2004). However, young Nrf2−/− mice did no present anemia (Chan et al., 1996). There are also conflicting reports on whether Nrf2 activation reduces hemolysis or not. Thus, the Nrf2 agonist dimethyl fumarate improved hematological parameters and reduced plasma-free Hb in SCD mice (Krishnamoorthy et al., 2017), whereas other authors showed that Nrf2 activation through ablation of its negative regulator Keap1 did not modify hemolysis and stress erythropoiesis in SCD mice, although it increased plasma heme clearance (Keleku-Lukwete et al., 2015). Our findings show no differences in serum free Hb and heme levels between Nrf2−/− and Nrf2+/+ mice or after Nrf2 activation with sulforaphane. Moreover, we do not observed differences in renal heme concentration according to Nrf2 genotypes or sulforaphane treatment. Altogether, these results seem to indicate that beneficial effects of Nrf2 on renal function are not related with a reduction in hemolysis or an increased heme catabolism in massive hemolysis induced by phenylhydrazine.

There is evidence that genetic or pharmacological Nrf2 activation may ameliorate systemic and vascular inflammation as well as lung injury and liver damage in mice with intravascular hemolysis (Keleku-Lukwete et al., 2015; Ghosh et al., 2016; Promsote et al., 2016; Belcher et al., 2017; Ghosh et al., 2018). However, none of these studies focused on renal injury, one of the major pathological consequences observed in patients with massive and recurrent hemolytic crises (Baddam et al., 2017; Mammen et al., 2017; Plewes et al., 2017). To investigate a potential beneficial effect of Nrf2 induction *in vivo*, we treated mice with sulforaphane before the onset of hemolysis. Remarkably, Nrf2 activation with sulforaphane ameliorated renal function and decreased oxidative and ER stress, cell viability, and tubular injury associated with massive hemolysis. Similar effects were observed in cultured tubular cells, where Nrf2 activation with sulforaphane or tBHQ reduced cellular and mitochondrial ROS production, the loss of GSH, and cell death induced by Hb and heme. These protective effects may be explained by an Nrf2-mediated induction of HO-1 and ferritin expression, thus decreasing heme-mediated toxic effects. In agreement with our data, Nrf2 activation also reduced cellular stress such as oxidative stress and ER stress and ameliorated renal function in experimental ischemia–reperfusion (Bayrak et al., 2008; Yoon et al., 2008; Trujillo et al., 2013) and rhabdomyolysis-associated AKI (Zhao et al., 2016; Guerrero-Hue et al., 2017). Interestingly, although activation of Nrf2 may induce UPR (Cullinan and Diehl, 2004; Liu et al., 2019), we did not observe significant changes in UPR-related genes in kidney from mice treated with sulforaphane compared with non-treated mice, suggesting that Nrf2 may activate UPR in the presence of ER stress but not in basal conditions. Moreover, different therapeutic approaches based on Nrf2 activation have preserved renal function in several past and ongoing clinical trials in patients with renal disease (Guerrero-Hue et al., 2017). Overall, our findings indicate for the first time that Nrf2 could be a potential target to prevent renal damage induced by intravascular hemolysis by ameliorating oxidative stress, ER stress, tubular cell death, and loss of renal function. These protective effects may be mediated almost in part by promoting the expression of antioxidant proteins involved in heme catabolism, including HO-1 and ferritin. Lack of cell-site-specific Nrf2 deletion in mice is a limitation of our study. Further studies must be performed to test whether specific Nrf2 deletion in tubular cells increases renal injury in hemolytic disorders.

In conclusion, our results show that Nrf2 plays a key regulatory role in limiting the severity of AKI triggered by intravascular hemolysis. Renal Hb accumulation promotes a loss of renal function and kidney injury, effects that are aggravated by the genetic deletion of Nrf2. Sulforaphane treatment induced Nrf2 activation as well as an increase of related proteins such as HO-1 and ferritin, decreasing the severity of kidney injury induced by intravascular hemolysis. Thus, Nrf2 could be a potential therapeutic target to limit Hb-induced toxicity in patients with intravascular hemolysis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Patients provided informed consent, and the biobank was approved by Instituto de Investigaciones Sanitarias-Fundacion Jimenez Diaz (IIS-FJD) ethics committee. All reported experiments with animals were conducted in accordance with the Directive 2010/63/EU of the European Parliament and were approved by Instituto de Investigaciones Sanitarias-Fundacion Jimenez Diaz Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AR-N and JM designed the study. AS, CY, and ER were involved in human samples collection. PC analyzed renal biopsies. AR-N, CV-C, CH, RL, PM, MG-H, SC, MG, JE, IC, CG-C, and BA performed research. AR-N, CV-C, MG-H, and JM performed data analysis and data interpretation. EG, AR-N, JE, AO, MP, and JM wrote the manuscript. All authors critically revised the manuscript for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00740/full#supplementary-material

FIGURE S1 | Nrf2 resulted activated in mice with intravascular hemolysis. Representative confocal microscopy images showing nuclear translocation of phosphorylated-Nrf2 (Ser40) (green) in C57Bl/6 wild type (Nrf2+/+) mice that were i.p. injected with saline (Vehicle) or phenylhydrazine (Phe, 2mg/10g of body weight) to induce intravascular hemolysis. Nuclei were stained with DAPI (blue), scale bar 100 μm.

FIGURE S2 | Expression of NGAL **(A)** and KIM-1 **(B)** determined by RT-qPCR in kidneys from control mice treated with sulforaphane (SFN, 12.5 mg/kg) for 3 days. These animals did not display evidence of tubular injury as there was no increase in the expression of these markers. **(C)** Quantification of total Nrf2 protein in kidney from control mice and treated with SFN (left panel). Representative western blot image showing total Nrf2 protein levels (right panel). Results are expressed as mean ± SE. *p<0.05 vs non-treated cells.

FIGURE S3 | (A) Expression of FtH mRNA expression measured by RT-qPCR in MCTs cells treated with heme for 6h. **(B)** Western blot image showing FtH expression in MCT cells treated with Heme (0-10 μM) for 24h. **(C)** FtH protein expression in MCT cells stimulated with Hb (0-500 μg/mL, 0-30 μM heme equivalents). FtH mRNA expression measured by RT-qPCR **(D)** and semiquantification of FtH protein expression determined by western-blot **(E)** of kidneys from wild type and Nrf2 -/- mice injected with phenylhydrazine or vehicle. FtH mRNA expression measured by RT-qPCR **(F)** and semiquantification of FtH protein expression determined by western-blot **(G)** of kidneys from wild type pre-treated with SFN and injected with phenylhydrazine or vehicle.

REFERENCES

Baddam, S., Aban, I., Hilliard, L., Howard, T., Askenazi, D., and Lebensburger, J. D. (2017). Acute kidney injury during a pediatric sickle cell vaso-occlusive pain crisis. *Pediatr. Nephrol.* 32 (8), 1451–1456. doi: 10.1007/s00467-017-3623-6

Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., et al. (1992). Ferritin: a cytoprotective antioxidant strategem of endothelium. *J. Biol. Chem.* 267 (25), 18148–18153.

Balla, J., Jacob, H. S., Balla, G., Nath, K., Eaton, J. W., and Vercellotti, G. M. (1993). Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc. Natl. Acad. Sci. U. S. A.* 90 (20), 9285–9289. doi: 10.1073/pnas.90.20.9285

Ballarin, J., Arce, Y., Torra Balcells, R., Diaz Encarnacion, M., Manzarbeitia, F., Ortiz, A., et al. (2011). Acute renal failure associated to paroxysmal nocturnal haemoglobinuria leads to intratubular haemosiderin accumulation and CD163 expression. *Nephrol. Dial. Transplant.* 26 (10), 3408–3411. doi: 10.1093/ndt/gfr391

Bayrak, O., Uz, E., Bayrak, R., Turgut, F., Atmaca, A. F., Sahin, S., et al. (2008). Curcumin protects against ischemia/reperfusion injury in rat kidneys. *World J. Urol.* 26 (3), 285–291. doi: 10.1007/s00345-008-0253-4

Belcher, J. D., Chen, C., Nguyen, J., Zhang, P., Abdulla, F., Nguyen, P., et al. (2017). Control of oxidative stress and inflammation in sickle cell disease with the Nrf2 activator dimethyl fumarate. *Antioxid. Redox Signal.* 26 (14), 748–762. doi: 10.1089/ars.2015.6571

Buehler, P. W., and Karnaukhova, E. (2018). When might transferrin, hemopexin or haptoglobin administration be of benefit following the transfusion of red blood cells? *Curr. Opin. Hematol.* 25 (6), 452–458. doi: 10.1097/MOH.0000000000000458

Buehler, P. W., D'Agnillo, F., and Schaer, D. J. (2010). Hemoglobin-based oxygen carriers: from mechanisms of toxicity and clearance to rational drug design. *Trends Mol. Med.* 16 (10), 447–457. doi: 10.1016/j.molmed.2010.07.006

Buendia, I., Navarro, E., Michalska, P., Gameiro, I., Egea, J., Abril, S., et al. (2015). New melatonin-cinnamate hybrids as multi-target drugs for neurodegenerative diseases: Nrf2-induction, antioxidant effect and neuroprotection. *Future Med. Chem.* 7 (15), 1961–1969. doi: 10.4155/fmc.15.99

Chan, K., Lu, R., Chang, J. C., and Kan, Y. W. (1996). NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. U. S. A.* 93 (24), 13943–13948. doi: 10.1073/pnas.93.24.13943

Chang, C. W., Chen, Y. S., Tsay, Y. G., Han, C. L., Chen, Y. J., Yang, C. C., et al. (2018). ROS-independent ER stress-mediated NRF2 activation promotes warburg effect to maintain stemness-associated properties of cancer-initiating cells. *Cell Death Dis.* 9 (2), 194. doi: 10.1038/s41419-017-0250-x

Cullinan, S. B., and Diehl, J. A. (2004). PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J. Biol. Chem.* 279 (19), 20108–20117. doi: 10.1074/jbc.M314219200

Deuel, J. W., Schaer, C. A., Boretti, F. S., Opitz, L., Garcia-Rubio, I., Baek, J. H., et al. (2016). Hemoglobinuria-related acute kidney injury is driven by intrarenal oxidative reactions triggering a heme toxicity response. *Cell Death Dis.* 7, e2064. doi: 10.1038/cddis.2015.392

Doss, J. F., Jonassaint, J. C., Garrett, M. E., Ashley-Koch, A. E., Telen, M. J., and Chi, J. T. (2016). Phase 1 study of a sulforaphane-containing broccoli sprout homogenate for sickle cell disease. *PLoS One* 11 (4), e0152895. doi: 10.1371/journal.pone.0152895

Escobar, G. A., Burks, D., Abate, M. R., Faramawi, M. F., Ali, A. T., Lyons, L. C., et al. (2017). Risk of acute kidney injury after percutaneous pharmacomechanical thrombectomy using AngioJet in venous and arterial thrombosis. *Ann. Vasc. Surg.* 42, 238–245. doi: 10.1016/j.avsg.2016.12.018

Feng, Y., Huang, R., Guo, F., Liang, Y., Xiang, J., Lei, S., et al. (2018). Selective histone deacetylase 6 inhibitor 23BB alleviated rhabdomyolysis-induced acute kidney injury by regulating endoplasmic reticulum stress and apoptosis. *Front. Pharmacol.* 9, 274. doi: 10.3389/fphar.2018.00274

Fervenza, F. C., Croatt, A. J., Bittar, C. M., Rosenthal, D. W., Lager, D. J., Leung, N., et al. (2008). Induction of heme oxygenase-1 and ferritin in the kidney in warm antibody hemolytic anemia. *Am. J. Kidney Dis.* 52 (5), 972–977. doi: 10.1053/j.ajkd.2008.07.012

Gall, T., Petho, D., Nagy, A., Hendrik, Z., Mehes, G., Potor, L., et al. (2018). Heme induces endoplasmic reticulum stress (HIER stress) in human aortic smooth muscle cells. *Front. Physiol.* 9, 1595. doi: 10.3389/fphys.2018.01595

Ghosh, S., Hazra, R., Ihunnah, C. A., Weidert, F., Flage, B., and Ofori-Acquah, S. F. (2018). Augmented NRF2 activation protects adult sickle mice from lethal acute chest syndrome. *Br. J. Haematol.* 182 (2), 271–275. doi: 10.1111/bjh.15401

Ghosh, S., Ihunnah, C. A., Hazra, R., Walker, A. L., Hansen, J. M., Archer, D. R., et al. (2016). Nonhematopoietic Nrf2 dominantly impedes adult progression of sickle cell anemia in mice. *JCI Insight* 1 (4). doi: 10.1172/jci.insight.81090

Gonzalez-Michaca, L., Farrugia, G., Croatt, A. J., Alam, J., and Nath, K. A. (2004). Heme: a determinant of life and death in renal tubular epithelial cells. *Am. J. Physiol. Renal Physiol.* 286 (2), F370–F377. doi: 10.1152/ajprenal.00300.2003

Guerrero-Hue, M., Farre-Alins, V., Palomino-Antolin, A., Parada, E., Rubio-Navarro, A., Egido, J., et al. (2017). Targeting Nrf2 in protection against renal disease. *Curr. Med. Chem.* 24 (33), 3583–3605. doi: 10.2174/0929867324666170511120814

Guerrero-Hue, M., Rubio-Navarro, A., Sevillano, A., Yuste, C., Gutierrez, E., Palomino-Antolin, A., et al. (2018). Adverse effects of the renal accumulation of haem proteins. Novel therapeutic approaches. *Nefrologia* 38 (1), 13–26. doi: 10.1016/j.nefro.2017.05.009

Gutteridge, J. M. (1986). Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett.* 201 (2), 291–295. doi: 10.1016/0014-5793(86)80626-3

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* 236 (2), 313–322. doi: 10.1006/bbrc.1997.6943

Jaiswal, A. K. (2004). Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radic. Biol. Med.* 36 (10), 1199–1207. doi: 10.1016/j.freeradbiomed.2004.02.074

Jia, Y., Buehler, P. W., Boykins, R. A., Venable, R. M., and Alayash, A. I. (2007). Structural basis of peroxide-mediated changes in human hemoglobin: a novel oxidative pathway. *J. Biol. Chem.* 282 (7), 4894–4907. doi: 10.1074/jbc.M609955200

Kamencic, H., Lyon, A., Paterson, P. G., and Juurlink, B. H. (2000). Monochlorobimane fluorometric method to measure tissue glutathione. *Anal. Biochem.* 286 (1), 35–37. doi: 10.1006/abio.2000.4765

Keleku-Lukwete, N., Suzuki, M., Otsuki, A., Tsuchida, K., Katayama, S., Hayashi, M., et al. (2015). Amelioration of inflammation and tissue damage in sickle cell model mice by Nrf2 activation. *Proc. Natl. Acad. Sci. U. S. A.* 112 (39), 12169–12174. doi: 10.1073/pnas.1509158112

Kim, I., Xu, W., and Reed, J. C. (2008). Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat. Rev. Drug Discov.* 7 (12), 1013–1030. doi: 10.1038/nrd2755

Kobayashi, M., and Yamamoto, M. (2006). Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* 46, 113–140. doi: 10.1016/j.advenzreg.2006.01.007

Krishnamoorthy, S., Pace, B., Gupta, D., Sturtevant, S., Li, B., Makala, L., et al. (2017). Dimethyl fumarate increases fetal hemoglobin, provides heme detoxification, and corrects anemia in sickle cell disease. *JCI Insight* 2 (20). doi: 10.1172/jci.insight.96409

Lee, J. M., Chan, K., Kan, Y. W., and Johnson, J. A. (2004). Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. *Proc. Natl. Acad. Sci. U. S. A.* 101 (26), 9751–9756. doi: 10.1073/pnas.0403620101

Li, C. Z., Jin, H. H., Sun, H. X., Zhang, Z. Z., Zheng, J. X., Li, S. H., et al. (2016). Eriodictyol attenuates cisplatin-induced kidney injury by inhibiting oxidative stress and inflammation. *Eur. J. Pharmacol.* 772, 124–130. doi: 10.1016/j.ejphar.2015.12.042

Liu, X., and Spolarics, Z. (2003). Methemoglobin is a potent activator of endothelial cells by stimulating IL-6 and IL-8 production and E-selectin membrane expression. *Am. J. Physiol. Cell Physiol.* 285 (5), C1036–C1046. doi: 10.1152/ajpcell.00164.2003

Liu, M., Grigoryev, D. N., Crow, M. T., Haas, M., Yamamoto, M., Reddy, S. P., et al. (2009). Transcription factor Nrf2 is protective during ischemic and nephrotoxic acute kidney injury in mice. *Kidney Int.* 76 (3), 277–285. doi: 10.1038/ki.2009.157

Liu, X., Zhang, R., Huang, L., Zheng, Z., Vlassara, H., Striker, G., et al. (2019). Excessive oxidative stress contributes to increased acute ER stress kidney injury in aged mice. *Oxid. Med. Cell. Longev.* 2019, 2746521. doi: 10.1155/2019/2746521

Mammen, C., Bissonnette, M. L., and Matsell, D. G. (2017). Acute kidney injury in children with sickle cell disease—compounding a chronic problem. *Pediatr. Nephrol.* 32 (8), 1287–1291. doi: 10.1007/s00467-017-3650-3

Maroti, Z., Nemeth, I., Turi, S., Karg, E., Ugocsai, P., and Endreffy, E. (2004). Heme oxygenase 1 expression in young uremic patients on hemodialysis. *Pediatr. Nephrol.* 19 (4), 426–431. doi: 10.1007/s00467-003-1384-x

McFaul, S. J., Bowman, P. D., Villa, V. M., Gutierrez-Ibanez, M. J., Johnson, M., and Smith, D. (1994). Hemoglobin stimulates mononuclear leukocytes to release interleukin-8 and tumor necrosis factor alpha. *Blood* 84 (9), 3175–3181.

Merle, N. S., Grunenwald, A., Figueres, M. L., Chauvet, S., Daugan, M., Knockaert, S., et al. (2018). Characterization of renal injury and inflammation in an experimental model of intravascular hemolysis. *Front. Immunol.* 9, 179. doi: 10.3389/fimmu.2018.00179

Moreno, J. A., Izquierdo, M. C., Sanchez-Nino, M. D., Suarez-Alvarez, B., Lopez-Larrea, C., Jakubowski, A., et al. (2011). The inflammatory cytokines TWEAK and TNFalpha reduce renal Klotho expression through NFkappaB. *J. Am. Soc. Nephrol.* 22 (7), 1315–1325. doi: 10.1681/ASN.2010101073

Moreno, J. A., Martin-Cleary, C., Gutierrez, E., Toldos, O., Blanco-Colio, L. M., Praga, M., et al. (2012). AKI associated with macroscopic glomerular hematuria: clinical and pathophysiologic consequences. *Clin. J. Am. Soc. Nephrol.* 7 (1), 175–184. doi: 10.2215/CJN.01970211

Mukaigasa, K., Tsujita, T., Nguyen, V. T., Li, L., Yagi, H., Fuse, Y., et al. (2018). Nrf2 activation attenuates genetic endoplasmic reticulum stress induced by a mutation in the phosphomannomutase 2 gene in zebrafish. *Proc. Natl. Acad. Sci. U. S. A.* 115 (11), 2758–2763. doi: 10.1073/pnas.1714056115

Naik, R. P., Derebail, V. K., Grams, M. E., Franceschini, N., Auer, P. L., Peloso, G. M., et al. (2014). Association of sickle cell trait with chronic kidney disease and albuminuria in African Americans. *JAMA* 312 (20), 2115–2125. doi: 10.1001/jama.2014.15063

Nath, K. A., Balla, G., Vercellotti, G. M., Balla, J., Jacob, H. S., Levitt, M. D., et al. (1992). Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J. Clin. Invest.* 90 (1), 267–270. doi: 10.1172/JCI115847

Nath, K. A., Balla, J., Croatt, A. J., and Vercellotti, G. M. (1995). Heme protein-mediated renal injury: a protective role for 21-aminosteroids *in vitro* and *in vivo*. *Kidney Int.* 47 (2), 592–602. doi: 10.1038/ki.1995.75

Nath, K. A., Grande, J. P., Croatt, A. J., Likely, S., Hebbel, R. P., and Enright, H. (1998). Intracellular targets in heme protein-induced renal injury. *Kidney Int.* 53 (1), 100–111. doi: 10.1046/j.1523-1755.1998.00731.x

Nath, K. A., Grande, J. P., Haggard, J. J., Croatt, A. J., Katusic, Z. S., Solovey, A., et al. (2001). Oxidative stress and induction of heme oxygenase-1 in the kidney in sickle cell disease. *Am. J. Pathol.* 158 (3), 893–903. doi: 10.1016/S0002-9440(10)64037-0

Ott, M., Gogvadze, V., Orrenius, S., and Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis* 12 (5), 913–922. doi: 10.1007/s10495-007-0756-2

Plewes, K., Kingston, H. W. F., Ghose, A., Maude, R. J., Herdman, M. T., Leopold, S. J., et al. (2017). Cell-free hemoglobin mediated oxidative stress is associated with acute kidney injury and renal replacement therapy in severe falciparum malaria: an observational study. *BMC Infect. Dis.* 17 (1), 313. doi: 10.1186/s12879-017-2373-1

Promsote, W., Powell, F. L., Veean, S., Thounaojam, M., Markand, S., Saul, A., et al. (2016). Oral monomethyl fumarate therapy ameliorates retinopathy in a humanized mouse model of sickle cell disease. *Antioxid. Redox Signal.* 25 (17), 921–935. doi: 10.1089/ars.2016.6638

Reiter, C. D., Wang, X., Tanus-Santos, J. E., Hogg, N., Cannon, R. O., 3rd, Schechter, A. N., et al. (2002). Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat. Med.* 8 (12), 1383–1389. doi: 10.1038/nm799

Rubio-Navarro, A., Carril, M., Padro, D., Guerrero-Hue, M., Tarin, C., Samaniego, R., et al. (2016). CD163-macrophages are involved in rhabdomyolysis-induced kidney injury and may be detected by MRI with targeted gold-coated iron oxide nanoparticles. *Theranostics* 6 (6), 896–914. doi: 10.7150/thno.14915

Rubio-Navarro, A., Sanchez-Nino, M. D., Guerrero-Hue, M., Garcia-Caballero, C., Gutierrez, E., Yuste, C., et al. (2018). Podocytes are new cellular targets of haemoglobin-mediated renal damage. *J. Pathol.* 244 (3), 296–310. doi: 10.1002/path.5011

Sastre, C., Rubio-Navarro, A., Buendia, I., Gomez-Guerrero, C., Blanco, J., Mas, S., et al. (2013). Hyperlipidemia-associated renal damage decreases Klotho expression in kidneys from ApoE knockout mice. *PLoS One* 8 (12), e83713. doi: 10.1371/journal.pone.0083713

Shelton, L. M., Park, B. K., and Copple, I. M. (2013). Role of Nrf2 in protection against acute kidney injury. *Kidney Int.* 84 (6), 1090–1095. doi: 10.1038/ki.2013.248

Stocker, R. (1990). Induction of haem oxygenase as a defence against oxidative stress. *Free Radic Res. Commun.* 9 (2), 101–112. doi: 10.3109/10715769009148577

Tracz, M. J., Alam, J., and Nath, K. A. (2007). Physiology and pathophysiology of heme: implications for kidney disease. *J. Am. Soc. Nephrol.* 18 (2), 414–420. doi: 10.1681/ASN.2006080894

Trujillo, J., Chirino, Y. I., Molina-Jijon, E., Anderica-Romero, A. C., Tapia, E., and Pedraza-Chaverri, J. (2013). Renoprotective effect of the antioxidant curcumin: recent findings. *Redox Biol.* 1, 448–456. doi: 10.1016/j.redox.2013.09.003

Tsuji, Y., Ayaki, H., Whitman, S. P., Morrow, C. S., Torti, S. V., and Torti, F. M. (2000). Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol. Cell Biol.* 20 (16), 5818–5827. doi: 10.1128/MCB.20.16.5818-5827.2000

Vasconcellos, L. R., Dutra, F. F., Siqueira, M. S., Paula-Neto, H. A., Dahan, J., Kiarely, E., et al. (2016). Protein aggregation as a cellular response to oxidative stress induced by heme and iron. *Proc. Natl. Acad. Sci. U. S. A.* 113 (47), E7474–E7482. doi: 10.1073/pnas.1608928113

Vogt, B. A., Alam, J., Croatt, A. J., Vercellotti, G. M., and Nath, K. A. (1995). Acquired resistance to acute oxidative stress. Possible role of heme oxygenase and ferritin. *Lab. Invest.* 72 (4), 474–483.

Wang, Z., Shah, S. V., Liu, H., and Baliga, R. (2014). Inhibition of cytochrome P450 2E1 and activation of transcription factor Nrf2 are renoprotective in myoglobinuric acute kidney injury. *Kidney Int.* 86 (2), 338–349. doi: 10.1038/ki.2014.65

Wetz, A. J., Richardt, E. M., Schotola, H., Bauer, M., and Brauer, A. (2017). Haptoglobin and free haemoglobin during cardiac surgery-is there a link to acute kidney injury? *Anaesth. Intensive Care* 45 (1), 58–66. doi: 10.1177/0310057X1704500109

Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., et al. (1999). Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J. Clin. Invest.* 103 (1), 129–135. doi: 10.1172/JCI4165

Yoon, H. Y., Kang, N. I., Lee, H. K., Jang, K. Y., Park, J. W., and Park, B. H. (2008). Sulforaphane protects kidneys against ischemia–reperfusion injury through induction of the Nrf2-dependent phase 2 enzyme. *Biochem. Pharmacol.* 75 (11), 2214–2223. doi: 10.1016/j.bcp.2008.02.029

Zarjou, A., Bolisetty, S., Joseph, R., Traylor, A., Apostolov, E. O., Arosio, P., et al. (2013). Proximal tubule H-ferritin mediates iron trafficking in acute kidney injury. *J. Clin. Invest.* 123 (10), 4423–4434. doi: 10.1172/JCI67867

Zhao, W., Huang, X., Zhang, L., Yang, X., Wang, L., Chen, Y., et al. (2016). Penehyclidine hydrochloride pretreatment ameliorates rhabdomyolysis-induced AKI by activating the Nrf2/HO-1 pathway and alleviating [corrected] endoplasmic reticulum stress in rats. The. *PLoS One* 11 (3), e0151158. doi: 10.1371/journal.pone.0151158

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