Hypoxia Induces Oxidative Injury and Apoptosis *via* Mediating the Nrf-2/Hippo Pathway in Blood Cells of Largemouth Bass (*Micropterus salmoides*)

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Investigating how aquatic animals respond to hypoxia brought about by changes in environmental temperature may be of great significance to avoid oxidative injury and maintain the quality of farmed fish in the background of global warming. Here, we investigated the effects of hypoxia on oxidative injury and environment-sensing pathway in blood cells of *Micropterus salmoides*. The total blood cell count (TBCC) and Giemsa staining showed that hypoxia could lead to damage of blood cells. Flow cytometry analysis confirmed that the apoptosis rate, Ca^{2+} level, NO production and ROS of blood cells were significantly increased under hypoxia stress. Environment-sensing pathways, such as Nrf2 pathway showed that hypoxia resulted in significant up-regulation of hiF-1 alpha subunit (Hif-1α), nuclear factor erythroid 2-related factor 2 (Nrf2) and kelch-1ike ECH- associated protein I (Keap1) expression. Meanwhile, the expression of Hippo pathway-related genes such as MOB kinase activator 1 (MOB1), large tumor suppressor homolog 1/2 (Lats1/2), yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ), protein phosphatase 2A (PP2A) were significantly increased in blood cells after hypoxia exposure. In addition, hypoxia stress also increased the expression of catalase (CAT) and glutathione peroxidase (GPx), but decreased the expression of superoxide dismutase (SOD). Consequently, our results suggested that hypoxia could induce oxidative injury and apoptosis *via* mediating environment-sensing pathway such as Nrf2/Hippo pathway in blood cells of *M. salmoides*.

Keywords: climate warming, hypoxia, *Micropterus salmoides*, blood cells, Nrf2/Hippo pathway

INTRODUCTION

The effects of global warming have become increasingly prevalent in marine ecosystems around the world, where consistently increasing seawater temperatures have exposed marine species to environmental stress conditions. Climate warming has already led to many changes in key environmental factors such as water temperature, seawater pH and oxygen concentration in the oceans (Breitburg et al., 2018; Vagner et al., 2019). Numerous factitious and environmental factors such as high temperature, water pollution and high-density fish farming in the water body will inevitably lead to hypoxic conditions in fish (Wang et al., 2012; Levin and Breitburg, 2015). Hypoxia is one of the main reasons that threatens the survival and growth of fish, it causes massive fish deaths and significant economic losses in the aquaculture industry, and seriously hinder the sustainable and healthy development of the aquaculture industry (Zhao et al., 2020).

Hypoxia may induce oxidative stress by breaking the balance between the production of reactive oxygen species (ROS) and the removal of ROS. Excess ROS are generated, which can damage critical cellular components and activate the antioxidant signaling pathway in fish (Azimi et al., 2017). Further, large amounts of ROS could cause apoptosis, lipid oxidation, tissue damage, and DNA and protein degradation (Jls et al., 2020). Nitric oxide (NO), as a cellular signaling molecule, has also been found to play a primary role of vasorelaxation in hypoxic situations and can be synthesized by nitric oxide synthase (NOS; Cao et al., 1981). When activated by pathogens or cytokines, such as ROS (Kumar et al., 2018), NO could be produced by NOS (Dimmeler and Zeiher, 1997). Further, NO reacts with ROS and forms peroxynitrite (ONOO−), which is highly reactive toward a wide variety of biomolecules (Curtin et al., 2002). There is also growing evidence that NO damages mitochondria, leading to the opening of transition pores, followed by cytochrome c release and caspase activation leading to apoptosis (Brüne, 2003). Earlier studies indicated that when compared with the population at sea level, the contents of NO and its products in Tibetan circulation are significantly higher, indicating that NO had effects on vascular tension, blood flow and oxygen delivery (Erzurum et al., 2007). Meanwhile, *Danio rerio* data support this possibility, accompanied by significant NO production, vasodilation and lower blood pressure (Jensen, 2007). Apoptosis is a critical component in maintaining homeostasis and growth in all tissues and plays a significant role in immunity and cytotoxicity, and it can initiate and clear damaged cells to maintain homeostasis when exposed to hypoxia (Luo et al., 2017; D'Arcy, 2019). However, the challenge that hypoxia poses to oxidative stress and the mechanisms that hypoxia induce oxidative injury and apoptosis in fish are not as well understood despite intensive research effort (Borowiec and Scott, 2020).

The antioxidant system can alleviate the negative effects of hypoxia on the body by preventing the production of ROS or by eliminating ROS, and it is mainly mediated by the transcription factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf-2), which can induce the expression of genes related to exogenous detoxification and cell protection (Barrera et al., 2021). As an environment-sensing pathway, Nrf-2 and its repressor, Kelch-like ECH-associated protein 1 (Keap1) can help the body defend against oxidative stress, thereby maintaining the body's homeostasis balance (Bellezza et al., 2018). Some research indicated that the interruption of Nrf-2-Keap1 signaling may occur during human aging and cause sarcopenia (Mondal et al., 2018). When oxidative stress occurs, Keap1 is oxidized at the active cysteine residue, causing Keap1 to inactivate, Nrf-2 to stabilize and translocate into the nucleus (Motohashi et al., 2002; Tonelli et al., 2018). The Hippo pathway also is an environment-sensing pathway, which always be a master regulator of tissue homeostasis (Hong et al., 2020). It is an evolutionarily conserved signal cascade that regulates many biological processes, including cell growth and fate determination, organ size control and regeneration (Ma et al., 2019). In previous studies, sterile 20-like kinase 1/2 (Mst1/2) kinases in Hippo pathway are associated with Salvador family WW domain containing protein 1 (SAV1) and Mps one binder kinase activator-like 1A and 1B (MOB1A/B or collectively, MOB1) to phosphorylate large tumor suppressor 1/2 (Lats1/2). Lats1/2 subsequently phosphorylate Yes-associated protein (YAP) and its paralog, WW domain–containing transcription regulator protein 1 (TAZ), causing them to bind *via* 14-3-3 (Zhao et al., 2007; Hao et al., 2008; Lei et al., 2008; Oka et al., 2008). Hereafter, YAP and TAZ are prevented from entering the nucleus, interacting with transcription factors (i.e., TEAD family members and others) in the cytoplasm and regulating downstream gene targets to initiate a series of signal cascades (Zhao et al., 2008; Zhang et al., 2009; Lehmann et al., 2016; Meng et al., 2016). Studies have shown that Nrf-2 could uses the Hippo pathway effector TAZ to induce tumorigenesis (Barrera et al., 2021). Meanwhile, the excessive ROS generated by hypoxia, superoxide dismutase (SOD) can efficiently convert superoxide anion (O_2^-) into H_2O_2 and O_2. After this process, H_2O_2 could be transformed into H_2O and O_2 under the combined action of catalase (CAT) and glutathione peroxidase (GPx), so that the body could be protected from hypoxic damage (Cao et al., 2012). However, there have been relatively few detailed examinations about how hypoxia induces oxidative damage and apoptosis through the Nrf-2/Hippo pathway in hypoxia-tolerant fish.

Largemouth bass (*Micropterus salmoides*), which is a world-famous economic fish, has been widely cultivated and become the major freshwater aquaculture species in China (Li et al., 2017; Yu et al., 2018). But the recent increase in global warming, high-density farming, and deterioration of water quality have increased the probability of hypoxia, which will inevitably lead to hypoxia stress (Sun et al., 2020c). Blood is not only an important part of the digestive and respiratory systems, but also plays important role in the immune system (Dichiera et al., 2020). Previous studies have also shown that acute hypoxic stress can lead to apoptosis and inflammatory responses in fish blood cells, and in severe cases can cause a dramatic reduction in the oxygen-carrying capacity of fish blood cells, resulting in mass mortality (Huang et al., 2019; Cai et al., 2020). Understanding the physiological changes of fish blood cells under

hypoxic conditions is one of the keys to understanding how environmental stress causes oxidative damage and cell apoptosis in fish. However, in recent years, research on largemouth bass under hypoxic stress has mainly focused on some other tissue such as liver and gill (Sun et al., 2020b). For example, hypoxia could induce an increase in anaerobic glycolysis in the liver and caused histological lesions in the gill tissue of the fish (Gaulke et al., 2014; Crans et al., 2015; Yang et al., 2017). It is little known about the mechanism of how hypoxia induces oxidative damage and apoptosis in blood cells of largemouth bass.

In this study, we simulated an acute hypoxia environment in the laboratory and analyzed the physiological parameters, environment-sensing pathways such as Nrf-2/Hippo pathway and antioxidant ability of largemouth bass exposed to acute hypoxic environment for various durations. The dynamic pattern of physiological parameters and the pattern of Nrf-2/Hippo pathway during hypoxic exposure would provide a theoretical basis for future studies on healthy aquaculture of largemouth bass and facilitate our understanding of the mechanisms in hypoxia stress and global warming adaptation.

MATERIALS AND METHODS

Animals

Largemouth bass were obtained from the Panyu aquaculture base of South China Normal University. Fish with no disease, no injury, and strong vigor were selected. The average weight of experimental fish was 108.4 ± 5.2 g and the average body length was 18.5 ± 3.1 cm. Before the trial, the water quality indicators were detected daily (pH 7.6 ± 0.2, temperature $25 \pm 1°C$, ammonia nitrogen ≤ 0.05 mg/L, and dissolved oxygen(DO) ≥ 6.5 mg/L). The fish were temporarily cultured with continuous ventilation for 2 weeks of domestication and were fed by Commercial feed (Guangdong Evergreen Feed industry Co., Ltd., Guangdong, China) during domestication. The fish were fasted for 24 h before the experiment.

Hypoxia Treatment and Sample Collection

After an 2-week acclimation period prior to the experiments, 160 largemouth bass which held in one big tank (1000 cm $\times$ 525 cm $\times$ 100 cm, water volume 520 L) were seperated into 16 plastic aquaria (50 cm $\times$ 35 cm $\times$ 30 cm, water volume 52 L). 10 largemouth bass were placed in each aquarium and each group contains four aquaria. The initial dissolved oxygen concentration of the culture water was around 6.50 mg/L. Before hypoxia treatments, three largemouth bass were randomly selected from four aquaria (Control group, DO = 6.50 mg/L) for sample collection. In order to start the hypoxia state, the aquaria were not subjected to oxygenation and circulation, and the mouth of aquaria were sealed with a thin film, and the dissolved oxygen in the tanks was measured once per 5 min. The oxygen in the aquaria gradually decreased with the consumption of largemouth bass. Three samples were collected from each experimental group after around 20 min

(H1 group, DO = 4.33 mg/L), around 40 min (H2 group, DO = 3.25 mg/L) of hypoxia exposure. After acute hypoxic stress, the remaining largemouth bass were re-oxygenated for 50 min and the dissolved oxygen reached 7.09 mg/L, which was recorded as the R group and then three fish were sampled. Blood cells were withdrawn from the tail vein and immediately transferred into individual plastic tubes. All samples were kept on ice to prevent blood cells clumping.

Total Blood Cell Count and Giemsa Staining

A glass microscope slide was placed on a horizontal surface, and 10 μL blood was added onto one end of the slide. A coverslip was used, lying across the glass slide and keeping the coverslip in contact with the blood. The coverslip was moved down the glass slide to the opposite end. The blood smear is made after air-dried. Blood smears were stained with Reisser-Giemsa dye (Biosharp Biotechnology Co., Ltd., Anhui, China). Garris-Giemsa A solution was dropped on the smear, and the whole smear was stained with the dye for 0.5 min. Then drop the Reisch-Jimsa B solution onto liquid A, blow the breeze with the mouth or ear ball to make the liquid surface ripple, make the two liquids fully mixed, dyeing for 1 min before being washed in distilled water. Finally, it was observed with polarized light microscope (Leica, Germany) and photographed after drying. At the same time, the blood cells after multiple dilutions were counted on the counting board under an ordinary microscope.

Flow Cytometry

The blood was centrifuged at 500 $\times$ *g*, 4°C for 10 min, and the supernatant was discarded after centrifugation. Then red blood cell lysis buffer was added to the cell pellet to lyse mature erythrocytes for 5 min. After lysis, the supernatant lysate was removed by centrifugation, and proper amount of PBS was added to re-precipitate the suspension. Then the blood cells were analyzed with a BD FACS Aria III flow cytometer (BD, NJ, United States).

Detection of Cell Apoptosis

Annexin V-FITC/PI Fluorescence double staining apoptosis assay kit (Elabscience Biotechnology Co., Ltd., Hubei, China) was used to stain the cells. The cells were suspended with 100 μL diluted 1 $\times$ Annexin V Binding Buffer, then 2.5 μL Annexin V-FITC and 2.5 μL PI staining solution were added to cell suspension. The mixed buffer were incubated for 15 min at room temperature in the dark. 400 μL of diluted 1 $\times$ Annexin V Binding Buffer was added to make the cell concentration 1.0×10^5 cells/mL and mixed the samples. The samples were placed on ice in darkness and tested by flow cytometry within 1 h and data were analyzed by the Flow Jo 10 software (version 10.2).

Intracellular Reactive Oxygen Species Measurement

Intracellular ROS was estimated by using a fluorescent probe, $2',7'$-dichlorodihydrofluorescein diacetate (DCFH-DA). The cells were stained by Meilun Reactive Oxygen Species Assay

Kit (Dalian Meilun Biotechnology Co., Ltd., Liaoning, China). After the cells were collected, the cells were suspended and added with an appropriate volume of diluted DCFH-DA working solution. The cells were incubated in a cell culture box at 37°C for 30 min under dark conditions, and mixed upside down every 5 min to make the probe and cells fully contact. After incubation, the cells were washed with PBS 3 times to fully remove DCFH-DA that did not enter the cells. Collected to make single-cell suspensions. Then the fluorescence was determined by flow cytometry.

Nitric Oxide Measurement

DAF-FM DA (NO fluorescent photoprobe) (Dalian Meilun Biotechnology Co., Ltd., Liaoning, China) was used to stain the cells, and the cells were suspended with diluted DAF-FM DA and incubated for 20 min in a cell culture box at 37°C. Mix upside down every 3–5 min so that the probe is in full contact with the cells. After incubation, the cells were washed with PBS 3 times to fully remove the DAF-FM DA that did not enter the cells. Then the cells were re-suspended by PBS to prepare single-cell suspension for flow cytometry.

Intracellular Calcium Levels Measurement

Fluo-3 and AM ester (eBioscience Biotechnology, MA, United States) were used to stain the cells. The calcium ion (Ca^{2+}) fluorescent probe Fluo-3 and AM ester were added to the cells and incubated at 37°C for 20 min in the dark. Cells were washed three times with PBS and collected to make single-cell suspensions. The intracellular fluorescence intensity measured by flow cytometry represented the Ca^{2+} level.

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from the blood cells of fish with Trizol (Takara Biotech, Kyoto, Japan) and their quality and quantity were determined by a Nanodrop2000 spectrophotometer (Thermo, MA, United States), and t the integrity was assessed by 1% agarose gel electrophoresis (Wang et al., 2015). The cDNA was synthesized by reverse transcription with PrimeScript reverse transcriptase (Takara Biotech, Kyoto, Japan) in a 20 μL reaction volume containing 1 μg total RNA. The reaction mixture was stored at −20°C for future use. Quantitative real-time RT-PCR (qRT-PCR) experiments were performed in a CFX96 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., CA, United States) using SYBR Mixture (Takara Biotech, Kyoto, Japan). Use β-actin as an internal reference gene. Each sample was paralleled three times. PCRs were performed on a total reaction volume of 20 μL containing 4 μL of cDNA, 0.5 μL of primer, 10 μL of SYBR Mixture, and 5 μL of ddH$_2$O. The thermal cycling program was as follows: activation at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, several annealing temperatures for 30 s, and 72°C for 32 s; melt curve detection of 60°C for 5 s to 95°C increments 0.5°C. Quantitative analyses were performed using the $2^{-\Delta\Delta Ct}$ method. The PCR cycling protocol was 95°C for 60 s, 40 cycles of 94°C for 5 s and 60°C for 30 s, followed by 95.0°C for

5 sec, followed by melting curve analysis from 65.0 to 95.0°C (increment 0.5°C, 0:05). Quantitative analyses were performed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are described in **Table 1**.

Statistical Analyses

All analyses were carried out using the SPSS software (version 26.0). The data were analyzed by single-factor analysis of variance (ANOVA) and multiple comparisons by LSD method. The data were expressed as mean $\pm$ standard deviation (SD, n = 3). $P < 0.05$ indicated significant differences.

RESULTS

Effect of Acute Hypoxia and Reoxygenation on Cell Morphology and Total Blood Cell Count

The results of the Giemsa staining (**Figure 1A**) of the blood cells showed a clearly significant decrease in the H1 and H2 group relative to control group. Interestingly, macrophage-like cells could be found in hypoxia groups, especially in H2 group.

After oxidative stress, compared to the control group ($1.70 \times 10^9 \pm 0.36 \times 10^9$ cell/ml), the TBCC of treatment group decreased at H1 ($1.26 \times 10^9 \pm 0.1 \times 10^9$ cell/ml) ($P < 0.05$) and H2 ($1.01 \times 10^9 \pm 0.21 \times 10^9$ cell/ml) ($P < 0.05$), and remain unchanged in the R group ($0.94 \times 10^9 \pm 0.04 \times 10^9$ cell/ml) ($P > 0.05$), while the TBCC of H1 group decreased more than H2

TABLE 1 | Sequences of primers used in qRT-PCR.

Gene		Primer sequence (5′–3′)
MOB1	F	ACACCCGAAACAGACGAGAC
	R	AATATCGCTGGCAGGACGAG
Lats1/2	F	GAGTCATGTGTCCAGCGGAA
	R	TTATCCGGCTCGGCATCTTC
YAP/TAZ	F	GCCAAAGTTTTGGTGTCGCA
	R	GAGTGTTTCCTCGGCTGTGA
14-3-3	F	AGATGGCAGTTTTGGGGACT
	R	AGTGTGAGCTCATGGAGGTTG
PP2A	F	ACGAAATCAGCGTGGACAGT
	R	GTTCACAGGACGTCACCCAT
TEAD	F	GAGTGTGTGGACATCAGGCA
	R	ATTGGCTGGTGACACCGTAG
Nrf-2	F	CAGACAGTTCCTTTGCAGGC
	R	AGGGACAAAAGCTCCATCCA
Keap1	F	CAGCATTACATGGCCGCATC
	R	CTTCTCTGGGTCGTAAGACTCC
SOD	F	CCACCAGAGGTCTCACAGCA
	R	CCACTGAACCGAAGAAGGACT
CAT	F	GTTCCCGTCCTTCATCCACT
	R	CAGGCTCCAGAAGTCCCACA
GPx	F	CCCTGCAATCAGTTTGGACA
	R	TTGGTTCAAAGCCATTCCCT
Hif-1a	F	CAGAGGACCTGTTGAATCGTT
	R	TTGTAGATGACAGTGGCTTGG

group (**Figure 1B**). And this result showed a similar trend which was consistent with Giemsa staining.

Effect of Acute Hypoxia and Reoxygenation on Apoptosis

After exposure to acute hypoxic conditions, the early (Q3 region) and late apoptosis (Q2 region) were increased in a dose dependent manner (**Figure 2A**). Further quantified these apoptosis cells by software, the number of apoptotic cells In Q2 and Q3 region were 22.72, 42.9 and 35.77% after treated with H1, H2, and R ($P < 0.05$, **Figure 2B**). Compared to the control group (10.52%), the proportion of apoptotic cells was demonstrated to be significantly increased from H1 to H2, peaking at H2, and subsequently declining (R). These results revealed that acute hypoxic conditions could promote apoptosis.

Effect of Acute Hypoxia and Reoxygenation on Concentration of Calcium

The level of Ca^{2+} fluorescence was low when the largemouth bass treated with control dissolved oxygen, while the level of Ca^{2+} fluorescence in H1 (52.83 ± 0.75) was two times higher than control group (20.53 ± 0.93). With the decrease of dissolved oxygen, the level of Ca^{2+} fluorescence was apparently increased at H1 and H2 group ($P < 0.05$, **Figure 3**), the level of Ca^{2+} fluorescence show the greatest peak in the H2 group, and the fluorescence intensity of the H2 group (61.63 ± 1.27) was
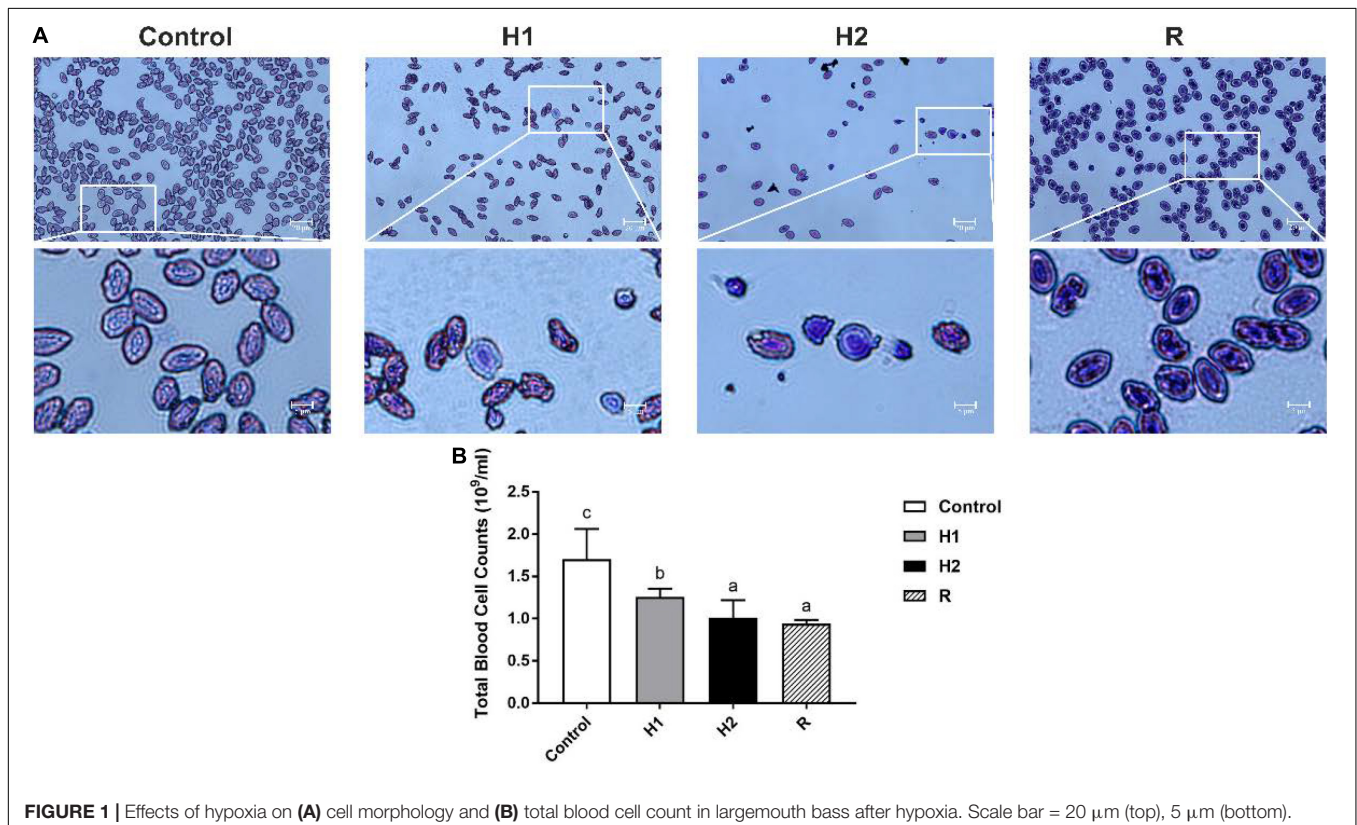
about three times that of the control group. In addition, the level of Ca^{2+} fluorescence was decelerated after reoxygenation (58.63 ± 0.68).
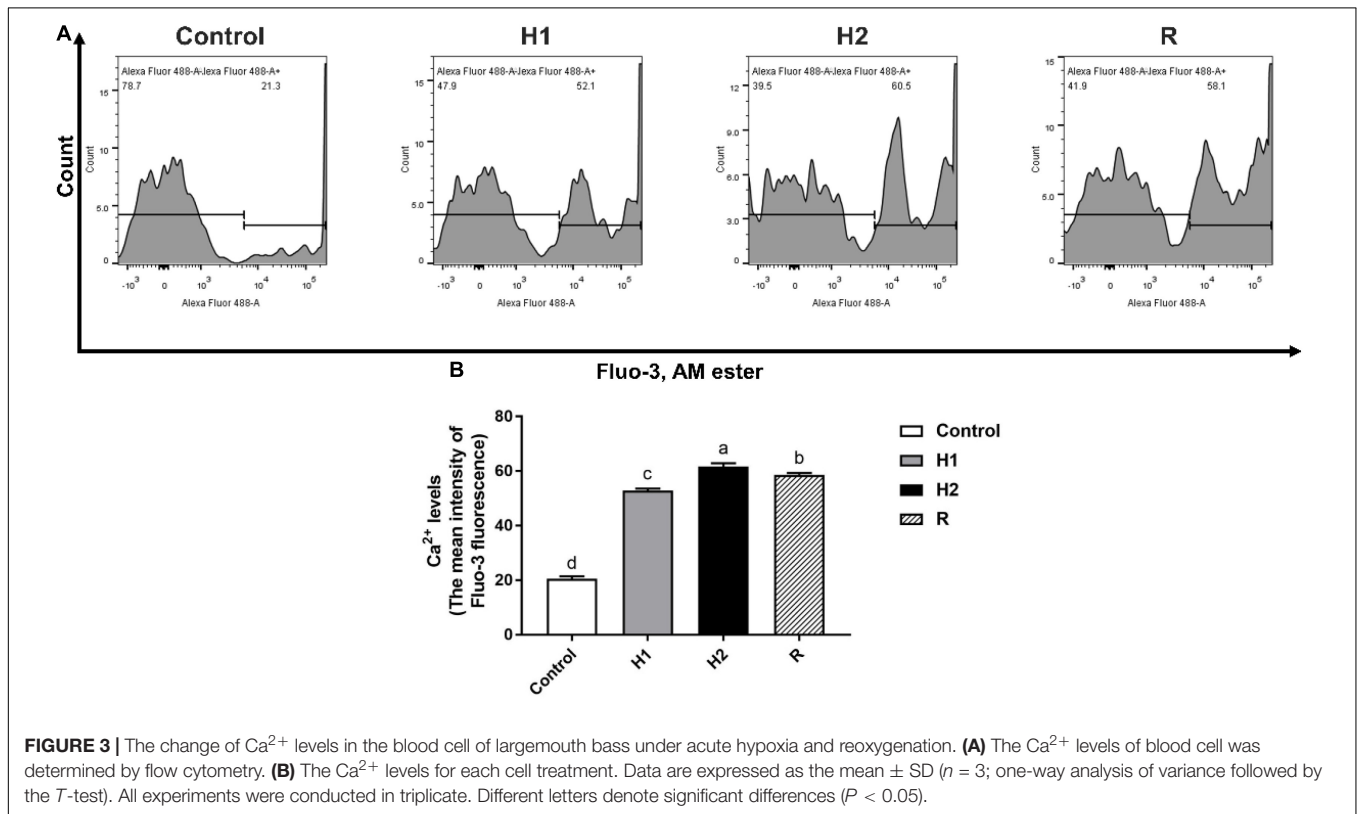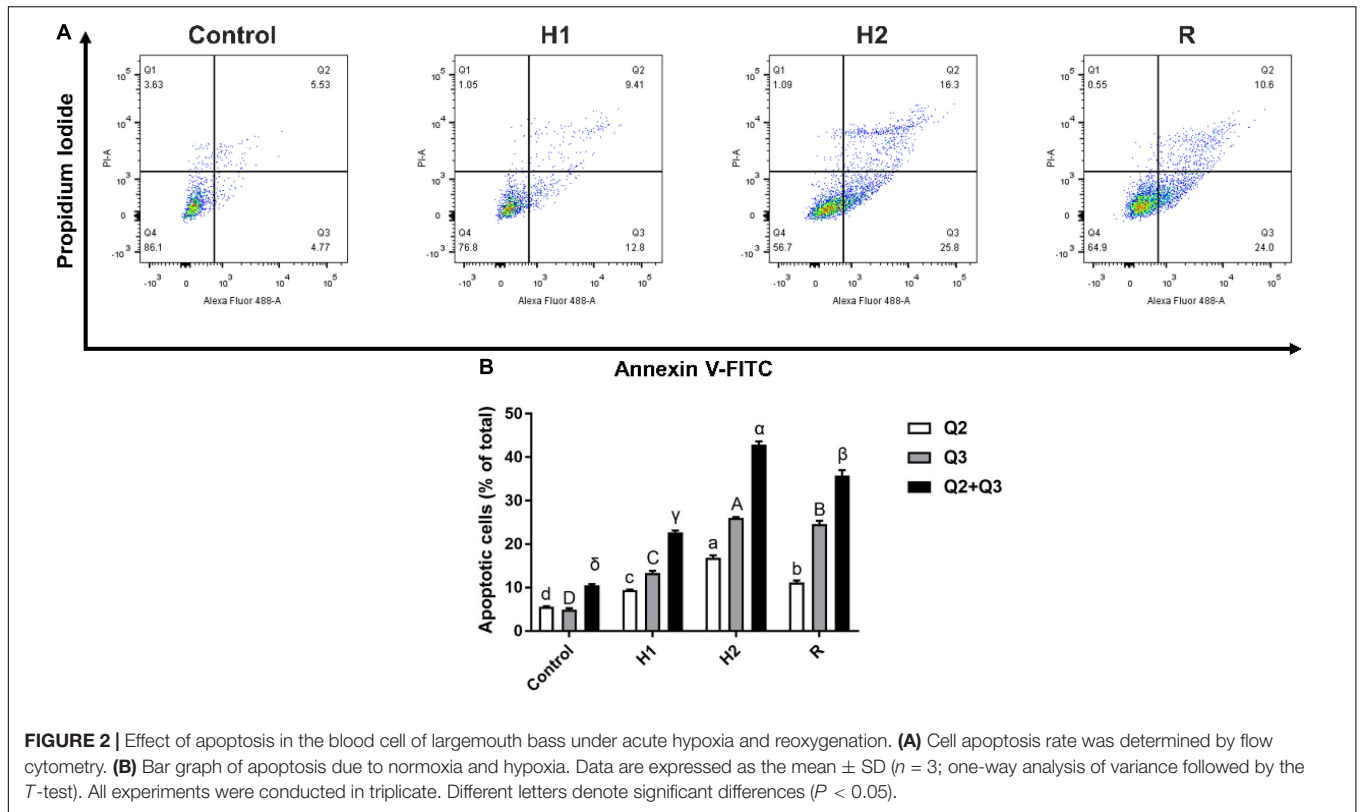
Effect of Acute Hypoxia and Reoxygenation on Concentration of Nitric Oxide

After largemouth bass treated with different dissolved oxygen, the level of Nitric oxide (NO) fluorescence was significantly increased when compared to the control group (20.87 ± 0.29) ($P < 0.05$, **Figure 4**). With the decrease of dissolved oxygen, the level of NO fluorescence was increased in a dependent manner at H1 and H2 group (26.7 ± 0.10, 35.47 ± 0.21). And after exposure to reoxygenation, the level of NO fluorescence was shown to be significantly decreased at R group (33.43 ± 0.25) ($P < 0.05$).

Effect of Acute Hypoxia and Reoxygenation on Reactive Oxygen Species Production

The level of ROS production of H1, H2 and R group were determined to be 37.80 ± 0.26, 45.83 ± 0.38 and 43.90 ± 0.26. In comparison with the control (31.97 ± 0.50), ROS formation significantly increase in H1 and H2 ($P < 0.05$, **Figure 5**); which the ROS formation was downregulated at R group. Although there was a subsequent upward trend, he level of ROS fluorescence remained significantly higher than those of control ($P < 0.05$).
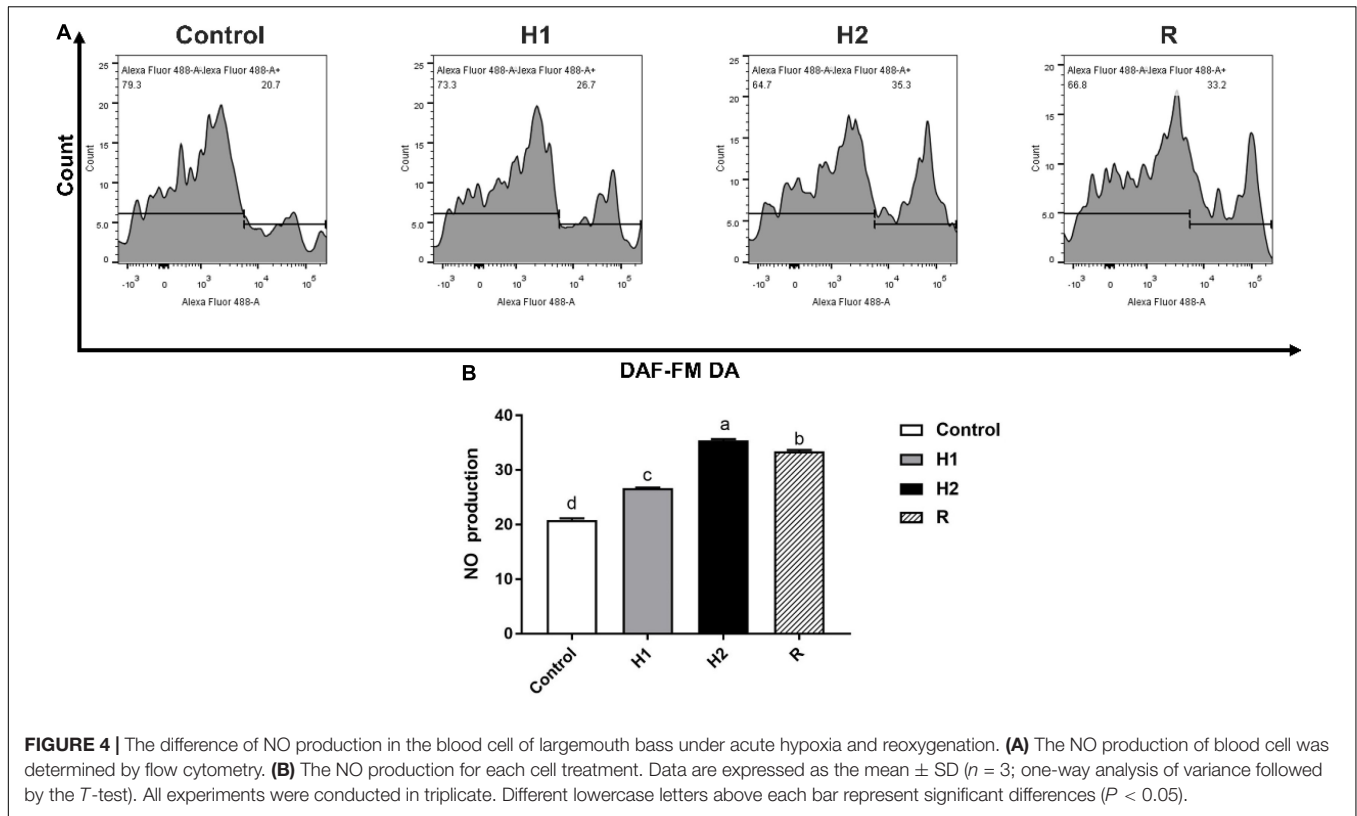

FIGURE 1 | Effects of hypoxia on **(A)** cell morphology and **(B)** total blood cell count in largemouth bass after hypoxia. Scale bar = 20 μm (top), 5 μm (bottom).

FIGURE 2 | Effect of apoptosis in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** Cell apoptosis rate was determined by flow cytometry. **(B)** Bar graph of apoptosis due to normoxia and hypoxia. Data are expressed as the mean $\pm$ SD ($n = 3$; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different letters denote significant differences ($P < 0.05$).

FIGURE 3 | The change of Ca^{2+} levels in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** The Ca^{2+} levels of blood cell was determined by flow cytometry. **(B)** The Ca^{2+} levels for each cell treatment. Data are expressed as the mean $\pm$ SD ($n = 3$; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different letters denote significant differences ($P < 0.05$).
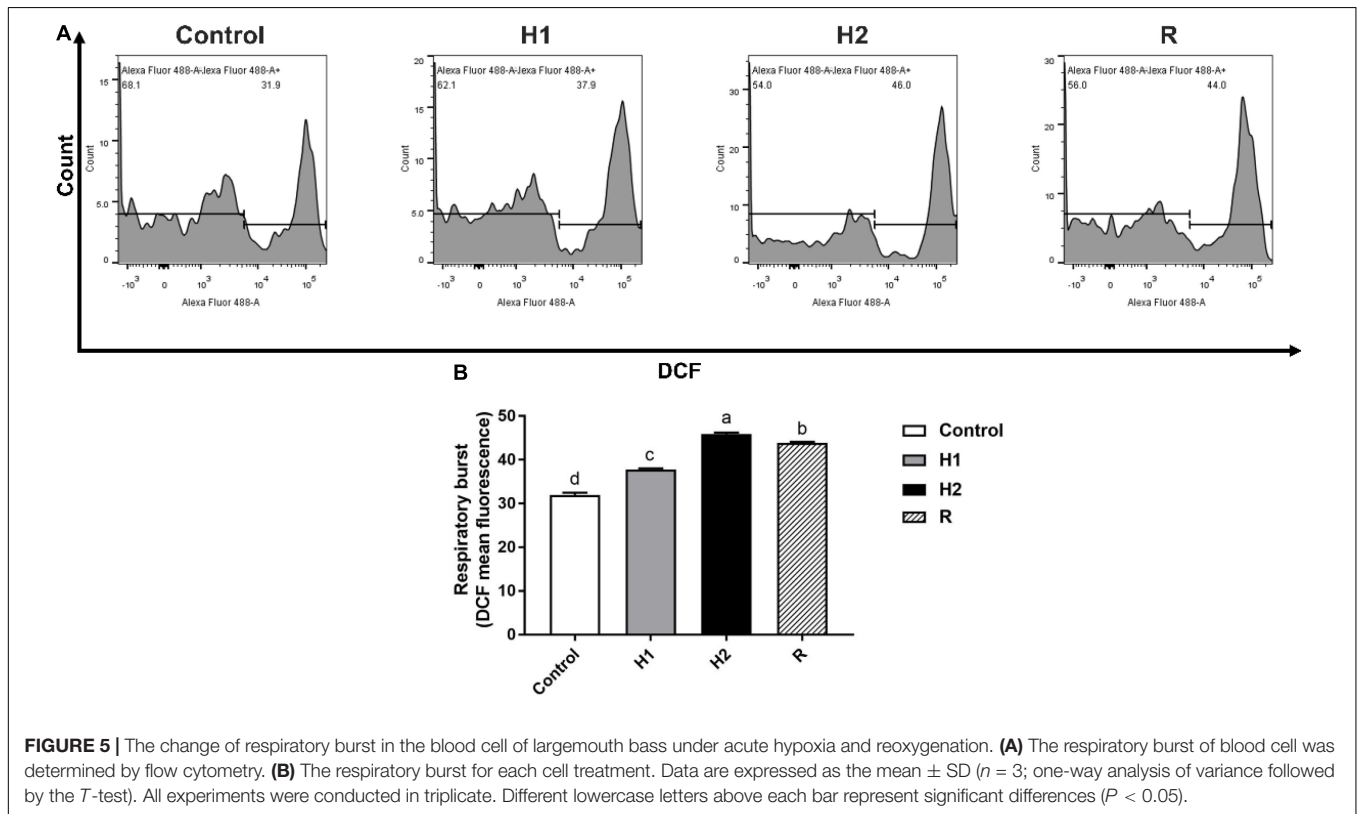
FIGURE 4 | The difference of NO production in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** The NO production of blood cell was determined by flow cytometry. **(B)** The NO production for each cell treatment. Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

FIGURE 5 | The change of respiratory burst in the blood cell of largemouth bass under acute hypoxia and reoxygenation. **(A)** The respiratory burst of blood cell was determined by flow cytometry. **(B)** The respiratory burst for each cell treatment. Data are expressed as the mean ± SD ($n = 3$; one-way analysis of variance followed by the T-test). All experiments were conducted in triplicate. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

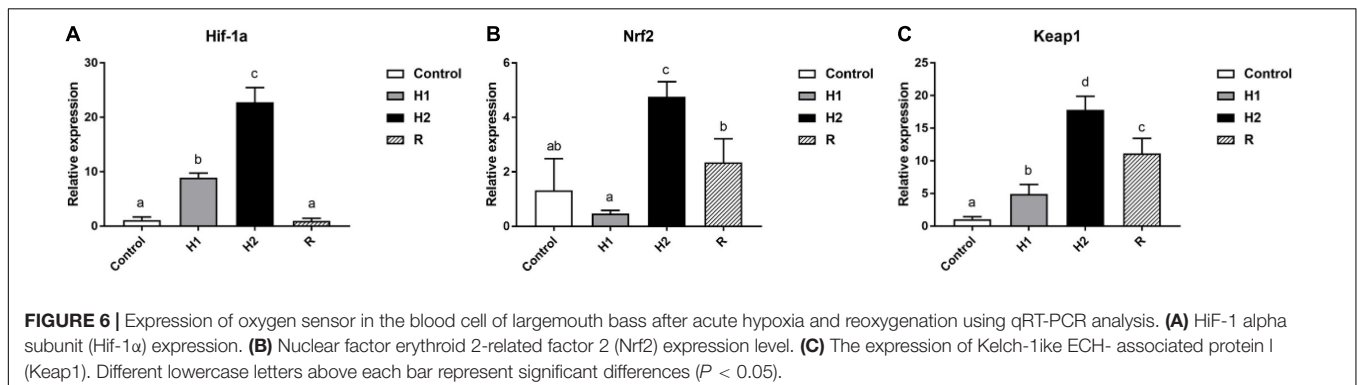## Effect of Acute Hypoxia and Reoxygenation on Nrf-2 Pathway Related Genes

The expression of hypoxic sensor protein Hypoxia-inducible factor-1 alpha (Hif-1α) gene was shown to be continuously increasing in H1 and H2 group during the hypoxic exposure stage, whereas decreasing to its lowest value at R group, which was not significantly different from that in Control ($P < 0.05$). Subsequently, the Hif-1α level was shown to be significantly increased in H1 and H2 group ($P < 0.05$), reaching its highest value in H2, with significant differences observed between Control, H1 and R ($P < 0.05$, **Figure 6A**). The expression level of Nrf-2 gene decreased to the lowest value in H1 first, then significantly increased ($P < 0.05$, **Figure 6B**), peaking at H2 group. During the hypoxic exposure stage, the Nrf-2 gene expression decreased obviously in R, which still higher than that in H1 and control group. Simultaneously, it was significantly different from that in H1 and H2 group ($P < 0.05$, **Figure 6B**). Expression of Keap1 gene was demonstrated to be continuously increased during normoxia to hypoxia stress, with H2 exhibiting the highest value, which was distinctly different from that in Control and H1 group ($P < 0.05$, **Figure 6C**). Nevertheless, after reoxygenation, the expression of Keap1 in Group R was significantly lower than that of H2 ($P < 0.05$, **Figure 6C**), but still obviously higher than that in Control and H1 ($P < 0.05$, **Figure 6C**). Throughout the study, the levels of Hif-1α and Keap1 showed similar trends. More noteworthy phenomenon was that the expression level of Keap1 in H1 was five times higher than that of Nrf-2, and the highest expression level of Keap1 was about three times higher than the highest expression level of Nrf-2. In addition, the expression level of Keap1 and Nrf-2 genes downregulated during the reoxygenation stage, while the expression level of Keap1 was still about five times higher than that of Nrf-2.
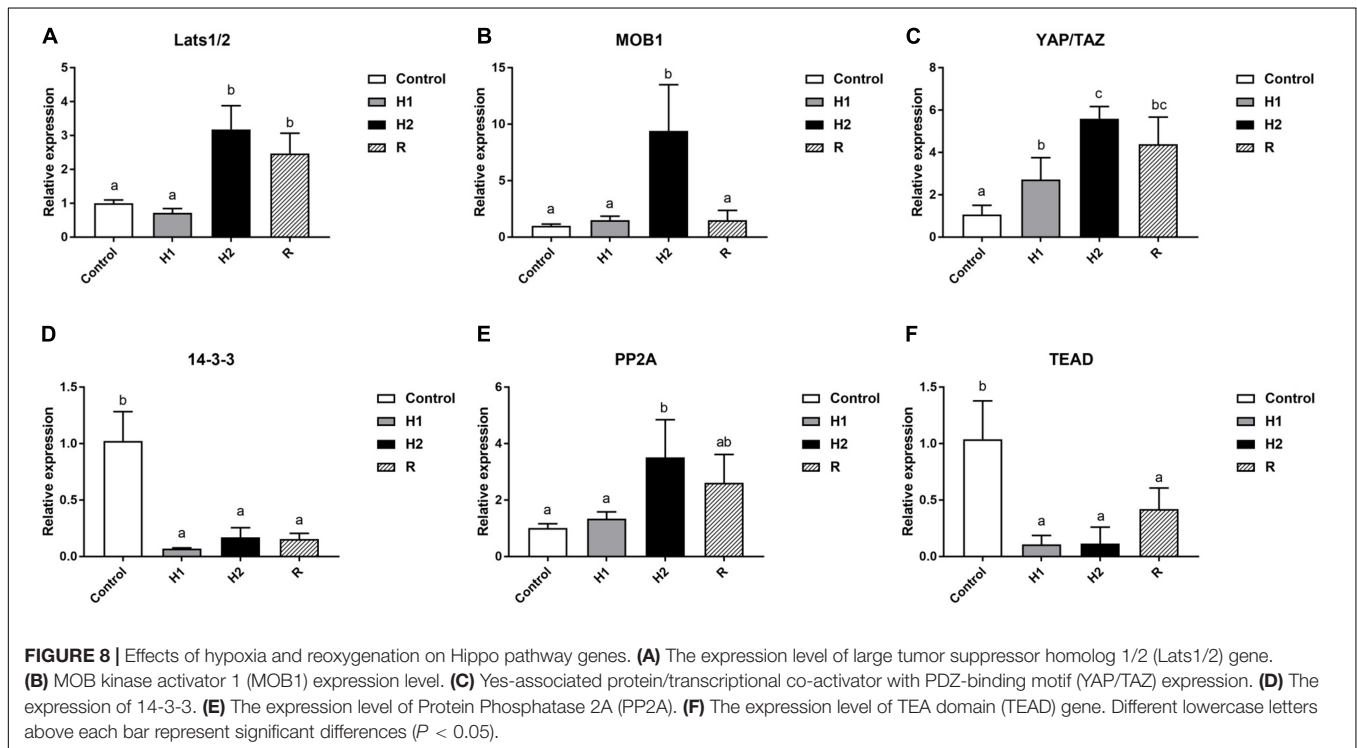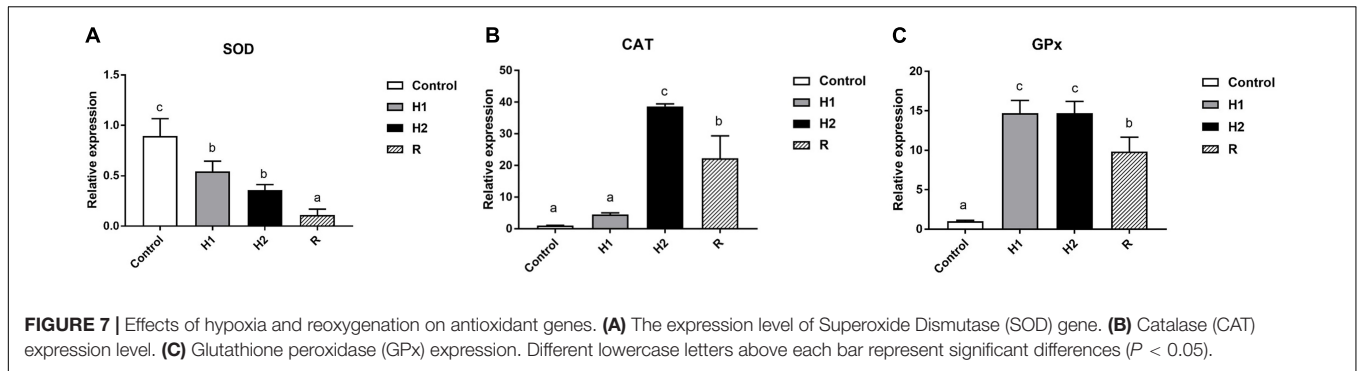
Furthermore, compared with the control group, the expression of SOD gene decreased obviously in H1 and H2 group ($P < 0.05$, **Figure 7A**), and there was no significant difference between H1 and H2 group ($P > 0.05$, **Figure 7A**). Moreover, the SOD gene expression in R Group reached the lowest level after reoxygenation ($P < 0.05$, **Figure 7A**). Besides, the CAT expression increased slightly in H1 first in hypoxia environment, with no significant difference with the control

group, however, the expression of CAT significantly upregulated in H2 group ($P < 0.05$, **Figure 7**), reaching its peak. In addition, the level of CAT gene subsequently declining in R during reoxygenation, meanwhile, its expression was significantly lower than H2 group ($P < 0.05$, **Figure 7B**), which was shown to be significantly higher than that in the control group and H1 ($P < 0.05$, **Figure 7B**). Ultimately, the expression of GPx gene increased rapidly at H1 and H2 group, which has no significant difference between them ($P > 0.05$, **Figure 7C**), and then downregulated in R group, with distinctly different from that in H1 and H2 ($P < 0.05$, **Figure 7C**). The level of GPx was significantly higher than that of control group throughout the experimental period ($P < 0.05$, **Figure 7C**). Most noteworthy, GPx expression increased significantly in H1 group ($P < 0.05$, **Figure 7**), about three times as much as that of CAT, while the expression of CAT upregulated distinctly in H2 group with its expression two times higher than that of GPx. Additionally, during hypoxia and reoxygenation stage, the expression of CAT and GPx was always higher than that of SOD, with the expression of CAT in H2 group was about 80 times higher than that of SOD as well as the expression level of GPx was about 30 times that of SOD in H2 group.

Effect of Acute Hypoxia and Reoxygenation on Hippo Pathway Related Genes

The expression levels of Hippo pathway-related genes were examined in blood cells from each group (**Figure 8**). Compared with the control group, the H1 group showed almost no significant change, but the H2 group showed a clear upward trend, and then the expression of the MOB1 decreased significantly in the R group. Moreover, MOB1 peaked and remarkably increased 10 times in H2 group ($P < 0.05$, **Figure 8B**), but no significant changes in the H1/R group ($P > 0.05$). Similar trend to MOB1, Lats1/2 expression levels peaked in the H2 group, followed by a decline in the R group, and increased by around 3 and 2.5 times, respectively, when compared to the control group ($P < 0.05$, **Figure 8A**). Moreover, in comparison with the control group, the trend of the YAP/TAZ expression levels was also highly similar to that of the MOB1. However, it is worth noting that expression levels of the H1 group of the YAP/TAZ has increased significantly by nearly 2-fold relative to the control group, and it

FIGURE 6 | Expression of oxygen sensor in the blood cell of largemouth bass after acute hypoxia and reoxygenation using qRT-PCR analysis. **(A)** HiF-1 alpha subunit (Hif-1α) expression. **(B)** Nuclear factor erythroid 2-related factor 2 (Nrf2) expression level. **(C)** The expression of Kelch-1ike ECH- associated protein I (Keap1). Different lowercase letters above each bar represent significant differences ($P < 0.05$).

FIGURE 7 | Effects of hypoxia and reoxygenation on antioxidant genes. **(A)** The expression level of Superoxide Dismutase (SOD) gene. **(B)** Catalase (CAT) expression level. **(C)** Glutathione peroxidase (GPx) expression. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

FIGURE 8 | Effects of hypoxia and reoxygenation on Hippo pathway genes. **(A)** The expression level of large tumor suppressor homolog 1/2 (Lats1/2) gene. **(B)** MOB kinase activator 1 (MOB1) expression level. **(C)** Yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ) expression. **(D)** The expression of 14-3-3. **(E)** The expression level of Protein Phosphatase 2A (PP2A). **(F)** The expression level of TEA domain (TEAD) gene. Different lowercase letters above each bar represent significant differences ($P < 0.05$).

reached a maximum in the H2 group, where YAP/TAZ expression rose sixfold, and then dropped a bit in the R group but was still four times as high as in the control group (all $P < 0.05$, **Figure 8C**). The trend of 14-3-3 expression levels was opposite to that of YAP/TAZ, with its expression significantly decreased nearly 2-fold in H1/H2/R group and reached its lowest point in the H1 group, followed by a slight increase in the H2 group and then remained almost the same as in the R group (all $P < 0.05$, **Figure 8D**). As shown in **Figure 8E**, no significant difference was observed between the control group and H1 group regarding the expression of PP2A ($P > 0.05$), while appreciably up-regulated by around four times in H2 group and reached the top, and then had slightly down-regulated in R group (all $P < 0.05$). Furthermore, PP2A expression in the R group was still nearly twice that of the control group. In contrast to the control group, TEAD expression significantly decreased to the lowest point in H1/H2 group and went up in R group, but decreased by around 1 and 0.5 times, respectively, when compared to the control group

(all $P < 0.05$, **Figure 8F**). The results indicated hypoxia could remarkably strengthen the expression of YAP/TAZ, a gene central to the Hippo pathway. Taken together, these data demonstrated hypoxia could effectually enhance the antioxidant response in blood cells of *M. salmoides*.

DISCUSSION

A large number of studies have shown that hypoxia can induce apoptosis (Saikumar et al., 1998; Sollid et al., 2003; Sun et al., 2020d). Studies have shown that an apoptotic and cell cycle arrest cascade downstream of HIF-1α involve transcriptional initiation of several genes, such as p53, BNip3 and BAX, which ultimately drive the cells into apoptosis (Harris, 2002). In previous study, the expression of Cyt-C, Caspase-9 and Caspase-3 in the stomach and intestinal tissues of Chinese sea perch (*Lateolabrax maculatus*) was initially increased and then decreased after acute hypoxia,

suggesting that hypoxia and reoxygenation induced apoptosis (Sun et al., 2020d). Our work reveals that hypoxic stress leads to an increase in the expression of relevant proapoptotic genes in largemouth bass, which in turn leads to apoptosis and activates inflammatory responses in blood cells, thus forcing largemouth bass to activate a series of resilience mechanisms to cope with and adapt to the adverse effects of hypoxia on its organism.

Studies have proved that hypoxia induced increase of Ca^{2+} in cardiac microvascular endothelial cells, which led to cell apoptosis (Zhang et al., 2016). Recent research found that elevation of intracellular Ca^{2+} level in human glioblastoma cells and mouse Schwann cells triggers ubiquitination of Merlin and activate Lats1,which is an important molecular in Hippo pathway (Wei et al., 2020). Our findings also indicate that hypoxia could induce Ca^{2+} over-produced in hypoxia-damaged blood cells, which show a consonant pattern with previous research. In other research, the mRNA expression levels of NOS did not change significantly in *Carassius carassius* L gills during hypoxia-induced transformation (Sollid et al., 2006). But our study showed that the level of NO fluorescence obviously affected by the amount of dissolved oxygen in the water. This suggests that there are differences in the production of NO induced by hypoxia in different tissues. Studies have shown that YAP/TAZ plays a certain role in ROS production by controlling mitochondrial respiration (White et al., 2019). Many results support the idea that hypoxia can induce ROS. Oxidative stress was caused by hypoxia stress in Przewalski's naked carp *(Gymnocypris przewalskii)*, and ROS production in telencephalon cells increased. It is consistent

with our findings which indicate that hypoxia induces ROS over-produced in hypoxia-damaged blood cells that may induce cells apoptosis under hypoxia.

Previous studies have demonstrated that hypoxia can cause metabolic disorders, decreased immunity, and respiratory dysfunction in aquatic animals (Han et al., 2017; Peruzza et al., 2018; Sun et al., 2018). Similarly, the negative effects of hypoxia on fishes have long been studied, including metabolic disorders, decreased immunity, and apoptosis (Sun et al., 2020a; Zhao et al., 2020; Wang et al., 2022). In hypoxic responses, the Hif-1α is a master regulator of oxygen sensitivity (Wang J. et al., 2021). Our study demonstrated that the activity of Hif-1α was significantly increased in the blood cell of largemouth bass after exposure to acute hypoxic conditions, consistent with the research on Mandarinfish *(Siniperca chuatsi)* (He et al., 2019; Sun et al., 2020a). Our study found significant differences in the content of Hif-1α with different levels of dissolved oxygen, which suggested that the expression level of Hif-1α may be regulated by the content of dissolved oxygen. Consequently, this study expected to deliberate the relationship between Hif-1α and Nrf-2 pathway, and further revealed the relationship between Hif-1α and antioxidant mechanism. The Nrf-2 is a key component of antioxidant system that its steady state levels are very low under un-stressed conditions, as Keap1 interacts with Nrf-2 primarily *via* its Neh2 domain and targets it for ubiquitin mediated degradation (Yamamoto et al., 2018). However, a variety of stressors such as excessive ROS acting on Keap1's redox-sensitive cysteine residues interfere with its inhibition of Nrf-2, allowing
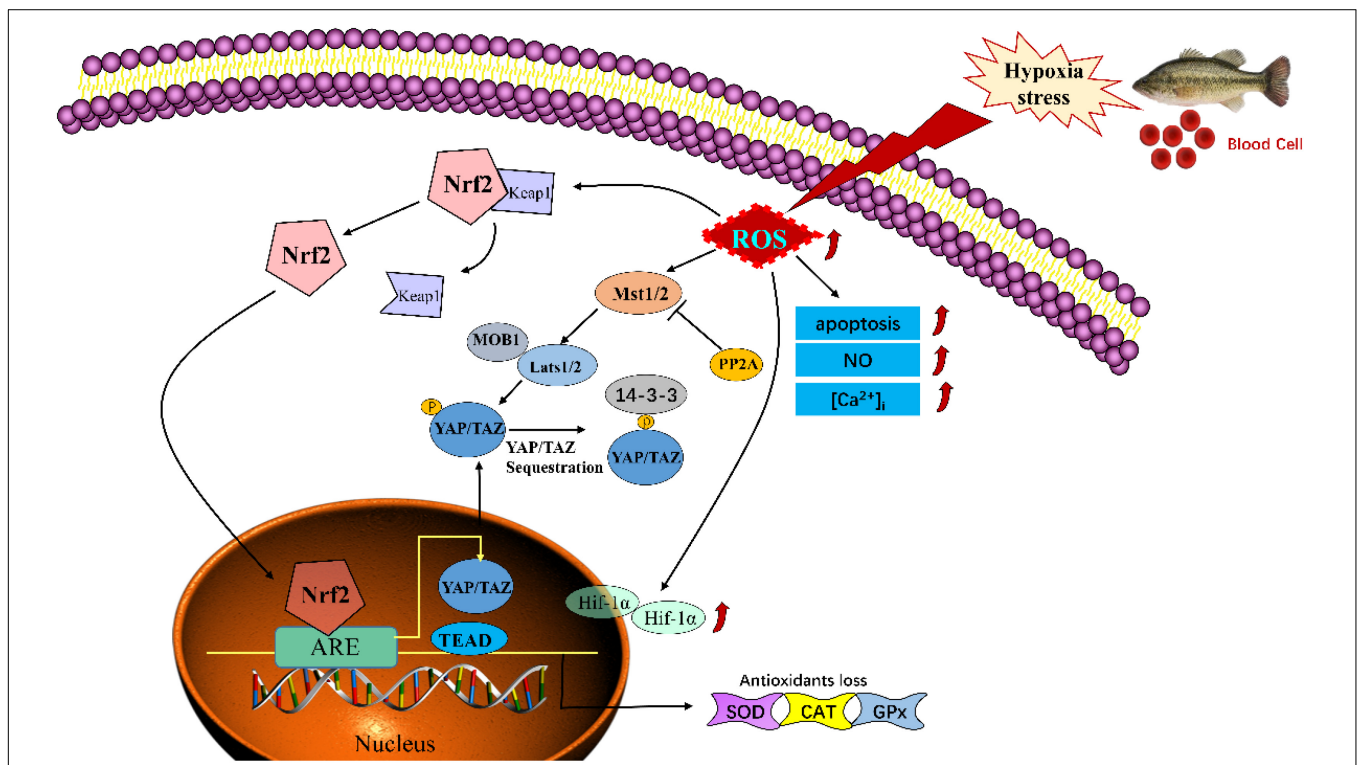

FIGURE 9 | Schematic model of hypoxia -induced injury *via* mediating the Nrf-2/Hippo pathway in blood cells of Largemouth bass (*Micropterus salmoides*).

it to assemble, move to the nucleus, and drive ARE-mediated gene expression, which include antioxidant, detoxification and proteostasis genes (Al-Mubarak et al., 2021). Keap1 and Nrf-2 genes expression was upregulated in blood cell under hypoxia condition, showing that the expression level of antioxidant enzyme genes was apparently consistent with that of Nrf-2. Faced with the excessive production of ROS, and the possible oxidative stress caused by the reoxygenation, the body will increase some antioxidant enzymes under hypoxic conditions to prepare in advance to reduce oxidative damage (Wang M. et al., 2021). In this study, the levels of GPx and CAT increased significantly in hypoxic condition, which was consistent with the previous study in the muscle tissue of largemouth bass (Yang et al., 2017). It is worth noting that the activity of CAT in blood cells did not increase significantly when the dissolved oxygen value was 4.29 mg·L^{-1}, while the expression of GPx upregulated obviously in 4.29 mg·L^{-1} of dissolved oxygen, suggesting that the mechanism of response to hypoxia injury in blood cells may be different from that in other tissues. Most noteworthy, the expression of CAT and GPx gene was higher than that of SOD gene during hypoxia stage, which suggested that SOD and CAT, GPx complement each other in hypoxia stress.

As an important pathway prevalent in living organisms, the Hippo pathway is not only a key regulator in controlling organ size and maintaining homeostasis within tissues, but recent studies have also shown that the Hippo pathway controls metabolic processes at the cellular and organismal levels (Ardestani et al., 2018). In the current study, we focused on the role of hypoxia in the regulation of Hippo pathway as well as its negative effect on blood cell of largemouth bass. Previous studies in mammals and Drosophila have established the centrality of YAP/TAZ in the Hippo pathway and found that hypoxia leads to upregulation of YAP/TAZ, which is in good agreement with our findings in blood cells of largemouth bass (Yan et al., 2014). According to the results, hypoxia significantly promoted the expression of YAP/TAZ and remained at a high level after reoxygenation, while MOB1 and Last1/2, upstream regulators of the Hippo pathway, which are highly associated with the regulation of YAP/TAZ expression, also showed a high level of expression in the H2 group. Subsequently, we also found that the expression of 14-3-3, which acts as a repressor of YAP/TAZ entry into the nucleus, was decreased in the hypoxic group, whereas the expression of PP2A, which dephosphorylates YAP/TAZ and thus enters the nucleus to bind to downstream target genes, was a greater increase. These results suggest that hypoxia is able to induce upregulation of YAP/TAZ, the core gene of the Hippo pathway, and further entry into the nucleus (Zhao et al., 2007; Mui et al., 2015). However, the expression of TEAD, which eventually binds to YAP/TAZ after nucleation, showed a dramatic decrease in the H1/H2 group. From our results, we conclude that hypoxia can cause dysregulation of the Hippo pathway and ultimately induce oxidative damage and apoptosis in largemouth bass blood cells. Similarly, results from the study of Dey et al. (2020) also suggested that dysregulated Hippo pathway and YAP/TAZ–TEAD activity is associated with various diseases, most notably cancer in human (Dey et al., 2020).

In conclusion, our findings have demonstrated that hypoxia could induce oxidative injury and apoptosis *via* mediating the Nrf-2/Hippo pathway in blood cells of Largemouth bass (*M. salmoides*) (**Figure 9**). These findings might provide a novel and potential therapeutic approach to understand the mechanisms in hypoxia stress and global warming adaptation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Animal experimental procedures were conducted under protocols approved by the Regulations for Animal Experimentation of South China Normal University.

AUTHOR CONTRIBUTIONS

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