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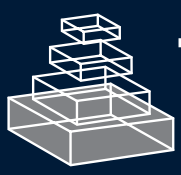
RESEARCH TOPICS

THE ROLE OF BILE PIGMENTS IN HEALTH AND DISEASE: EFFECTS ON CELL SIGNALING, CYTOTOXICITY, AND CYTOPROTECTION

Hosted by
Jaime Kapitulnik and Mahin D. Maines



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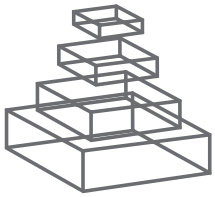

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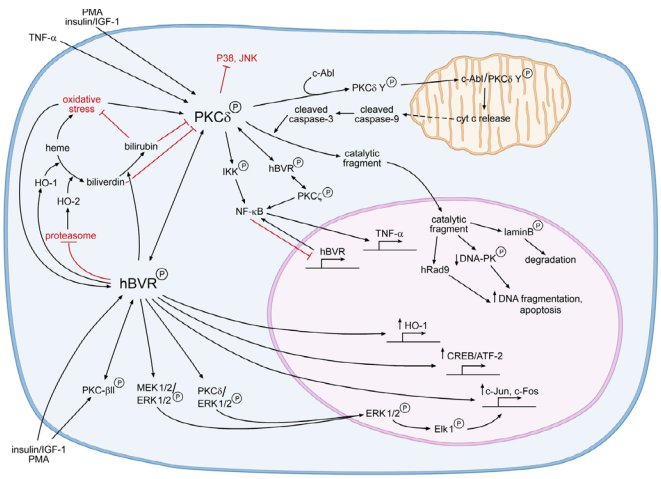
THE ROLE OF BILE PIGMENTS IN HEALTH AND DISEASE: EFFECTS ON CELL SIGNALING, CYTOTOXICITY, AND CYTOPROTECTION

Hosted By
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel
Mahin D. Maines, University of Rochester School of Medicine, USA



Degradation of heme involves its conversion to biliverdin by heme oxygenase followed by reduction of biliverdin to bilirubin by biliverdin reductase. There is ample evidence for the pleiotropic functions of biliverdin reductase in cell signaling and regulation of gene expression. This enzyme plays a major role in glucose uptake and the stress response. Bilirubin has been shown to behave as a "double-edged sword". It can exert either cytotoxic or cytoprotective effects, depending on the blood and/or tissue concentration of its free fraction, the nature of the target cell or tissue, and the cellular redox state. Its antioxidant effect is the basis for the proposed cardioprotective effect of relatively low blood concentrations of bilirubin in humans with moderate hyperbilirubinemia. This Special Topic forum is intended to serve as a platform for updating information and presenting advances in basic and clinical research in the above and related subjects.

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EDITORIAL
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The role of bile pigments in health and disease: effects on cell signaling, cytotoxicity, and cytoprotection

Jaime Kapitulnik[1]* and Mahin D. Maines[2]

[1] *Laboratory of Drug Metabolism, Institute of Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel*
[2] *Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, NY, USA*
**Correspondence: jaimek@savion.huji.ac.il*

Edited by:
Théophile Godfraind, Université Catholique de Louvain, Belgium

Reviewed by:
Théophile Godfraind, Université Catholique de Louvain, Belgium

Degradation of heme involves its conversion to biliverdin by heme oxygenase followed by reduction of biliverdin to bilirubin by biliverdin reductase. There is ample evidence for the role of heme oxygenase in protecting cells from the toxic effects of heme, as well as for the pleiotropic functions of biliverdin reductase in cell signaling and regulation of gene expression. This enzyme plays a major role in glucose uptake and the stress response. Bilirubin has been shown to behave as a "double-edged sword." It can exert either cytotoxic or cytoprotective effects, depending on the blood and/or tissue concentration of its free fraction, the nature of the target cell or tissue, and the cellular redox state. The central nervous system is particularly sensitive to the neurotoxic effects of bilirubin. Its antioxidant effect is the basis for the proposed cardioprotective effect of bilirubin in humans with moderate hyperbilirubinemia, as is the case in subjects with the Gilbert syndrome.

This Research Topic forum is intended to serve as a platform for updating information and presenting advances in basic and clinical research in the above and related subjects. The topic is discussed by leading experts in the field of bile pigments, and presented in 15 Reviews, 3 Original Research articles and 1 Opinion article. It covers important aspects related to the enzymes involved in the heme catabolic pathway: the role of heme oxygenase in inflammation and fibrosis (Lundvig et al., 2012) as well as in atherosclerosis (Araujo et al., 2012) and immune-mediated inflammatory diseases (Larsen et al., 2012), the regulation of cell signaling by biliverdin reductase and its peptide fragments (Gibbs et al., 2012), and the regulation of bilirubin clearance (Bock, 2011).

The role of glial cells and inflammation in bilirubin neurotoxicity (Brites, 2012) and the transport and metabolism of bilirubin at blood-brain interfaces and neural cells (Gazzin et al., 2012) illustrate the complex nature of bilirubin-induced brain damage. The effects of bilirubin vary with age (Dennery, 2012), and metalloporphyrins have been suggested to reduce excessive hyperbilirubinemia and brain damage in newborns (Schulz et al., 2012).

The regulatory properties of bile pigments and the role of biliverdin reductase in mediating their antioxidative (Jansen and Daiber, 2012) and anti-inflammatory effects (Wegiel and Otterbein, 2012), and their role in aging and age-related diseases (Kim and Park, 2012), are only part of the known protective functions of bile pigments. Bilirubin displays antiviral activity (Santangelo et al., 2012; Schmidt et al., 2012), ameliorates renal hemodynamics and blood pressure in an animal model of hypertension (Stec et al., 2012), and has beneficial effects in pulmonary and vascular diseases (Ryter, 2012). The protective effects of bilirubin in the vasculature include inhibition of neointima formation and reduction of vascular smooth muscle cell proliferation and migration (Peyton et al., 2012). In microvascular endothelial cells, low ("physiological") bilirubin concentrations induce apoptosis, which is exacerbated under hyperglycemic conditions. Endothelial cells of the blood-brain barrier are particularly sensitive to these effects of bilirubin (Kapitulnik et al., 2012).

CONCLUSION

Bilirubin, which has been considered for decades to be a toxic waste product of heme catabolism, is now recognized as an endogenous cytoprotective compound at low ("physiological") concentrations. However, its protective effects have been demonstrated *in vitro*, mainly in peripheral tissues, while its neurotoxicity remains unchallenged.

Although moderately elevated plasma bilirubin levels (as those of subjects with Gilbert syndrome) have been shown in retrospective and prospective clinical studies to be associated with a decreased risk of cardiovascular diseases (Vitek, 2012), there is still no general concensus in applying this knowledge in manipulating bilirubin levels for the prevention of cardiovascular and associated diseases. Stimulation of biliverdin reduction and/or inhibition of bilirubin conjugation are highly questionable with regards to their safety in humans, particularly in the scenario of a life-long treatment. We hope that the work presented in this Research Topic will stimulate further basic and clinical research in the area of bile pigments and its pathological as well as therapeutic implications.

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The evolving landscape of neurotoxicity by unconjugated bilirubin: role of glial cells and inflammation

Dora Brites [1,2] *

[1] *Neuron Glia Biology in Health and Disease Unit, Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal*
[2] *Department of Biochemistry and Human Biology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal*

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel
Cesare Mancuso, Catholic University School of Medicine, Italy
Andreas Daiber, University Medical Center Mainz, Germany

***Correspondence:**
Dora Brites, Faculdade de Farmácia da Universidade de Lisboa, Avenida Professor Gama Pinto, 1649-003 Lisbon, Portugal.
e-mail: dbrites@ff.ul.pt

Unconjugated hyperbilirubinemia is a common condition in the first week of postnatal life. Although generally harmless, some neonates may develop very high levels of unconjugated bilirubin (UCB), which may surpass the protective mechanisms of the brain in preventing UCB accumulation. In this case, both short-term and long-term neurodevelopmental disabilities, such as acute and chronic UCB encephalopathy, known as kernicterus, or more subtle alterations defined as bilirubin-induced neurological dysfunction (BIND) may be produced. There is a tremendous variability in babies' vulnerability toward UCB for reasons not yet explained, but preterm birth, sepsis, hypoxia, and hemolytic disease are comprised as risk factors. Therefore, UCB levels and neurological abnormalities are not strictly correlated. Even nowadays, the mechanisms of UCB neurotoxicity are still unclear, as are specific biomarkers, and little is known about lasting sequelae attributable to hyperbilirubinemia. On autopsy, UCB was shown to be within neurons, neuronal processes, and microglia, and to produce loss of neurons, demyelination, and gliosis. In isolated cell cultures, UCB was shown to impair neuronal arborization and to induce the release of pro-inflammatory cytokines from microglia and astrocytes. However, cell dependent sensitivity to UCB toxicity and the role of each nerve cell type remains not fully understood. This review provides a comprehensive insight into cell susceptibilities and molecular targets of UCB in neurons, astrocytes, and oligodendrocytes, and on phenotypic and functional responses of microglia to UCB. Interplay among glia elements and cross-talk with neurons, with a special emphasis in the UCB-induced immunostimulation, and the role of sepsis in BIND pathogenesis are highlighted. New and interesting data on the anti-inflammatory and antioxidant activities of different pharmacological agents are also presented, as novel and promising additional therapeutic approaches to BIND.

Keywords: unconjugated hyperbilirubinemia, neurotoxicity, reactive astrocytes, activated microglia, sepsis, pro-inflammatory cytokines, oxidative stress, myelin

INTRODUCTION

Jaundice occurs during the first week of life in most infants due to elevated levels of unconjugated bilirubin (UCB) by increased breakdown of fetal erythrocytes, deficient human serum albumin (HSA) transport to the liver, and inefficient conjugation. Although the outcome for the majority is benign, untreated neonates, or newborns with very high UCB levels can develop acute encephalopathy or kernicterus. The most disabling neurological findings in kernicterus are auditory and motor sequelae. At autopsy of kernicteric infants, UCB is macroscopically visible within globus pallidus, hippocampus, lateral ventricular walls, cerebellum, and subthalamic nuclei, reflecting a preferential deposition of UCB in specific brain areas. Unfortunately, no established criteria recognize whether neurological or developmental problems result from hyperbilirubinemia. Nevertheless, patient history, physical examination, and laboratory findings allow the categorization of kernicterus as mild, moderate, or severe. Recently, Shapiro (2010) has suggested the term of subtle kernicterus for the less severe injuries, including minor neurological abnormalities. This milder form of bilirubin induced neurological dysfunction (BIND) in the central nervous system (CNS) needs to be distinguished from other known causes of neurodevelopmental disabilities. Further studies looking at potential alterations produced by UCB on neurodevelopmental programs and lasting neurological deficits that may involve vulnerability to aging and neurodegenerative diseases are needed.

To reduce BIND incidence, normograms, and guidelines were proposed (Bhutani et al., 2008) for jaundiced newborn infants of 35 or more weeks of gestation. However, in sick and preterm infants, the absence of precise data on hyperbilirubinemia prevalence and the lack of proven predictive indices has made impossible to establish such guidelines. Therefore, the problem is even more worrying for premature babies and low birth weight infants, mainly when infection/sepsis is associated, but also because they are particularly vulnerable to BIND (Bhutani et al., 2004; Okumura et al., 2009; Moll et al., 2011), probably due to neurodevelopmental maturity differences. In fact, temporal windows of CNS vulnerability to UCB toxicity have been suggested and

the neurodevelopmental age at the time of UCB brain injury influences the location of the selective neuropathological damage (Keino and Kashiwamata, 1989; Conlee and Shapiro, 1997). While auditory predominant kernicterus subtype prevails in infants with peak levels at earlier gestational ages of exposure to total serum bilirubin (TSB), motor kernicterus subtype usually develops in infants with more than 34 weeks of gestation (Shapiro, 2010). TSB is the sum of UCB plus the fraction of the pigment that has already been conjugated in the liver. Interestingly, if we look into two different mice models with equivalent TSB levels, results indicate that when hyperbilirubinemia develops in the first week of life, such as in the Ugt1$^{-/-}$ model (Nguyen et al., 2008), all the animals succumb, while if occurring in the second week of life as in the Tg(UGT1A1*28)Ugt1$^{-/-}$ model (Fujiwara et al., 2010), only 10% of the mice dye. These findings suggest an acquired resistance with the postnatal age. Therefore, high levels of UCB during early neurodevelopment may determine injuries that can be diverse in nature, severity, and enduring effects from those produced a week later.

Neonatal jaundice, if prolonged or severe, could have an impact on the infant's learning and memory, and even moderate levels of UCB have been associated with developmental delay, attention-deficit disorder, autism, and isolated neural hearing loss (Gkoltsiou et al., 2008; Jangaard et al., 2008; Shapiro, 2010). However, the establishment of these casual relationships was never demonstrated with certainty because of the two decades that separate neonatal jaundice from signs of mental disorders at puberty. Moreover, neonatal hyperbilirubinemia, mainly in prematures, could be one of the multifactorial risk factors leading to long-lasting deficits as recently evidenced for moderate systemic inflammation (Favrais et al., 2011). Thus, elucidation of structural–functional relationship might help in understanding the pathological processes and developmental difficulties experienced by jaundiced babies.

In vitro experiments have been elucidating the mechanisms of UCB neurotoxicity (Brites et al., 2009; Brites, 2011), while *in vivo* studies have almost exclusively focused on homozygous (jj) Gunn rats (Rice and Shapiro, 2008; Daood et al., 2009), the kernicterus classic model. Hyperbilirubinemic Gunn rats have a congenital deficiency of the bilirubin liver conjugating enzyme uridine-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) and present neurological abnormalities similar to kernicterus (Billing, 1972) when acute bilirubin encephalopathy is induced by the administration of phenylhydrazine (Rice and Shapiro, 2008) or sulfadimethoxine (Daood et al., 2009). Indeed the model, although indicated as mimicking the Crigler–Najjar I syndrome due to a defect in the UGT1A1 isoform of the UDPGT gene (Johnson et al., 1959), evidence less severe hyperbilirubinemia, and the natural course of the disease is milder in the Gunn rat than in human Crigler–Najjar I syndrome (Shapiro, 2003). Interestingly, Gunn rats with mild concentrations of UCB at 2 months of age (∼3 mg/dL) have exhibited behavioral alterations and neuropathological changes similar to those found in schizophrenia (Hayashida et al., 2009).

Hence, clarification of the molecular mechanisms of UCB neurotoxicity is an emerging area of research as it may open new perspectives for therapeutic approaches at circumventing potential enduring injurious effects of neonatal brain insults by UCB. The aim of this review is to highlight the most recent and relevant information on the determinants and widespread effects of UCB neurotoxicity, the potential molecular mechanisms involved and the associated neurodevelopmental susceptibilities, as well as the glia immune-inflammatory responses and the most promising neuropharmacological agents based on *in vitro* experimental models.

DETERMINANTS OF BRAIN BILIRUBIN ENTRANCE AND LOAD

Several factors in the first week of postnatal life may concur to increase the amount of UCB in the circulation, which when surpassing the albumin-binding capacity for UCB lead to an increase of the unbound (free) fraction of UCB (Bf). Although UCB entrance into the brain is prevented by the brain microvascular endothelial cells tightly jointed by the elaborated junctional complexes that form the blood–brain barrier (BBB; Cardoso et al., 2010; **Figure 1A**), Bf diffuses into the brain (Ostrow et al., 2003b), and its accumulation in the CNS will depend on the total amount of Bf in the systemic circulation, on the efficacy of BBB transporters, and on the presence of acidosis, which increases binding of Bf to the brain parenchyma and the risk of kernicterus (Cashore et al., 1983; Meisel et al., 1988), due to its decreased solubility in acidic aqueous solutions (less than 1 nM at pH = 7 and about 0.1 μM at pH = 8; Ostrow and Celic, 1984).

ATP-dependent cellular export of UCB by P-glycoprotein (Pgp) and multidrug resistance-associated protein 1 (MRP1/Mrp1), which are highly expressed in BBB and in CSF (Gazzin et al., 2011), respectively, limit Bf passage across the BBB (the first one) and its intracellular accumulation (mainly the last one; Sequeira et al., 2007; Daood et al., 2008; Bellarosa et al., 2009). However, the lower expression of Pgp in early-life (Daood et al., 2008; Gazzin et al., 2011) may be critical in preventing UCB entrance into the CNS. Similarly, Mrp1 down regulation by long exposure to elevated concentrations of UCB, recently observed at the blood–CSF barrier in the Gunn rats, might favor BIND (Gazzin et al., 2011). UCB can even compromise the integrity of BBB lining by disrupting glutathione (GSH) homeostasis and increasing endothelial nitric oxide synthase (NOS) expression followed by nitrite (NO) production and cytokine release (Palmela et al., 2011), thus favoring UCB brain uptake in prolonged hyperbilirubinemia. Formation of NO, originally described as the endothelial-derived relaxing factor (Bellefontaine et al., 2011), requires NOS, in which there are three isoforms: two constitutive (i) neural-type NOS I (nNOS) and (ii) endothelial-type III (eNOS), and one inducible NOS II (iNOS) isoform. Interestingly, upregulation of eNOS expression was more pronounced in the first 4 h incubation and showed to increase with the concentration of UCB (Palmela et al., 2011). The elevated levels of NO generated may increase microvascular permeability (Kim and Jung, 2011), thus favoring passage of UCB into the brain.

Once in the brain, preferential deposition and neurotoxicity of UCB may result from cell specific susceptibilities and CNS regional vulnerabilities, while time of exposure will be critical in extending UCB load and toxicity (Wennberg et al., 2006). Localization of Mrp1 in astrocytes and of Pgp in those associated to BBB may help in restricting UCB injury to cells located at BBB proximity and to maintain BBB properties in mild hyperbilirubinemia

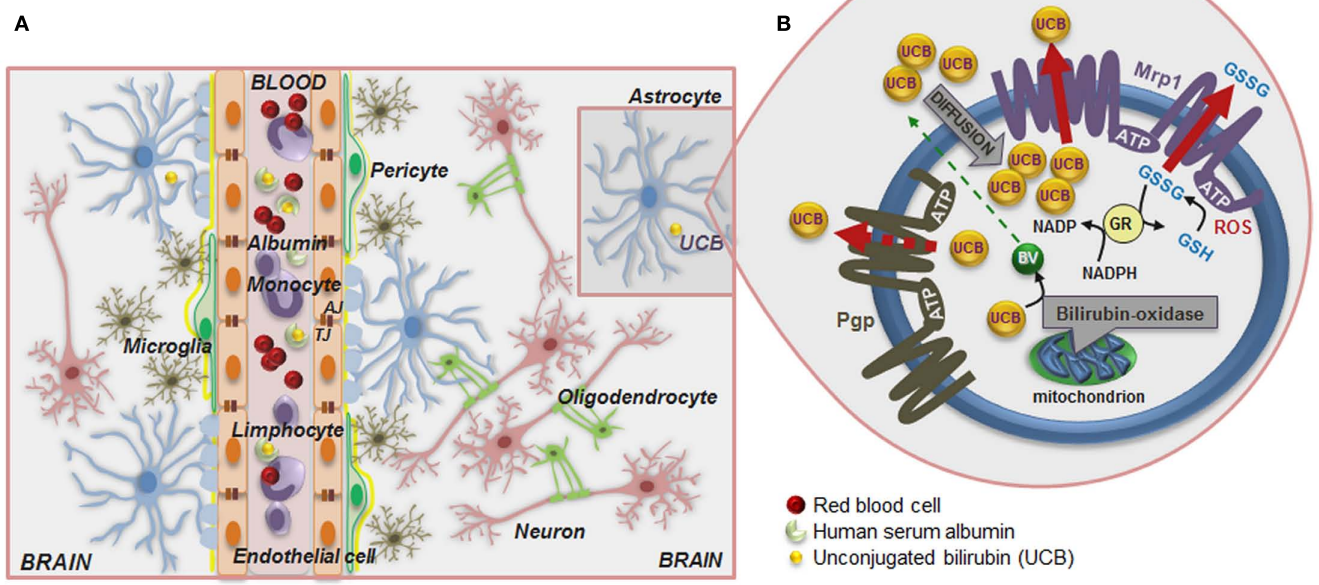

FIGURE 1 | Schematic of the blood–brain barrier (BBB) in a condition of mild hyperbilirubinemia (A) and overview of the mechanisms that prevent the accumulation of unconjugated bilirubin (UCB) in astrocytes (B). (A) The normally functioning BBB maintains the central nervous system homeostasis by separating the brain from the systemic blood circulation and regulating the exchange of various compounds. The BBB is composed by endothelial cells (orange) and their tight junctions (TJ), located more apically, and adherens junctions (AJ), located underneath, as well as by astrocyte end-feet (light blue spots), perivascular microglia (greenish-brown), pericytes (light-green), and the basement membrane (yellow). Integrity of all BBB elements is essential for neuron (pink) function and for oligodendrocytes (solid-green) to exert protection of axon function and promotion of neuronal survival. Circulation of UCB in the blood is provided by its binding to human serum albumin. Thus, decreased levels of albumin, the lower capacity for UCB binding, and the high UCB concentrations favor the entrance of unbound (free) UCB (Bf) fraction by passive or facilitated diffusion into the brain. In a condition of mild hyperbilirubinemia the Bf levels in the plasma rarely surpass 100 nM and in the brain are usually lower than 10 nM; entrance restriction to UCB is afforded by the P-glycoprotein (Pgp) at the BBB, which is an ATP-dependent cellular export protein of UCB. **(B)** At the brain cells, UCB is a substrate for the efflux pump multidrug resistance-associated protein 1 (Mrp1) and together with Pgp limit the intracellular accumulation of UCB, mainly in astrocytes. Reduced expression of Pgp and Mrp1 in young astrocytes and neurons, as well as in Gunn rats' pups, probably account to the vulnerability of premature babies to UCB. A high intracellular concentration of glutathione (GSH) protects against reactive oxygen species (ROS). GSH is regenerated from glutathione disulfide (GSSG) in a reaction catalyzed by glutathione reductase (GR) using NADPH as a second substrate. Astrocytes release GSSG under oxidative stress via Mrp1. Another protective mechanism against UCB toxicity is the mitochondrial membrane enzyme bilirubin oxidase which in the presence of molecular oxygen converts UCB to biliverdin (BV) and other products that are much less toxic than UCB. Changes in BBB integrity will lead to UCB entrance into the brain, and failure of cell defensive mechanisms against UCB toxicity will favor brain injury.

(Watchko et al., 2001). Rigato et al. (2004) have demonstrated that Mrp1 mediates ATP-dependent cellular export of UCB by astrocytes (**Figure 1B**). GSH protects astrocytes from reactive oxygen species (ROS) by directly reacting with radicals and by reducing peroxides generating oxidized glutathione (GSSG). In order to maintain a reduced thiol reduction potential under oxidative stress, Mrp1 mediates GSSG release from astrocytes (Hirrlinger et al., 2001). Mrp1 expression was also shown in neurons (Falcão et al., 2007a). Moreover, it was observed that immature neurons and astrocytes exhibit low levels of the protein as compared with mature cells, in line with what was observed for Pgp, and that inhibition of Mrp1 expression with MK571 increases UCB toxicity to neurons and astrocytes (Falcão et al., 2007a), as well as to human neuroblastoma SH-SY5Y cells when RNA interference technology was used (Corich et al., 2009).

Another important defense mechanism resides on the ability of UCB oxidation by brain mitochondrial membranes (**Figure 1B**), at a rate of 100–300 pM UCB/mg protein/min (Hansen, 2000a). The enzyme activity showed to be cytochrome *c* dependent, but it is not influenced either by GSH or GSSG (Hansen et al., 1999). Once it is less expressed in immature brains, as well as in undifferentiated cells, and in neurons than in glia (Hansen and Allen, 1997), it is hypothesized that it could be related to the increased vulnerability of the newborn to UCB, mainly if premature.

Microscopic evaluation of icteric stained brain sections revealed that UCB interacts with neurons, neuronal processes, and glia elements (Martich-Kriss et al., 1995), but the contribution of each cell type to BIND is not clear, mainly when considering the UCB-induced nerve cell pathological alterations as an integrated phenomena, i.e., by using organotypic slice cultures. If neurons are highly specialized for the processing and transmission of cellular signals, glial cells, which include astrocytes, microglia, and oligodendrocytes (OLGs), are essential in the maintenance of the neuronal network, in neuronal migration during development and in the generation of myelin, respectively. Therefore, once such cells are important players in neuronal dysfunction they should

be considered as promising therapeutic targets in BIND. Moreover, one must consider glial damage not as a marginal event or a secondary consequence of neuronal injury, but rather as an intrinsic participant and determinant of the UCB-induced neuropathological processes.

BIOMARKERS AND PREDICTORS OF BILIRUBIN NEUROTOXICITY

Classical and subtypes of kernicterus associated with kernicterus and BIND, respectively, have been related to auditory impairment (Shapiro and Popelka, 2011). Intriguingly, it was observed that from a total of 13 neonates with severe hyperbilirubinemia, six infants had audiologic findings of acute auditory neuropathy spectrum disorder, while only two from the six had clinical signs and symptoms of acute UCB encephalopathy (Saluja et al., 2010). Therefore, abnormal neurological function with auditory brainstem responses (ABRs), also known as brainstem auditory evoked potentials (BAEPs), or responses (BAERs), is considered a very objective and sensitive test for the diagnosis of kernicterus and UCB neurotoxicity, once these auditory regions are too small to be seen on conventional magnetic resonance imaging (MRI) scans (Gupta and Mann, 1998; Shapiro, 2010). Actually, audiological diagnostic work-up in neonatal hyperbilirubinemia above 20 mg/dL, and even for UCB at <20 mg/dL, has been recommended (Sheykholeslami and Kaga, 2000; Nickisch et al., 2009).

Aberrant ABRs have been consistently used as a non-invasive method to identify UCB toxicity in Gunn rats. However, UCB by itself is not able to cause hearing impairment in jaundiced Gunn rats (Levi et al., 1981), unless combined with induced asphyxia (Silver et al., 1995a). Other sensitive markers for the massive entry of UCB into the nervous system include the somatosensory evoked potential (Silver et al., 1996) and visual evoked potential abnormalities (Silver et al., 1995b), both observed in the Gunn rats, in the absence or in the presence of sulfadimethoxine, respectively.

Nevertheless, in what concerns biomarkers to evaluate the risk of BIND, Bf is considered a more sensitive predictor (Ahlfors et al., 2009b; Lee et al., 2009) than either bilirubin–albumin molar ratio (UCB/HSA) or abnormal ABR maturation (Amin et al., 2001) and, in contrast to TSB levels, revealed to be associated with ABRs results (Ahlfors and Parker, 2008). In sum, Bf or TSB plus Bf, instead of TSB alone, are more rational indicators (Wennberg et al., 2006). However, due to the difficulties of Bf assessment in the clinical practice it has been referred the use of TSB/HSA instead of UCB alone (Hulzebos et al., 2008). Additionally, the HSA (Ahlfors et al., 2009a) and the "reserve HSA binding capacity" (McDonagh, 2010) determinations are indicated as complementary, and a Bf of 20 nM proposed as the risk threshold for UCB toxicity in term infants (Wennberg et al., 2006). In fact, while the values of TSB only varied between 116 and 615 μM in infants with birth weight higher or equal to 2.5 kg, greater variations in Bf concentrations (from 0.9 to 130 nM) were encountered, reinforcing the need of individualized jaundice management (Ahlfors et al., 2009b). Finally, the Bf/TSB ratio is even believed to be a best displayer of abnormal BAEPs than Bf alone (Ahlfors et al., 2009a). However, when the challenge resides in relating Bf or TSB with abnormal neurodevelopmental and lasting neurological changes, alterations in ABRs are considered the top clinical approach.

Recently, it was suggested that serum tau and S100B protein levels are equally promising biomarkers, which besides being correlated with TSB, were found to be more elevated in infants with auditory neuropathy, neurologic defects, or electroencephalogram abnormalities (Okumus et al., 2008), and consequently with UCB-induced brain sequelae.

HOW DOES BILIRUBIN MAKE NEURONS SICK?

Most published studies on the neurotoxicity of UCB have been performed either at too high UCB/HSA molar ratio (>3) or at UCB values in the absence of serum or albumin, Bf, vastly higher (>1 μM) than those seen in jaundiced neonates at risk of kernicterus or BIND (between 100 and 500 nM), and thus are of questionable relevance to the clinical manifestations of neurotoxicity (Ostrow et al., 2003a). In fact, those Bf concentrations in the absence of HSA form microsuspensions, followed by coarser aggregates that precipitate spontaneously, mainly at the level of the cellular membranes. Some data suggest that metastable aggregates begin to occur at concentrations of Bf as low as 1–2 μM and probably not at 500 nM (Mukerjee et al., 2002; Ostrow et al., 2003a). Once bilirubin has been referred as a neuroprotective compound, as well, it deserves to be noted that only Bf concentrations below 100 nM are able to protect CNS against oxidative damage (Doré et al., 1999; Dora Brites lab, unpublished results). Therefore, we have privileged in this section the studies addressing the neurotoxic effects produced by moderately supersaturated Bf levels (between 100 nM and 1 μM) and/or UCB/HSA molar ratios ≤3.

Neurons are more susceptible than astrocytes to UCB-induced demise, probably as a result of lower GSH content (Brito et al., 2008b). Indeed, neurons generally show higher levels of ROS, protein oxidation, and lipid peroxidation upon UCB exposure than astrocytes, despite the different vulnerability of neuronal subpopulations (Conforti et al., 2007). One of the most challenging issues is to define the earliest steps of injury once it can differ on time, severity, and site between one cell and another (Muramatsu et al., 2009). Years ago, Chen et al. (1971) suggested, based in their experimental kernicterus model, that after absorption of UCB from astrocytes at the dendritic level, UCB is transported from the distorted microtubules to the Golgi complex, before the enlarged vesicles coming from the Golgi reach the granular endoplasmic reticulum (ER), which in turn show vesiculation, vacuolation, and dilated cisternae.

Interaction of UCB with the cells occurs first at the cell membrane (**Figure 2A**), and although UCB rapidly diffuses through membranes (Zucker et al., 1999), it was shown that it causes increased polarity and fluidity at carbon numbers 5 and 7 (Rodrigues et al., 2002c), ultimately perturbing the neuronal cell membrane structure. These features showed to be associated with oxidative injury to membrane lipids and were not sensed in the more hydrophobic regions. Similar effects were observed on membranes of astrocytes (Rodrigues et al., 2002c) and erythrocytes (Brito et al., 2001), as well as on mitochondrial membrane (Rodrigues et al., 2002b). This increased membrane fluidity favors the externalization of phosphatidylserine to the outer leaflet, which allows phagocytic recognition. This finding, also observed

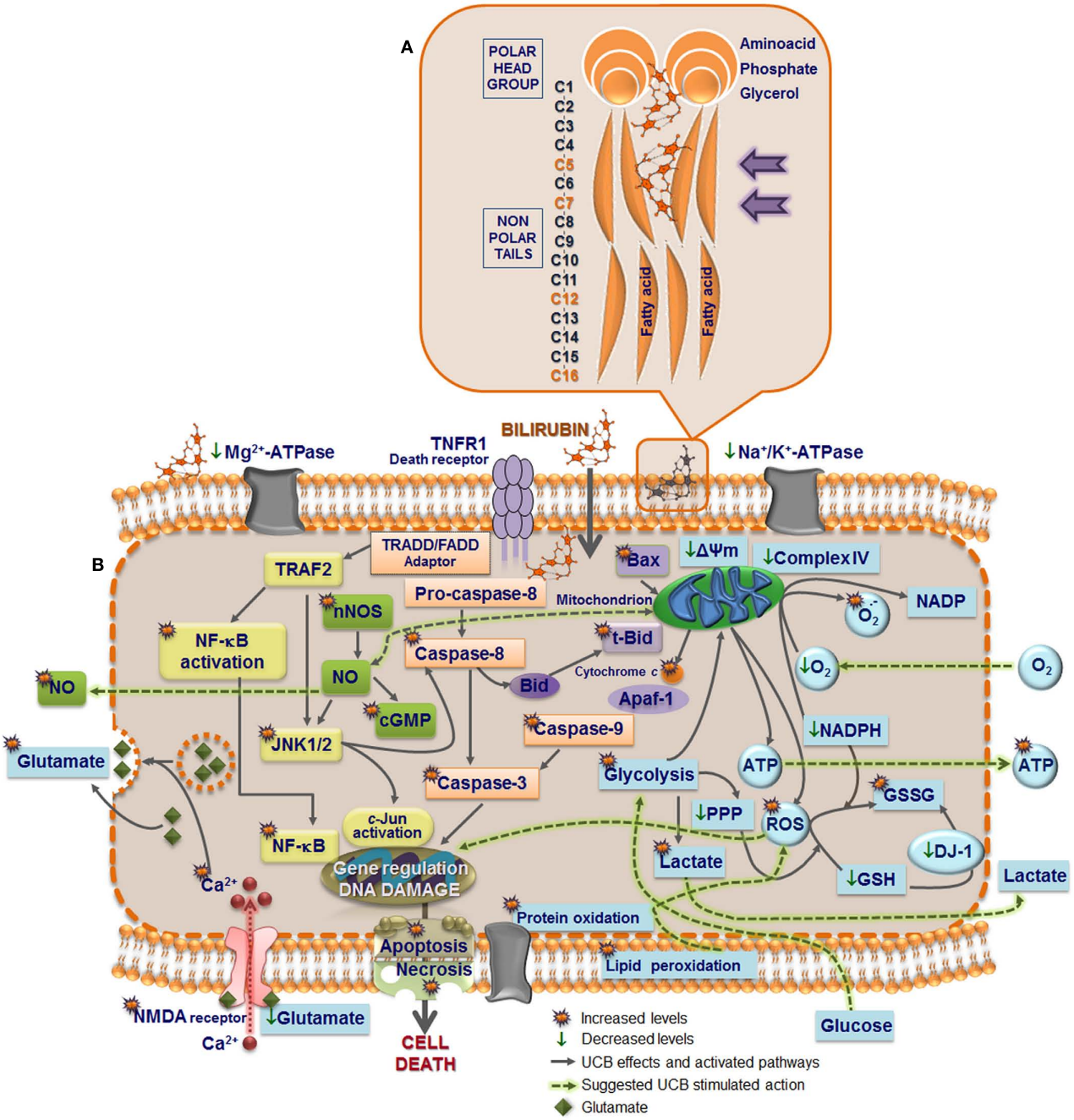

FIGURE 2 | Model of UCB interaction with neural plasma membrane (A) and pathways by which UCB may affect neuronal function (B). Cell membrane includes a bilayer of packed phospholipids, cholesterol, and integral proteins. **(A)** The inset represents a phospholipid of the outer membrane containing a phosphatidyl-glycerol polar (hydrophilic head) and two fatty acids (hydrophobic tails). The phosphate group is attached to the glycerol and form ester links with a variety of other molecules. UCB decreases phospholipid mobility and the polarity at carbon (C) 5, while increased it at C7, and less markedly at C12 and C16 (orange). The intercalation at C5 may trigger the release of phospholipids and cholesterol, as well as the externalization of the phosphatidylserine, a recognition signal for phagocytosis. **(B)** UCB-induced protein oxidation and lipid peroxidation may arise from increased reactive oxygen species (ROS) production. UCB impairs Mg^{2+}-ATPase activity, responsible for the maintenance of phosphatidylserine in the inner leaflet, and Na^{+}, K^{+}-ATPase, which may trigger the intracellular intrusion of calcium (Ca^{2+}). Opening of Ca^{2+} channels, as well as the activation of NMDA receptors by UCB, recognized to modulate the exocytosis of glutamate, can also account to it. Changes in membrane polarity and fluidity, equally produced at the mitochondrial membrane by UCB, lead to the collapse in the mitochondrial transmembrane potential ($\Delta\Psi m$), translocation of proapoptic Bax to the mitochondria, inhibition of cytochrome *c* oxidase activity (complex IV of the respiratory chain), and efflux of cytochrome *c*. Then, activation of caspases 3 and 9 (intrinsic apoptotic cascade) takes place, as well as UCB-induced

(Continued)

FIGURE 2 | Continued
activation of caspase-8 (extrinsic apoptotic cascade) and the cleavage of Bid into truncated Bid (*t*Bid). Oxidation of GSH to GSSG and inability to be restored is associated with the increased ROS generation, which may determine DNA damage. DJ-1 that is early up-regulated, to diminish UCB-induced oxidative stress, is latter on oxidized and proteolitically degraded due to the reduced levels of GSH. Increased superoxide anion radical production ($O_2^{\bullet -}$) and decreased NADPH upon exposure to UCB also account to the oxidative stress. Glycolysis upregulation by UCB lead to increased intracellular lactate content and inhibition of the pentose phosphate pathway (PPP), providing ATP to support the bioenergetic crisis. UCB-asscociated nitrosative stress derives from the increased expression of nNOS and generation of NO and cyclic guanosine 3′,5′-monophosphate (cGMP). Actually, NO signaling, c-Jun N-terminal kinases (JNK1/2) stimulation, activation of nuclear factor-kappaB (NF-κB) with translocation to the nucleus, ROS generation, and activation of caspases are key players in the induction of genes involved in UCB-induced neuronal demise by necrosis and apoptosis.

in erythrocyte membranes (Brito et al., 2002) and synaptosomes (Brito et al., 2004) after exposure to UCB, was accompanied by reduced activities of Mg^{2+}-ATPase aminophospholipid translocase (flippase) and Na^+, K^+-ATPase, together with enhanced intracellular levels of calcium (Ca^{2+}; **Figure 2B**). These effects can lead to cell destruction, as evidenced by the hemolysis of erythrocytes (Brites et al., 1997; Khan and Poduval, 2011). Ca^{2+} influx or increased release of Ca^{2+} from ER or other stores may contribute to the intracellular increase of Ca^{2+}. However, Zhang et al. (2010) demonstrated that UCB mainly induces the opening of the Ca^{2+} channel. Ca^{2+} influx into cortical neurons was shown to regulate the expression of nNOS mediated by a cAMP response element-binding (CREB) family transcription factor-dependent mechanism (Sasaki et al., 2000). Later it was shown that UCB-induced phosphorylation of CREB, induction of nNOS, and generation of NO, was indeed dependent of extracellular Ca^{2+} (Mancuso et al., 2008). Both extracellular signal regulated kinases (ERK1/2) (Mancuso et al., 2008) and c-Jun N-terminal kinases (JNK1/2) (Vaz et al., 2011b) phosphorylation is mediated through the NOS/NO pathway, but at least ERK activation seems to not depend from extracellular Ca^{2+} (Mancuso et al., 2008).

Developing neurons are particularly susceptible to UCB interaction which causes neurogenesis impairment, neuritic atrophy, and cell death by both necrosis and apoptosis (**Figures 3A,B**; Brito et al., 2002; Falcão et al., 2007c) that is enhanced by associated hypoxia (Vert et al., 2001). Impact results in sudden and lasting decrease in neuronal arborization, as well as in reduced number of dendritic spines and synapses (Fernandes et al., 2009b), and cells stayed sensitized and increasingly vulnerable to a secondary toxic stimulus (Falcão et al., 2007c). The observed neurite outgrowth impairment is consistent with UCB-induced neurodevelopmental abnormalities, by interfering with neural plasticity, and hippocampal susceptibility corroborates the brain regional specificity toward UCB in bilirubin encephalopathy (Jangaard et al., 2008). It deserves to be noted that even in more differentiated neurons (10 days *in vitro*) impairment in neurite outgrowth by UCB is still observed after 12–24 h of exposure (Zhang et al., 2010). However, it was reported that cells are able to reverse some of the UCB-mediated effects when it is used a shorter (6 h), instead of a longer exposure time (12 or 24 h; Hankø et al., 2006b).

Alterations of UCB at neuronal arborization derive, at least in part, from cytoskeleton changes elicited in microtubule associated proteins (MAP) in immature neurons. Extended MAP2 entry in the axonal shaft (**Figures 4A,B**) and increased Tau1 expression by UCB, promotes Tau1 binding to microtubules and its release into the cytoplasm (Fernandes et al., 2009a, 2010; **Figures 4C,D**). Tau is localized in the axon and MAP2 is somato-dendritically compartmentalized. Mislocalization of Tau to dendritic spines and subsequent synaptic impairment appears to precede neuronal loss (Hoover et al., 2010) and its release into the extracellular space, from where it can reach the CSF and the plasma (Qiang et al., 2006). Therefore, the increased Tau levels observed in serum are considered to be a predictive indicator of neurodegeneration (Wang and Liu, 2008; Noguchi-Shinohara et al., 2011) and Okumus et al. (2008) have proposed that it should be considered a biomarker of BIND. Further studies should elucidate on this and other promising biomarkers of early neuronal dysfunction by UCB, as well as on the temporal window of reversibility, so that clinical detection and rescue intervention treatments can be achieved.

In addition, UCB also causes synaptotoxicity that has been related with potentiation of inhibitory synaptic transmission (Shi et al., 2006), as well as with impairment of the induction of long-term potentiation (LTP) and long-term depression (LTD; Chang et al., 2009). Interestingly, UCB was shown to decrease the expression of synaptophysin and SNAP-25 (Silva et al., 2012), proteins that participate in synapse establishment and neurotransmitter release (Wang and Tang, 2006). Accordingly, UCB was demonstrated to cause presynaptic degeneration in the Gunn rat, while preserving postsynaptic neurons (Haustein et al., 2010).

Mitochondria swelling by UCB (Solá et al., 2002), as well as Bax translocation, mitochondrial depolarization, release of cytochrome *c*, activation of caspase-3, and degradation of poly(ADP)ribose polymerase (Rodrigues et al., 2002a) were shown to be implicated in neuronal apoptosis by UCB (**Figure 2B**). Inhibition of cytochrome *c* oxidase in mitochondria isolated from mice brain (Malik et al., 2010) and in immature cortical neurons obtained from rats, in which UCB evidenced to affect cell respiration due to the low oxygen consumption, account for the decrease in mitochondrial functional activity by UCB (Vaz et al., 2010). In addition, while low and moderate concentrations of UCB may favor a delayed apoptosis in cultured human NT2-N neurons, moderate and high levels preferentially trigger early necrosis (Hankø et al., 2005). Cell death prevalence in immature (3–4 days *in vitro*) neurons, mostly by apoptosis (Falcão et al., 2006; **Figure 3B**), involves a bioenergetic and oxidative crisis (Vaz et al., 2010). Oxidative stress derives from the increased superoxide anion radical production ($O_2^{\bullet -}$) (**Figure 2B**) together with an elevation of the GSSG relatively to the total glutathione (GSH plus two times GSSG), as well as from a decrease in NADPH concentration. It is conceivable that the upregulation of glycolysis by UCB will be an attempt to provide ATP to support the bioenergetic crisis, while ATP release resulting from the NO production and oxidative stress should be associated with apoptosis.

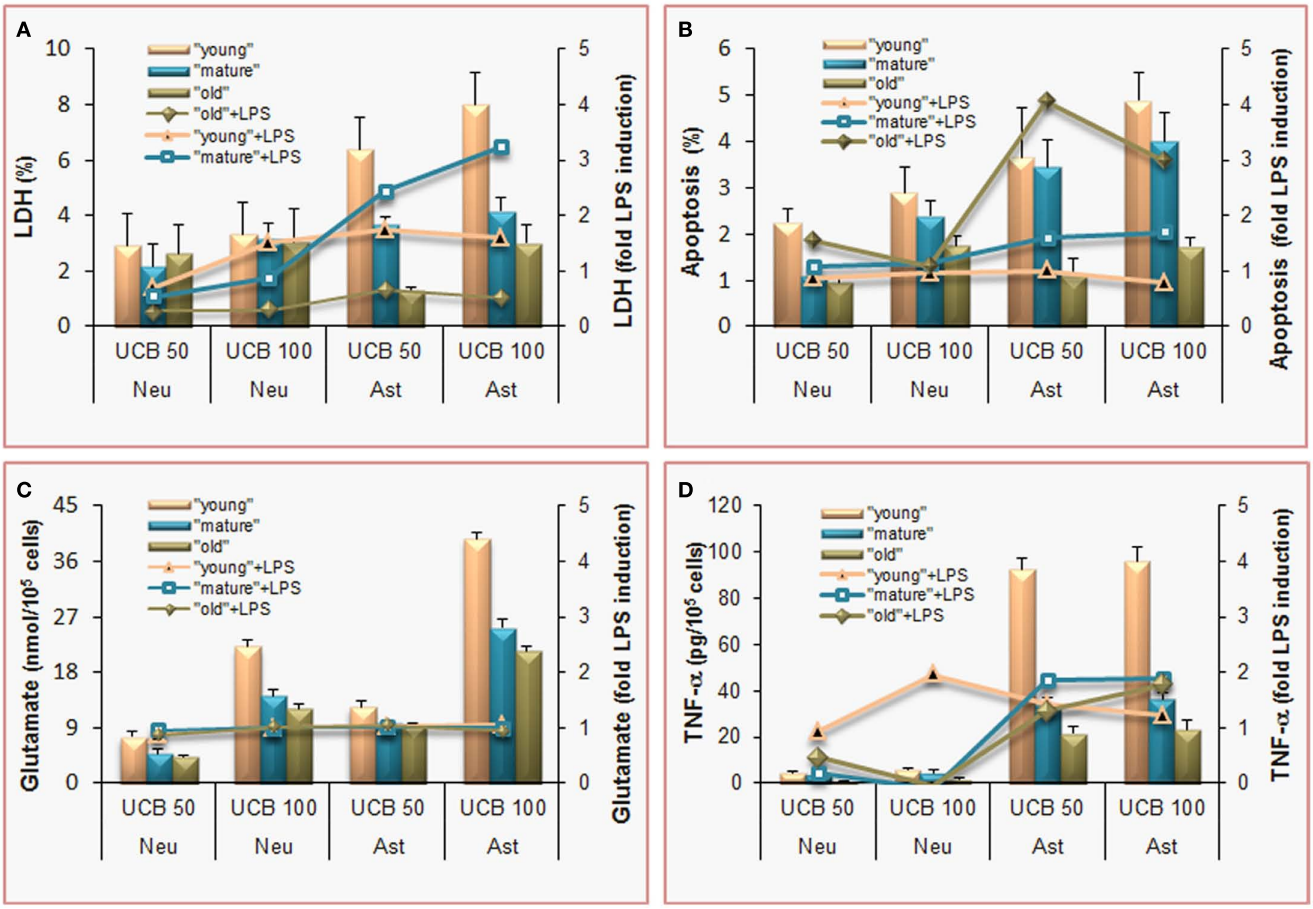

FIGURE 3 | Effects of unconjugated bilirubin (UCB) are concentration-dependent, more pronounced in astrocytes (Ast) than in neurons (Neu), and most sensed by undifferentiated (young) cells, which also show increased demise by co-incubation with lipopolysaccharide (LPS). To mimic young (immature), mature, and old cells, and to achieve the same stage of differentiation in both, rat cortical astrocytes and neurons were cultured for 5, 10, and 20 days *in vitro* (DIV) or 4, 8, and 18 DIV, respectively. Cells were incubated with 50 or 100 μM purified UCB, or no addition (control), in the presence of 100 μM human serum albumin, at pH 7.4, for 4 h, at 37°C. In sister experiments, cells were co-incubated with 1 ng/L LPS and results were expressed as fold LPS induction. **(A)** Results of lactate dehydrogenase (LDH) release by non-viable cells are expressed as percentage of cell death relatively to total cell lysis. Astrocytes are the most susceptible cells, and LPS effects are similarly produced in both type of cells, although mature astrocytes are the most vulnerable. **(B)** Apoptosis was estimated by analysis of nuclear morphology following staining with Hoechst 33258 dye. Immature and mature cells reveal enhanced vulnerability to UCB and old astrocytes greater apoptosis by associated LPS. **(C)** Release of glutamate to the incubation medium proportionally increases with UCB concentration and it is not modified by LPS co-incubation. **(D)** The pro-inflammatory cytokine tumor necrosis factor (TNF)-α is released to the culture medium mainly by astrocytes and always increase by LPS addition. Mean values ± SEM are differences from the respective control, for all the parameters except for the fold increase by LPS, which data are means. Data derived from Fernandes et al. (2004) and Falcão et al. (2005, 2006, 2007c).

The *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxic mechanism is another pathway leading to UCB-induced neuronal injury, providing the necessary increase in intracellular Ca^{2+} required for activation of nNOS (Bellefontaine et al., 2011). In fact, UCB was demonstrated to raise the extracellular concentration of glutamate, by enhancing its release mainly from immature neurons (Falcão et al., 2006; **Figures 2B** and **3C**), thus engendering overstimulation of NMDA receptors (Ostrow et al., 2004; Falcão et al., 2006; Brites et al., 2009). In addition, prolonged UCB exposure led to a decrease in presynaptic transmitter release but also impaired the postsynaptic NMDA receptor function (Chang et al., 2009). Therefore, the use of NMDA receptor antagonist MK-801 was shown to prevent the expression of nNOS, the consequent release of NO, the production of cyclic guanosine 3′,5′-monophosphate (cGMP), as well as cell dysfunction and demise (Hankø et al., 2006a; Brito et al., 2010), but not the extracellular accumulation of glutamate in primary cultures of cortical neurons (Brito et al., 2010). Conflicting results were observed for MK-801 in hippocampal neurons treated with high UCB levels (Shapiro et al., 2007), not considered clinically relevant by surpassing UCB aqueous solubility (Ostrow et al., 2003b; Zhou et al., 2010), and in Gunn rats pups, where no protection against BAEPs abnormalities was observed (Shapiro et al., 2007).

Increased expression of nNOS by upregulation at both the mRNA and the protein level and the generation of NO were shown to depend from neurotrophins (Mancuso et al., 2008).

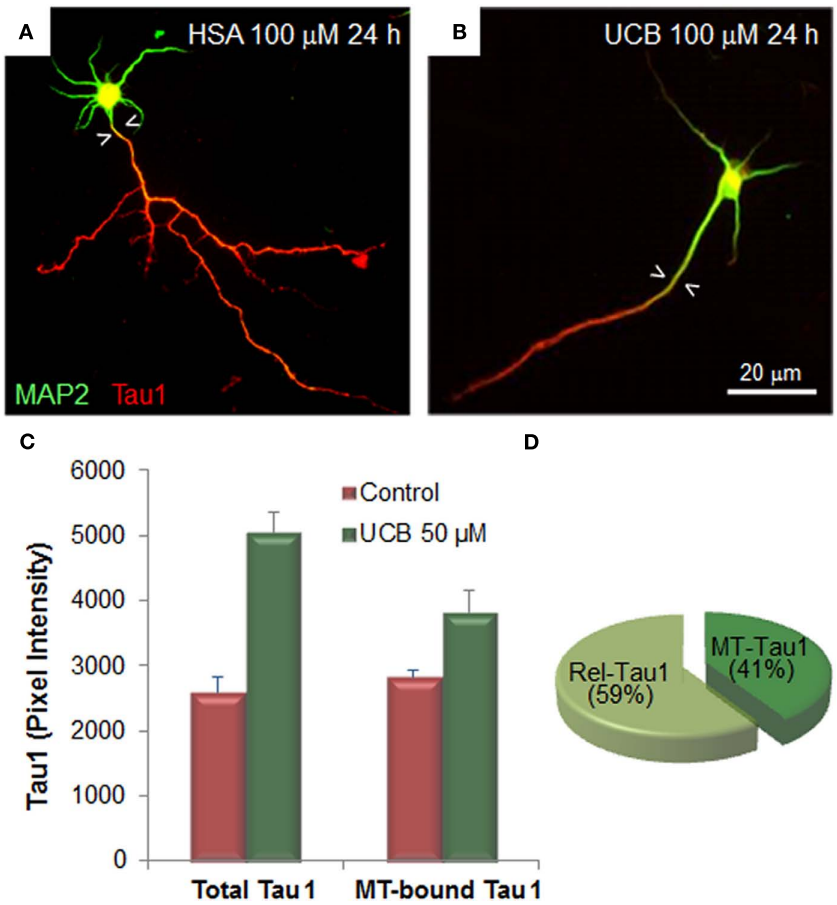

FIGURE 4 | Exposure of immature hippocampal neurons to unconjugated bilirubin (UCB) impairs neuronal arborization by reducing axonal ramification and dendritic output, while increasing microtubule stability by changing the expression and localization patterns of the microtubule (MT) associated protein 2 (MAP2) and Tau1. Embryonic hippocampal neurons were treated with vehicle (human serum albumin, HSA) alone (control) or with UCB 100 µM (UCB/HSA = 1) for 24 h at 1 day *in vitro* (DIV) and fixed at 3 DIV. Representative images of hippocampal neurons immunostained with MAP2 (green) to identify the cell body and dendrites, and Tau1 (red) to identify the axon, are shown in **(A)** for control and **(B)** for UCB. UCB induces MAP2 axonal entry, evidenced by the white arrowheads pointing to the portion of axon with MAP2 entry, and a decrease in the number of axonal and dendritic branches when compared with HSA alone. Graph bars representing total and MT-bound Tau1 are shown in **(C)** illustrating that UCB induces axonal expression of Tau1. Percentage of Rel-Tau1 and MT-Tau1 in the pie chart in **(D)** depicts that part of Tau1 will be increasingly bound to microtubules (MT-Tau) **(C,D)**, although the major portion is released (Rel-Tau), as illustrated in **(D)**. Data derived from Fernandes et al. (2009a, 2010).

These Authors have shown that 10 µM Bf reduces the formation of H_2O_2-induced ROS, contributing to the inhibition of the nerve growth factor and the brain-derived neurotrophic factor signaling to Akt and ERK1/2 decreased phosphorylation. Intriguingly, in the absence of endogenous growth factors, UCB evidenced to activate an NO-dependent cascade leading to ERK activation, actions that the same Authors hypothesize to result from cell membrane perturbation and calcium influx in PC12 cells. Therefore, UCB neurotoxicity would derive in the perspective of these Authors from the presence of neurotrophins which predominate in hippocampus and cerebellum on the perinatal life in order to promote neuronal development and differentiation. Recently, it was observed an increased expression of nNOS by UCB in hippocampal neurons, as compared to cerebellar or cortical neurons (Vaz et al., 2011a), indicating a particular susceptibility of hippocampus to nitrosative stress. Most important, acute hyperbilirubinemia by administration of sulfadimethoxine to the Gunn rat was evidenced to lead to a presynaptic failure of synaptic transmission, considered to be in the basis of UCB-induced deafness and related to the upregulation of nNOS and generation of NO. In fact, the administration of a nNOS antagonist has shown to protect the loss of auditory function (Haustein et al., 2010), highlighting the role of NO in the neurotoxic mechanisms of UCB. Additional studies are necessary to further explore the UCB-dependent neurotoxicity from growth factor availability, as proposed by Mancuso et al. (2008).

Both glutamate (Silva et al., 2012) and oxidative stress (Vaz et al., 2011a) were shown to mediate the disruption of neuronal dynamics by UCB. Interestingly this effect, similarly to nNOS induction, was also more evident in neurons from the hippocampus than in those from the cortex and the cerebellum. To that should account the decreased levels of DJ-1, a protein with a

protective role against oxidative damage (Lev et al., 2008; Kahle et al., 2009), the increased nitrosative stress, ROS production and GSSG evidenced by hippocampal neurons when treated with UCB (Vaz et al., 2011a). Similarly to other studies pointing to DJ-1 overexpression as a mechanism to protect cells from UCB-induced cell death (Deganuto et al., 2010), our data also evidenced an early upregulation effect (Vaz et al., 2011a), probably as an initial effort to diminish UCB-induced oxidative stress. However, in this last study it only lasted 4 h instead of the 24 h observed by Deganuto et al. (2010). These different results may be caused by the use of different models, in the case the human neuroblastoma cell line SH-SY5Y and not hippocampal neurons from Wistar rats as we used. The low levels of DJ-1 later observed (24 h) may result from its oxidation due to the decreased levels of GSH (Miyama et al., 2011) and degradation through proteolysis (Saeed et al., 2010). Inability to restore GSH from GSSG is related to the increased production of ROS and oxidative damage of proteins and lipids (green broken line, **Figure 2**), leading to DNA damage (Wiseman and Halliwell, 1996), which was evidenced to be produced by UCB in the neuroblastoma cell line (Deganuto et al., 2010). However, it was recently shown that a subpopulation of SH-SY5Y cells appear to survive to the initial damage of 140 nM UCB by increasing GSH content, thus becoming more resistant to oxidative stress (Giraudi et al., 2011).

Finally, even though that microglia and astrocytes are the predominant sources of pro-inflammatory cyto/chemokines, neurons can also express these factors in disease settings (Ohtori et al., 2004; Janelsins et al., 2008; Nazmi et al., 2011). Nevertheless, far smaller concentrations of tumor necrosis factor (TNF)-α than the ones produced by astrocytes were secreted from UCB-treated neurons (Falcão et al., 2006; **Figure 3D**).

Once glial cells degeneration has direct deleterious consequences on the function and survival of neurons, which will be deprived of their optimal microenvironment, UCB-induced glial damage cannot be simply considered as marginal events or secondary reactions to neuronal injury, but rather should be jointly included in the BIND processes. Therefore, in the following sections we will describe how glial cells sense and respond to UCB as important players in neuronal dysfunction and promising therapeutic targets.

GLIAL REACTIVITY AND DYSFUNCTION BY BILIRUBIN

The survival and proper function of neurons is ensured by the large number of glial cells (Streit, 2002). They have acquired a special relevance in brain injuries and include OLGs and astrocytes, as well as microglial cells which share features with immune cells. Microglia and astrocytes communicate each other through the cytokine IL-1 and ATP and are recognized as active participants in various pathological conditions, reason why their activation is associated with the development and severity of diseases. Once microglia are activated earlier than astrocytes, it is believed that they promote the activation of astrocytes (Liu et al., 2011a).

Interestingly, both astrocytes and microglia have lately been referred to be involved in "synaptic stripping" after axon lesion (Cullheim and Thams, 2007). Recently, it was observed that microglia make contacts with neuronal synapses at a rate of about one per hour with a lasting time of 5 min (Wake et al., 2009), being considered vital members of the "quadripartite synapse," which also includes presynaptic and postsynaptic neurons, astrocytes, and extracellular matrix (Sykova and Nicholson, 2008). Therefore, we should consider that microglia–neuronal cross-talk is a key point in guiding microglial activation and that the cytokines released by activated microglia influence not only neighboring neurons but also astrocytes.

Interaction of all these cell types thus fulfills brain function. *In vitro* cell models have been used to unravel specific cell dysfunction mechanisms by UCB and the intricate signaling processes comprised, as well to screen target-driven therapeutic agents, in an attempt to incorporate this heterogeneity to better understand the basis of BIND pathogenesis. In the following sections, we will describe the research that is being undertaken using specific cell populations.

ASTROCYTES ARE KEY PLAYERS IN BILIRUBIN INJURY

Astrocytes that are the most abundant type of glia (Nair et al., 2008), outnumbering neurons by over fivefold (Sofroniew and Vinters, 2010), have multiple homeostatic properties (Orellana et al., 2009). They are closely associated to neurons, and most synapses are intimately ensheathed by astrocytes processes. Astrocytes, like microglia, may have either a neuroprotective or a neurotoxic role, where the release of pro-inflammatory cytokines, glutamate, ATP, and free radicals may decrease neuronal survival and increase their susceptibility to neurotoxins. In fact, injuries in astrocyte function have been recognized as highly contributing to neuronal dysfunction (Allaman et al., 2011).

First reports on the UCB toxicity to astroglia came out in the 1960s, either in rat cerebellum cultures (Silberberg and Schutta, 1967) or in experimental kernicterus (Chen et al., 1969), in which the Authors observed swelling of the perivascular astrocyte foot and evidence of impending cell damage. Access of UCB to neurons was later proposed on the basis of a first absorption from astrocytes foot processes followed by diffusion from presynaptic astrocytes processes into the dendrites.

In primary cultures of fetal rat glial cells (Amit and Brenner, 1993), as well as in astrocytes (Falcão et al., 2006), toxic effects of UCB were noticed on cell morphology, cell viability, and mitochondrial function (**Figure 5A**). Astrocytes, similarly to neurons exhibited an age-dependent sensitivity to UCB toxic effects. Decreased expression of Mrp1 and Pgp, observed in immature astrocytes when compared to more differentiated cells (Watchko et al., 2001; Falcão et al., 2007a; Sequeira et al., 2007), surely contributes for the increased susceptibility of immature astrocytes to UCB. Moreover, induction of necrotic-like cell death by UCB was higher in astrocytes than in neurons, in immature than in mature cells, and more elevated than its own apoptosis (**Figures 3A,B**). In addition, both hypoxia and combined oxygen–glucose deprivation (OGD) followed by reoxygenation demonstrated to increase astrocyte susceptibility to UCB injury (Falcão et al., 2007b), accounting as risk factors of BIND.

Similarly to neurons, UCB determines a fast increase in the extracellular glutamate content (Fernandes et al., 2004), mainly in immature astrocytes (**Figure 3C**; Falcão et al., 2005) that appears to result from the inhibition of its uptake by UCB (Silva et al., 1999), more effective in these glial cells than in neurons (Brito

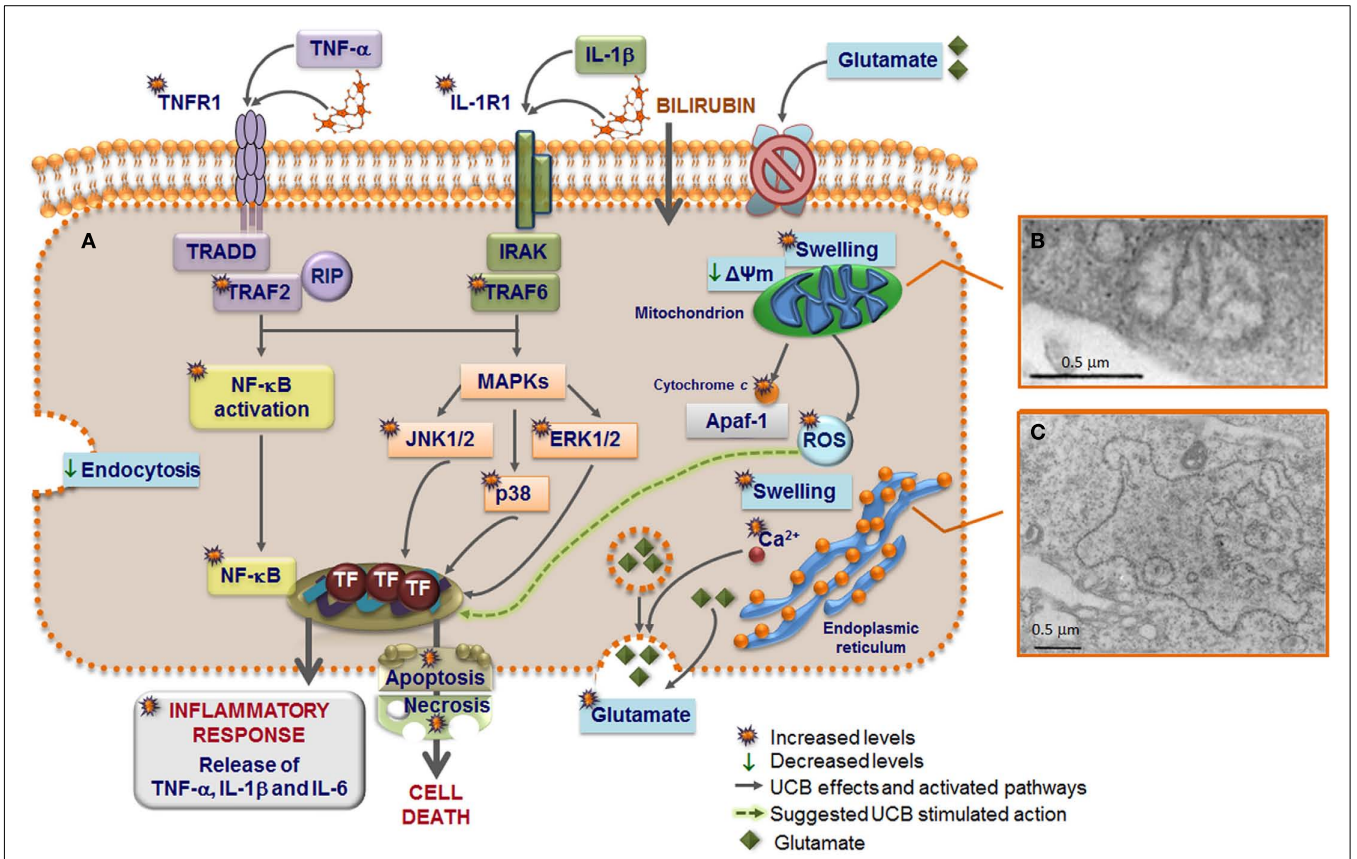

FIGURE 5 | Model of astrocytic reactivity to unconjugated bilirubin (UCB) evidencing the pathways involved in the release of pro-inflammatory cytokines and in cell demise (A) mediated by mitochondrial (B) and rough endoplasmic reticulum (ER) (C) abnormalities. UCB causes inhibition of glutamate uptake and elevation of intracellular calcium (Ca^{2+}) levels that modulate the exocytosis of glutamate, thus increasing the extracellular content of glutamate. High concentrations of UCB disrupt endocytosis in astrocytes and produce mitochondria swelling, mediated by the opening of the permeability transition megapore, and ER enlargement probably preceded by ER stress response and release of Ca^{2+}. UCB-induced apoptosis include mitochondrial permeabilization and cytochrome *c* efflux. UCB induces the secretion of both tumor necrosis factor (TNF)-α and interleukin (IL)-1β, which stimulate the synthesis of IL-6 after the first 4 h of interaction. The primary inflammatory stimulus causes a rapid and transient activation of the mitogen-activated protein kinase (MAPK) pathways that include p38, c-Jun N-terminal kinases (JNK) 1/2, and extracellular signal regulated kinases (ERK) 1/2, as well as of the nuclear factor-kappaB (NF-κB) that is translocated to the nucleus. Then, downstream transcription factors (TF) are activated and the expression of genes involved in cytokine release and cell death is induced. UCB interacts with the cytoplasmatic membrane of astrocytes inducing a rapid and sustained upregulation of both TNF-α receptor (TNFR)1 and IL-1β receptor (IL-1R)1. TNFR1 activation triggers the recruitment of the receptor-associated factor (TRAF)2, TNF receptor-associated death domain (TRADD), and receptor-interacting protein (RIP). Formation of an active IL-1R1 signaling complex mediate the recruitment of IL-1 receptor-associated kinases (IRAK) and leads to the recruitment of TRAF6 that is required for NF-κB mobilization to the nucleus and to the phosphorylation of the three MAPKs. Activation of this intracellular cascade, upon direct UCB challenge or indirectly by the increased secretion of TNF-α and IL-1β, lead to a number of astrocytic death paradigms, which cover a spectrum from apoptosis to necrosis.

et al., 2002), but also from enhanced reactive astrocyte secretion (Falcão et al., 2006), which may either occur directly from the cytosol or via regulated exocytosis (**Figure 5A**). Abnormally prolonged exposure to glutamate causes neuronal injury, and such "excitotoxicity" can be deleterious to neurons and OLGs and cause disease. Interestingly, this glutamate elicits a fast communication between astrocytes and adjacent cells in the brain (De Keyser et al., 2008) and although glutamate-induced Ca^{2+} influx can trigger DNA damage by a mitochondrial ROS-mediated mechanism, the $Ca^{2+}$was indicated to simultaneously enhance DNA repair capability, thereby protecting neurons against injury and disease (Yang et al., 2011).

To the best of our knowledge, our lab provided the first evidence that elevated concentrations of UCB trigger the secretion of TNF-α (**Figure 6A**) and IL-1β by astrocytes (**Figure 6B**; Fernandes et al., 2004) through the increase of TNF-α and IL-1β mRNA expression and activation of TNF-α-converting enzyme (TACE) and IL-1β-converting enzyme (ICE), known as caspase-1, raising the conversion of the cytokine pro-forms into active forms (Fernandes et al., 2006). Again, immature astrocytes were the main producers of TNF-α (**Figure 3D**) and IL-1β (Falcão et al., 2006).

Effects of UCB at the level of the membrane of astrocytes also include the inhibition of cell endocytosis (Silva et al., 2001a) and the upregulation of both TNF-α receptor (TNFR)1 and IL-1β receptor (IL-1R)1 (Fernandes et al., 2011), triggering the recruitment of receptors' molecular adaptors TRAF2 and TRAF6, respectively (**Figure 5**). Activation of the three mitogen-activated

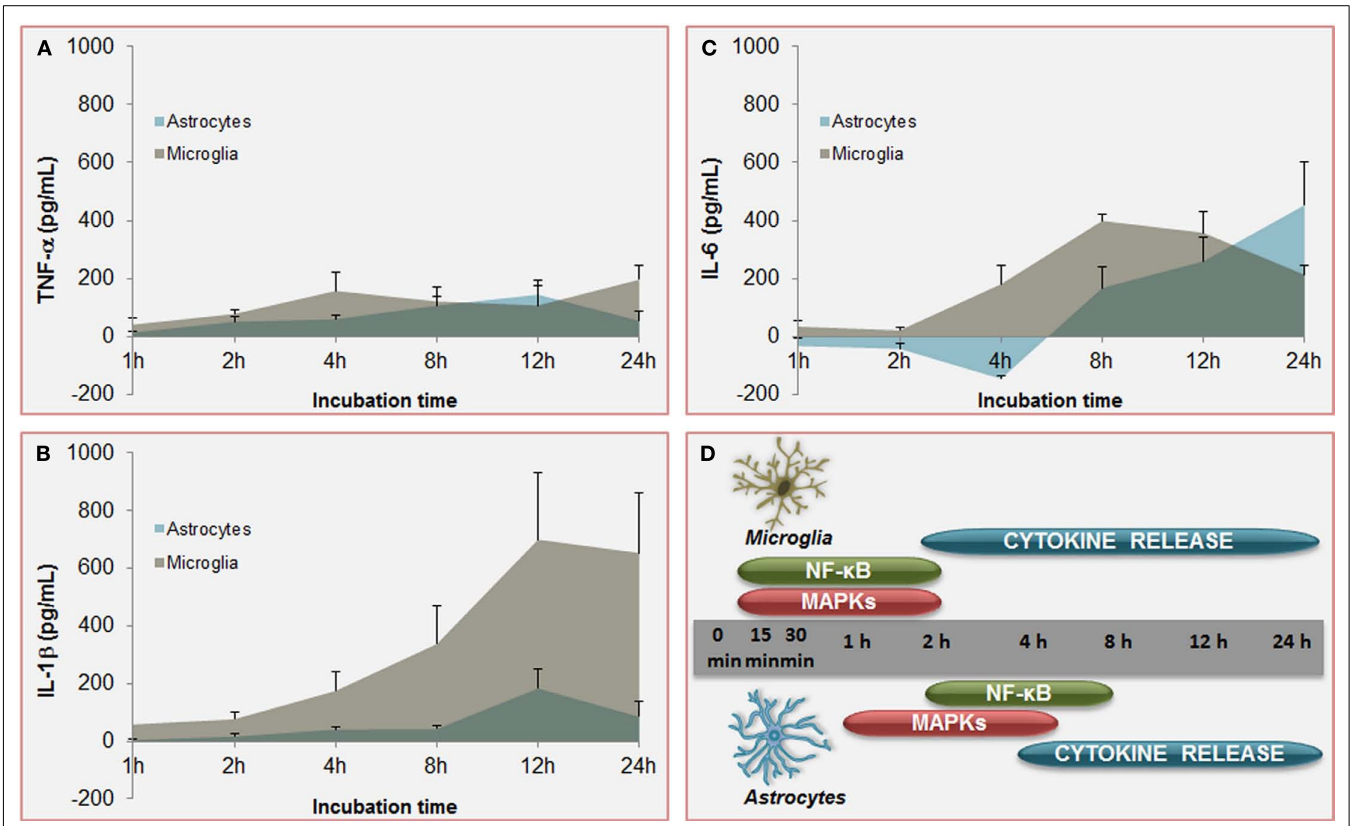

FIGURE 6 | Temporal profile of unconjugated bilirubin-induced (UCB) activation of inflammatory pathways: a comparison between microglia and astrocytes. Increased and equivalent release of tumor necrosis factor (TNF)-α **(A)** is determined by UCB on both astrocytes and microglia. However, secretion of interleukin (IL)-1β **(B)** by astrocytes is much higher in microglia and earlier produced upon UCB challenge than in astrocytes. Initial effects of UCB at the level of IL-6 release **(C)** are of opposite signals. While IL-6 production by astrocytes is depressed in the first 4 h of incubation with UCB before being enhanced, IL-6 secretion by microglia is almost immediately induced by UCB. These timely differences in the secretion of pro-inflammatory cytokines between astrocytes and microglia should reside in the former activation of nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) **(D)** in microglia than is astrocytes. These features corroborate the belief that microglia is activated earlier than astrocytes, thus promoting astrocytic activation. Data, presented as mean ± SEM, derived from Fernandes et al. (2006) and Silva et al. (2010).

protein kinases (MAPK) pathways, p38, JNK1/2, and ERK1/2 is subsequently achieved, inducing the pro-inflammatory cytokine gene expression and the release of TNF-α and IL-1β. Although IL-6 expression was initially down-regulated by UCB, it substantially increased after 6 h of incubation with UCB (**Figure 6C**; Fernandes et al., 2006). Similarly, UCB activated nuclear factor-kappaB (NF-κB) signaling pathway (**Figures 5A** and **6D**) via activation of TNF-α/TNFR1 and IL-1β/IL-1R1 cascades (Fernandes et al., 2006, 2007a).

Injury of astrocytes by high levels of UCB determines ER and mitochondria morphological changes (**Figures 5B,C**; Silva et al., 2001a), which have been related with oxidative stress and cell death in other neurological diseases (Higgins et al., 2010). Even considering the higher GSH levels and the elevated resistance to UCB-induced oxidative stress in astrocytes, as compared to neurons, depletion of GSH was still observed after incubation with Bf concentrations of 0.1 and 1 μM (Brito et al., 2008b).

Because all these aspects may contribute to the degeneration of astrocytes in brain parenchyma, compromising neuronal survival, and functional recovery after BIND, we propose that astrocytes should be considered one of the targets when searching for novel therapeutics for preventing neurological sequelae by UCB in conditions where the most used clinical approaches evidence to fail.

MICROGLIA IN THE PROCESS OF BILIRUBIN-INDUCED INFLAMMATION

Microglia reside within the CNS parenchyma, in both gray and white matter (WM), and constitute about 12% of total glial cell population (Aloisi, 2001; Ladeby et al., 2005). In the surveillant/vigilating state, previously designed as resting state, microglia through their fine processes have an elevated motility, higher than that of astrocytes, allowing them to constantly monitor their microenvironment (Davalos et al., 2005; Nimmerjahn et al., 2005). Therefore, microglia were shown to be involved in neurogenesis, postlesional tissue repair, and "synaptic stripping" (Graeber, 2010). Microglia should be considered an additional target to protect in hyperbilirubinemia because they play a major role in synaptic pruning during postnatal development, as suggested by data obtained in mice during normal brain development (Paolicelli et al., 2011). Moreover, phagocytosis of tissue debris by microglia is essential for tissue homeostasis and is considered to be associated

to a neuroprotective outcome. The first reaction of microglia toward UCB insult is the increase in phagocytosis (**Figure 7**), probably as an attempt to constrain the lesion extent, although only transiently exerted once increased levels of apoptosis were shown to follow until the 12 h of incubation (Silva et al., 2010).

However, microglia can also be activated or overactivated, depending on the stimuli, thus becoming neurotoxic (Carson et al., 2007). When activated by UCB microglia change from an elongated morphology to an amoeboid-appearance (Gordo et al., 2006) and even can dye by several pathways (Silva et al., 2010). Maximal levels for caspase-8 (extrinsic pathway), caspase-9 (intrinsic) and the effector caspase-3 were obtained at 6 h of treatment. Moreover, the cells release pro-inflammatory cytokines such as TNF-α (**Figure 6A**), IL-1β (**Figure 6B**), and IL-6 (**Figure 6C**), which parallels the alterations in cell morphology. Similarly to astrocytes, UCB activates MAPKs and NF-κB in microglia. MAPKs involvement and NF-κB engagement in UCB-challenged microglia occurs at an early time point (**Figure 6D**), and appear to underlie the phagocytic activity observed at 4 h incubation and the inflammatory phenotypes initiated at 2 h with peak levels at 4 and 24 h for TNF-α, at 8 h for IL-6, and at 12 h IL-1β (**Figure 6D**). TNF-α has shown to have a critical role in several neuropathologic disorders. Indeed, TNF-α lies at the beginning of a signal cascade that may lead to neuronal cell death, potentiates glutamate neurotoxicity, and can stimulate the production of IL-1β, IL-6, and other cytotoxic cytokines (Lee et al., 1993; Chao and Hu, 1994). Moderate upregulation of cyclooxygenase (COX)-2 and of matrix metalloproteinase (MMP)-2 and -9 activities were induced by UCB only after 12 and 24 h incubation. The most marked effect of UCB on microglia was shown to consist in the elevated levels of IL-1β what was curiously related with increased Tau phosphorylation (Li et al., 2003) and loss of neuronal integrity (Depino et al., 2005).

Microglia, similarly to neurons and astrocytes (Falcão et al., 2006), also release the cytotoxic molecule glutamate (Piani et al., 1991) when exposed to UCB (Gordo et al., 2006). The levels were found to be threefold enhanced when compared to those in astrocytes and sixfold to those in neurons (Brites et al., 2009). Release of glutamate by microglia was observed by other neurotoxins, is related to disorders of synaptic plasticity (Patrizio and Levi, 1994; Maezawa and Jin, 2010) and indicated to participate in microglial neurotoxicity (Barger and Basile, 2001; Liang et al., 2008).

What triggers increased microglial activation is still unclear but oxidative stress, resulting from increased ROS, appears to have a determinant role (Floyd and Hensley, 2002; Lu et al., 2004). As it can be seen in **Figure 7**, UCB is implicated in ROS generation by neurons. After CNS injury, microglia can rapidly populate the site of injury (Morgese et al., 1983) and migration of these cells is triggered by compounds released by the damaged tissue (Kurpius et al., 2007), such as ATP and NO (Duan et al., 2009). Actually, ATP was shown to be released from neurons treated with UCB (Vaz et al., 2010), and NO generated by OLGs (Genc et al., 2003), neurons (Vaz et al., 2011b), and microglia (Silva et al., 2012) upon exposure to UCB (**Figure 7**). Therefore, since microglial cells themselves can release ATP, it is possible that a positive feedback mechanism perpetuates migration and a gradient of NO will direct migration to the site of injury, as the stopping location for microglial cells reparative functions (Samuels et al., 2010). These include phagocytosis of cellular debris leading to the axonal sprouting and reestablishment of synaptic contacts (Morgese et al., 1983; Ngu et al., 2007). Nevertheless, microglial phagocytosis of stressed neurons also contributes to their loss. Moreover, excessive microglial production of NO induced by UCB in hippocampal slices (Silva et al., 2012) may lead to increased cell death. In fact, and in contrast to the "protective" effects exerted by microglia in preventing excessive glutamate, microglia additionally contribute to the generation of NO, an important mediator in microglia-induced neuron death (Golde et al., 2002; Graber et al., 2012). Generation of NO may derive from iNOS activation, which once activated produces moderate levels of NO chronically without any requirement for further activation (Brown, 2010), or from constitutive NOS, probably originating in the case of UCB an overproduction that may inhibit phagocytosis (Kopec and Carroll, 2000), as we observed following the 4 h of incubation (Silva et al., 2010).

Furthermore, microglia exhibit partial or complete fragmentation of cytoplasm, as well as nuclear condensation, when exposed to UCB for longer periods of time (Silva et al., 2010), thus compromising any beneficial effect that they could have in BIND protection. This feature is designed as dystrophy of microglia (Streit et al., 2008), or cytorrhexis (Fendrick et al., 2007; Hasegawa-Ishii et al., 2011) and is an indication of microglia degeneration and senescence (Streit et al., 2004).

Collectively, we may assume that the release of cytokines and, eventually other soluble products from microglia activated by damaged neurons, contributes to the initiation and maintenance of astrogliosis observed in kernicterus (Steinborn et al., 1999), and an underlying component of a diverse range of diseases and associated neuropathologies. Thus, development of targeted anti-microglial activation therapies might help to attenuate reactive astrogliosis and generate a more beneficial environment to protect brain from UCB-induced neurotoxicity.

BILIRUBIN DAMAGES MYELINATING GLIA

In the CNS, WM is primarily comprised of axonal bundles ensheathed with myelin, which reduces axonal loss. The cells forming these sheaths are the OLGs that spirally wrap segments of axons during late fetal and early postnatal stages. Just before and after birth, oligodendrocyte precursor cells (OPCs) multiply rapidly, differentiate into myelinating OLGs, and develop processes, which are then involved in the formation of myelin (Arai and Lo, 2009; Piaton et al., 2010). Reciprocal communication between neurons and OLGs are essential for myelin biogenesis and myelin repair (Piaton et al., 2010). When OPCs and OLGs are damaged there is a loss of myelin synthesis and interruption of proper axonal function and, consequently, of neuronal connectivity and function (Arai et al., 2009). However, compared to the mechanisms of neuronal injury in gray matter, WM pathophysiology remains relatively understudied and poorly understood, probably accounting for the scarce information about its influence on BIND and kernicterus.

Moderate perinatal systemic inflammation and hypoxia–ischemia, and perhaps most important, combinations of these, are related to deficient OPCs maturation, with reduced density of myelinating OLGs and disruption of the expression of several transcription factors important in OPCs maturation, thus causing

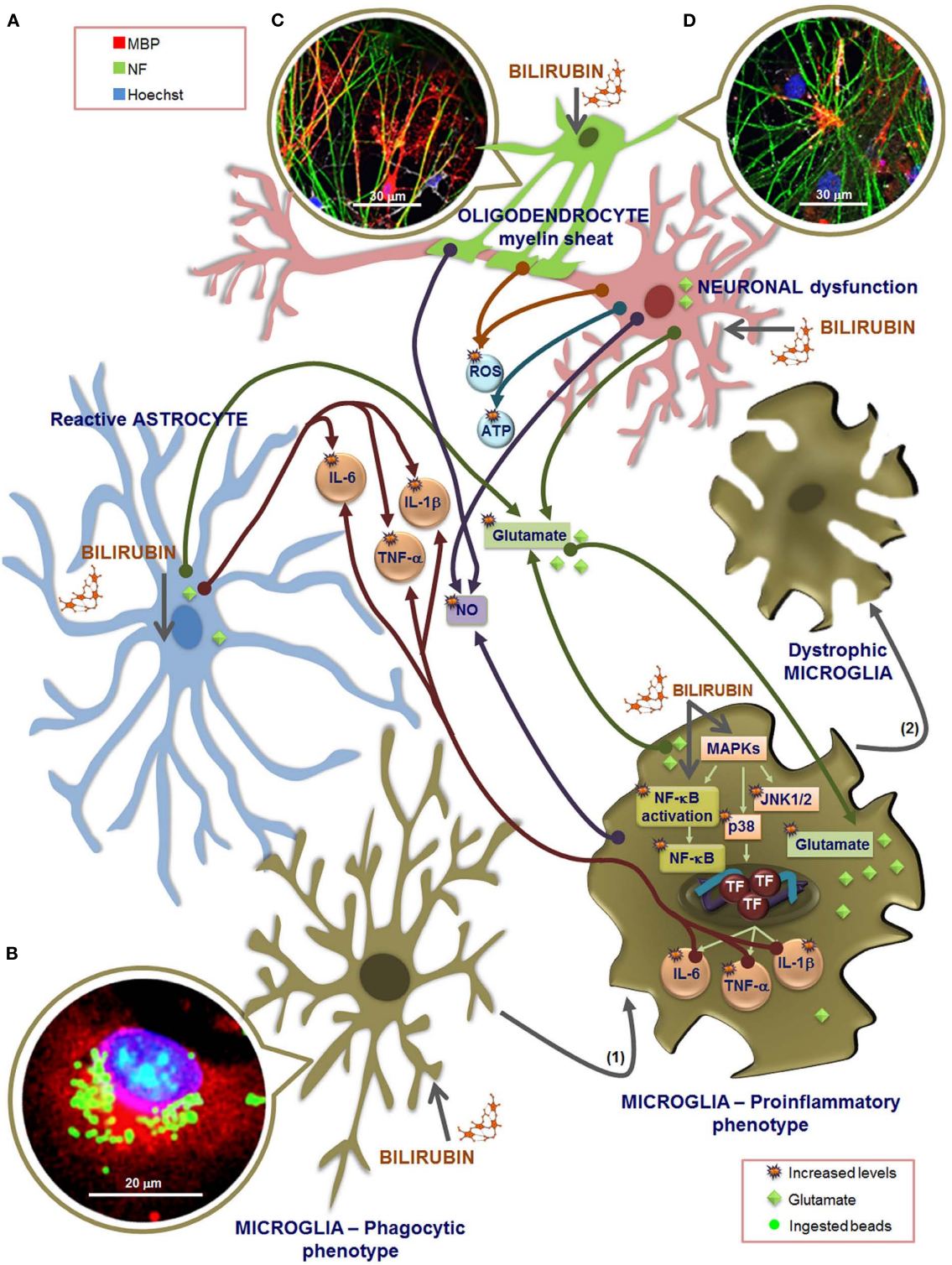

FIGURE 7 | Overview of the effectors that may be involved in microglia dialog with other glial cells and neurons, upon exposure to unconjugated bilirubin (UCB). Extracellular increased levels of glutamate by UCB-induced secretion from astrocytes, microglia, and neurons, as well as by astrocytic uptake inhibition, together with the release of inflammatory mediators, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β from astrocytes and microglia **(A)**, are included in cross-talk effectors. Also comprised are members of the oxidative stress, including the reactive oxygen species (ROS) produced by neurons and oligodendrocytes (OLGs), as well as nitrosative stress by nitric oxide (NO) generated by microglia, neurons, and OLGs, and elevated release of ATP from neurons, during exposure to UCB. **(B)** Phagocytosis upon short stimulation with UCB is visualized in microglia immunostained with an antibody against Iba1 (red) and using Hoechst 33258 stain to visualize the nucleus (blue), by counting the number of ingested fluorescent latex beads (green). Microglia intervenes in glutamate tissue

(Continued)

FIGURE 7 | Continued
homeostasis by both releasing and retaining glutamate when exposed to UCB. But microglia also reacts to UCB stimulus, very rapidly, through the activation of mitogen-activated protein kinases (MAPKs), mainly the c-Jun N-terminal kinases (JNK)1/2 and p38, and of the nuclear factor-kappaB (NF-κB) that is translocated to the nucleus, determining the activation of transcription factors (TF) and further release of cytokines. These pathways may mediate both the phagocytic and the inflammatory phenotypes and are related with a shift from an elongated morphology to a large and amoeboid shape **(1)**. Longer exposure to UCB leas to fragmented and condensed cytoplasm, a feature of dystrophic microglia **(2)** indicative of cell degeneration and senescence. The multifaceted profile of microglia is determined by the stimulus type and duration, and dialog between cells. Although microglia activation may promote astrocytic reactivity, the release of pro-inflammatory cytokines, glutamate, and NO from these cells may exacerbate microglia reactivity, which by propagating inflammation will sensitize neurons and decrease their survival upon UCB exposure. Moreover, astrocytic deregulation and degeneration by UCB determine inaccurate neuron–astrocyte interactions, causing neuritic arborization and synaptic transmission impairment, favoring brain damage. OLGs do not play a role in promoting inflammation although, like neurons, are damaged by UCB and associated inflammatory processes.**(C)** OLGs and dorsal root ganglion neurons co-cultures incubated for 24 h with 100 μM human serum albumin (HSA) at 7 days *in vitro* evidence an increased number of myelinating OLGs. **(D)** Incubation with 50 μM UCB plus 100 μM HSA reveal a delayed myelination once a number of neurofilaments are still not myelinated when compared to the image in **(C)**. To evaluate myelination, OLGs were fixed at 21 days *in vitro* and immunolabeled for myelin basic protein (MBP, red) and neurofilament (NF, green). Nuclei were counterstained with Hoechst 33258 dye (blue).

long-lasting effects (Favrais et al., 2011; Volpe et al., 2011). In these circumstances, susceptible microglia during early postnatal development may modify their functional capacity, similarly to a "primed" state, and become more susceptible to a secondary reaction by injury or to the effects of aging (Harry and Kraft, 2012).

Several years ago, it was reported that UCB adversely affects myelination in tissue cultures of rat cerebellum (Silberberg and Schutta, 1967). Binding of UCB to myelin basic protein (MBP) was later on evidenced (Gurba and Zand, 1974) and suggested to provide a mechanism for retention of UCB in the brain, while causing the inhibition of cerebellar protein synthesis by an undefined mechanism. Curiously elevated concentrations of UCB were found in the CNS myelin fraction after injection of UCB into rats (Hansen et al., 2001). Recently, by using cranial ultrasound and MRI scans, Gkoltsiou et al. (2008) observed abnormal WM signal intensity in 10/11 later scans in infants that have shown to be in risk of kernicterus, as well as delayed myelination in some of them. These authors suggested that reaction to UCB neurotoxicity in extremely immature brains may be delayed as compared to term or near term infants, determining the observation of changes only in late scans. Impairments in myelination and WM involvement in UCB encephalopathy were also observed at autopsy (Ahdab-Barmada and Moossy, 1984; Koo and Roessmann, 1988). Furthermore, separation of the myelin lamellae was visualized in a model of experimental kernicterus (Chen et al., 1971), and myelin figures surrounding vacuoles, bits of cytoplasm, and other intracytoplasmic debris were identified in the Gunn rat (Jew and Williams, 1977). In addition, a decreased density of myelinated fibers was recently evidenced in the cerebellum of a preterm infant who died from kernicterus (Brito et al., 2012).

In vitro studies have indicated that UCB is responsible for decreased OPCs (Barateiro et al., 2010) and OLGs viability (Genc et al., 2003). OPC displayed increased apoptosis (Barateiro et al., 2010), and necrosis-like cell death upon UCB exposure, mediated by early signals of ER stress followed by mitochondrial dysfunction (**Figures 8A,B**). Indeed, UCB activates the unfolded protein response in OPCs by up-regulating the expression of GRP78 and GRP94. This is not without precedent once recent reports have shown that both UCB and Bf up-regulate many genes involved in ER stress both in SH-SY5Y (Calligaris et al., 2009) and Hepa 1c1c7 cells (Oakes and Bend, 2010). Other effects produced by UCB on OPCs include intracellular Ca^{2+} overload, ROS generation and JNK activation (Barateiro et al., 2010), while NO production

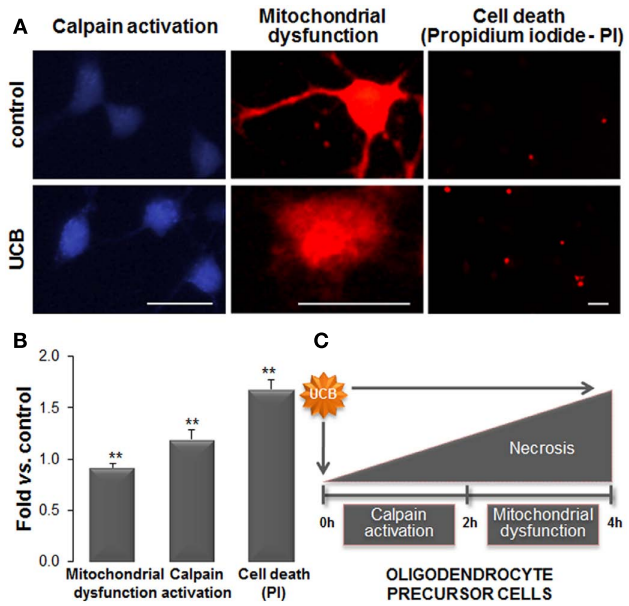

FIGURE 8 | Exposure of oligodendrocyte precursor cells (OPCs) to unconjugated bilirubin (UCB) leads to mitochondrial dysfunction, followed by calpain activation, and cell death. Primary cultures of OPCs were isolated from mixed glial cultures and stimulated to differentiate. OPCs were treated with 100 μM of human serum albumin (HSA; control) or 50 μM UCB plus 100 μM HSA for 24 h (UCB). Mitochondrial functionality was evaluated by Mitotracker Red. Endoplasmic reticulum stress was assessed by the activity of calcium-dependent calpain using a specific calpain substrate. Necrotic-like cell death was evaluated by propidium iodide (PI) through the emission of red fluorescence. **(A)** Representative results of one experiment. **(B)** Graph bars represent the fold increase in staining intensity comparatively to control (mean ± SEM) in labeled active mitochondria (red), in calpain activity (blue), and in PI^+ cells (red) from at least three independent experiments performed in duplicate. **(C)** Effects produced by UCB in OPCs start by mitochondrial dysfunction that induces the increase in intracellular calcium levels and activation of calpains. This cascade of events culminates in OPCs demise. Impairment of OPCs differentiation into oligodendrocytes by UCB will determine defective myelination. Scale bar represents 20 μm. $^{**}p < 0.01$ vs. control. Data derived from Barateiro et al. (2010).

via UCB-induced iNOS mRNA expression was observed in OLGs (Genc et al., 2003). Interestingly, no release of TNF-α, IL-1β, or glutamate could be noticed in OPCs cultured with UCB (Barateiro

et al., 2010). A consequence of the sustained high concentration of intracellular Ca^{2+} is the activation of calpains by UCB, compromising cellular specific functions, and promoting cell death (**Figure 8C**). Moreover, UCB also delays differentiation of OPCs enhancing the number of immature ($NG2^{+}$) cells and reducing that of OLGs (MBP^{+}) when compared to vehicle-treated OPCs. These findings indicate that UCB, besides compromising OPCs proliferation, may also delay myelination (**Figures 7C,D**). Therefore, hyperbilirubinemia during the early phase of developmental myelination, where an unusual synthesis rate of myelin structural proteins are necessary (Nave, 2010), may impair OPCs differentiation into OLGs triggering defective myelination and neurological damage.

Collectively, these findings indicate that UCB compromises myelinogenesis and determines degeneration and eventual loss of functional myelin sheaths, features that should be taken in consideration for BIND neuroprotective strategies, mainly in premature babies. Indeed, neuroprotection cannot be truly attained without effective oligoprotection (Arai and Lo, 2009). Thus, OPCs count among the most vulnerable cells of the CNS due to their complex differentiation program (Bradl and Lassmann, 2010), and neuroinflammation, and OLGs dysfunction were shown as a main cause of axonal loss (Edgar et al., 2010). Dysregulation of myelination pathways by UCB surely deserves further investigation.

ROLE OF NEUROINFLAMMATION IN BIND

Some past studies have evidenced that UCB inhibits the function of the polymorphonuclear leukocytes and lymphocytes (**Table 1**; Thong and Rencis, 1977; Thong et al., 1979; Iwanaga et al., 1987; Haga et al., 1996a,b). Inflammatory properties by UCB have lately been observed in a mice model of intracerebral hemorrhage (Loftspring et al., 2011) and immunotoxic effects on both bone marrow and spleen cell suspensions obtained from mice (Khan and Poduval, 2011). As previously commented for neural cells, UCB has shown to trigger intrinsic and extrinsic pathways activation, GSH depletion, p38MAPK activation, and oxidative stress in splenocytes. Production of systemic pro-inflammatory mediators ultimately activates microglia, as well as it causes neuronal apoptosis and disruption of BBB and neuroinflammation (Morandi et al., 2011; Daulatzai, 2012). This alteration disrupts the delicate balance between neuro-glial interactive components resulting in deficient neuritic arborization and synaptic transmission (Rossi and Volterra, 2009), as well as in impaired memory, neural plasticity, and neurogenesis (Yirmiya and Goshen, 2011).

As commented before, UCB is able to cause the reactivity of rat cortical astrocytes and microglia (**Table 1**), which in turn exacerbate inflammation (Sofroniew and Vinters, 2010) and increase BBB permeability by acting on endothelial cells and tight junctions (Nair et al., 2008). While the secreted levels of TNF-α (**Figure 6A**) are at the same order either in astrocytes or in microglia exposed to UCB, those of IL-1β and IL-6 are increasingly produced by microglia (**Figures 6B,C**) and earlier secreted (**Figure 6D**), suggesting that microglia participate in astrocyte reactivity by UCB and corroborating microglia faster response to stimuli (Liu et al., 2011a). Release of pro-inflammatory cytokines, as well as of ROS and NOS, can disrupt nerve terminals activity causing dysfunction and loss of synapses by UCB (Haustein et al., 2010). Therefore, UCB-induced inflammatory processes have emerged as a critical concept to understand the neurotoxic effects of UCB.

Unconjugated bilirubin-induced immunostimulation involves the activation of NF-κB signal transduction pathway in both astrocytes (Fernandes et al., 2006, 2007a, 2011) and microglia (Silva et al., 2010), one of the most important determinants of inflammatory gene expression and cytokine synthesis in glia (**Table 1**; **Figure 7**). Activation of NF-κB was mainly evidenced in immature astrocytes and increase by risk factors common in neonatal life (Falcão et al., 2007b), pointing to an added determinant for the susceptibility of preterm infants. Interestingly, activation of this transcription factor evidenced to precede MAPK activation and to be directly associated with TNF-α and IL-1β secretion, as well as with the loss of cell membrane integrity, but not the release of glutamate by astrocytes (Fernandes et al., 2006, 2007a; Brites et al., 2009). As already noticed for MAPKs activation and cytokine release, activation of NF-κB was faster in microglia than in astrocytes, as well (**Figure 6D**). It should be emphasized that activation of NF-κB, although not as markedly as in astrocytes and microglia, was also induced in neurons cultured with UCB (**Figure 2**), mainly in the immature ones (Falcão et al., 2006). However, only mild levels of IL-6, even less of TNF-α, and undetectable of IL-1β secretion were obtained under UCB exposure. Actually, NF-κB has diverse functions in the CNS, depending on the cellular context. Activation of NF-κB in neurons is implicated in axon growth, which is important for long-term memory (Kaltschmidt and Kaltschmidt, 2009; Teng and Tang, 2010). In this context, NF-κB in the synapse can be activated and transported retrogradely to the cell nucleus to regulate neural development, plasticity, and even neurogenesis (Gutierrez and Davies, 2011; Yakovleva et al., 2011). Thus, it is recognized as a major regulator of the growth and morphology of process initiation to stages when neurons are establishing functional connections and dendritic spines (Gutierrez and Davies, 2011). Collectively, these findings indicate that while NF-κB inhibition in glia might ameliorate disease, induced activation in neurons might enhance memory.

Associated conditions, such as sepsis, during, or following moderate to severe hyperbilirubinemia, are believed to contribute to BIND (Connolly and Volpe, 1990; Kaplan and Hammerman, 2005) and bacterial infection is considered a risk factor in the development of kernicterus (Pearlman et al., 1980; Yeung and Ngai, 2001; **Figure 9**). Moreover, in a Nigerian study, sepsis was the sole cause of hyperbilirubinaemia in 5% of the cases with severe neonatal jaundice (Dawodu et al., 1984). In *in-vitro* experimental conditions, addition of lipopolyssacharide (LPS) to cultures of astrocytes or neurons was shown to increase UCB-induced cell death (**Figures 3A,B**), as well as the release of TNF-α (**Figure 3D**) and IL-1β by astrocytes (Fernandes et al., 2004).

Addition of LPS to astrocytes has revealed to increase UCB-induced necrosis mainly in mature cells (**Figure 3A**), apoptosis in old ones (**Figure 3B**; Falcão et al., 2005), and the release of IL-1β (Fernandes et al., 2004) and TNF-α at all cell ages (Falcão et al., 2005). Interestingly, increased secretion of TNF-α by LPS was also evidenced in immature neurons when co-incubated with UCB (**Figure 3D**). Although LPS has previously shown to inhibit

Table 1 | Immunostimulant and immunotoxic effects of unconjugated bilirubin (UCB) in cell primary cultures, cell suspensions, and animal models.

Model	Cell	Effect	Reference
UCB	Polymorphonuclear leukocytes	↓ Bactericidal activity	(Thong and Rencis, 1977)
UCB	Polymorphonuclear leukocytes	↓ Microbicidal function	(Thong and Rencis, 1977)
UCB	Polymorphonuclear leukocytes	↓ Fungicidal capacity	(Thong et al., 1979)
UCB	Cytotoxic T lymphocytes	↓ Activity	(Haga et al., 1996a)
UCB	Lymphocytes	↓ MHC-unrestricted cytotoxicity	(Haga et al., 1996b)
UCB	Astrocytes	↑ Release of IL-1β, TNF-α, and IL-6	(Fernandes et al., 2004)
UCB	Astrocytes	↑ Activation of NF-κB and MAPK signaling cascades	(Fernandes et al., 2006, 2007a)
UCB	Microglia	↑ Release of IL-1β, TNF-α, and IL-6	(Gordo et al., 2006)
UCB	Microglia	↑ Activation of NF-κB ↑ Activation of MAPK Activation ↑ Metalloproteinases 2 and 9 ↑ COX-2	(Silva et al., 2010)
UCB + LPS vs. UCB	Astrocytes	↑ Necrosis ↑ TNF-α and IL-1β	(Fernandes et al., 2004; Falcão et al., 2005)
UCB + LPS vs. UCB	Neurons	↑ Apoptosis ↓ Neurite extension	(Falcão et al., 2007c)
UCB + TNF-α vs. UCB	Neurons	↑ Apoptosis	(Falcão et al., 2007c)
UCB + TNF-α + IL-1β vs. UCB	Neurons	↑ nNOS ↑ NO ↑ Apoptosis (caspases 3, 8, 9 and *t*Bid) ↑ P-JNK1/2 ↓ Viability	(Vaz et al., 2011b)
UCB	Bone marrow cell suspension	↑ Apoptosis ↑ Necrosis	(Khan and Poduval, 2011)
UCB	Spleen cell suspension	↑ Apoptosis ↑ Necrosis	(Khan and Poduval, 2011)
UCB injection		↓ Viability of bone marrow cells ↓ Viability of splenocytes ↓ Splenocyte proliferative response ↑ p38MAPK activation ↑ ROS ↓ GSH	(Khan and Poduval, 2011)
Mice model of intracerebral hemorrhage		↑ Edema ↑ ICAM-1 expression ↑ Neutrophil infiltration ↓ Perihematomal microglia immunoreactivity	(Loftspring et al., 2011)

MHC, major histocompatibility complex; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; NF-κB, nuclear factor-kappaB; MAPK, mitogen-activated protein kinase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; tBid, truncated BH3-interacting domain death agonist; P-JNK1/2, phospho-c-jun NH2-terminal kinase1/2; ROS, reactive oxygen species; GSH, reduced glutathione; ICAM-1, intercellular adhesion molecule-1.

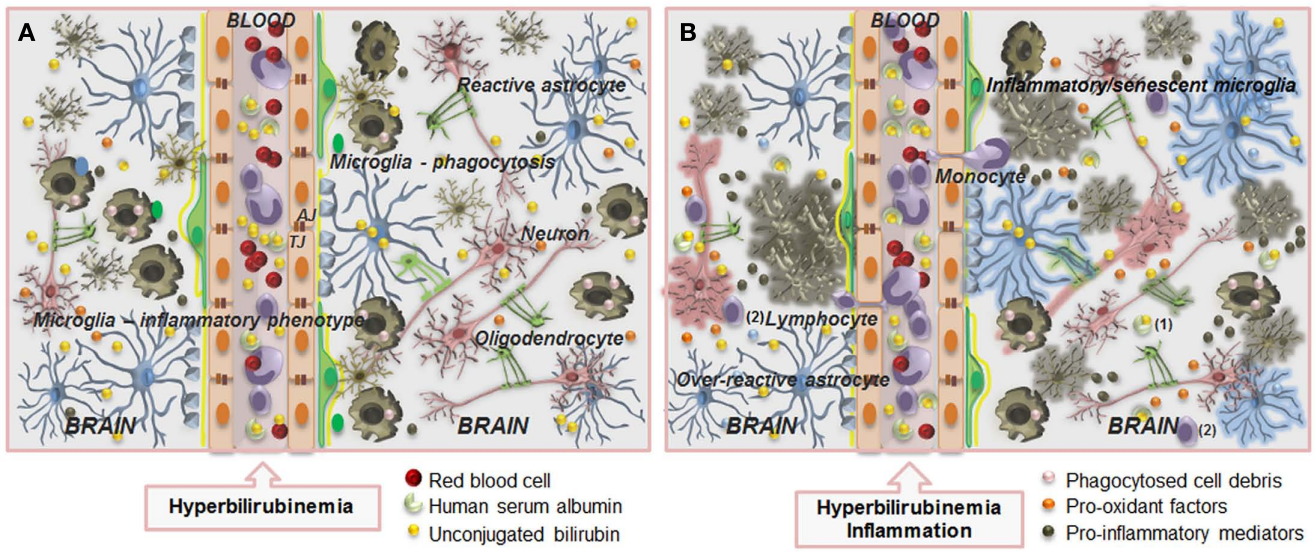

FIGURE 9 | Overview of the main alterations occurring at the blood–brain barrier (BBB) and in the interplay between neurons and glial cells during severe hyperbilirubinemia (A) and associated infection (B). The BBB is composed by endothelial cells (orange) and their tight (TJ) and adherens junctions (AJ), astrocyte end-feet (light blue spots), perivascular microglia (greenish-brown), pericytes (light-green), and the basement membrane (yellow). Circulation of unconjugated bilirubin (UCB) in the blood (yellow bullets) is provided by its binding to human serum albumin, but when the UCB levels greatly increase, interaction of unbound (free) fraction of UCB (Bf) with the BBB is enhanced, as well as its passage into the brain **(A)**, by passive or facilitated diffusion. Entrance will be also facilitated by the increased number of caveolae and caveolin-1 upregulation. Then, mainly after long-term exposure, UCB decreases zonula occludens-1 and β-catenin levels, and thus TJ strands and cell-to-cell contacts, favoring UCB passage across the BBB. Pericytes will be damaged and glutathione homeostasis disruption, together with increased nitric oxide synthase expression, nitrite production, and cytokine release will cause endothelial cell degeneration. In the brain parenchyma, elevated levels of UCB will surpass protective mechanisms (see **Figure 1**) and will cause neuron (pink) degeneration and release of pro-oxidant factors (orange bullets), microglia activation that will first phagocyte debris (pink bullets), and together with astrocytes (blue) reactivity will secrete pro-inflammatory mediators (greenish-brown bullets). In addition, decreased number of myelinating oligodendrocytes (solid-green) is also observed. **(B)** When sepsis is associated to hyperbilirubinemia, increased alterations at structural and functional levels are produced. Endothelial cell and astrocyte end-feet degeneration, modified basement membrane composition, tight junction, and transporter system impairment, vascular protein leakage, extensive extracellular edema, and activation of metalloproteinases are hallmarks of BBB impairment. Facilitated entrance of blood-borne harmful substances may accelerate neuron degeneration and activate microglia and reactive astrocytes leading to the release of cytokines further damaging neurons and detrimentally affecting vascular endothelium. Cross-talk between monocytes and endothelial cells in this inflammatory state leads to reciprocal activation of the two cell types, allowing the diapedesis of monocytes and lymphocytes. Leukocytes extravasation across the activated endothelium includes its arrest and adhesion, as well as alterations in their morphology and transmigration. Alterations in BBB dynamic properties may favor the presence of albumin carrying UCB **(1)** and increased passage of lymphocytes **(2)** into the brain parenchyma. In this condition, astrocytes become over-reactive, microglia will acquire inflammatory/senescent phenotypes and both neurons and oligodendrocytes will degenerate. Thus, therapeutic strategies directed at controlling the activation of microglia and astrocytes, and the excessive production of pro-inflammatory mediators and pro-oxidant factors may add on the prevention and recovery of neurologic sequelae resulting from unconjugated hyperbilirubinemia.

glutamate uptake (Parry et al., 2010), elevation of extracellular glutamate when neurons, astrocytes, and microglia were simultaneously treated with LPS and UCB was not sensed (**Figure 3C**; Fernandes et al., 2004; Falcão et al., 2005). The same was observed in astrocytes when co-incubated with UCB and TNF-α (Fernandes et al., 2004). However, when neurons were treated with UCB plus TNF-α + IL-1β, these cytokines were shown to have cumulative effects on the generation of NO and activation of nNOS, JNK1/2, and caspase cascades (Vaz et al., 2011b). Increased cleavage of Bid into truncated Bid (*t*Bid), as well as cytotoxic potential, was also observed (**Figure 2**; **Table 1**). These events provide a reason for the risk of sepsis in BIND and point it as a potential target for therapeutic intervention in the BIND prevention. Also remarkable was the observation that neurons previously exposed to UCB evidence an increased susceptibility to a subsequent inflammatory insult with LPS or TNF-α (Falcão et al., 2007c), suggesting that priming of neurons by UCB may result in long-lasting neurological dysfunction, as observed for early-life seizures through enhanced and persistent glial activation in adult life (Somera-Molina et al., 2007). Inflammatory priming was also shown to influence dopaminergic neurons sensitivity to subsequent environmental toxins and suggested to contribute to neurodegenerative diseases (Mangano and Hayley, 2009). Moreover, microglial priming by acute or chronic stress was shown to predispose the brain to neurodegeneration and to affect disease progression as well (Frank et al., 2012; Ramaglia et al., 2012). Microglia activation was observed in the Gunn rats at 8 weeks of age and suggested to contribute to chronic neuronal inflammation (Liaury et al., 2012) and behavioral abnormalities similar to schizophrenia (Hayashida et al., 2009). These findings may have a high relevance in the evaluation of potential lasting sequelae by unconjugated hyperbilirubinemia and surely deserve further investigation.

Prostaglandin E (PGE), also considered one mediator in neuroinflammatory processes, originates from the degradation of arachidonic acid through the sequential actions of COX-1 and COX-2, and three terminal PGE synthases (PGES) one cytosolic and two membrane associated PGES, the mPGES-1 and the mPGES-2. COX-1 is expressed constitutively at very low levels in the brain, while COX-2, the inducible isoform, is acutely expressed in several cell types after brain injury (Zhuang et al., 2003). COX-2 is induced by pro-inflammatory challenges (Font-Nieves et al., 2012) and pointed as a defensive response to the excess of glutamate, at least in neurons (Bidmon et al., 2000). LPS was shown to significantly induce PGES-1 in microglia (de Oliveira et al., 2008), as well as PGE2 release from astrocytes by the stimulation of both the constitutive and inducible COX isoforms (Pistritto et al., 1998, 1999). Intriguingly, studies in COX-deficient cells and using COX inhibitors evidenced that COX-2 mediated the production of PGE2, and COX-1 deficiency further increased PGE2 production after LPS treatment (Font-Nieves et al., 2012). In fact, the contribution of these enzymes varies accordingly with the stimuli and cell type. While UCB/HSA = 0.5 revealed to moderately increase COX-2 in microglia (Silva et al., 2010) no effects on basal PGE2 release were produced by 10 μM Bf on primary cultures of rat hypothalamic astrocytes (Mancuso et al., 1997). Nevertheless, in rat cortical astrocytes the same Bf amount was able to increase PGE2 production (Vairano et al., 2001). Since no other studies on PGE2 increased production by UCB in the presence or in the absence of HSA can be found in the literature, further studies should attest on the effects of UCB on the PGE2 production, on whether it should be taken or not as an index of inflammation, and on its invariable coupling to COX-2 activation, since LPS-induced mPGES-1 synthesis showed to not strictly be coupled to the synthesis of COX-2 in activated primary rat microglia (de Oliveira et al., 2008).

Neuronal and glial interplay may reinforce the cascade of inflammatory signaling in astrocytes and microglia interfering with the reactivity of each other and enhancing detrimental outcomes in neurons (**Figure 7**). Conditioned media obtained from either UCB-treated neurons (NCM) or UCB-treated astrocytes (ACM) revealed to be able to modulate microglia response, leading to an enhanced secretion of IL-6 while decreasing that IL-1β (Silva et al., 2011). NCM also showed to down-regulate TNF-α production by microglia, and to increase NO production and MMPs activities, thus accounting to microglia demise. In contrast, ACM protected microglia from loss of viability by UCB, in line with the property of reactive astrocytes in facilitating activation of distant microglia, while inhibiting microglial activities (Liu et al., 2011a) and preventing overactivation (Renault-Mihara et al., 2008). These results emphasize the ability of neurons and astrocytes to modulate the pro-inflammatory properties of microglia. However, evaluation of neuroprotective or neurotoxic responsiveness of cells to a certain stimulus is only possible if unrestricted dialog between neurons and glial cells is achieved, as the one allowed by organotypic-cultured slices.

Brain slice models offer unique advantages over other *in vitro* platforms in that interactions between neurons or between neurons and glial cells are fundamentally preserved replicating many aspects of the *in vivo* context (Cho et al., 2007; Lossi et al., 2009). In hippocampal slices incubated with UCB microglia showed to contribute to the release of NO, thus potentiating nitrosative stress. However, and alike astrocytes (Matute et al., 2007), microglia seem to participate in the homeostasis of glutamate by promoting its uptake (Silva et al., 2012), as a way of contributing to restoration of homeostasis (Shaked et al., 2005). In fact, microglia express the glutamate transporter 1 (Nakajima et al., 2001), thus reinforcing the neuroprotective role of microglia (see Role of Neuroinflammation in BIND). By using organotypic-cultured hippocampal slices, depleted, or not-depleted in microglia (Silva et al., 2012), the highest extracellular levels of glutamate were obtained in microglia-depleted slices, thus corroborating the participation of microglia in the homeostasis of glutamate, protecting neurons against excitotoxicity. Indeed, it was recently considered that depending on the stimuli, microglia can adopt a phenotype that facilitates rather than impairs glutamate clearance, as a way of contributing to restoration of homeostasis (Shaked et al., 2005). Nevertheless, other studies underscore glutamate's key role in microglial neurotoxicity (Barger and Basile, 2001; Liang et al., 2008) that may be related to a more "silent" microglia resulting from detrimental effects over the course of the injury upon various regulatory processes (Sargsyan et al., 2011; Harry and Kraft, 2012).

Once degeneration of glial cells has direct deleterious effects on neuronal function and survival, it will be then important to look for UCB-induced pathophysiological alterations as integrated phenomena (**Figure 7**), only achieved with organotypic slice cultures. These cultures will be ideal for looking at the mechanisms of neuroinflammation and the death of neurons. Their potential for drug discovery is substantial once microglial and astrocytic activation could be specifically targeted to preserve cross-talk between neurons and glia, constituting new strategic therapeutics to prevent BIND.

BIND: IN SEARCH OF POTENTIAL NEUROTHERAPEUTICS

Incidence of kernicterus varies from 1:30 000 to 1:100 000 births (Manning et al., 2007), but can be higher in developing countries. Excessive hyperbilirubinemia in human neonates may cause permanent dysfunction of the auditory system, as assessed with BAEPs (Rice et al., 2011). Phototherapy has been a widely and successfully used therapy to reduce the levels of UCB by transforming UCB in the skin into water-soluble isomers that are cleared without the need of liver conjugation (Stokowski, 2011). Guidelines for the management of hyperbilirubinemia in newborn infants and recommendations to use phototherapy were published by the American Academy of Pediatrics (2004) and Bhutani (2011), respectively. If phototherapy fails in reducing UCB levels, or at the readmission of very ill infants, aggressive management, as exchange transfusion (ET), is usually recommended. Administration of albumin 1 h prior to ET was shown to significantly reduce the tissue-bound UCB and to increase ET efficacy (Shahian and Moslehi, 2010; Mitra et al., 2011). However, ET is not always successful in preventing acute bilirubin encephalopathy and adverse outcome (Mukhopadhyay et al., 2010), and complications such as thrombocytopenia and seizure were indicated to be present in 21.5% of jaundiced neonates performing such intervention (Davutoglu et al., 2010). Since kernicterus continues to occur whereas it ought to be avoidable (Hansen, 2011), recognition

of new therapeutic modulation capacities as adjunctive therapies may contribute to a more consistent regimen for treatment and prevention of kernicterus.

Furthermore, once the threshold for UCB neurotoxicity is unknown, and kernicterus was only evidenced in 5% of yellow brains ensuing from jaundiced infants at autopsy, as described by Schmorl and cited by Hansen (2000b), these other cases probably resultant from moderate levels of UCB may also have harmful consequences. We speculate that neonatal hyperbilirubinemia, like perinatal infection/inflammation (Favrais et al., 2011), may disrupt the neurodevelopmental program and lead to potential lifetime effects, mainly in preterm infants, warranting an intensified search for neuroprotective actions.

Minocycline can be an effective treatment in neurological diseases associated with both oxidative stress (Zhong and Lee, 2007) and inflammation (Chu et al., 2007), by inhibiting microglia activation (Tikka et al., 2001; Suzuki et al., 2010), caspase-1 expression (Kim and Suh, 2009), and both caspase-dependent and -independent programmed cell death (Ossola et al., 2012; **Figure 10**). It also induces autophagic cell death (Liu et al., 2011b) and decreases glutamate excitotoxicity (Maier et al., 2007), NO production (Levkovitz et al., 2007), activation of NF-κB (Cai et al., 2011), formation of ROS (Schildknecht et al., 2011), MMP-9 expression (Guo et al., 2011), and the delayed responses of microglia (Yamada and Jinno, 2011). Its efficacy has been tested in Gunn rats, where it was shown to almost completely block the loss of Purkinje and granule neurons, probably by inhibiting p38 activation (Lin et al., 2005). Minocycline has a graded neuroprotective capability in acute UCB neurotoxicity induced by sulfadimethoxine in Gunn rat pups; while minocycline administration 30 min post-sulfadimethoxine totally prevented alterations in BAEPs, the minocycline 120 min post-sulfadimethoxine did not

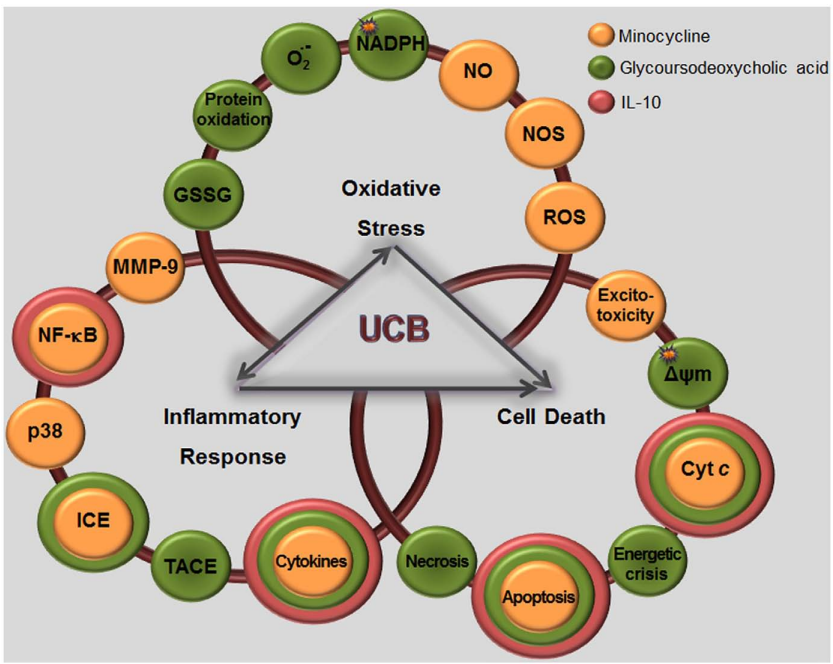

FIGURE 10 | Shematic representation of some potential neuroprotective, antioxidant, and anti-inflammatory adjunctive therapies for preventing bilirubin-induced neurological dysfunction. Given the marked oxidative stress, neuroinflammatory response, and cell death elicited by unconjugated bilirubin (UCB) in neurons and glial cells, it will be interesting to revise the potentialities of some particular antioxidants and immunomodulatory agents such as the interleukin (IL)-10, the bile acid glycoursodeoxycholic acid (GUDCA), and the antibiotic minocycline. IL-10 revealed to reduce the UCB-induced release of cytokines such as tumor necrosis factor (TNF)-α and IL-1β, as well as nuclear factor-kappaB (NF-κB) activation and translocation to the nucleus. In other conditions, IL-10 prevented apoptosis through the inhibition of the cytochrome *c* (Cyt*c*) release and of the caspase-3 cleavage, pointing to its antiapoptic properties. Neuroprotective effects of the synthetic tetracycline minocycline include: (i) cytoprotective, by decreasing glutamate and excitotoxicity; (ii) anti-apoptotic, by reducing the release of Cyt*c* and therefore apoptosis; (iii) anti-inflammatory by decreasing the activity of the metalloproteinase-9 (MMP-9), of caspase-1 or IL-1β converting enzyme (ICE) and of NF-κB, and specifically directed to UCB immunostimulation by preventing p38 phosphorylation, cytokine release, and cell death; (iv) antioxidant by reducing reactive oxygen species (ROS) formation, as well as nitric oxide synthase (NOS) expression and NO production. Concerning GUDCA benefits, it prevents the inflammatory effects of UCB by reducing both TNF-α and IL-1β release through the inhibition of TNF-α converting enzyme (TACE) and ICE inhibition. GUDCA also prevented UCB-induced energetic crisis by counter acting cytochrome *c* oxidase or complex IV inhibition, oxygen consumption, extracellular ATP increase, intracellular lactate, fructose-2,6-bisphosphate, oxidized glutathione (GSSG) increase, protein oxidation, production of superoxide anion radical ($O_2^{\bullet-}$) and NADPH reduction, as well as necrosis and apoptosis by avoiding the collapse in the mitochondrial transmembrane potential (ΔΨm) and caspase activity. Taken together, minocycline and GUDCA may prove to have additional benefits over IL-10 in preserving neural cells functionality in cases of lasting unconjugated hyperbilirubinemia, by abolishing and/or decreasing the dangerous determinants of UCB brain injury, and reestablishing the compromised ones, marked with stars. However, in what concerns minocycline, applications in clinical settings dealing with pregnant women and children are restricted and contentious.

work anymore (Rice et al., 2011). In addition, it deserves to be noticed that minocycline has several side effects such as gastrointestinal symptoms, pediatric tooth discoloration, and dizziness, among many others (Smith and Leyden, 2005). Thus, applications in clinical settings dealing with pregnant women and children are restricted and contentious.

Ursodeoxycholic acid (UDCA) is an endogenous bile acid used for the treatment of hepatobiliary disorders (Lazaridis et al., 2001). Following oral administration, it is conjugated originating tauroursodeoxycholic acid and, mostly, glycoursodeoxycholic acid (GUDCA), which acquires the highest clinical relevance (Rudolph et al., 2002). Recent data indicate that unconjugated and conjugated UDCA species have anti-apoptotic, antioxidant, and anti-inflammatory effects in nerve cells (Fernandes et al., 2007b; Solá et al., 2007; Brito et al., 2008a), pointing to their therapeutic potential for CNS disorders (Parry et al., 2010; Fonseca et al., 2012). UDCA has also shown to have beneficial effects on itching and in preventing liver failure in children with progressive familial intrahepatic cholestasis (Davit-Spraul et al., 2010), as well as in recovering liver function in patients with intrahepatic cholestasis of pregnancy, a condition with fetal poor prognosis resulting from increased transfer of bile acids from mother to fetus (Brites et al., 1998c). In this disorder, UDCA is administered until labor, decreasing the content of bile acids in the maternal serum, amniotic fluid, cord blood serum, meconium and colostrum, improving fetal prognosis and showing to be safe for the mother and babies (Brites and Rodrigues, 1998; Brites et al., 1998a,b; Brites, 2002). Thus, no secondary effects were observed in newborns from UDCA-treated mothers, which showed a better liver function than the non-treated babies (Mazzella et al., 2001). Interestingly, UDCA has shown to decrease unconjugated hyperbilirubinemia in the Gunn rat model, supporting its application in preventing BIND (Cuperus et al., 2009).

In the neurotoxic effects produced by UCB *in vitro*, UDCA revealed ability to prevent both necrosis and apoptosis in cultured rat astrocytes and neurons (**Figure 10**; Silva et al., 2001b). Experiments with GUDCA produced similar results in astrocytes (Fernandes et al., 2007b). Interestingly, GUDCA also prevented the UCB-induced release of TNF-α and IL-1β, but only reduced the TNF-α mRNA induction. Curiously, it was observed that UCB increases TACE and ICE activities, which were counteracted in the presence of GUDCA, leading to the increase of the cytokine pro-forms at the cytosol. Thus, whereas UCB promotes the maturation of the cytokine pro-forms and their consequent release, GUDCA inhibits their activation. Nevertheless, GUDCA did not modulate either glutamate release or NF-κB activation. In addition, it was observed that it prevents the decrease of GSH produced by UCB in neurons together with the oxidation of proteins (Brito et al., 2008a). GUDCA also completely precludes the inhibition of the cytochrome *c* oxidase activity by UCB, the increase of the $O_2^{\bullet -}$ production and the GSSG formation, as well as the decrease in NADPH concentration and in oxygen consumption (Vaz et al., 2010). The ability of GUDCA to prevent UCB-induced energetic crisis and mitochondria dysfunction further supports the efficacy of this compound as a potential preventive therapy for BIND.

However, despite the beneficial effects that UDCA may have in early-life as a neuroprotectant, fundamental aspects of investigation are still outstanding. Harnessing the potential of UDCA for use in neonates clearly requires further investigation to identify definitively the window of opportunity for UDCA intervention once shortcoming in that most of the studies performed have been by administration to the mother in the course of the intrahepatic cholestasis of pregnancy.

IL-10 is another anti-inflammatory compound that was tested in *in vitro* conditions mimicking BIND. It did not produce any effect in both apoptotic and necrotic cell death of astrocytes, neither in glutamate release (Fernandes et al., 2007b). In contrast, it prevented UCB-induced release of TNF-α and IL-1β by down-regulating their expression, as well as abrogated NF-κB translocation to the nucleus. In a recent study of spinal cord injury it was used a herpes simplex virus-based vector to express IL-10 in spinal cord *in vivo* (Zhou et al., 2009). In this case, the cytokine was able to inhibit cytochrome *c* release and caspase-3 cleavage, pointing to the possibility that IL-10 may present separate effects from the anti-inflammatory properties. However, the anti-apoptotic effect may derive from the suppression of glial activation as previously observed by Pang et al. (2005).

The aminoacid taurine is not only a neurotransmitter, but also a trophic factor in CNS development. It maintains the structural integrity of the membrane and regulates calcium transport and homeostasis (Wu and Prentice, 2010). Taurine was initially indicated to have benefits in stroke together with anti-inflammatory effects (Yamori et al., 2010; Sun et al., 2012). In 2010, taurine has shown to prevent the loss of neuronal viability induced by UCB, the changes in neurite outgrowth and the influx of Ca^{2+} from external microenvironment, but with limited efficacy on the release of Ca^{2+} from ER (Zhang et al., 2010). Later, Gao et al. (2011) observed the same properties in a jaundiced baby mice model established by intraperitoneal injection with UCB. When these mice were pretreated with taurine for 4 h, intracellular levels of Ca^{2+} were inhibited and down regulation of caspase-3 activity was achieved. Besides this anti-apoptotic effect, the new perspective for the use of taurine for preventing and/or treating neural damage in neonatal jaundice comes from its anti-inflammatory potential.

CONCLUSION

Although we have still a long way to envisage the complex and diverse reactivity of astrocytes and microglia to UCB, and their cross-talk with neurons in BIND conditions, in the present review we attempted to provide information on how individual cell response depends on the time of exposure to UCB, on the associated stimuli and on the presence of each type of neural cell. Receptor activation by UCB and signaling cascades followed by injury due to lipid peroxidation and protein oxidation starts at the cell membrane level. Targeted subcellular compartments by UCB are ER, mitochondria and nucleus that orchestrate cell death by apoptosis and necrosis. Alterations induced by UCB on neurogenesis, neuritogenesis, spinogenesis, and on axonal cytoskeleton dynamics are suggestive of synaptic plasticity abnormalities and lasting neurodevelopmental disabilities that should be further investigated for their relevance and priming effects. The extracellular increase in glutamate, in pro-oxidant factors, and the presence of sepsis or inflammation that further enhances the susceptibility

of neurons to UCB, even more in immature cells explain, at least in part, the vulnerability of premature infants to BIND. How temporal windows of neurodevelopment will influence and determine increased brain damage by BIND and different type or sequelae at early ages surely deserve additional studies, but preliminary data suggest that infants in the first postnatal week are more susceptible to BIND than in the second week of life. UCB-induced reactivity of microglia, although initially triggering a neuroprotective phagocytosis of damaged neurons, decreasing their number but restricting the area of damage, later it leads to the release of pro-inflammatory mediators that activate TNF-α and IL-1β receptor signaling pathways in astrocytes, culminating in an additional production of pro-inflammatory cytokines mediated by MAPKs and NF-κB activation pathways. How far is the microglia reactivity modulated by the other glial cells and neurons during unconjugated hyperbilirubinemia is just beginning to be studied. By interfering with OPCs differentiation and proliferation, UCB can directly damage myelinogenesis, although activated microglia can similarly produce the same effects, thus probably contributing to enhance the damage of UCB to OPCs. Therefore, therapeutic strategies such as GUDCA, IL-10, taurine, and minocycline directed at controlling the activation of microglia and astrocytes, and the excessive production of pro-inflammatory and pro-oxidant factors, may be valuable to control BIND and prevent long-lasting vulnerabilities to elderly and adult neurodegenerative diseases resultant from glial sensitization or priming. Further studies using the organotypic slice culture model will contribute to better identify risks, targets, and determinants of BIND and to improve our knowledge on nerve cell pathological alterations as integrated phenomena. It also will have an important role in the pharmacological assessment of novel preventive and curative approaches, as well as strategies based on new therapeutic indications. In closing, dysregulation of "neurodevelopmental programming" by moderate and high levels of UCB, enduring impacts on brain microglial function, and risk for cognitive aging disorders warrant further research that should lead to innovative treatments.

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Transport and metabolism at blood–brain interfaces and in neural cells: relevance to bilirubin-induced encephalopathy

Silvia Gazzin[1], Nathalie Strazielle[2], Claudio Tiribelli[1] and Jean-François Ghersi-Egea[3]*

[1] *Italian Liver Foundation, AREA Science Park Basovizza, Trieste, Italy*
[2] *Brain-i, Lyon, France*
[3] *Neurooncology and Neuroinflammation Team, INSERM U1028, CNRS UMR5292, Lyon Neuroscience Research Center, Lyon-1 University, Lyon, France*

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Dora Brites, University of Lisbon, Portugal
Xavier Decleves, University Paris Descartes, France

***Correspondence:**
Jean-François Ghersi-Egea, Faculté de Médecine Laennec, Neurooncology and Neuroinflammation Team, INSERM U1028, Lyon Neuroscience Research Center, 7 rue G. Paradin, 69372 Lyon Cedex 08, France.
e-mail: jean-francois.ghersi-egea@inserm.fr

Bilirubin, the end-product of heme catabolism, circulates in non-pathological plasma mostly as a protein-bound species. When bilirubin concentration builds up, the free fraction of the molecule increases. Unbound bilirubin then diffuses across blood–brain interfaces (BBIs) into the brain, where it accumulates and exerts neurotoxic effects. In this classical view of bilirubin neurotoxicity, BBIs act merely as structural barriers impeding the penetration of the pigment-bound carrier protein, and neural cells are considered as passive targets of its toxicity. Yet, the role of BBIs in the occurrence of bilirubin encephalopathy appears more complex than being simple barriers to the diffusion of bilirubin, and neural cells such as astrocytes and neurons can play an active role in controlling the balance between the neuroprotective and neurotoxic effects of bilirubin. This article reviews the emerging *in vivo* and *in vitro* data showing that transport and metabolic detoxification mechanisms at the blood–brain and blood–cerebrospinal fluid barriers may modulate bilirubin flux across both cellular interfaces, and that these protective functions can be affected in chronic unconjugated hyperbilirubinemia. Then the *in vivo* and *in vitro* arguments in favor of the physiological antioxidant function of intracerebral bilirubin are presented, as well as the potential role of transporters such as ABCC1 and metabolizing enzymes such as cytochromes P-450 in setting the cerebral cell- and structure-specific toxicity of bilirubin following hyperbilirubinemia. The relevance of these data to the pathophysiology of bilirubin-induced neurological diseases is discussed.

Keywords: ABC transporters, astrocyte, biliverdin, blood–brain barrier, choroid plexus, glutathione-*S*-transferase, OATP, UDP-glucuronosyltransferase

INTRODUCTION

Unconjugated bilirubin (UCB) is the end-product of heme catabolism, and is eliminated from the body following hepatic conjugation to glucuronic acid. When UCB clearance is inefficient, plasma UCB concentration increases markedly, and brain damage may ensue. This most frequently occurs in severe jaundice of the neonate, or in cases of severe congenital impairment of bilirubin conjugation, such as in the Crigler–Najjar syndrome. In adults, the average total plasma UCB concentration is $<20\,\mu M$. This value is higher in newborns, due to the immaturity of transport and metabolic processes in the liver, which is faced with a higher production rate. This typically results in a mild "physiological" neonatal jaundice (UCB concentrations up to $170\,\mu M$), which is not harmful to newborns and may even be beneficial to the organism owing to the antioxidant properties of the pigment (for review, see Jansen, 1999; Wennberg et al., 2009). When serum concentrations increase further, UCB may become neurotoxic, leading to clinical manifestations of encephalopathy, sometimes resulting in irreversible brain damage when jaundice is especially severe or prolonged (Jansen, 1999; Wennberg et al., 2009).

The prevalent explanation for the existence of a sharp threshold plasma concentration of UCB setting its neurotoxicity is based on the ability of UCB to bind with a high affinity to plasma proteins, mostly to albumin. The slow dissociation rate of the albumin/bilirubin complex and the short transit time of plasma in the brain do not favor the cerebral penetration of bound UCB (Robinson and Rapoport, 1986). Numerous early works (reviewed in Wennberg, 2000) demonstrated experimentally that the pigment accumulates into the brain and neurotoxicity occurs when the circulating free (unbound) UCB increases abnormally. This happens especially when plasma protein binding to UCB approaches saturation, or when UCB is dissociated from albumin by drugs competing for albumin binding sites. UCB is a dicarboxylic acid which has remarkably high pK_a values (8.1 and 8.4), and is thus mostly non-ionized at the physiological plasma pH of 7.4. The relative hydrophobicity of UCB allows it to readily permeate the cell layers that form the tight blood–brain interfaces (BBIs) by passive diffusion (Zucker et al., 1999; Vitek and Ostrow, 2009). Within the brain some regions (mainly the basal ganglia, selected brain stem areas, hippocampus, and cerebellum) show a greater sensitivity to UCB toxicity (Shapiro, 2010). UCB alters neuronal cell functions and causes cell death by mechanisms that seem to involve synaptic dysfunction, but also oxidative stress, impairment of mitochondrial oxidative phosphorylation and apoptosis (reviewed in Watchko, 2006). In this pathophysiological scheme, BBIs merely acts as structural barriers limiting the penetration of

the carrier protein, while allowing unrestricted passive diffusion of free UCB into the brain, and neural cells are considered as passive targets of UCB toxicity.

Yet, our current knowledge on transport and metabolic processes in the brain supports the concepts that (1) the implication of BBIs in the occurrence of bilirubin encephalopathy is more complex than a simple role as a diffusion barrier to carrier proteins, and (2) neural cells such as astrocytes and neurons appear to play an active role in controlling the balance between the neuroprotective and neurotoxic properties of UCB.

Relevant to the first point, BBIs may actively participate in the regulation of the cerebral bioavailability of UCB. First, the binding affinity of UCB to human serum albumin has been shown to be strongly influenced by both albumin and chloride concentrations and free UCB concentration is actually much higher than previously thought (Weisiger et al., 2001). This suggests that parameters other than the rate of diffusional influx of UCB exist at the BBIs to account for the very low physiological UCB intracerebral concentrations (Daood and Watchko, 2006; Zelenka et al., 2008) and the absence of neurotoxicity. Furthermore, the numerous transporters and detoxifying enzymes that have been described in BBIs may confer an active role to the barriers in limiting the entry of free UCB into the CNS, and/or in increasing its elimination from the CNS. In line with this latter possibility, Levine et al. (1985) reported that bilirubin efflux from brain and cerebrospinal fluid (CSF) was an important factor of UCB cerebral homeostasis. An alteration of the neuroprotective functions of the BBIs is another aspect that may have been overlooked in bilirubin-induced encephalopathy. The BBIs are key elements in establishing the cerebral homeostasis required for proper brain development and neuronal function throughout life. In addition to supplying the brain with nutrients and micronutrients (Davson and Segal, 1996; Redzic and Segal, 2004), the BBIs protect the cerebral tissue by decreasing brain exposition to numerous circulating drug and toxic substances, and by their antioxidant properties. The cells forming the BBIs are the first cerebral cells exposed to plasma free UCB, so that any impairment of their antioxidant and neuroprotective functions, especially during the postnatal period when the developing neuropil is highly sensitive to toxic insults, may contribute to the occurrence of bilirubin encephalopathy.

Relevant to the second point, UCB is present in the brain under normal conditions at nanomolar concentrations (40–50 nM in normobilirubinemic rats). UCB confers neuroprotection against oxidative stress to cultured neuronal cells at similar (20–50 nM) concentrations (Doré and Snyder, 1999), and become toxic for neuronal cells at extracellular concentrations only slightly higher (Ostrow et al., 1994). Physiologically, the main fraction of cerebral UCB derives from the peripheral degradation of hemoglobin, but the pigment is also produced within the brain by catabolism of other heme-containing proteins. Neural cells seem to possess a biochemical machinery to maintain the intracellular concentration of UCB within a range compatible with the beneficial effect of the pigment, and damage occurs only when these mechanisms are overrun by elevated UCB brain extracellular concentrations such as during chronic unconjugated hyperbilirubinemia. In this situation, the brain UCB content increases to micromolar levels throughout the brain as shown in animal models (Zelenka et al., 2008; Gazzin et al., 2012) while neuronal damage occurs with a selective topography (Shapiro, 2010; Gazzin et al., 2012). This suggests that factors other than intracerebral UCB concentration are involved in the regional selectivity of UCB toxicity.

The following sections review these complex facets of bilirubin interactions with the BBIs as well as with glial and neuronal cells (**Figure 1**). They raise some questions that still need to be solved in view to optimize the development of efficient prophylactic and therapeutic treatments to protect the brain against the accumulation of excessive levels of UCB and prevent the neurotoxic effect of the pigment.

BILIRUBIN INTERACTIONS WITH METABOLIC/TRANSPORT PROCESSES AT THE BLOOD–BRAIN INTERFACES

SPECIFIC PROPERTIES OF THE BLOOD–BRAIN AND BLOOD–CEREBROSPINAL FLUID BARRIERS

The BBIs, together with the CSF circulatory system, are responsible for the homeostasis of the cerebral extracellular fluid necessary to neuronal functions. The interface between the blood and the brain parenchyma proper, referred to as the blood–brain barrier (BBB), is located at the endothelium of the cerebral microvessels. Pericytes ensheathed in the endothelial basal membrane and astrocytic end-feet apposed to this membrane are thought to be essential for the induction of the barrier phenotype characteristic of the brain endothelium. Occasional macrophages are also associated with the vasculature. Another barrier component is the blood–CSF barrier (BCSFB), formed by the epithelium of the four choroid plexuses (CPs; **Figure 1**). Located in brain ventricles, the CPs display the highest local blood flow rate among all cerebral structures, and are responsible for the secretion of CSF. The CP–CSF system adds a degree of complexity to the mechanisms that set the cerebral bioavailability of both endogenous and exogenous bioactive compounds. The CSF circulatory pathways, the precise histological organization of these barriers and the interplay between the BBB, BCSFB, and CSF have been described elsewhere (Strazielle and Ghersi-Egea, 2000a; Ghersi-Egea et al., 2009a,b).

The molecular mechanisms that support the neuroprotective functions of BBIs are multiple. The tight junctions efficiently restrict the entry of blood-borne polar compounds into the brain, with the exception of nutrients, polypeptides, and hormones required for brain function, that are carried by selective influx transport systems. The penetration of more lipid-soluble molecules capable of diffusing across the cell membranes is limited by binding to serum albumin and influenced by transport proteins present in both barriers. These transport systems include P-glycoprotein, Breast Cancer Resistance Protein, or Multidrug-associated Resistance Proteins (MRPs)[1], all belonging to several subfamilies of ATP Binding Cassette (ABC) efflux transporters. They are also organic anion and cation transporters of the Solute Carrier (SLC) superfamily. All these systems display broad substrate specificities, and a pattern of expression specific to each barrier. Being distributed between the blood-facing and brain-facing

[1] Capitalized characters are used when referring to human transporters and enzymes or to transporters and enzymes listed in a general context. Minor characters are used when specifically referring to rat or mouse transporters.

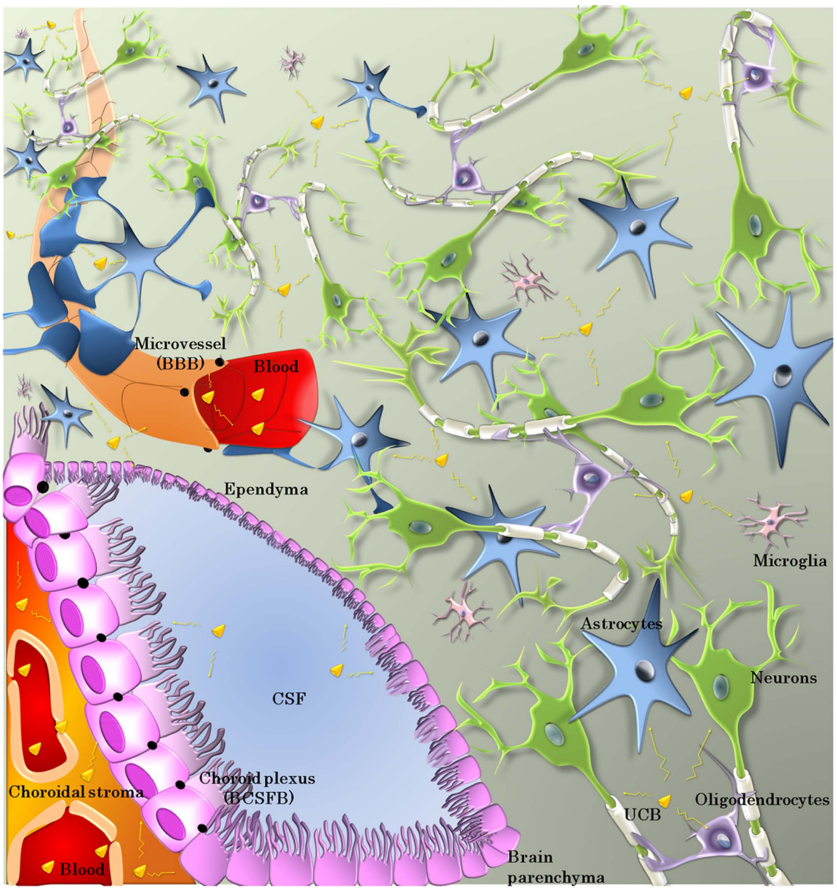

FIGURE 1 | Cellular effectors involved in the bioavailability and toxicity of UCB. Bilirubin exchanges between blood and brain occur mainly across the microvessel walls forming the blood–brain barrier (BBB) or the choroid plexus epithelium forming the blood–cerebrospinal fluid barrier (BCSFB). The cells forming these barriers are sealed by tight junctions (black dots and lines). The fenestrated choroidal vessels allow extensive exchanges between the blood and the choroidal stroma. CSF–brain exchanges take place across the ependyma, or the pia–glia limitans (not shown here). Within the neuropil, neurons are the terminal target of bilirubin toxicity. Astrocytes, microglia, and oligodendrocytes all play a role in controlling bilirubin toxicity-over-benefit balance.

membranes in both endothelial and epithelial cells, they work in concert to achieve highly efficient neuroprotection (Strazielle et al., 2004; Leslie et al., 2005; Strazielle and Ghersi-Egea, 2005; Gazzin et al., 2008). Finally, detoxifying enzymes present at BBIs can influence the cerebral availability of toxic compounds (Ghersi-Egea et al., 1994; Dauchy et al., 2008). In particular, CPs possess a high conjugation capacity based on phase II drug metabolizing enzymes such as glutathione-*S*-transferases, as well as a high epoxide hydrolase activity and antioxidant capacity, hence fulfilling "liver-like" functions within the brain (Ghersi-Egea et al., 1995, 2006; Strazielle et al., 2004).

Within the CNS, diffusion between the CSF and the parenchyma occurs more freely across the ependyma bordering the ventricles, or the pia–glia limitans separating the neurons from the subarachnoid and cisternal spaces as the cells forming these interfaces are not linked by tight junctions. However, exchanges between the different CNS compartments may be influenced by glutathione-*S*-transferases and ABC transporters, which have been described in the ependymal and glial cells (Strazielle and Ghersi-Egea, 2000b; Mercier et al., 2004; see infra).

The structural features of the barriers are established early on during brain development. The paracellular pathway is already impeded in the fetal brain, pointing to the efficiency of the BBIs in protecting the developing brain. In addition the BBIs may fulfill fence and transport functions which are specific to the immature brain (Ek et al., 2006; Johansson et al., 2008; Daneman et al., 2010). Less is known about the developmental regulation of the efflux transporters and enzymes involved in detoxification processes (see infra).

It is well established that in the liver a complex interplay between ABC efflux pumps, detoxifying enzymes, and possibly SLC transporters (**Table 1**) allows controlling the plasma UCB level. Similar interactions between UCB and these families of proteins may occur at the BBIs, hence influence UCB cerebral bioavailability.

MECHANISMS CONTROLLING BILIRUBIN PERMEABILITY AT BBIs

Transporters

Different studies (reviewed in Ghersi-Egea et al., 2009a) pointed out UCB as a substrate for various transporters. These include

Table 1 | Main potential mechanisms involved in bilirubin detoxification and elimination.

Binding (diminishes free fraction of UCB in cytosol)	Bilirubin binds to glutathione-*S*-transferases. Alpha class subunits display the highest affinity for UCB
Metabolism	UCB is conjugated to glucuronic acid. This is catalyzed by UDP-glucuronosyltransferase UGT1A1 Bilirubin is oxidized by cytochrome-P-450-dependent monooxygenases (CYP1A1, CYP1A2, CYP2A5 (Cyp2a3 in rodents) isoenzymes)
Cellular export	UCB is a high affinity substrate for MRP1 (ABCC1) UCB is a potential substrate for P-glycoprotein (ABCB1) Bilirubin glucuronoconjugates are exported by several members of the ABCC family, such as ABCC2 and ABCC3, and with less affinity ABCC11
Cellular uptake (allows further metabolism or elimination)	UCB can enter some polarized cells by a carrier-mediated process, possibly involving SLC21 (OATP) transporters

Modified from Ghersi-Egea et al. (2009a).

the ATP-dependent ABCC1 transporter which is a member of the multidrug-resistance associated protein family also called MRP1, the ATP-dependent multidrug-resistance protein ABCB1 (human)/Abcb1a/b(rodents; see text footnote 1) also called P-glycoprotein or MDR1, which displays a lower affinity than MRP1, and finally transporters of the SLC21/Organic Anion Transport Polypeptide (OATP) family[2], such as OATP1B3 (also called OATP8, SLC21A8), or OATP1B1 (OATP-C, OATP2, SLC21A6) in human.

P-glycoprotein and MRP1 are two major efflux transporters involved in neuroprotection. They prevent access to, or increase elimination from the brain of various endo- and xenobiotics (Schinkel et al., 1996; Wijnholds et al., 2000; Schinkel and Jonker, 2003). P-glycoprotein is almost exclusively located at the blood-facing luminal membrane of the endothelium forming the BBB, while MRP1 is mainly located at the basolateral membrane of the choroidal epithelium. This mirror image of the relative abundance of P-glycoprotein and MRP1 between the two interfaces was confirmed in human (Gazzin et al., 2008). Different members of the OATP family have also been localized at the BBB and BCSFB in both laboratory animals and human. For most of these OATPs, the precise cellular localization, directionality of transport and functional significance remain to be assessed (for details, see Strazielle et al., 2004; Strazielle and Ghersi-Egea, 2005; Ghersi-Egea et al., 2009a). OATP1A2 can transport opioid peptides across the human BBB (Gao et al., 2000; Lee et al., 2005), and at the BCSFB, Oatp1a5 (Oatp3, Slc21a7) located at the apical membranes of the rodent choroidal epithelium, transports organic anions from the CSF into the choroidal epithelial cells, for further efflux into the blood. All P-glycoprotein, MRP1, and those of the OATP transporters located at brain-facing membranes, are ideally localized to prevent the entry of blood-borne substrates, including UCB into the brain, or to allow their elimination from the central nervous system.

In line with this hypothesis, a role of P-glycoprotein in decreasing UCB penetration into the brain has been highlighted in Abcb1a/b knockout mice which display a cerebral UCB concentration 1.8-fold higher than wild type animals after a 5-min intravenous UCB infusion. Importantly, this treatment resulted in a high plasma total UCB that was not different between the two types of animals (Watchko et al., 1998). Brain-to-plasma bilirubin ratios were also increased in wild type rats treated with P-glycoprotein inhibitors (Hanko et al., 2003). The significance of a P-glycoprotein-dependent efflux process limiting free UCB diffusion from plasma into the neuropil remains to be assessed at lower clinically relevant UCB concentrations and in the developing brain. Indeed, a role for P-glycoprotein in the canalicular efflux of UCB in the liver could not be established, and the amount of P-glycoprotein associated to microvessels isolated from 9-day-old animals is only one-fifth of that measured in capillaries from adult animals (Gazzin et al., 2008). No study has specifically investigated the involvement of MRP1 in UCB transport across the BCSFB. A considerable uptake of UCB by isolated rabbit CP has been described, which is inhibited by iodipamide, a substrate of organic anion transport systems (Jakobson, 1991). Which OATP or other transport proteins located at the brain-facing membranes of the BCSFB are involved in the clearance of UCB from the CNS remains an open question. It is however tempting to hypothesize that the carrier-mediated uptake of UCB at the apical membrane of the choroidal epithelium, coupled to active efflux into the blood via the basolateral MRP1 pump forms a pathway accelerating the clearance of UCB from the CNS. Such mechanism would be of special interest during early postnatal life, as the CP is a brain structure maturing early during development, and already expresses high levels of MRP1 at birth (Gazzin et al., 2008; Johansson et al., 2008).

Metabolizing enzymes

In the liver, UCB interacts mainly with three families of detoxifying enzymes, cytochrome P-450-dependent mixed function oxygenases (CYPs), glutathione-*S*-transferases (GSTs), and UDP-glucuronosyltransferases (UGTs).

Unconjugated bilirubin taken up by hepatocytes binds to cytosolic isoforms of GST, which act as a sink that maintains the gradient of free UCB and limits its backflux into plasma. There are several GST classes, those best characterized in mammals being the alpha, mu, and pi classes (see Mannervik et al., 2005 for GST

[2]See Hagenbuch and Meier (2004) for review on new and old protein and gene OATP nomenclatures.

nomenclature in human, rat, and mouse). GSTs are homo- or heterodimer proteins. The GSTA (alpha class) subunits have the highest affinity for free UCB. Dimers of GSTA1 and/or GSTA2 form ligandin, the main GST isoform involved in hepatic UCB binding. This binding is independent of the enzymatic activity (bilirubin is not conjugated to glutathione), and contributes to protect the cells by maintaining the intracellular pool of free UCB at low level. It may also facilitate UCB transfer to the smooth endoplasmic reticulum for further catabolism. The three main classes of cytosolic GSTs of all three alpha, mu, and pi classes are present in BBIs, although GST pi rather than GST alpha seems to be the major GST proteins in adult human brain capillaries (Shawahna et al., 2011). GST-dependent activities are especially high in CPs isolated from both animal and human developing brain (reviewed in Ghersi-Egea et al., 2006, 2009a). Thus, in the course of UCB transport across BBI cells, binding of UCB to GST subunits, especially of the alpha class, may contribute to an overall barrier effect. The developmental profile of GST proteins at the interfaces differs between the pi, mu, and alpha classes. In rat, transcripts of mu and pi subunits are higher in CPs from newborn compared to adult animals, while those of the alpha subunits which display the highest affinity for UCB are absent or down regulated in newborn pups (unpublished data). The alpha class subunits were detectable by immunohistochemistry only 10 days after birth in mouse choroidal cells (Beiswanger et al., 1995), or in adult but not fetal CP in human (Carder et al., 1990). Hence, at CSF-contacting interfaces, UCB binding to GST subunits is likely to be less efficient during development than at the adult stage.

Bilirubin metabolic pathways include oxidation by CYPs, namely CYP1A1 and CYP1A2 (Kapitulnik and Gonzalez, 1993), as well as CYP2A5 (Abu-Bakar et al., 2005; Cyp2a3 in rodents). Activities of CYP isoenzymes appear modest in cells forming the BBIs (Strazielle and Ghersi-Egea, 2000b; Miksys and Tyndale, 2002). Total cytochrome P-450 has been detected by spectrophotometry in microvessels isolated from both rat and human brains (Ghersi-Egea et al., 1993). Gene and protein expression studies conducted in human microvessels showed that CYP1B1 was the main endothelial CYP, while CYP1A1 and 2 expression levels were low or undetected (Dauchy et al., 2008; Shawahna et al., 2011). Cyp1a1 protein has been detected in cultured mouse brain endothelial cells only after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, an inducer acting via the Ah receptor (Filbrandt et al., 2004). Cyp1a1 mRNA was detected in isolated rat capillaries only following *in vivo* treatment with Ah receptor-dependent inducers but the Cyp1a1 protein was not detected (Jacob et al., 2011). Another study performed by Western blot detected the protein in brain microvessels following Ah receptor activation (Wang et al., 2011). This apparent discrepancy may have come from differences in the composition of the microvessels preparations between the two studies. Indeed previous *in vivo* works had shown that Cyp1a1 was detected in mouse cerebral veins rather than capillaries following treatment with Ah receptor-dependent inducers. Induced Cyp1a1 was also detected in rat and mouse choroidal sinusoidal vessels (Brittebo, 1994; Morse et al., 1998; Dey et al., 1999; Granberg et al., 2003). No information is available concerning CYP2A5 in brain barriers. Overall, CYP activities likely play only a minor role in UCB metabolism at BBIs, unless specific inductive mechanisms occur in the course of hyperbilirubinemia (see infra).

The main pathway of bilirubin detoxification in the liver is conjugation with glucuronic acid. It is specifically catalyzed by the UGT1A1 isoenzyme (Kadakol et al., 2000). This pathway leads to the production of the more water soluble mono and di-glucuronide metabolites which are mainly transported from the liver into the bile by ABCC1, 2, and 3 proteins (Keppler, 2011). In the rat brain, Ugt activity toward planar compounds is several times higher in microvessels than in the cerebral parenchyma, and is notably comparable to the hepatic activity in the choroidal epithelium, even in developing animals (Ghersi-Egea et al., 1994; Strazielle and Ghersi-Egea, 1999). In both barriers these activities increase following *in vivo* treatment of rats with exogenous inducers (Ghersi-Egea et al., 1988; Leininger-Muller et al., 1994). The UGT isoenzyme responsible in rat BBIs for the conjugation of planar compounds is likely to be Ugt1a6 (Gradinaru et al., 2009). Ugt1a1 mRNA has been reported in whole rat brain extract (Shelby et al., 2003), but it is not known whether Ugt1a1 is associated with BBIs in rodents. Neither UGT1A6 nor UGT1A1 mRNA was detected in human brain capillaries (Shawahna et al., 2011). As the CPs fulfill "liver-like" functions for the brain, and as the conjugation to glucuronic acid within the epithelial cells, coupled to a basolateral efflux of the resulting glucuronides forms a functional metabolic barrier preventing the entry of selected substrates into the CSF (Strazielle and Ghersi-Egea, 1999), the choroidal tissue might prove to be an important site of UCB detoxification in the brain. This and the presence of different ABCC proteins located in both brains barriers (reviewed in Ghersi-Egea et al., 2009a), call for more investigations on UCB and bilirubin conjugate disposition at the BBIs.

CSF proteins

The protein concentration is of 3.2 g/l in CSF at birth in rat and decreases thereafter to reach adult values as low as 200 mg/l. This is not due to an immaturity of the BBIs allowing non-specific leakage of plasma proteins into the brain, but rather to a regulated mechanism involving a specialized transport function of the BCSFB during development (Johansson et al., 2008). This opens new fields of investigation to appreciate the relevance of CSF and ICF proteins in sequestering UCB within the extracellular space in the brain during early postnatal development, and to decipher the choroidal mechanisms that control protein CSF concentration in normal and jaundiced brain.

Photoisomers

Finally, the BBIs may play a significant role in the mechanisms behind the efficacy of phototherapy, a treatment degrading the light sensitive plasma UCB in jaundiced children. During phototherapy, both photooxidation and photoisomerization of UCB occur. While the former is a slow process, photoisomers are detected in blood within minutes after starting the treatment, and although the reaction is reversible, the major 4Z, 15E photoisomer represents 20–30% of total bilirubin after 2 h. Total bilirubin concentration is not affected as rapidly in cases of phototherapy in jaundiced newborn, and does not decrease in blood during the 4-h following treatment initiation (Mreihil et al., 2010). Yet, because

the isomers have lower lipid solubility relative to the parent UCB species, and should be restricted to a greater extent by the BBIs, an immediate phototherapy benefit should be achieved before UCB plasma levels are normalized. This hypothesis remains to be proven experimentally (McDonagh, 2010).

ALTERATION OF THE NEUROPROTECTIVE FUNCTIONS OF BBIs BY HYPERBILIRUBINEMIA

At low concentration UCB behaves as an antioxidant agent and may represent a transitional antioxidative mechanism during physiological jaundice (see infra). However, higher concentrations of bilirubin are harmful. *In vitro* studies have shown that a prolonged exposure of cells to free bilirubin at concentrations that were within the range of aqueous solubility, i.e., similar to plasma free bilirubin concentrations reached in moderate jaundice, elicited cytotoxic effects toward different cell types (Chuniaud et al., 1996 and reviewed in Ostrow et al., 2002). The molecular mechanisms of UCB cytotoxicity, assessed mostly *in vitro*, are not fully unraveled. Besides neuron-specific mechanisms, key events in UCB-induced toxicity would involve both oxidative injury and mitochondrial damage. The latter is probably triggered by bilirubin-membrane interactions and leads to an impairment of oxidative phosphorylation (reviewed in Watchko, 2006; Ghersi-Egea et al., 2009a).

This mechanism is especially relevant when evaluating the role of BBIs in bilirubin cerebral toxicity, because the cells forming the BBIs are the first cerebral cells exposed to blood-borne free bilirubin, and many of the key functions of the barriers are energy-dependent. Mitochondria are numerous in cells forming the BBIs, especially the choroidal epithelial cells (Schlosshauer, 1993; Cornford et al., 1997). Tight junction protein complexes undergo continuous molecular remodeling, which is linked to the energy status of the cells (Eckert and Fleming, 2008; Shen et al., 2008). ABC efflux transporters involved in neuroprotection are unidirectional, and require ATP-dependent energy to transport substrates against the concentration gradient. Several other organic anion and cation transporters are secondarily dependent on inorganic anion gradients generated by the Na^+K^+ATPase, as demonstrated at the BCSFB (Pritchard et al., 1999). Finally, at the CP, CSF secretion is also a tightly controlled process linked to Na^+K^+ATPase.

These different BBI-related neuroprotective mechanisms have hardly been evaluated in the context of bilirubin encephalopathy. Experimental hyperbilirubinemia obtained by short-term continuous infusion of bilirubin does not lead to any prominent alteration of the barrier integrity (Roger et al., 1993, 1995). Alterations secondary to mitochondrial dysfunction are likely to develop only after sustained exposure to elevated levels of UCB. Accordingly, a decrease in the expression of occludin, one of the proteins forming the tight junctions in BBB and BCSFB, was observed in microvessel fractions prepared from brains of rats subjected to obstructive jaundice for several days. The direct involvement of bilirubin in this process was however not evaluated (Faropoulos et al., 2010). Exposition of bovine brain endothelial cells in culture to UCB concentrations generating high levels of extracellular free bilirubin-induced cell apoptosis (Akin et al., 2002). A similar increase in apoptotic/necrotic processes was more recently described in an immortalized cell line and in primary cultures of human brain endothelial cells exposed for several hours to lower and more physiopathologically relevant free bilirubin concentrations (Palmela et al., 2011). In this study, the cell loss in bilirubin-treated cultures was twice that in untreated cells after 24 h. The relevance of these *in vitro* data to *in vivo* UCB–induced brain endothelial cell lethality remains to be established. In these cells, bilirubin only marginally affected inflammatory mediators, and induced an oxidative stress reflected by a large increase in nitrite production by comparison with untreated cells. The effect of such UCB-induced alteration of the redox status on the barrier properties of the endothelial cell monolayers was not assessed. The potential influence of bilirubin-activated perivascular astrocytes and macrophages on BBB properties would also deserve attention.

The effect of UCB on the BCSFB has been investigated using monolayers of rat choroidal epithelial cells cultured in bicameral devices that maintain the barrier and transport properties of this barrier (Strazielle and Ghersi-Egea, 1999; Strazielle and Preston, 2003; Ghersi-Egea et al., 2006). The cells were exposed at their basolateral (blood-side) membrane to 40 and 140 nM free bilirubin, concentrations respectively below and above the aqueous solubility of the pigment and which mimic levels evaluated in physiological and pathological jaundices, respectively (Rigato et al., 2005; Ahlfors et al., 2009). Treatment was applied for six consecutive days to approach the *in vivo* prolonged postnatal exposure. This treatment did not alter the integrity of the barrier, as assessed by the measurement of the paracellular permeability of the monolayers to sucrose (Gazzin et al., 2011). The high antioxidant capacity of the choroidal tissue (Tayarani et al., 1989; Ghersi-Egea et al., 2006), in concert with efflux transporters, may allow maintaining the integrity of the barrier under pathophysiologically relevant UCB-challenge.

By contrast, ABC transporters are affected by sustained UCB exposure. The homozygous hyperbilirubinemic jj Gunn rat bears a congenital inherited deficiency of the hepatic UDP-glucuronosyltransferase enzyme and is used as an animal model of Crigler–Najjar type I syndrome and neonatal jaundice. P-glycoprotein expression is increased up to twofold in brain microvessels from developing jj Gunn rats as compared to heterozygous littermates; however, it does not reach the high levels observed in microvessels of adult animals. More strikingly, the level of MRP1 protein, already high in CPs of newborn animals, is decreased by 50% in jj Gunn rats compared to heterozygous littermates. This decrease in protein content was a direct effect of bilirubin, because it could be reproduced by treating choroidal epithelial cells for 6 days with UCB at a dose generating a 140-nM free bilirubin concentration in the medium (Gazzin et al., 2011), the latter concentration mimicking the levels measured in pathological jaundice in Gunn rats 9 days after birth (Gazzin et al., personal communication). Mrp1 is involved in regulating the cerebral availability of endogenous biologically active compounds and of circulating potentially toxic xenobiotics. Hence the UCB-induced decrease in the efflux transport activity of choroidal MRP1 may critically impact on normal brain maturation.

Finally, the CPs strongly participate to thyroid hormone homeostasis which is crucial for brain maturation (reviewed in Zheng, 2001; Ghersi-Egea et al., 2009a). UCB competitively inhibits

cellular uptake of thyroid hormones in hepatocytes (Lim et al., 1993; Hennemann et al., 2001). Whether hyperbilirubinemia also disturbs cerebral thyroid hormone transport and homeostasis needs to be assessed in jaundiced neonates.

CONTROL OF BILIRUBIN INTRACELLULAR CONCENTRATION AND TOXICITY IN THE NEUROPIL

ANTIOXIDANT FUNCTION OF BILIRUBIN: THE BILIVERDIN/BILIRUBIN REDOX CYCLE IN NEURAL CELLS

Among all body organs, the brain shows the highest rate of oxygen metabolism, consuming 20% of the whole body oxygen while weighting only 2% of the total body (Clarke and Sokoloff, 1999). Oxygen reactive species (ROS) are continuously generated at a high rate, and oxidative stress does occur to a certain degree in the absence of any pathology. ROS may damage sugars, proteins, DNA, and lipids. The brain is rich in unsaturated fatty acids which are main targets for lipid peroxidation. Relevant to perinatal diseases, birth is accompanied by a sudden exposition to oxygen, leading to an increased oxidative stress. In premature newborns, antioxidant defenses are not fully efficient because their maturation occurs during the late gestation period (Saugstad, 1989; Friel et al., 2004). Thus newborns and especially premature infants exhibit a unique sensitivity to oxidant injury.

The brain possesses only moderate antioxidant superoxide dismutase and glutathione peroxidase activities compared to the liver (Cooper et al., 1997; Ho et al., 1997), with the exception of the CP (Tayarani et al., 1989). Besides the reduced/oxidized glutathione redox cycle functioning as a cell antioxidant mechanism, the bilirubin-dependent redox cycle also seems to play a role in cell protection against oxidative stress in brain, for the following reasons.

Bilirubin is present in brain tissue under normal conditions in nanomolar concentrations (20–50 nM; Gazzin et al., 2012). The dynamic of bilirubin movement between extracellular and intracellular compartment is complex, and may involve both transporter-mediated processes and diffusion. Hence data on UCB concentration in tissue do not inform on the actual intracellular concentration in different neural cells. Bilirubin concentrations in the overall brain represent less than 0.1% of the level of the antioxidant molecule glutathione (Baranano et al., 2002). Nevertheless the pigment ability to protect against oxidative stress is well acknowledged (Dennery et al., 1995; Dohi et al., 2005; Vitek, 2005a,b), and evidence that this holds true for brain cells has been documented. Bilirubin is the reduction product of biliverdin. The biliverdin/bilirubin and glutathione redox cycles share some similarities. Biliverdin synthesis results from the degradation of heme-containing proteins by heme oxygenases (HO). The inducible HO-1 is expressed in glial cells (Dwyer et al., 1995) and is induced by oxidative stress (Calabrese et al., 2006). The constitutive HO-2 accounts for the main portion of this enzymatic activity in brain, where it seems to be expressed in neuronal populations in several regions (Ewing and Maines, 1997; Mancuso, 2004). HO-2 impairment results in a loss of bilirubin in cells and a higher susceptibility to different CNS damages (Chen et al., 2005). Doré et al. (1999) described a correlation between an activation of HO-2 by protein kinase C-mediated phosphorylation, increased cellular bilirubin levels (sixfolds after 24 h), and resistance to H_2O_2 induced stress in cultured hippocampal and cortex neurons.

Biliverdin is reduced to bilirubin by the cytosolic enzyme biliverdin reductase (BVR), the depletion of which strongly induces (20–40%) the apoptosis of cells cultured from hippocampal/cortical structures (Sedlak et al., 2009). BVR activity requires free SH groups and NADH or NADPH (at pH of 6.8 and 8.7, respectively; Maines and Trakshel, 1993). BVR pattern of expression in normal brain coincides with HO gene expression (Ewing et al., 1993). BVR is also involved in cell signaling (Kapitulnik and Maines, 2009), and can transport the transcription factor hematin from the cytoplasm to the nucleus, thereby allowing hematin-dependent HO-1 gene transcription (Tudor et al., 2008). Silencing BVR leads to a depletion of cellular bilirubin, increases cellular ROS and promotes apoptotic death in neuronal cultures (Baranano et al., 2002). Finally BVR can increase bilirubin production from heme degradation during oxidative stress (Miralem et al., 2005). In turn, the increase in UCB inhibits BVR and HO activity, maintaining a balanced intracellular biliverdin/bilirubin ratio (Maines, 2005). When produced intracellularly, bilirubin may act as ROS scavenger by quenching reactive radicals (Nag et al., 2009) before being reoxidized to biliverdin (Baranano et al., 2002). In brain homogenate, biliverdin, and bilirubin have been reported to inhibit lipid peroxidation induced by hemoglobin. This was not observed *in vivo* after intrastriatal injection of hemoglobin, probably because of its conversion into biliverdin and bilirubin by the endogenous enzymatic machinery (Van Bergen et al., 1999).

Finally, approaches using bilirubin antioxidant properties for therapeutic purposes have been evaluated experimentally. The administration of exogenous biliverdin (immediately converting into bilirubin) to rat following transient cerebral artery occlusion, significantly reduced the volume of infarct tissue as well as superoxide production and lipid peroxidation (Deguchi et al., 2008). In autoimmune encephalomyelitis, an animal model for multiple sclerosis, treatment with BVR (10 μg/day) ameliorates the outcome of the pathology more efficiently than optimal treatments with catalase or HO-1, while treatments with superoxide dismutase and glutathione reductase have no significant effect (Liu et al., 2006).

Overall, these sets of data indicate that biliverdin production and the biliverdin/bilirubin cycle are effective in the brain, and can play a role in the regulation of oxidative stress. The huge capacity of bilirubin to protect against oxidative stress (10 nM UCB protects against 75 μM H_2O_2 in cell culture, Doré and Snyder, 1999), seems to result from the high turn-over rate of the biliverdin/bilirubin cycle (Baranano et al., 2002) by comparison to the glutathione redox system (Hollensworth et al., 2000). The two redox systems appear complementary. Unlike glutathione, bilirubin binds avidly to albumin and to cell membranes, especially myelin rich membranes (Mustafa and King, 1970; Brodersen, 1981), where it may prevent lipid peroxidation and protect the proteins inside the bilayer (Van Bergen et al., 1999). Reduced glutathione and bilirubin have thus been hypothesized to be complementary in their effects against oxidative stress. Reduced glutathione may serve as a protectant for cytoplasmic ROS targets, while bilirubin acts preferentially against lipid peroxidation. This was demonstrated in cultured HEK293 cells (Sedlak et al., 2009). The sixfold increase in

protein oxidation in H_2O_2 treated cells compared to control cells was reversed by addition of reduced glutathione but not bilirubin, while a 2.5- to 3-fold increase in lipid peroxidation was obtained by silencing BVR activity, but not by BSO-induced glutathione depletion. A confirmation of this hypothesis was obtained *in vivo* by comparing wild type and HO-2 deleted mice (Sedlak et al., 2009).

Besides complementing each other, the glutathione redox cycle and the biliverdin/bilirubin cycle undergo complex interactions. On one hand, the reduced/oxidized glutathione equilibrium acts as a sensor for redox stress. Reduced glutathione depletion activates HO-1 gene transcription, probably by mitogen-activated protein kinases, and thereby could favor the bilirubin/biliverdin cycle. On the other hand, molecules bearing free thiol groups are necessary to BVR activity. Thus a decrease in the concentration of reduced glutathione and other molecules bearing SH groups might impact on the efficacy of the bilirubin/biliverdin cycle.

A threshold UCB concentration sets the switch between anti- and pro-oxidant effects of bilirubin. Doré and Snyder (1999) reported that its maximal neuroprotective effects in hippocampal cultures was reached at nanomolar concentrations (10–50 nM), while at higher pigment concentrations the pro-oxidant effects of bilirubin started to dominate. A similar dual effect was reported in primary cultures of oligodendrocytes (Liu et al., 2003). The exact concentration thresholds between anti- and pro-oxidant effects of bilirubin in different brain structures and cells *in vivo* are however not well estimated, and need further investigation.

METABOLIC/TRANSPORT PROCESSES CONTROLLING BILIRUBIN CONCENTRATION AND TOXICITY IN GLIAL AND NEURONAL CELLS

The pro-oxidant activity of UCB is probably the most studied aspect of bilirubin encephalopathy and kernicterus in which UCB content in tissue increases from protective nanomolar to toxic micromolar concentrations, mainly as a result of UCB influx from blood (Zelenka et al., 2008; Gazzin et al., 2012). Neural cells possess regulatory mechanisms that allow controlling UCB intracellular concentrations to some extent. Even if expressed at lower levels than in BBI (see supra), transporters, and detoxifying enzymes such as those described in **Table 1** might act also at the brain parenchyma level.

Among the two ABC proteins that transport bilirubin, P-glycoprotein level of expression is at least 20 times lower in whole brain parenchyma compared to cerebral capillaries, indicating that P-glycoprotein is almost exclusively located at the BBB (Gazzin et al., 2008) and is unlikely to play a role in bilirubin transport in neural cells. Of note, P-glycoprotein has been identified in cultured microglia (Lee et al., 2001) and astrocytes (Ronaldson et al., 2004). The high affinity bilirubin transporter Mrp1 (Rigato et al., 2004) is present in rat brain parenchyma, where the protein content represents 6% of that measured in the BCSFB (Gazzin et al., 2008). Mrp1 is not enriched in the capillary fraction compared to parenchyma, indicating its presence in neural cells. Accordingly, it has been identified on brain tissue sections in rat astrocytes and in the glia limitans (Mercier et al., 2004). *In vitro*, the expression of MRP1 was shown in microglia primary cultures or cell lines (Dallas et al., 2003), in oligodendrocytes (Hirrlinger et al., 2002), and in rat, mice, and human primary cultures of astrocytes (Decleves et al., 2000; Hirrlinger et al., 2002, 2005; Spiegl-Kreinecker et al., 2002; Gennuso et al., 2004; Falcão et al., 2007). The simultaneous up-regulation and redistribution of the transporter from the perinuclear region to the plasma membrane after exposure to UCB was interpreted as a mechanism to confer cellular protection by allocating the UCB transporter in the most adequate subcellular position to face pigment entry (Gennuso et al., 2004). Mrp1 was also detected in primary cultures of embryonic rat neurons, even if in amounts lower than in astrocytes (Falcão et al., 2007). An inverse correlation between Mrp1 expression and sensibility to UCB, was reported in neurons and astrocytes, possibly explaining the higher UCB toxicity observed in cultured neurons which display the lowest levels of Mrp1 (Falcão et al., 2007). In agreement with a protective function of MRP1, mouse embryonic fibroblast cells derived from Mrp1 KO mice (Calligaris et al., 2006), and SH-SY5Y cells in which the transporter activity was silenced (Corich et al., 2009), displayed a higher mortality after bilirubin treatment compared to their respective controls. Cell culture conditions substantially modulate the expression of several ABCs transporters, however, and the observations obtained from *in vitro* experiments may not be fully applicable to the *in vivo* scenario. Accordingly, the actual levels of expression and functional relevance of UCB transporters in brain parenchyma *in vivo* are still debated.

The potential relationship between GST isoforms present in astrocytes and bilirubin toxicity has not been investigated, but in one study that showed higher cytosolic GST activity and level of alpha class GST subunits in cerebellum of hyperbilirubinemic jj Gunn rats compared to the Jj heterozygous animals. Within this distinct structure, the alterations in GST levels were region-specific, developmentally regulated, and inversely related to region-specific cerebellar hypoplasia (Johnson et al., 1993). This calls for additional investigation on the potential implication of GSTs in the control of long-term hyperbilirubinemic cerebral intoxication.

Cytochrome P-450 monooxygenases are involved in controlling bilirubin concentration in tissues and cells, as originally shown by Kapitulnik and colleagues. Selected hepatic Cyps isoforms (Cyp1a1 and 1a2) are able to compensate for the lack of hepatic glucuronidation in Gunn rat (Kapitulnik et al., 1987; Kapitulnik and Gonzalez, 1993), by increasing UCB body clearance following its oxidation (Zaccaro et al., 2001; De Matteis et al., 2006). CYP isoenzymes display a highly heterogeneous expression among different regions or cellular types in the CNS. For instance it is proposed that certain cellular subsets might express CYPs at levels as high as in the liver (Chinta et al., 2005b). Thus, it is conceivable that local CYP-dependent metabolic pathways have considerable effects on brain pathology and physiology. CYP 1A1 and 1A2 seems to be largely expressed in brain, predominantly in cerebral cortical neurons, Purkinje, and granule cells of hippocampus (Chinta et al., 2005a). CYP1A2 pattern of expression in human brain seems to be diffuse (Farin and Omiecinski, 1993). The role played by CYPs in brain resistance to bilirubin has just started to be investigated.

The expression of CYPs was investigated in the context of typical kernicterus which occurs only when an acute shift of bilirubin from blood to tissues is generated. It was reproduced experimentally in homozygous Gunn rats exposed to sulfadimethoxine to displace bilirubin from albumin. In this model, ABC proteins

appear inefficient to control the sudden increase in tissue free bilirubin concentrations (Gazzin et al., 2012). By contrast, a strong correlation was observed between the time-course and extent of induction of the Cyp genes involved in bilirubin oxidation on one hand, and the kinetic of tissue UCB disappearance on the other hand, in the different brain regions. Cyp mRNAs increased up to 70-fold in some regions, but returned to basal levels within 48–72 h, indicating that these changes are transitory. Overall, this set of data points to the possible role of UCB-mediated CYP induction in limiting UCB accumulation in selected brain regions, and suggests that these enzymes may protect selected brain areas from bilirubin neurotoxicity (Gazzin et al., 2012).

UNDERSTANDING CELLULAR AND REGIONAL SELECTIVITY OF BILIRUBIN TOXICITY

A decade ago, based on the observation that yellow staining of the basal nuclei is an extremely rare event, even in newborns that die from severe neonatal jaundice, Hansen challenged the concept that hyperbilirubinemia always leads to kernicterus by UCB accumulation in distinctive brain areas. In fact, only few cases of "real kernicterus" strictly describing bilirubin accumulation in specific brain regions have been reported in humans (Hansen, 2000). This conclusion was recently corroborated experimentally using the Gunn rat as an animal model for this disease. Bilirubin concentrations measured in four different brain regions in hyperbilirubinemic animals were found identical (Gazzin et al., 2012). Along the same line, the local cerebral blood flow that could influence the net blood-to-brain UCB flux is more homogeneous among regions in both human and rodent during early postnatal development than in adult, and is not correlated to the regional susceptibility to UCB toxicity (reviewed in Ghersi-Egea et al., 2009a). Similarly P-glycoprotein-dependent efflux processes which could marginally influence the net UCB flux in brain structures (see supra) is rather homogeneous among brain regions at least in the human adult (Eyal et al., 2010).

Despite the absence of a typical kernicterus, no doubt exists, however, as to the localization of damage to specific brain structures in both the human pathology and its experimental models (Shapiro, 2010). One hypothesis to explain this selective topography of bilirubin-induced injury would be based on a superimposable selective regionalization of the threshold concentrations between the anti- and pro-oxidant effects of the pigment.

Evidence for such a heterogenous sensitivity to bilirubin toxicity has already been reported between organs, regions within a given organ, or cells types in the same region. In hyperbilirubinemic Gunn rats, the bilirubin content in organs involved in heme metabolism such as the liver and spleen is several folds higher than that determined in brain (Zelenka et al., 2008; Gazzin et al., 2012), yet UCB-induced tissue damage has never been reported in the formers. In the brain, UCB-induced toxicity leads mainly to neuronal loss (especially Purkinje and granular cells from cerebellum; Keino et al., 1985; Conlee and Shapiro, 1997) and myelination defects (Brito et al., 2012). In line with this, astrocytes displayed a greater resistance than neurons to UCB toxicity (Brito et al., 2008), and the myelin-forming oligodendrocytes and their precursors have also emerged as possible sensitive targets in bilirubin encephalopathy (Kapitulnik, 2004). The latter hypothesis is corroborated by *in vitro* studies showing that oligodendrocyte viability decreases after exposure to UCB (Genc et al., 2003). It is in agreement with both the myelin alterations described in hyperbilirubinemic Gunn rats (O'Callaghan and Miller, 1985) and the loss of myelin fibers described in a preterm infant with kernicterus (Brito et al., 2012). With respect to regional differences, when primary cultures of neurons isolated from the cerebral cortex, the hippocampus or the cerebellum were exposed to UCB, hippocampal cells presented more oxidative damage associated with lower reduced glutathione levels, impairment of neurite outgrowth, and maximal cell death compared with cerebellar and cortical neurons (Vaz et al., 2011). This points to differences in the intrinsic sensitivity to UCB between neuronal sub-populations.

As bilirubin detoxification mechanisms are inducible/repressible processes, a differential modulation of transporters and enzymes following exposure to UCB must also be considered when searching for factors involved in the cell- and tissue-specific toxicity. The initial data described above such as those obtained for ABC transporters in the BBIs and neural cells, or for CYPs and GSTs in the neuropil, support a link between the expression level of these genes and bilirubin-induced toxicity, which needs to be further investigated.

Finally, the vulnerability of the brain to UCB intoxication is dependent on the timing of UCB exposure. The maximal sensitivity is observed during early postnatal development (Rice and Barone, 2000), at a time when the CNS undergoes extensive remodeling and maturation with temporal differences between the various cerebral structures. Thus the developmental age at the time of insult may determine the neural cell sensitivity to UCB intoxication, and possibly the efficiency of neuroprotective pathways in the different brain regions, and thereby influence the regional pattern of damage.

FUTURE CHALLENGES

An in-depth characterization of the detoxification and transport processes at BBIs and in the different neural cells of the developing brain are probably the most important basic knowledge we need to acquire in order to better appreciate the functional basis of selective brain structure damage following exposure to elevated UCB levels. The phenotype, the magnitude of symptoms, and the outcome of the disease vary with the developmental age at the time of bilirubin exposition. This heterogeneity thus calls for studies defining the developmental profile of the various effector molecules. These include all transporters and enzymes involved in UCB detoxification or in the regulation of CSF protein level and those exerting neuroprotective functions (antioxidants). Furthermore, these key actors should be investigated in the context of the pathology to evaluate the possible UCB-induced alteration in their expression or activity. Due to the complexity of the brain architecture and the developmental pattern specific to each cerebral structure, it is reasonable to speculate that several "sensible targets" are yet to be identified, and that those targets are developmentally regulated.

From a therapeutic point of view, conventional treatments include phototherapy and blood exchanges for hyperbilirubinemic infants, and liver transplantation or gene therapy for patients with the Crigler–Najjar syndrome. While waiting for new therapeutic targets to be identified, neuroprotective therapies that complement the conventional treatments in their initiation phase would be highly beneficial to the final outcome of the disease.

A current approach is to target the perturbations of the neural cell redox status. Minocycline, a drug with properties similar to Vitamin E (Kraus et al., 2005), and already in use in several other central nervous system disorders, inhibits the cerebellar hypoplasia in Gunn rats (Lin et al., 2005). Unfortunately, its use in bilirubin-induced neurological disorders is impeded by side effects in pediatric patients (Yong et al., 2004). This highlights the difficulty of developing therapeutic strategies for this category of patients and stresses out the real need to fully understand the differences between adult and children in the factors setting the cerebral bioavailability of drugs, including drug transporters at BBIs. Among milder therapeutic approaches, essential fatty acid supply may be considered. Polyunsaturated fatty acids are major components of brain membranes and especially of myelin, and fatty acid depletion impairs nervous system functions leading to learning, motor, vision, and auditory abnormalities (Yehuda et al., 2005). Omega-3 and -6 lipid supplementation by oral administration has proved beneficial in several neurodegenerative pathologies that involve oxidative stress (review in Kim et al., 2010). Since myelin is a target for UCB, this therapeutic approach could be relevant to the prevention of UCB-mediated toxicity in children. In addition oral administration is possible, and side effects are not expected. To the best of our knowledge, this approach has never been tested.

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Heme cytotoxicity and the pathogenesis of immune-mediated inflammatory diseases

Rasmus Larsen, Zélia Gouveia, Miguel P. Soares and Raffaella Gozzelino*

Instituto Gulbenkian de Ciência, Oeiras, Portugal

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Stefan W. Ryter, Harvard Medical School, USA
Stephan Immenschuh, Hannover Medical School, Germany

***Correspondence:**
Raffaella Gozzelino, Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2780-156 Oeiras, Portugal.
e-mail: rgozzelino@igc.gulbenkian.pt

Heme, iron (Fe) protoporphyrin IX, functions as a prosthetic group in a range of hemoproteins essential to support life under aerobic conditions. The Fe contained within the prosthetic heme groups of these hemoproteins can catalyze the production of reactive oxygen species. Presumably for this reason, heme must be sequestered within those hemoproteins, thereby shielding the reactivity of its Fe-heme. However, under pathologic conditions associated with oxidative stress, some hemoproteins can release their prosthetic heme groups. While this heme is not necessarily damaging *per se*, it becomes highly cytotoxic in the presence of a range of inflammatory mediators such as tumor necrosis factor. This can lead to tissue damage and, as such, exacerbate the pathologic outcome of several immune-mediated inflammatory conditions. Presumably, targeting "free heme" may be used as a therapeutic intervention against these diseases.

Keywords: heme, heme oxygenase, cytotoxicity, programmed cell death, immune-mediated inflammatory diseases

PHYSIOLOGICAL ROLE OF HEME

Heme acts as prosthetic group in a range of hemoproteins that play a pivotal role in many essential biological processes. These include gas transport and storage, mitochondrial electron transport chain, drug metabolism, signal transduction and regulation of gene expression (Chapman et al., 1997).

STRUCTURE AND BIOCHEMISTRY OF HEME

Heme is a hydrophobic metallo-compound containing a Fe atom within its protoporphyrin ring. It is composed of four methane-bridged pyrroles (tetrapyrrole ring) bound to the central Fe atom through nitrogen atoms (Reedy and Gibney, 2004; Kumar and Bandyopadhyay, 2005; Tsiftsoglou et al., 2006). Ferrous (Fe^{2+}) heme has a neutral chemical charge, whereas ferric (Fe^{3+}) heme is positively charged, and can bind anions (Kumar and Bandyopadhyay, 2005). Although ferrous and ferric states are the most common within the heme structure, oxidative states of Fe-heme can vary from Fe^{2+} to Fe^{5+}(Ogura et al., 1996; Karpefors et al., 2000; Dey and Ghosh, 2002).

Heme exists mainly as *a*, *b*, and *c* variants, which differ by subtle chemical modifications (Chapman et al., 1997; Tsiftsoglou et al., 2006; Smith et al., 2010). One of the vinyl groups of heme *b*, is replaced by a 17-hydroxyethylfarnesyl group in heme *a*. One of the methyl groups of heme *b* is also replaced by a formyl group in heme *a* (Caughey et al., 1975; Han et al., 1991). In heme *c*, both vinyl groups of heme *b* are replaced by sulfhydryl groups (Bowman and Bren, 2008). Heme *b* is the most common variant in mammals, present, among others, in hemoglobin (Hb) and myoglobin (Fermi et al., 1984; Evans and Brayer, 1990; Paoli et al., 1996; Park et al., 2006). Heme *a* and *c* are found in cytochrome *c* oxidase and cytochrome *c*, respectively (Bushnell et al., 1990; Tsukihara et al., 1995). Whereas heme *c* can bind covalently to proteins, via two thioether bonds, this is not the case for heme *b* and *a*, eventually allowing for their release from hemoproteins (Allen et al., 2003).

HEMOPROTEINS

Hemoproteins have a diverse spectrum of biological functions. These include transport and storage of diatomic gaseous molecules such as oxygen (O_2), nitric oxide (NO) and carbon monoxide (CO; Chay and Brillhart, 1974a,b; Aono, 2008; Kakar et al., 2010). They are also essential for electron transfer reactions (Makinen et al., 1983; Gray and Winkler, 1996) as well as for modulation of gene transcription (Tahara et al., 2004a,b; Youn et al., 2006; Hira et al., 2007), such as involved in the regulation of circadian rhythms (Kaasik and Lee, 2004). Prosthetic heme groups have also been implicated in the regulation of signal transduction pathways (Igarashi and Sun, 2006; Zenke-Kawasaki et al., 2007), ion-channel functions (Tang et al., 2003; Horrigan et al., 2005), micro-RNA processing (Faller et al., 2007) and the metabolism of xenobiotics and drugs (Bernhardt, 1996; Rowland et al., 2006).

Under physiologic conditions, heme is found mainly in the "heme pockets" of hemoproteins. These are characterized by a high frequency in aromatic amino acids (**Table 1**), such as phenylalanine (F), tyrosine (Y) or tryptophan (W) and by few or no charged amino acids (Li et al., 2011), conferring the hydrophobicity required to promote stable heme binding to hemoproteins. Five highly conserved amino acids, including histidine (H), methionine (M), cysteine (C), tyrosine (Y) and lysine (K) can act as axial heme ligands in the heme pockets of hemoproteins (Li et al., 2011). Of these, H is frequently found in hemoproteins containing heme *c* or *b* (**Table 1**). Moreover, C often promotes binding of heme *b*, whereas M binds heme *c* (Fufezan et al., 2008; Li et al., 2011; **Table 1**). Other hydrophobic amino acids such as leucine (L), isoleucine (I) and valine (V) can also create interactions with the porphyrin structure of heme, whereas arginine (R) and other

Table 1 | Selected representatives of Mammalian hemoproteins and heme-sensing proteins.

Function	Representative hemoprotein	Number/type of heme	Axial ligand	References
Electron transport	Cytochrome c	1/c-type	His, Met	Mirkin et al. (2008)
	Cytochrome b5	1/b-type	His, His	Wirtz et al. (2000)
	Cytochrome c oxidase	2/a-type	His/Vacant, His/His	Tsukihara et al. (1995)
	Cytochrome c reductase	2/b-type, 1/c-type	His/His, His/His, His/Met	Xia et al. (1997); Zhang et al. (1998)
Gas carriers	Hemoglobin	4/b-type	His, Vacant	Park et al. (2006)
	Myoglobin	1/b-type	His, Vacant	Evans and Brayer (1988); Vojtechovsky et al. (1999)
Catalytic activity – enzyme	Catalase I	1/b-type	Tyr, Vacant	Putnam et al. (2000)
	Cytochrome P450	1/b-type	Cys, Vacant or water	Rowland et al. (2006)
	Indoleamine 2,3-dioxygenase	1/b-type	His, ligand	Sugimoto et al. (2006)
	NO synthase	2/b-type	Cys, Vacant	Crane et al. (1997)
	Cystathionine β-synthase	1/b-type	Cys/His	Meier et al. (2001)
	Tryptophan 2,3-dioxygenase	4/b-type	His/Vacant	Zhang et al. (2007b)
Transient heme binding	Heme oxygenase	1/b-type	His, Water	Lad et al. (2003)
	Hemopexin	1/b-type	His/His	Paoli et al. (1999)
	Albumin	1/b-type	Tyr/Vacant	Wardell et al. (2002)
Heme-sensing/ heme regulated	NPAS2	2/b-type	His/Cys or His/His, His/?	Dioum et al. (2002); Uchida et al. (2005)
	Iron regulatory protein 2 (IRP2)	1	Cys/?	Jeong et al. (2004)
	Soluble guanylyl cyclase	1/b-type	His/Vacant	Ma et al. (2007)

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positively charged amino acids can interact with the negatively charged heme propionate groups (Schneider et al., 2007).

Several heme-binding motifs have been described, namely CXXCH and CXXCK that bind primarily heme *c* and in which "X" refers to any amino acid (Fufezan et al., 2008; Smith et al., 2010; Li et al., 2011). The GX[HR]XC[PLAV]G heme-binding motif is associated mainly with heme *b* (Li et al., 2011). Another related FXXGXXCXG motif found in mammal, cytochrome P450, as well as in plant and bacterial hemoproteins binds to heme *b* (Li et al., 2011). Other heme-binding motifs can be formed by only two amino acids (such as the CP motif; Li et al., 2011).

In some hemoproteins, the Fe and the protoporphyrin IX ring of heme can undergo chemical and/or electronic modifications, such as demonstrated for peroxidases in which heme uses hydrogen peroxide (H_2O_2) to undergo several Fe oxidoreductase cycles associated with conformational alterations of its protoporphyrin ring. This can lead to the oxidation of neighboring molecules, e.g., indoles, phenols, aromatic amines, lignin, Mn^{2+} ions, halide ions or proteins (Badyal et al., 2006; Zederbauer et al., 2007; Battistuzzi et al., 2010). In other hemoproteins, heme does not undergo alterations in its Fe redox state or protoporphyrin conformation (Poulos, 2007). This is the case for hemoproteins that bind gaseous molecules such as guanylate cyclase, Hb, myoglobin or the transcription factor neuronal PAS domain-containing protein 2 (NPAS2), among others (Chay and Brillhart, 1974a,b; Dioum et al., 2002; Podstawka and Proniewicz, 2004; Uchida et al., 2005). Binding of NO or CO to these hemoproteins modulates their biologic activity, e.g., increased production of cGMP in the case of guanylate cyclase (Ma et al., 2007), regulation of O_2 transport in the case of Hb or gene transcription in the case of CO binding to NPAS2 (Dioum et al., 2002). In other instances, biologic activity is regulated by heme binding itself, as it is the case for the transcriptional repressor Bach1 (Tahara et al., 2004a,b; Hira et al., 2007).

HEME METABOLISM

Heme intracellular content must be tightly controlled to prevent heme-driven cytotoxicity while assuring that heme is available to be incorporated into nascent apo-hemoproteins. This is particularly relevant for cells with very high hemoprotein content such as erythroid or muscle cells expressing Hb and myoglobin, respectively. This regulatory mechanism is ensured largely through control of heme synthesis, transport and catabolism.

HEME SYNTHESIS

Heme *de novo* synthesis occurs through a sequence of eight enzymatic steps, four of which take place in the cytoplasm and four in the mitochondria (**Figure 1**; Heinemann et al., 2008). The first and rate-limiting step is catalyzed in the mitochondria by δ-amino levulinic acid (ALA) synthase (ALAS), an enzyme that converts glycine and succinyl-CoA into ALA. In most cells, heme synthesis is down-regulated by heme, via inhibition of ALAS mRNA

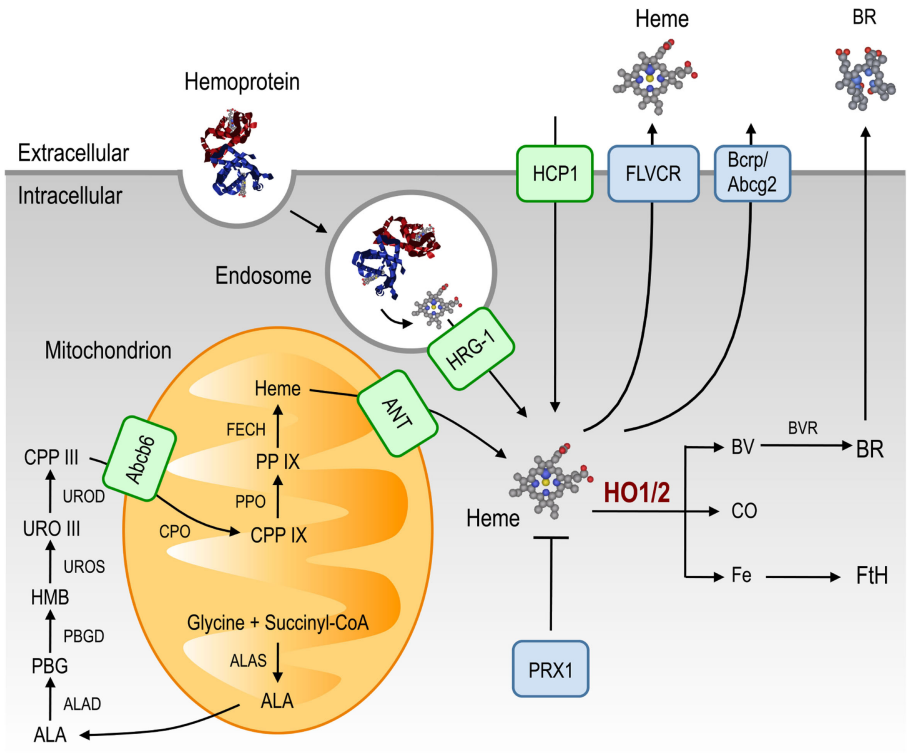

FIGURE 1 | Heme metabolism. Heme is synthesized from glycine and succinyl-CoA, through eight enzymatic steps that take place in the cytoplasm and the mitochondrion. Heme and heme precursors are transported between these compartments by ANT and Abcb6, respectively. In addition to the heme produced via this pathway, extracellular heme can be imported. Hemoproteins can be taken up and denatured in endosomes, liberating heme that can be transported to the cytoplasm via HRG-1, whereas cell-free heme is taken up by the cell membrane-associated HCP1. Cytoplasmic heme can be scavenged by PRX1, which neutralizes its pro-oxidant effects. Excess heme can be exported via FLVCR and Bcrp/Abcg2 or catabolized by HO-1/2 into equimolar amounts of BV, CO and free Fe. BV can be converted into BR by BVR while labile cellular Fe induces the expression of FtH that scavenges excess cellular Fe. Abbreviations: Abcb6, ATP-binding cassette b6; ALA, δ-aminolevulinic acid; ALAD, ALA dehydratase; ALAS, ALA synthase; ANT, adenine-nucleotide translocator; Bcrp/Abcg2, breast cancer resistance protein/ATP-binding cassette g2; BR: bilirubin; BV, biliverdin; BVR, BV reductase; CO, carbon monoxide; CPP, coproporphyrinogen; CPO, CPP oxidase; Fe, iron; FECH, ferrochelatase; FLVCR, feline leukemic virus receptor; Fth, ferritin; HCP1, heme carrier protein-1; HMB, hydroxymethylbilane; HO, heme oxygenase; HRG-1, heme responsive gene-1; PBG, porphobilinogen; PBGD, PBG deaminase; PP IX, protoporphyrin IX; PPO, protoporphyrinogen oxidase; PRX1, peroxiredoxin-1; URO, uroporphyrinogen; UROD, URO decarboxylase; UROS, URO III synthase.

expression (Hamilton et al., 1991) or ALAS translocation from the cytoplasm into the mitochondrion (Lathrop and Timko, 1993).

HEME TRANSPORT

Being hydrophobic, heme was assumed to diffuse passively across cellular membranes. However, heme cellular trafficking appears to be controlled by a range of evolutionary conserved heme transporters (**Figure 1**; reviewed in Schultz et al., 2010; Khan and Quigley, 2011). Some of these heme transporters, e.g., mitochondrial ATP-binding cassette (ABC) family member, Abcb6, and the adenine-nucleotide translocator (ANT), control the translocation of heme as well as that of heme precursors between the cytoplasm and the mitochondria (**Figure 1**; Krishnamurthy et al., 2006; Azuma et al., 2008). Other heme transporters, including HRG-1 expressed in endosomes and lysosomes (Rajagopal et al., 2008) or the heme carrier protein (HCP1) expressed in the cytoplasmic membrane, control extracellular heme import (**Figure 1**; Shayeghi et al., 2005; Latunde-Dada et al., 2006; **Figure 1**). Heme can also be exported from cells via the ATP-binding cassette (Abcg2; Krishnamurthy et al., 2004; Krishnamurthy and Schuetz, 2006) and the feline leukemic virus receptor (FLVCR; **Figure 1**; Quigley et al., 2000, 2004; Schultz et al., 2010).

HEME CATABOLISM

Heme degradation can be catalyzed by the enzymatic activity of heme oxygenases (HO; Tenhunen et al., 1968. There are two isoforms, i.e., HO-1 and HO-2, encoded by the *HMOX1* and *HMOX2* genes in humans, respectively (**Figure 1**; Maines et al., 1986; Trakshel et al., 1986). HO-2 is constitutively expressed by most cells (Trakshel et al., 1986) and is thought to carry out heme catabolism under homeostatic conditions. In contrast, excess intracellular heme induces the expression of the stress-responsive HO-1 isoform (see HO-Catalyzed Heme Degradation), increasing the rate of heme catabolism and, as such, preventing its putative cytotoxic effects (Gozzelino et al., 2010). HO activity catalyzes the oxidative cleavage of the heme protoporphyrin IX ring, yielding biliverdin (BV), concomitant with the release of equimolar amounts of labile Fe and carbon monoxide (CO; **Figure 1**; Tenhunen et al., 1968).

Biliverdin is converted into bilirubin (BR) by BV reductase (Kutty and Maines, 1981; **Figure 1**). The physiological roles of these end products of heme catabolism, are discussed in further detail under Section "Cellular Control of Free Heme."

PATHOPHYSIOLOGICAL EFFECTS OF FREE HEME

Under pathophysiological conditions, heme *b* can be released from hemoproteins to which it binds non-covalently (Bunn and Jandl, 1968; Hebbel et al., 1988; Pamplona et al., 2007). This produces "free heme," that is, heme that is not bound to the heme pockets of hemoproteins. Free heme can act as a potent cytotoxic pro-oxidant (Balla et al., 1992, 1993) owing to the Fe atom contained within its protophorphyrin ring (Seixas et al., 2009; Gozzelino and Soares, 2011).

Immune-mediated inflammatory diseases can be associated with the accumulation of free heme in the circulation (Reiter et al., 2002; Pamplona et al., 2007; Seixas et al., 2009; Andrade et al., 2010a,b; Larsen et al., 2010). The observation that free heme can sensitize non-hematopoietic cells, e.g., hepatocytes, to undergo programmed cell death (PCD) in response to a variety of pro-inflammatory agonists (Seixas et al., 2009; Larsen et al., 2010; Gozzelino and Soares, 2011), suggests that free heme may participate functionally in the pathogenesis of immuno-mediated inflammatory diseases (**Figure 2**; Francis et al., 1997), in which the host component of inflammation would exacerbate the deleterious effects of free heme (Seixas et al., 2009; Larsen et al., 2010; Gozzelino and Soares, 2011). Taking this into account, the amount of circulating free heme, as a result of the overall heme metabolism, would dictate the outcome of these pathologic conditions. While free heme also appears to exert pro-inflammatory effects (Figueiredo et al., 2007; Porto et al., 2007; Fernandez et al., 2010) that might participate in the pathogenesis of immune-mediated inflammatory diseases these will not be covered in detail hereby.

Presumably, the cytotoxic effect of free heme is mediated, to a large extent, via the unfettered production of free radicals leading to oxidative stress. This notion is supported by the observation that pharmacologic antioxidants can confer cytoprotection against free heme (Seixas et al., 2009; Larsen et al., 2010). Sustained free radicals production can prolong the activation of the c-Jun N-terminal Kinase (JNK) signaling transduction pathway (Ventura et al., 2004; Kamata et al., 2005), via inhibition of phosphatases controlling JNK activation in response to tumor necrosis factor (TNF; Davis, 2000; Sanchez-Perez et al., 2000; Masuda et al., 2001; Tanoue et al., 2001; Kamata et al., 2005; **Figure 2**). The cytotoxic effect of free heme is owed to this sustained JNK activation, as suggested by the observation that heme-driven free radical production leads to sustained JNK phosphorylation/activation (**Figure 2**) and that inhibition of JNK activation affords cytoprotection against free heme (Raffaella Gozzelino et al., unpublished results). The cytotoxic effect of free heme is also associated with cleavage of caspase-3, suggesting that heme sensitizes non-hematopoietic cells, e.g., hepatocytes, to undergo PCD by apoptosis. This notion is supported by the demonstration that pharmacologic caspase inhibition, including specific inhibition of caspase-3, confers cytoprotection against free heme (**Figure 2**; Seixas et al., 2009). More recently, free heme has also been shown to trigger necroptosis in hematopoietic cells, as demonstrated for macrophages (Fortes et al., 2012).

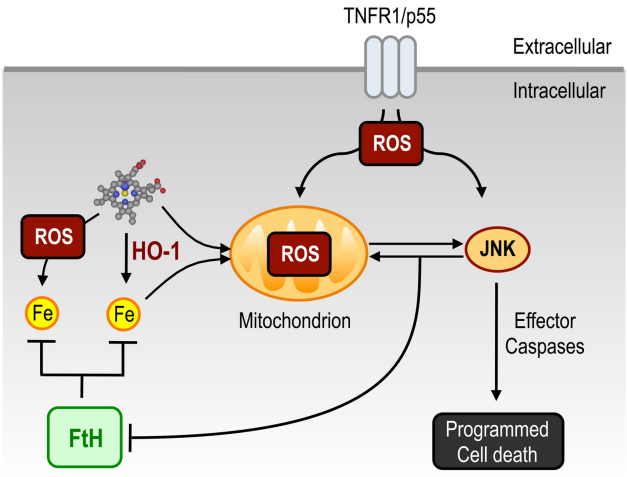

FIGURE 2 | Pathophysiological effects of heme. Free heme can sensitize parenchyma cells to undergo PCD in response to pro-inflammatory signals such as TNF. Under homeostatic conditions, cellular heme is degraded via HO-1 activity and the labile Fe released in this manner, is scavenged by FtH, thus preventing oxidative stress. Under a variety of inflammatory conditions, the concentration of intracellular free heme can exceed the rate of heme catabolism by HO-1. In addition, accumulation of cellular labile Fe can also overcome FtH scavenging capacity. When this occurs, free heme and/or labile Fe accumulate in cells, thereby boosting mitochondrion-driven ROS. Activation of the TNF receptor leads to further generation of ROS, resulting in sustained JNK activation which promotes FtH degradation (Antosiewicz et al., 2007), leading to further labile Fe overload and hence to further ROS generation via Fenton chemistry. Moreover, sustained JNK activation lead to the activation of effector caspases thereby inducing PCD. Abbreviations: FtH, ferritin; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; ROS, reactive oxygen species; TNF, tumor necrosis factor; TNFR1, TNF receptor-1.

PHYSIOLOGIC MECHANISMS OF PROTECTION AGAINST FREE HEME

Under pathophysiologic conditions associated with intravascular hemolysis, significant amounts of Hb can be released from red blood cells (RBCs). The resulting circulating cell-free Hb undergoes oxidation, releasing its prosthetic heme groups (Bunn and Jandl, 1968; Hebbel et al., 1988; Pamplona et al., 2007; Ferreira et al., 2008). Similarly, oxidation of other hemoproteins, e.g., myoglobin, may also contribute to the unwarranted accumulation of cytotoxic free heme, such as associated with rhabdomyolysis (Nath et al., 1992).

Protective mechanisms are in place, on systemic as well as cellular levels, to deal with this challenge, as described in this section.

SYSTEMIC CONTROL OF FREE HEME

Cell-free Hb can be scavenged by haptoglobin (Hp), an acute phase protein synthesized in the liver and present at relatively high concentrations (0.3–3 mg/mL) in plasma (Levy et al., 2010). Binding of Hb to Hp prevents Hb oxidation (Melamed-Frank et al., 2001)

and thereby the release of its heme moiety (**Figure 3**). Hb:Hp complexes binds to CD163 (Kristiansen et al., 2001) expressed in macrophages and hepatocytes (Philippidis et al., 2004; Quaye, 2008), leading to endocytosis and degradation of the Hb:Hp complex (**Figure 3**). Polymorphisms in the human *HPT* gene can act as independent risk factors for several immune-mediated inflammatory diseases (Quaye, 2008), suggesting that Hb scavenging by Hp modulates the pathogenesis of these diseases.

Under pathophysiological conditions associated with more or less extensive intravascular hemolysis, such as malaria or different forms of hemolytic anemia (see Heme and the Pathogenesis of Infectious Diseases and Heme and the Pathogenesis of Non-Infectious Immune-Mediated Inflammatory Conditions), extracellular Hb can lead to the depletion of circulating Hp (Muller-Eberhard et al., 1968; Rother et al., 2005). When Hb accumulates in the circulation it becomes oxidized, releasing its heme groups (Bunn and Jandl, 1968; Hebbel et al., 1988; Pamplona et al., 2007; Ferreira et al., 2008). A number of circulating proteins can scavenge free heme in plasma (Bunn and Jandl, 1966; **Figure 3**), including hemopexin (Paoli et al., 1999; Tolosano et al., 2010), albumin (Fasano et al., 2007), α1-microglobulin (Allhorn et al., 2002) and high- and low-density lipoproteins (Miller and Shaklai, 1999).

Hemopexin (Hx) is an acute phase protein present at high concentrations in plasma (0.6–1.2 mg/mL), with the highest binding affinity for free heme ($K_d < 10^{-12}$ in humans) of any described protein (Paoli et al., 1999; Tolosano et al., 2010). Heme:Hx complexes, which inhibits Fe-heme reactivity (Eskew et al., 1999), are recognized by the macrophage CD91 receptor (Hvidberg et al., 2005), allowing for intracellular heme catabolism (**Figure 3**; Alam and Smith, 1989). Hx is not degraded by macrophages, to any significant extent, being secreted back into the circulation (**Figure 3**; Hvidberg et al., 2005). As a consequence, Hx is less prone to complete depletion from the circulation, as compared to Hp.

Mice deficient in Hx and/or Hp ($Hpx^{-/-}Hpt^{-/-}$) develop severe renal damage when subjected to hemolysis, suggesting that Hx is part of a systemic protective mechanism against the deleterious effects of circulating free heme (Tolosano et al., 1999). The reason for which the kidney appears to be the first target of heme cytotoxicity *in vivo* is not clear, but is in keeping with the observation that induction of heme catabolism prevents kidney injury driven by rhabdomyolysis, where the hemoprotein, myoglobin, is released into circulation (Nath et al., 1992).

Albumin can bind heme with an affinity 10^4 times lower than Hx ($K_d = 10^{-8}$; Little and Neilands, 1960). However, the high

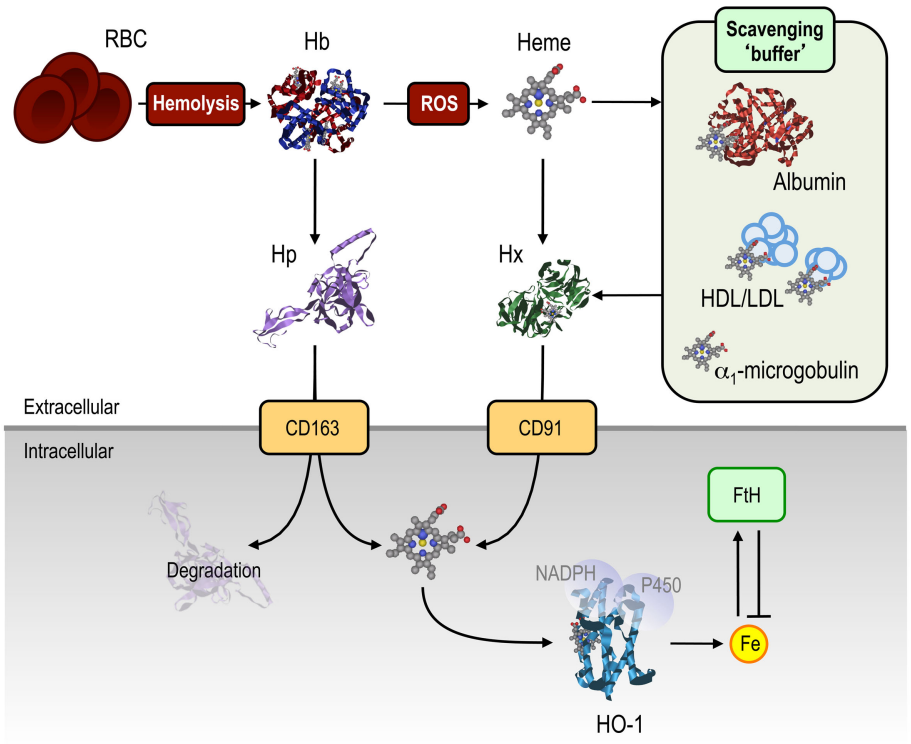

FIGURE 3 | Mechanisms of protection against free heme. A range of extracellular mechanisms confer protection against cell-free Hb and heme. Under hemolytic conditions, Hb is released from RBC into the circulation where it is scavenged by Hp, thereby preventing Hb oxidation, and allowing for the uptake of Hb:Hp complexes via the CD163 receptor. Once the scavenging capacity of Hp is exhausted, Hb becomes oxidized, releasing its heme prosthetic groups. These can be scavenged by albumin, HDL and LDL, α1-microglobulin, acting as "scavenging buffer," and ultimately by Hx, which has the highest affinity for free heme. Hx:heme complexes are taken up via the CD91 receptor, delivering heme for catabolism by HO-1. Intracellular heme, whether originating from the extra- or intracellular compartment, is degraded via HO-1. The labile Fe released from heme catabolism is subsequently scavenged by FtH, thus conferring cytoprotection against heme-Fe. Abbreviations: Fe, iron; FtH, ferritin; Hp, haptoglobin; Hb, hemoglobin; HDL/LDL, high/low-density lipoprotein; Hx, hemopexin; ROS, reactive oxygen species.

concentration of albumin in plasma (∼43 mg/mL) might compensate to some extent for its low affinity (**Figure 3**). Heme binding to albumin can neutralize the pro-oxidant effect of free heme, which may explain the protective effect of albumin infusion to individuals developing severe forms of sepsis (Delaney et al., 2011) or malaria (Maitland et al., 2005; Akech et al., 2006), the pathogenesis of which are driven to a significant extent by free heme (Pamplona et al., 2007; Ferreira et al., 2008; Seixas et al., 2009; Larsen et al., 2010; Raffaella Gozzelino et al., unpublished results).

α1-Microglobulin is a member of the lipocalin protein superfamily, that binds small lipophilic ligands (Flower et al., 2000). Cell-free Hb can cleave α1-microglobulin, into a truncated protein that binds ($K_d = 10^{-6}$) and converts free heme into a yellow-brown proteinaceous chromophore, subsequently cleared by renal filtration (Allhorn et al., 2002). Binding of α1-microglobulin to heme neutralizes its pro-oxidant effect, suggesting that α1-microglobulin acts as a heme scavenger in circulation (**Figure 3**; Allhorn et al., 2002). Whether under pathophysiologic conditions α1-microglobulin plays a protective role against heme is unknown.

High- and low-density lipoproteins (HDL and LDL, respectively) can bind heme ($K_d \sim 10^{-11}$–10^{-12}) with faster kinetics to those of Hx or albumin (Miller and Shaklai, 1999), thereby acting as the initial heme scavengers in circulation (Balla et al., 1991; Jeney et al., 2002). Once bound to HDL and LDL, heme can be transferred to Hx (**Figure 3**; Morgan et al., 1976; Hvidberg et al., 2005). In this manner, HDL and LDL may function as an initial "buffer" for circulating heme. Binding of heme to HDL or LDL oxidizes HDL and LDL that are then cleared via the CD36 and CD68 scavenger receptors expressed on macrophages (Camejo et al., 1998; Miller and Shaklai, 1999). Of note, however, oxidized HDL and LDL are cytotoxic (Jeney et al., 2002; Stocker and Perrella, 2006), suggesting that heme-mediated lipid peroxidation might be a key mechanism via which heme exerts its deleterious effects (Jeney et al., 2002; Stocker and Perrella, 2006). This also indicates that the beneficial role of HDL and LDL in circulation would be dependent on their rapid clearance that might otherwise be detrimental. Considering its high affinity for heme, Hx acts most probably as an ultimate heme scavenger in plasma, targeting heme bound to other carrier proteins or not, and allowing for heme delivery for intracellular degradation by the HO system (see HO-Catalyzed Heme Degradation).

CELLULAR CONTROL OF FREE HEME

Heme incorporation into hemoproteins

The rate of *de novo* heme synthesis must correspond accurately to its rate of incorporation into newly synthesized apo-hemoproteins. This is regulated at different levels via the control of heme (see Heme Metabolism) as well as apo-hemoprotein synthesis. Free heme can induce the transcription and hence the expression of several apo-hemoproteins, as demonstrated for Hb, myoglobin and neuroglobin in erythroid, muscle and neuronal cells, respectively (Bruns and London, 1965; Tahara et al., 1978; Graber and Woodworth, 1986; Zhu et al., 2002a,b). This evolutionary conserved strategy prevents intracellular heme accumulation, presumably limiting heme cytotoxicity.

HO-catalyzed heme degradation

Whereas HO-2 is responsible for homeostatic heme catabolism (see Heme Catabolism), HO-1 can be induced by different forms of stress (Choi and Alam, 1996). Oxidative stress can lead to heme release from hemoproteins (*reviewed in* Gozzelino et al., 2010), requiring a cellular adaptive response maintaining intracellular free heme below a cytotoxic threshold level. This is accomplished, to a large extent, via the induction of *HMOX1* transcription and HO-1 expression (Alam et al., 1994, 1995; Choi and Alam, 1996; Alam and Cook, 2007). In line with this notion, mouse cells deficient in HO-1 expression ($Hmox1^{-/-}$), and thus unable to increase heme catabolism in response to different forms of stress, display exacerbated cytotoxicity in response to oxidative stress (Poss and Tonegawa, 1997a,b; Yet et al., 2003; Bishop et al., 2004). Whether this is, as speculated hereby, due to the accumulation of free heme or whether HO-1 acts in a cytoprotective manner independently of its ability to neutralize the cytotoxic effect of free heme, remains unclear.

Induction of HO-1 expression in response to infection can be essential to sustain host survival, as demonstrated for systemic polymicrobial (Larsen et al., 2010) as well as for *Plasmodium* infection (Pamplona et al., 2007; Seixas et al., 2009), the causative agent of malaria. Accumulation of heme in the circulation is cytotoxic, thus leading to tissue damage and, as such, exacerbating the pathologic outcome of these diseases (Pamplona et al., 2007; Seixas et al., 2009; Larsen et al., 2010). HO-1 neutralizes this cytotoxic effect and hence provides tissue damage control and host protection against infections (Pamplona et al., 2007; Larsen et al., 2010; *reviewed in* Gozzelino et al., 2010). This host defense strategy acts irrespectively of pathogen load, a phenomenon referred to as host tolerance to infection (Medzhitov et al., 2012).

The cytoprotective action of HO-1 (Vile et al., 1994; Soares et al., 1998) resides not only in the degradation of cytotoxic heme, but also in the production of the different end products of heme catabolism, namely CO, BV, and labile Fe (**Figure 3**). CO has a variety of biological effects that mitigate the pathogenesis and/or progression of immune-mediated inflammatory diseases (Otterbein et al., 1999b; Fujita et al., 2001; Sato et al., 2001). These include its ability to bind the Fe atom in the prosthetic heme groups of hemoproteins, thereby modulating their function (Kim et al., 2006; Piantadosi, 2008; Mustafa et al., 2009). This mechanism of action probably underlies the cytoprotective (Brouard et al., 2000; Silva et al., 2006) and "anti-inflammatory" effects of CO (Otterbein et al., 2000) as well as the ability of this gasotransmitter to inhibit platelet activation and aggregation (Brune and Ullrich, 1987). In addition, binding of CO to the Fe-heme prevents the oxidation of hemoproteins, thereby slowing down heme release. When exerted over cell-free Hb (Hebbel et al., 1988; Pamplona et al., 2007), CO prevents heme release from Hb, suppressing the pathogenesis of severe forms of malaria (Pamplona et al., 2007; Ferreira et al., 2008). Presumably this mechanism should contribute to prevent the pathogenesis and/or progression of other immune-mediated inflammatory diseases associated with heme release from hemoproteins (*reviewed in* Ferreira et al., 2008; Gozzelino et al., 2010).

Biliverdin is converted into lipid phase antioxidant BR (Stocker et al., 1987) by BV reductase (BVR; **Figure 3**; Singleton and Laster,

1965; Kutty and Maines, 1981). The BV/BR system affords cytoprotection against reactive oxygen species (ROS; Dore et al., 1999; Baranano et al., 2002). Both free heme and BR are lipophilic, suggesting that the antioxidant action of BR may limit lipid oxidation by free heme. Presumably, the cytoprotective effect of BV/BR contributes to their overall protective effect against a range of immune-mediated inflammatory diseases (Yamashita et al., 2004; Ollinger et al., 2005, 2007; Sarady-Andrews et al., 2005; Overhaus et al., 2006; Wang et al., 2006).

The Fe released from the protoporphyrin IX ring of heme (**Figure 3**) can catalyze the generation of ROS via Fenton chemistry (Fenton, 1894). Cells can neutralize this cytotoxic effect by secreting Fe via Fe efflux pumps (Ferris et al., 1999; Baranano et al., 2000) or by storing Fe inside ferritin (**Figure 3**; Eisenstein et al., 1991; Harrison and Arosio, 1996; Baker et al., 2003), a multimeric protein complex composed of a heavy/heart chain (FtH) and a light/liver chain (FtL) that store up to 4500 Fe molecules (Harrison and Arosio, 1996). The FtH subunit possesses ferroxidase activity that allows it to catalyze the conversion of Fe^{2+} into Fe^{3+}, required for Fe storage. FtH is cytoprotective against TNF (Ferris et al., 1999; Berberat et al., 2003; Cozzi et al., 2003; Xie et al., 2005) as well as heme-driven cytotoxicity (Balla et al., 1992). This salutary effect relies on the ability of FtH to neutralize the pro-oxidant activity of labile Fe, thereby preventing sustained JNK activation (Pham et al., 2004; Raffaella Gozzelino et al., unpublished results). Presumably, the cytoprotective effect of FtH prevents the pathogenesis of immune-mediated inflammatory diseases, such as demonstrated originally for ischemia–reperfusion injury (IRI; Berberat et al., 2003) and more recently for malaria (Raffaella Gozzelino et al., unpublished results).

Heme transport and secretion

Proteins involved in heme transport or intracellular heme scavenging (see Heme Transport) should regulate the pro-oxidant effects of heme (**Figure 1**; reviewed in Schultz et al., 2010; Khan and Quigley, 2011). Expression of the intracellular heme-binding thioredoxin-dependent peroxidase (PRX-1) is induced transcriptionally by heme as well as by oxidative stress (Immenschuh et al., 1995, 2005). PRX1 negates the pro-oxidant effect of free heme, preventing its deleterious effects (Immenschuh et al., 1995, 2005). Expression of the ubiquitous heme exporter, FLVCR, can also provide protection against cytotoxic free heme (Quigley et al., 2004; Keel et al., 2008; reviewed in Khan and Quigley, 2011). The Abcg2 transporter can export intracellular PPIX, delivering heme to extracellular albumin (Szatmari et al., 2006) and as such may be considered, together with FLVCR, as a therapeutic target to promote cell survival against heme-mediated cytotoxicity.

HEME AND THE PATHOGENESIS OF INFECTIOUS DISEASES

Failure to control the deleterious effects of free heme can contribute to the pathogenesis of a number of pathologies, associated with heme release from Hb or other hemoproteins.

SEVERE SEPSIS

Severe sepsis develops from an exacerbated inflammatory response to microbial infection, leading to systemic refractory hypotension, disseminated intravascular coagulation, multiple end-stage organ failure and ultimately to death (*reviewed in* Cohen, 2002; Hotchkiss and Karl, 2003). The mechanisms underlying the pathogenesis of severe sepsis remains poorly understood (*reviewed in* Cohen, 2002; Riedemann et al., 2003; Ulloa and Tracey, 2005). We have recently identified heme as a central component in the pathogenesis of this acute inflammatory disease (Larsen et al., 2010).

Severe sepsis driven by polymicrobial infection can be induced experimentally in mice by cecal ligation and puncture (CLP; Rittirsch et al., 2009). Disease progression is associated with RBC deformation (poikilocytosis), accumulation of cell-free Hb and free heme as well as with decreased concentration of Hp and Hx in plasma (Larsen et al., 2010). Poikilocytosis (Piagnerelli et al., 2003; Bain, 2005) and decreased concentration of Hx in plasma (Larsen et al., 2010) are also observed in human sepsis. This indicates that in the absence of overt hemolysis, significant amounts of Hb can be released from RBC resulting in heme accumulation in the circulation. A number of factors can contribute to this (Kempe et al., 2007; Lang et al., 2008), including deregulated plasma osmolarity, acidosis, bacterial hemolysins (particularly *Clostridium perfringens*; Gutierrez et al., 1995), or free heme itself (Sil et al., 2004). The pathophysiologic relevance of these findings is supported by the observations that lethality driven by severe sepsis is associated with decreased levels of Hx in plasma and that administration of purified Hx, after the onset of lethal sepsis in mice, improves host survival (Larsen et al., 2010). This suggests that targeting free heme, such as using Hx, might be used therapeutically to treat severe forms of sepsis.

As discussed above (see Pathophysiological Effects of Free Heme) free heme sensitizes cells in parenchymal tissues to undergo PCD in response to a variety of pro-oxidant and/or inflammatory compounds (Seixas et al., 2009; Gozzelino et al., 2010; Larsen et al., 2010). This cytotoxic effect might contribute to the development of organ failure associated with severe sepsis. This may also explain why therapeutic targeting of single pro-inflammatory molecules, e.g., the cytokine TNF (Cohen and Carlet, 1996) or IL-1 (Opal et al., 1997) have failed as a treatment for severe sepsis, while targeting free heme may be a valuable therapeutic option, since heme acts as a cytoxic catalyst for these as well as other pro-inflammatory molecules.

It should be noted that the accumulation of free heme in the circulation of septic patients and mice does not have to derive exclusively from the oxidation of cell-free Hb. It is possible that heme *b* is released from the oxidation of other hemoproteins to which it binds non-covalently, such as myoglobin released during rhabdomyolysis associated with septic shock (Kumar et al., 2009).

MALARIA

Together with sepsis, malaria remains a major cause of morbidity and mortality worldwide, in particular in subtropical countries where it affects millions of individuals every year (Murray et al., 2012). Malaria develops in response to *Plasmodium* infection, at a specific stage of the life cycle of this protozoan parasite associated with RBC lysis (Miller et al., 2002). This so-called "blood stage" of infection is associated with hemolysis and hence with oxidation of cell-free Hb and production of free heme (Ferreira et al., 2008).

All the clinical manifestations of malaria, from fever to respiratory distress, circulatory collapse, abnormal bleeding, jaundice,

hemoglobinuria, severe anemia, convulsions, prostration, and/or impaired consciousness are associated with the blood stage of infection (Idro et al., 2005) and as such with the accumulation of cell-free Hb and heme in plasma. When an individual presents with one or more of these symptoms, with no cause of disease other than *Plasmodium* infection, he is defined as developing a severe form of malaria (Marsh et al., 1995; Maitland and Marsh, 2004).

That non-Hb bound heme contributes to the pathogenesis of severe forms of malaria is suggested by the following observations in mice. Severity of *Plasmodium* infection correlates with the accumulation of cell-free Hb and non-Hb bound free heme in plasma (Pamplona et al., 2007; Ferreira et al., 2008; Andrade et al., 2010a). The same is true in humans (Andrade et al., 2010a). Free heme is sufficient to elicit the onset of severe forms of malaria in *Plasmodium* infected mice (Pamplona et al., 2007; Ferreira et al., 2008, 2011; Seixas et al., 2009). Heme catabolism by HO-1 suppresses the pathogenesis of severe forms of malaria in mice (Pamplona et al., 2007; Seixas et al., 2009; Ferreira et al., 2011) and is associated with the pathologic outcome of malaria in humans as well (Andrade et al., 2010a; Sambo et al., 2010; Walther et al., 2012).

The pathologic effect of free heme is most probably driven by the Fe atom released from the protoporphyrin ring of heme, either through catabolism by HO-1 (Tenhunen et al., 1968) or via non-enzymatic oxidative degradation (Schaefer et al., 1985; Nagababu and Rifkind, 2000, 2004). The deleterious effects of labile Fe can be countered by FtH (Balla et al., 1992), probably explaining why FtH prevents the onset of severe forms of malaria in mice (Raffaella Gozzelino et al., unpublished results).

HEME AND THE PATHOGENESIS OF NON-INFECTIOUS IMMUNE-MEDIATED INFLAMMATORY CONDITIONS

HEMOLYTIC DISORDERS

A number of hereditary diseases associated with defects in RBC function can lead to premature RBC senescence, associated with increased hematophagocytosis as well as with the release of Hb and heme into circulation. Heme toxicity may contribute to the clinical complications of these diseases, as illustrated in this section for sickle cell anemia and G6PD deficiency.

Sickle cell disease

Sickle cell disease is a molecular disease (Pauling et al., 1949) caused by a single point mutation in the β chain of Hb (β6Glu > Val), leading to the synthesis of a modified Hb variant generally referred to as HbS (Williams et al., 2005a; Jallow et al., 2009). Other sickle mutations include HbC (β6Glu > Lys; Modiano et al., 2001) and HbE (β6Glu > Lys; Hutagalung et al., 1999).

When present in the homozygous form, the Hb β6Glu > Val sickle mutation becomes pathologic, leading under low oxygen pressure to Hb polymerization, RBC deformation (sickle shape), shortened RBC half-live and eventually to hemolysis. This is associated with Hb and heme release into circulation (Hebbel et al., 1988; Reiter et al., 2002). The disease manifests by a range of clinical outcomes that include episodes of vascular occlusion/dysfunction eventually leading to the development of stroke and/or renal failure. Sickle cell anemia is also associated with tissue Fe overload, presumably driven by chronic accumulation of free heme in the circulation (Hebbel et al., 1988; Reiter et al., 2002).

Cell-free Hb is thought to contribute to the pathogenesis of sickle cell anemia, essentially by scavenging NO (Reiter et al., 2002), thereby promoting vasoconstriction, platelet aggregation and expression of adhesion molecules associated with endothelial cell activation (Belcher et al., 2003). Interaction of NO with cell-free Hb also catalyzes the production of free radicals, promoting Hb oxidation and heme release. Further oxidation of cell-free Hb can lead to the formation of ferrylHb aggregates acting as a pro-inflammatory agonist in the vascular endothelium (Silva et al., 2009).

Expression of HO-1 or administration of exogenous CO counters the pathological outcome of sickle cell disease (Belcher et al., 2006). Presumably this protective effect is mediated via inhibition of endothelial cell activation (Soares et al., 2004; Seldon et al., 2007). In addition, this salutary effect may also be mediated via inhibition of Hb oxidation, preventing heme release from cell-free Hb. If this proves to be the case, then targeting cell-free Hb or free heme in plasma using Hp or Hx should ameliorate the pathological outcome of sickle cell disease as well.

When present in the heterozygous form, the Hb β6Glu > Val sickle mutation is not pathogenic, conferring instead a net survival advantage to human population living in endemic areas of malaria (Williams et al., 2005b). This protective effect acts via a mechanism involving the accumulation of low (non-cytotoxic) levels of free heme in plasma that induce the expression of HO-1 via activation of the transcription factor NF-E2-related factor 2 (Nrf2; Ferreira et al., 2011). The CO produced via heme catabolism by HO-1 binds to cell-free Hb and prevents the accumulation of high levels (cytotoxic) of circulating free heme following *Plasmodium* infection, thus suppressing the pathogenesis of severe forms of malaria (Ferreira et al., 2011). This protective effect does not interfere with parasite load revealing that sickle Hb confers tolerance against *Plasmodium* infection (Ferreira et al., 2011). That is, it promotes host survival irrespectively of its pathogen load (Medzhitov et al., 2012).

Glucose-6-P dehydrogenase deficiency

RBC lack mitochondria and as such cannot generate NAPDH via oxidative phosphorylation. Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step of the pentose phosphate pathway of glucose metabolism, and is a critical mechanism for the generation of reducing power via RBC NADPH. More than 140 G6PD mutations have been reported, leading to RBC oxidative stress and eventually to varying degrees of hemolysis (*reviewed in* Cappellini and Fiorelli, 2008). The clinical outcomes associated with G6PD deficiency are revealed clinically by fatigue, jaundice (i.e., hepatic damage), increased circulating unconjugated BR and lactate dehydrogenase as well as reticulocytosis (Cappellini and Fiorelli, 2008). Presumably, some of these clinical outcomes can be explained by the release of Hb and heme into circulation, associated to chronic or acute episodes of hemolysis.

G6PD deficiency is one of the most common enzyme mutation in human populations (Mason, 1996) and is particularly prevalent in geographical areas where malaria is endemic. This suggests that mutations leading to the functional silencing of the human *G6PD*

gene were naturally selected through evolution on the basis of the survival advantage they provide against malaria, a major driving force in human evolution (Cappellini and Fiorelli, 2008). The molecular mechanism via which *G6PD* mutations confer protection against *Plasmodium* infection are not clear but, as discussed in the previous section, this is likely to be related to the mechanism involved in the protection conferred by sickle Hb against malaria.

PATHOLOGICAL COMPLICATIONS OF PREGNANCY

The early stages of pregnancy in mammals are associated with the induction of high levels of HO-1 expression by placental trophoblasts, as demonstrated in mice, rats, and humans (Ihara et al., 1998; Barber et al., 2001; Sollwedel et al., 2005; Zenclussen et al., 2011). This suggested that expression of HO-1 might be required to support early stages of pregnancy, a notion in keeping with the observation that *Hmox1* deletion causes pre-natal lethality in mice (Poss and Tonegawa, 1997b; Yet et al., 1999; Tzima et al., 2009). Several studies have demonstrated that reduced HO-1 expression and/or activity can promote miscarriages in rodents as well as in humans (Zenclussen et al., 2003, 2005), which is also the case for pre-eclampsia, probably the most severe pathological complication associated to human pregnancy (Ahmed et al., 2000).

A more detailed analysis of the mechanisms involved in pre-natal lethality associated with *Hmox1* deletion in mice revealed that HO-1 is required to sustain placental formation and function and hence fetal development and success of the early stages of pregnancy (Zenclussen et al., 2011). Presumably, the salutary effect of HO-1 relies on its ability to prevent the accumulation of free heme, generated in the placenta from the oxidation of cell-free Hb. This notion is supported by the observation that impairment of intrauterine fetal survival, driven by *Hmox1* deletion, is associated with the accumulation of free heme in plasma, while heme administration to wild type ($Hmox1^{+/+}$) mice is sufficient *per se* to impair fetal survival (Zenclussen et al., 2011). CO can be used therapeutically to prevent miscarriages in mice, inhibiting heme release from cell-free Hb and preventing the accumulation of circulating free heme (Zenclussen et al., 2011). This is, to the best of our knowledge, the first demonstration that heme can participate in the pathogenesis of an immune-mediated inflammatory condition that is not driven by infection.

ISCHEMIA AND REPERFUSION INJURY

Ischemia and reperfusion injury act as a major cause of pathology leading to myocardial infarction, stroke or damage associated with organ transplantation, among others. Ischemia refers to a more or less pronounced restriction of blood supply to a given organ, associated with lower than physiological supply of several essential molecules, including O_2. In the event that blood flow is re-established, i.e., reperfusion, the ischemic tissue is confronted with an abrupt delivery of O_2 that cannot be readily used by the mitochondrial electron transport chain. The excess O_2 becomes available to oxidative enzymes, e.g., NADPH oxidase, catalyzing the generation of ROS, i.e., superoxide ($O_2\bullet$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\bullet$). Accumulation of ROS above a certain threshold level causes oxidative stress, promoting PCD and tissue damage (Zhang et al., 2007a).

Expression of HO-1 exerts strong protective effects against IRI (*reviewed in* Soares and Bach, 2007), via a mechanism that is not clear but is likely to involve the catabolism of deleterious free heme, which is in keeping with the notion that hemolysis might act as an inherent component of the pathogenesis of IRI. Presumably, the cytotoxic effect of free heme contributes to promote tissue damage associated with IRI, explaining the salutary effects of HO-1 (Shen et al., 2005), CO (Fujita et al., 2001; Akamatsu et al., 2004) or FtH (Berberat et al., 2003) against this pathologic process.

TRANSPLANTATION

Mouse grafts can survive indefinitely when transplanted into immunosuppressed rats (Miyatake et al., 1998). Long-term survival relies on a cytoprotective response elicited in the transplanted organ, a phenomenon termed by Fritz H. Bach as "accommodation" (Soares et al., 1999). HO-1 expression in the transplanted organ is required to promote accommodation (Soares et al., 1998, 1999), a salutary effect mediated at least partially by CO (Sato et al., 2001; reviewed in Soares and Bach, 2007). While there are probably several mechanisms that can contribute to explain the salutary effects of HO-1 and CO in the context of organ transplantation, these are likely mediated by the capacity of HO-1 to prevent the deleterious effect of free heme (see HO-Catalyzed Heme Degradation above). This remains however to be established experimentally.

TARGETING FREE HEME THERAPEUTICALLY TO TREAT IMMUNE-MEDIATED INFLAMMATORY DISEASES

SYSTEMIC HEME TARGETING

Targeting heme proteins

While CO appears to be a major component of the protective action exerted by HO enzymatic activity (Otterbein et al., 1999b; Brouard et al., 2000, 2002; Fujita et al., 2001; Sato et al., 2001; Akamatsu et al., 2004; Soares et al., 2004), the mechanism underlying the salutary effects of this gasotransmitter remain unclear. CO can bind Fe^{2+} within the prosthetic heme groups of hemoproteins and hence modulate their biologic function (see HO-Catalyzed Heme Degradation). Presumably, this underlies the capacity of CO to act in a cytoprotective manner (Brouard et al., 2000, 2002; Silva et al., 2006) as well as to modulate macrophage activation (Otterbein et al., 2000) and to control cell proliferation (Otterbein et al., 2003).

There is another major biological output associated with CO binding to the Fe atom in the prosthetic heme groups of hemoproteins that contributes to the salutary effects of this gasotransmitter. Namely, CO can inhibit Fe-heme oxidation, preventing as well hemoprotein oxidation. This inhibits heme release from hemoproteins, as demonstrated for Hb (Pamplona et al., 2007; **Figure 4**), which is sufficient *per se* to explain how this gasotransmitter prevents the pathological outcome of malaria (Pamplona et al., 2007; Ferreira et al., 2008; Rosenthal, 2011). This suggests that CO may be used pharmacologically as a supportive therapy for the treatment of life-threatening complications of malaria.

It is likely that in a similar manner to Hb, CO may also prevent heme release from other hemoproteins such as myoglobin. Whether this would limit the pathological outcome of diseases associated with the accumulation of cell-free myoglobin, such

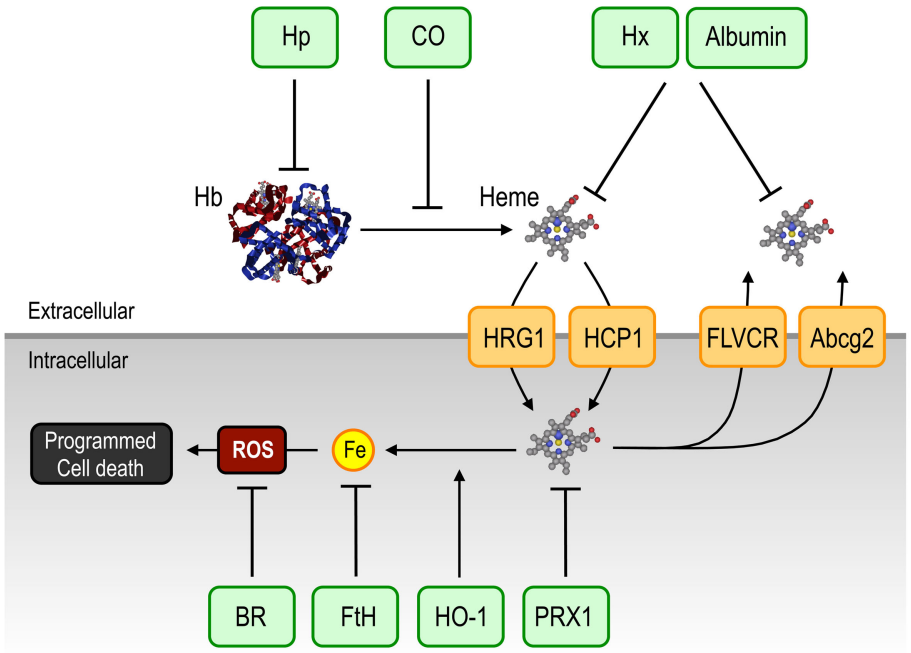

FIGURE 4 | Therapeutic targeting of free heme. Free heme, and its pathophysiological effects, might be targeted directly and indirectly, extra- and intracellularly, by a range of mechanisms. Extracellularly, albumin and the two naturally occurring acute phase proteins, Hp and Hx, can scavenge cell-free Hb and free heme, respectively, whereas CO can prevent the release of heme from Hb. Intracellularly, induction of HO-1 or FtH, might target excess heme and labile Fe, respectively. Antioxidants such as BR and the heme carrier protein PRX1, could prevent the participation of heme-Fe in the generation of unfettered production of free radicals (ROS). Specific heme transport between the intra- and extracellular compartments, including Abcg2, FLVCR, HCP1 and HRG-1, might be considered as potential, therapeutic targets to afford protection against heme cytotoxicity. Abbreviations: Bcrp/Abcg2, breast cancer resistance protein/ATP-binding cassette g2; BR, bilirubin; CO, carbon monoxide; Fe, iron; FLVCR, feline leukemic virus receptor; FtH, ferritin; Hb, hemoglobin; HCP1, heme carrier protein-1; Hp, haptoglobin; HO-1, heme oxygenase-1; HRG-1, heme responsive gene-1; Hx, hemopexin; ROS, reactive oxygen species.

as during rhabdomyolysis (Kumar et al., 2009), remains to be established.

While the evidence for the therapeutic application of CO in a number of diseases is solid, the clinical application of this gaseous molecule poses a number of practical challenges. Recently, the development of CO-releasing molecules (CORMs) received considerable attention as an alternative means of CO delivery (Foresti et al., 2005; Motterlini and Otterbein, 2010). A number of studies have demonstrated that the therapeutic benefits of CORMs mimic or can even excel those of CO inhalation (Pena et al., 2012).

Targeting free heme using natural heme scavengers

Under pathologic conditions associated with severe, acute, or chronic hemolysis, as occurs under certain infectious and non-infectious inherited diseases (see Heme and the Pathogenesis of Infectious Diseases and Heme and the Pathogenesis of Non-Infectious Immune-Mediated Inflammatory Conditions), the physiologic Hb and heme scavengers become saturated (Muller-Eberhard, 1970; Larsen et al., 2010). Therefore, replenishing the circulating stores of heme scavengers, thereby compensating for the loss of heme scavenging capacity in plasma, may be used as a therapeutic approach to target circulating free heme and prevent its deleterious effects.

Two clinical trials demonstrated that the administration of albumin, a heme scavenger, provides a significant survival benefit to children developing severe forms of malaria (Maitland et al., 2005; Akech et al., 2006). Similar data has recently been reported in the context of severe sepsis (Delaney et al., 2011). Whether the therapeutic benefit of albumin is due to its heme scavenging capacity remains to be established.

We have recently shown that exogenously administered Hx can prevent the lethal outcome of severe sepsis in mice (Larsen et al., 2010), suggesting that this may also be a viable therapeutic intervention against sepsis and possibly against other pathologies associated with a hemolytic component, such as malaria, sickle cell disease or other inherited hemolytic disorders (**Figure 4**).

CELLULAR HEME TARGETING

Cellular targeting of hemoproteins or heme transporters may counter the deleterious effects of intracellular accumulation of free heme and, as such, limit tissue damage and ameliorate the pathological outcome of immune-mediated inflammatory diseases. This may be accomplished by the pharmacological use of CO that in a similar manner to other gasotransmitters can diffuse freely into cells and, as such, target intracellular hemoproteins. In keeping with this notion, the cytoprotective effect of CO (Brouard et al., 2000) may in this manner limit tissue damage associated with the pathological outcome of immune-mediated inflammatory diseases (Otterbein et al., 2000; Fujita et al., 2001; Sato et al., 2001; Akamatsu et al., 2004; Pamplona et al., 2007; Ferreira et al., 2011).

Given that the cytoprotective effect of CO acts via a mechanism targeting the mitochondria (Queiroga et al., 2010; Wang et al., 2011) it is possible that CO would also target mitochondrial hemoproteins that play a central role in the execution of PCD, e.g., cythocrome c.

Targeting heme transport may also be used therapeutically to prevent heme-driven tissue damage (**Figure 4**). This type of approach is based on the assumption that the cytotoxic effects of free heme are mediated via a mechanism that involves its transport by dedicated heme transporters. If this proves to be the case, then targeting these transporters may provide salutary effects against immune-mediated inflammatory diseases. The general principle may be that favoring the activity of heme exporters, such as FLVCR or Abcg2 may allow cells to secrete heme and, as such, to prevent its pro-oxidant effects leading to PCD and tissue damage. In a similar manner, inhibiting cellular heme import such as by targeting HRG-1 and/or HCP1 may also be used to limit intracellular heme accumulation (**Figure 4**; Gozzelino et al., 2010).

Finally, intracellular heme might be targeted by the modulation of HO-1 expression and/or activity. This has been shown using recombinant virus-mediated overexpression of HO-1 in animal models of disease. These include vasospasm following subarachnoid hemorrhage in rats (Ono et al., 2002), transplantation of myogenic precursor cells in pigs (Laumonier et al., 2008), IRI following liver transplant in rats (Coito et al., 2002), hyperoxia-induced lung injury in rats (Otterbein et al., 1999a), and atherosclerotic diseases (Stocker and Perrella, 2006), as reviewed in (Abraham et al., 2007).

Alternatively, the deleterious effects of cellular heme can be neutralized by the therapeutic induction of endogenous HO-1, via the delivery of non-cytotoxic levels of heme or heme-containing proteins, e.g., by so-called preconditioning. In this manner, the administration of Hb in rats prior to challenge, was shown to improve survival in response to endotoxic shock (Otterbein et al., 1995), liver injury and kidney failure following rhabdomyolysis (Nath et al., 1992). Interestingly, a physiological example of heme preconditioning is represented by sickle cell trait, in which individuals carry the HbS mutation (see Sickle Cell Disease) in the heterozygous form (Williams et al., 2005a,b; Jallow et al., 2009). This mutation confers protection against severe forms of malaria due to the continuous release of low concentrations of heme into circulation, thereby leading to induction of endogenous HO-1 expression and protection against *Plasmodium* infection (Ferreira et al., 2011).

CONCLUSION

Although not highly cytotoxic *per se*, free heme sensitizes non-hematopoietic cells to undergo PCD in response to a variety of pro-inflammatory agonists. This unique pathophysiologic feature relies on the capacity of heme to promote the production of free radicals in an unfettered manner. Several protection mechanisms, regulating heme synthesis, catabolism and transport, can be considered as possible therapeutic targets to prevent the deleterious effects of free heme. In keeping with this notion, tissue damage control afforded by genes involved in heme and Fe metabolism, such as HO-1 and FtH, provide a "proof of principle" for the therapeutic benefits associated with neutralization of heme-Fe cytotoxicity in the context of immune-mediated inflammatory diseases.

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Heme oxygenase, inflammation, and fibrosis: the good, the bad, and the ugly?

Ditte M. S. Lundvig[1], Stephan Immenschuh[2]* and Frank A. D. T. G. Wagener[1]*

[1] *Department of Orthodontics and Craniofacial Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands*
[2] *Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany*

Edited by:
Mahin D. Maines, University of Rochester School of Medicine, USA

Reviewed by:
Roberto Motterlini, Institut National de la Santé et de la Recherche Médicale, France
Rene Schmidt, Freiburg University Medical Center, Germany

****Correspondence:***
Stephan Immenschuh, Institute for Transfusion Medicine, Hannover Medical School, Carl Neuberg-Street 1, 30625 Hannover, Germany.
e-mail: immenschuh.stephan@mh-hannover.de;
Frank A. D. T. G. Wagener, Department of Orthodontics and Craniofacial Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, Netherlands.
e-mail: f.wagener@dent.umcn.nl

Upon injury, prolonged inflammation and oxidative stress may cause pathological wound healing and fibrosis, leading to formation of excessive scar tissue. Fibrogenesis can occur in most organs and tissues and may ultimately lead to organ dysfunction and failure. The underlying mechanisms of pathological wound healing still remain unclear, and are considered to be multifactorial, but so far, no efficient anti-fibrotic therapies exist. Extra- and intracellular levels of free heme may be increased in a variety of pathological conditions due to release from hemoproteins. Free heme possesses pro-inflammatory and oxidative properties, and may act as a danger signal. Effects of free heme may be counteracted by heme-binding proteins or by heme degradation. Heme is degraded by heme oxygenase (HO) that exists as two isoforms: inducible HO-1 and constitutively expressed HO-2. HO generates the effector molecules biliverdin/bilirubin, carbon monoxide, and free iron/ferritin. HO deficiency in mouse and man leads to exaggerated inflammation following mild insults, and accumulating epidemiological and preclinical studies support the widely recognized notion of the cytoprotective, anti-oxidative, and anti-inflammatory effects of the activity of the HO system and its effector molecules. In this review, we address the potential effects of targeted HO-1 induction or administration of HO-effector molecules as therapeutic targets in fibrotic conditions to counteract inflammatory and oxidative insults. This is exemplified by various clinically relevant conditions, such as hypertrophic scarring, chronic inflammatory liver disease, chronic pancreatitis, and chronic graft rejection in transplantation.

Keywords: heme oxygenase, heme, HO-effector molecules, fibrosis, therapy

INTRODUCTION

Tissue injury can occur after physical, chemical, or infectious insults, such as burns, trauma, and toxins. Wound healing is a dynamic and complex process involving the coordinated action of different cell types. The quality of the wound healing process is dependent on different factors, including the severity of the insult and the inflammatory and redox status of the wound. Pathological wound healing can lead to two extreme cases: chronic, non-healing wounds or fibrosis, which ultimately may lead to excessive scarring. Both types of complications related to pathological wound healing have major impact on patient quality of life in terms of aesthetical and functional problems.

Numerous clinical and experimental observations have demonstrated that prolonged inflammation and oxidative stress may cause pathological wound healing and the development of fibrosis. However, the underlying mechanisms still remain largely unclear. Inflammation is a complex response of the innate immune system in vascularized tissues initiated to protect the organism against invading pathogens as well as to restore tissue homeostasis at the wound site.

WOUND HEALING AND FIBROSIS – WHEN GOOD TURNS BAD

The primary goal of tissue repair is to restore tissue integrity and homeostasis. Hereto, wounding initiates a complex cascade of events to stop blood loss, to eliminate invading pathogens, and, ultimately, to promote tissue integrity and homeostasis (Chettibi et al., 1999; Hunt et al., 2000).

Wound healing occurs in three distinct, but overlapping phases: (1) inflammation, (2) regeneration, and (3) remodeling, and involves a well-coordinated sequence of cellular responses.

The *inflammatory phase* is preceded by blood clot formation and coagulation to stop blood loss and to reduce pathogen invasion. This fibrin-containing clot also serves as an early provisional extracellular matrix (ECM) to provide structural support for cellular attachment and proliferation. Coagulation also triggers the production of pro-inflammatory agents, and activation of the complement system. This leads to increased blood vessel permeability, chemokine expression, vascular adhesion molecule expression, and recruitment of immune cells. At the wound site, granulocytes and macrophages are pivotal for the innate immune system-mediated elimination of invading pathogens through the generation of reactive oxygen species (ROS; Fialkow et al., 2007), and the production of pro-inflammatory chemokines and cytokines (Ryan et al., 2004).

The temporal and spatial pattern of inflammation resolution is crucial for proper wound healing and is characterized by reduced levels of pro-inflammatory adhesion molecules and cytokines and a decreasing number of inflammatory cells at the site of injury.

This is followed by the initiation of the *regenerative phase* that mediates re-epithelialization, neovascularization, and wound closure. In particular, fibroblasts produce and deposit ECM components to substitute the provisional matrix. Also, recruited keratinocytes and endothelial cells are crucial to this process.

When the wound area is fully re-epithelialized, *remodeling* takes over from regeneration. Fibroblasts differentiate into myofibroblasts causing wound contraction and ECM reorganization by synthesis and deposition of ECM components and by providing matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), which ultimately leads to wound closure. At the end of this phase, myofibroblasts present in the granulation tissue disappear by apoptosis, producing a rather acellular scar. It is important to realize that scar formation is a natural part of the wound healing process, as normal scar tissue, which mainly consists of connective tissue, represents a stable restoration of the skin (for a recent review, see Guo and Dipietro, 2010).

Prolonged inflammatory conditions accompanied by oxidative stress may interfere with the normal wound healing process, leading to an extended presence of myofibroblasts and excessive scar formation, a process known as fibrosis. Fibrosis is not only restricted to dermal wound healing, but also occurs in palatal tissue, lungs, heart, liver, intestine, and joints, and causes major medical problems ranging from disfigurement to progressive disability and even death.

Despite the fact that disorders that are associated with fibrosis account for up to 45% of deaths in the developed world, no successful anti-fibrotic therapies exist (Wynn, 2008).

Extensive research has shown that the heme–heme oxygenase (HO) system is closely involved in the regulation of various (patho)physiological processes, in particular in cellular adaptation to oxidative stress, and the anti-inflammatory response.

In this review, we address the role of heme, HO-1 and HO-effector molecules in inflammation and fibrosis with an emphasis on hypertrophic scarring, chronic inflammatory liver disease, chronic pancreatitis, and transplantation complications.

INFLAMMATION AND SCARRING – WHEN BAD TURNS UGLY

A prolonged inflammatory phase is considered a major cause of fibrosis and excessive scar formation. A clear link between inflammation and fibrosis has been revealed through embryonic studies. Mammalian embryos heal without scar formation, if wound healing occurs without influx of inflammatory cells (Ashcroft et al., 1999), indicating that inflammatory cell recruitment to the wound site is a prerequisite for scar formation. Wound healing studies in different knockout and transgenic mouse models (Fathke et al., 2004; Duffield et al., 2005; Oakley et al., 2005; Tabibiazar et al., 2006; Saito et al., 2008; Goren et al., 2009), athymic mice (Gawronska-Kozak et al., 2006), and *in vivo* antisense knockdown studies (Mori et al., 2006, 2008; Mathew et al., 2007) further support this notion. Also, an overwhelming amount of data have shown that inflammatory cells emigrating from blood, such as granulocytes, macrophages, and helper T cells, provide signals that promote granulation and fibrosis, including ROS, ECM deposition, and chemokines and cytokines (reviewed in Martin and Leibovich, 2005).

Clinical data demonstrate elevated inflammatory profiles in patients with pathological scarring (Harty et al., 2003; Martin and Leibovich, 2005). Also, chronic wounds demonstrate impaired resolution of the inflammatory phase, and thus remain in a chronic inflammatory state (Loots et al., 1998). Time is a critical factor for abnormal scar formation, as long-lasting wound healing significantly increases the risk of hypertrophic or excessive scarring (Deitch et al., 1983).

The continuous presence of inflammatory mediators results in increased secretion of growth factors, extending the proliferative phase via the prolonged presence of myofibroblasts (Singer and Clark, 1999). This in turn causes excessive deposition of ECM components, exaggerating the outcome of the proliferative phase and contributing to hypertrophic scarring or fibrosis (Aarabi et al., 2007). Thus, the inflammatory cell-derived ROS generated during the early inflammatory phase must be tightly controlled by cellular anti-oxidative and anti-inflammatory systems, as impaired ROS clearance exacerbates wound healing (Frantz et al., 1993; Steiling et al., 1999).

The unifying hallmark of fibrotic disorders is abnormal and excessive deposition of ECM components. As exemplified in the following sections, the broad range of affected organs, the progressive and often irreversible nature of fibrosis, and the large number of affected patients combined with ineffective treatment poses a challenge to the development of novel therapeutic approaches toward limiting fibrosis.

HYPERTROPHIC SCARRING

The skin is the organ with the largest surface area in the body, functioning as a barrier against the external milieu, and is important in protecting the body against pathogens and dehydration. Other functions include temperature regulation, tactile sensation, and vitamin D synthesis.

Upon cutaneous injury, dermal wound healing is crucial for restoration of the damaged skin barrier. In most cases, wound healing will result in the formation of a visible scar. However, prolonged inflammation and high levels of oxidative stress (e.g., due to infections) may result in excessive deposition of predominantly collagen I (Sidgwick and Bayat, 2012), leading to fibrosis and excessive scarring that in skin is manifested as a hypertrophic or a keloid scar. Both keloids and hypertrophic scars are characterized by excessive collagen deposition and are discolored with a rough, stiff appearance, but whereas hypertrophic scars are confined to the original wound area, keloids overgrow these boundaries. Hypertrophic scars may occur in persons of any age and at any site, whereas keloids develop with higher risk in certain ethnic groups (Brissett and Sherris, 2001). Almost all abnormal scars are associated with prolonged inflammation caused by foreign body reaction, bacterial infections, tattoos, burns, injections, bites, cuts, vaccination, trauma, surgery, or infections. Importantly, immunological alterations have been reported in hypertrophic scars, including abnormal growth factor and cytokine levels (van der Veer et al., 2009).

Often patients report itching and pain as major issues besides the aesthetical and functional problems (Bock et al., 2006). These complications may require surgical corrections, which may then lead to further scarring complications.

FIBROSIS IN CHRONIC INFLAMMATORY LIVER DISEASE AND CHRONIC PANCREATITIS

Liver and pancreas are two major abdominal organs which have specific exocrine and endocrine functions within the gastrointestinal tract and in control of the body's metabolic homeostasis.

The liver is not only a major regulator of systemic metabolism but also has key functions in detoxification of endogenous and exogenous toxins. Hepatic fibrosis is mainly caused by chronic hepatitis B and C, alcoholic liver disease, and fatty liver disease/non-alcoholic steatohepatitis (extensively reviewed in Hernandez-Gea and Friedman, 2011). Albeit from different origins, these disorders have similar early pro-inflammatory features, which can progress into hepatic fibrosis and ultimately into end stage liver cirrhosis. Hepatic fibrosis can be regarded as a result of the hepatic wound healing response to repeated injury. If hepatic injury sustains, failure of liver regeneration occurs and results in fibrosis, in which activated hepatic stellate cells (HSCs) play a key role via excessive production of ECM components. More specifically, HSC activation is the result of a complex sequence of events such as secretion of pro-inflammatory cytokines by other hepatic cells such as liver tissue macrophages (Kupffer cells). Ultimately, hepatic fibrosis and cirrhosis lead to a replacement of healthy hepatic tissue with scars and regenerative nodules, causing loss of liver function.

The pancreas is a major gland organ and has crucial exocrine functions in the gastrointestinal digestive tract. In addition, it is a major endocrine organ and produces the glucose-regulating peptide hormones insulin and glucagon. Chronic pancreatitis is a progressive inflammatory disease (Witt et al., 2007), which is most commonly caused by chronic alcoholism or is of idiopathic origin. Similar to the liver, pancreatic stellate cells (PSCs) play a key role in pancreatic fibrogenesis (Omary et al., 2007). Normally, PSCs regulate the synthesis and degradation of ECM proteins and, thereby, the maintenance of the healthy tissue architecture. Upon pancreatic injury, PSCs differentiate into an activated phenotype which is secreting excessive amounts of ECM components and leads to the formation of fibrotic tissue (Omary et al., 2007). Persistent inflammation of the pancreas causes permanent structural deterioration with tissue fibrosis and ductal obstruction, and subsequently irreversible, declined exocrine and endocrine function (pancreatic insufficiency).

FIBROSIS ASSOCIATED WITH GRAFT REJECTION

During transplantation, cells, tissues, or organs are transferred between organisms from one individual to another or from one site to another in the same individual to correct the loss or dysfunction of an organ (e.g., kidney, liver, skin). A major problem in transplantation biology is the immunological barrier between donor and recipient, which may cause graft rejection. Based on clinical and pathological criteria, graft rejection has been classified into three major forms: hyperacute, acute, and chronic graft rejection (Azimzadeh et al., 2011). Hyperacute rejection starts within minutes of transplantation and exhibits thrombosis of graft vessels and ischemic necrosis due to circulating recipient antibodies, and organ failure occurs hours after transplantation. Moreover, acute graft rejection generally occurs within days or weeks after organ transplantation and leads to graft failure within the first year post-transplantation. This form of rejection has been primarily linked to a T cell-mediated reaction of graft destruction. Finally, chronic transplant rejection (CTR) is less well defined and results from multifactorial, pathological events, involving both immunological and non-immunological factors. Immune cells such as T cells and macrophages, chemokines, pro-inflammatory cytokines, and allo-antibodies have all been linked to the initiation and progression of the rejection process (Cornell et al., 2008; Ashoor and Najafian, 2012). Organs subjected to CTR generally display fibrotic scarring and vascular damage due to transplant vasculopathy (TV), which has similar features to atherosclerosis (Mitchell, 2009; Racusen and Regele, 2010).

Together, impaired resolution of inflammation and/or elevated levels of ROS may facilitate fibrosis and scar formation. It is now commonly accepted that the heme–HO system is a key player in the control of the inflammation and oxidative stress (Keyse and Tyrrell, 1989; Willis et al., 1996; Rushworth and O'Connell, 2004; Pae et al., 2008).

THE HEME–HO SYSTEM

The microsomal enzyme heme oxygenase (HO) catalyzes the oxidative degradation of free heme, and generates carbon monoxide (CO), ferrous iron (Fe^{2+}), and biliverdin (Tenhunen et al., 1968; Maines, 1988). Two genetically distinct HO isoforms exist: an inducible form, HO-1, and the constitutively expressed HO-2 (Immenschuh and Ramadori, 2000). Accumulating data demonstrate that the HO enzymes execute anti-inflammatory, anti-apoptotic, and anti-proliferative functions through the effector molecules generated by heme catabolism (reviewed in Willis et al., 1996; Abraham and Kappas, 2008). Biliverdin is almost instantaneously converted into bilirubin by biliverdin reductase, and the free iron is rapidly scavenged by co-induced ferritin (Wagener et al., 2003a). CO, Fe^{2+}/ferritin, and biliverdin/bilirubin affect different biological processes (Abraham and Kappas, 2008), including resolution of inflammation (Willoughby et al., 2000); however, the executed effects depend on the generated amounts and the microenvironment (Wagener et al., 2003b).

HEME AS A MOLECULAR SWITCH

Heme (iron protoporphyrin IX) is composed of an iron atom conjugated to a porphyrin group. Heme is synthesized by every mammalian cell, and enables in physiological concentrations a wide range of essential biological functions, by acting as the prosthetic group for hemoproteins (hemoglobin, cyclooxygenases, peroxidases) and cellular signaling (reviewed in Wagener et al., 2003b).

However, upon injury, free heme is released from hemoproteins, causing severe tissue damage (Nath et al., 1995; Balla et al., 2000; Ryter and Tyrrell, 2000; Jeney et al., 2002), predominantly by generating ROS through the Fenton reaction (Halliwell and Gutteridge, 1984) and oxidative modifications of proteins (Nath et al., 1995; Jeney et al., 2002). Also, local accumulation of high levels of free heme, e.g., in blood clots, or atherosclerotic lesions (Hasan and Schafer, 2008), overwhelms cellular detoxification systems by prolonged oxidative stress, which may cause ROS-dependent oxidation of lipids, proteins, and DNA, subsequently damaging cells and tissues (Balla et al., 1991, 1993; Jeney et al., 2002).

Furthermore, accumulating evidence shows that heme besides being a pro-oxidant in high concentration also possesses pro-inflammatory properties, because heme increases the expression of vascular adhesion molecules ICAM-1, VCAM-1, and E-selectin (Wagener et al., 1997, 1999, 2001a; Tolosano et al., 2002; Belcher et al., 2006), upregulates vascular permeability (Wagener et al., 2001b), promotes leukocyte recruitment (Wagener et al., 2001b; Porto et al., 2007), and induces the release of pro-inflammatory cytokines (Natarajan et al., 2007; Cosgrove et al., 2011; Hao et al., 2011). Administration of large amounts of heme has been demonstrated to not only result in oxidative and inflammatory stress (Wagener et al., 2001a,b; Tolosano et al., 2002), but also to exacerbate different disease settings (Pamplona et al., 2007; Seixas et al., 2009; Larsen et al., 2010). Together, this heme-induced injury has been associated with pathological manifestations of different conditions, e.g., malaria (Pamplona et al., 2007). Interestingly, increased heme levels have also been associated with fibrosis formation (Tolosano et al., 2002; Kovtunovych et al., 2010).

Furthermore, independent studies have supported a role for heme in wound healing. In moderate concentrations, heme stimulates vasoconstriction, pro-coagulation, complement activation, platelet aggregation, and cell differentiation (Nakajima et al., 1999). Also, low concentrations of free heme down-regulates the levels of pro-inflammatory cytokines, contributing to the resolution of inflammation, likely by inducing HO-1 expression (Ma et al., 2007; Cambos et al., 2010; Cambos and Scorza, 2011).

Consequently, heme can be considered as a molecular switch, since different concentrations of free heme generate different cellular responses (Wagener et al., 2003a). In small amounts, it provides essential cellular functions and cytoprotection via HO-1 induction, whereas in high concentrations, free heme can cause severe tissue injury. It is thus of importance to control the levels of free heme at sites of injury.

THE HEME–HO SYSTEM AND INFLAMMATION – FRIENDS OR FOE?

The dose-dependency of the effects mediated by free heme underscores the importance of proper control of the cellular levels hereof by the HO system.

High levels of free heme in the vascular system have been suggested to be involved in the initiation and progression of atherosclerosis, a chronic inflammatory vascular disease with fibrotic plaque pathology (Hasan and Schafer, 2008). Heme-induced oxidative stress induces expression of immediate early gene early growth response (Egr)-1 protein (Hasan and Schafer, 2008) that has been directly linked to vascular pathologies (McCaffrey et al., 2000; Blaschke et al., 2004). Importantly, this induction is inhibited by CO (Hasan and Schafer, 2008), underscoring the importance of the heme–HO feedback loop as a mechanism to counteract pathological levels of free heme. Heme also triggers vascular pro-inflammatory processes by promoting foam cell formation through oxidative modification of low-density lipoprotein (LDL) and apolipoprotein B100, which are major risk factors for the development of atherosclerosis (Tsimikas and Miller, 2011).

In contrast, free heme is an inducer of the expression of stress-sensitive genes, including HO-1, ferritin, Hsp70 as well as chemokines, and adhesion molecules (Theodorakis et al., 1989; Wagener et al., 1997; Kanakiriya et al., 2003; Iwasaki et al., 2006). Also, the local release of large amounts of free heme upon injury induces inflammatory processes, suggesting that heme acts as a danger signal (Wagener et al., 2003a; Figueiredo et al., 2007).

Furthermore, the induction of HO-1 by increased levels of heme at the site of injury also functions as a feedback system by improving the anti-inflammatory response, as HO activity counteracts a diverse range of cellular stresses (Abraham et al., 1988).

Recently, we and others have demonstrated a clear link between HO activity and diverse (pathological) cellular processes, as HO activity has been shown to be cytoprotective and anti-apoptotic as well as to reduce oxidative stress and inflammation in a multitude of different cellular and rodent models (Willis et al., 1996; Soares et al., 1998; Brouard et al., 2000; Rucker et al., 2001; Wagener et al., 2003a, 2010; Ryter and Choi, 2009; Gozzelino et al., 2010). These effects are mediated through the actions of the effector molecules generated by HO activity (Abraham and Kappas, 2008), as biliverdin and bilirubin possess strong anti-oxidant properties and CO is implicated in different signaling cascades and vasodilatation (Siow et al., 1999).

Moreover, overexpression of HO-1 counteracts the cytotoxic, pro-oxidative and pro-inflammatory effects caused by heme via down-regulating inflammatory adhesion molecules and abrogating tissue influx of leukocytes (Wagener et al., 1999, 2001b, 2003b; Rucker et al., 2001). On the contrary, inhibition of HO activity intensifies the heme-mediated oxidative and inflammatory injury *in vitro* and *in vivo* (Hayashi et al., 1999; Vachharajani et al., 2000; Rucker et al., 2001; Takahashi et al., 2007, 2009; **Figure 1**).

This is further exemplified by studies in HO-1 knockout mice and a patient with genetic HO-1 deficiency, in which an increased expression of vascular adhesion molecules and more severe inflammation was observed (Yachie et al., 1999; Kawashima et al., 2002; Wagener et al., 2003a; Kartikasari et al., 2009; Ferenbach et al., 2010; Radhakrishnan et al., 2011). Recent studies also

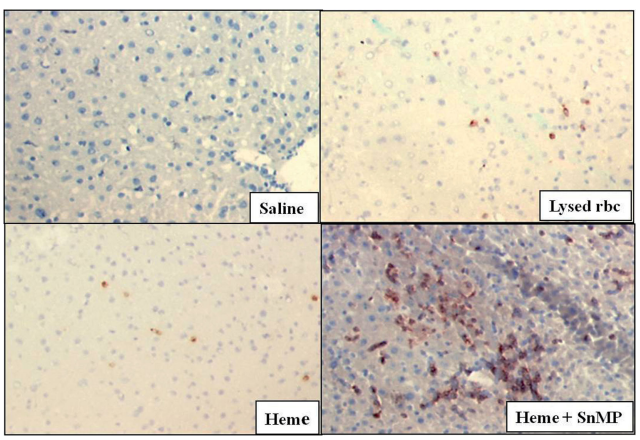

FIGURE 1 | One hour after administration of heme (750 μM) or lysed red blood cells (rbc) to mouse livers, influx of leukocytes was evident by immunohistological staining. After saline administration, no leukocytes were detected. Importantly, inhibition of HO activity before heme exposure (24 h pretreatment, SnMP) exacerbated leukocyte influx (for more details, see Wagener et al., 2001b).

suggest that HO-2 is important for the resolution of inflammation, as HO-2 deficient mice demonstrate slower corneal epithelial wound healing and an amplified inflammatory response (Bellner et al., 2008, 2009, 2011; Halilovic et al., 2011; Marrazzo et al., 2011).

In contrast, transgenic HO-1 mice are better protected from oxidative stress, inflammation, and vascular dysfunction as well as fibrotic tissue formation post-infarction (Wang et al., 2010).

Also, clinical studies have suggested that swift activation of HO-1 gene expression might be important to cope with cellular stresses. Several HO-1 gene promoter polymorphisms have been discovered, determining the levels of HO-1 induction in humans (Exner et al., 2004). The clinical relevance of the HO-1 promoter variability has been demonstrated in independent studies and higher HO-1 induction has been associated with lower incidence for cardiovascular and inflammatory diseases (Wagener et al., 2008; Grochot-Przeczek et al., 2012).

Thus the degradation of the pro-inflammatory and pro-oxidative heme molecules by HO, the signaling actions of CO, the anti-oxidant properties of bilirubin combined with the scavenging of the pro-oxidant free iron by ferritin may all contribute to the anti-inflammatory effects of the HO system.

By degrading heme, the HO system efficiently converts the pro-oxidative and pro-inflammatory heme molecule into anti-inflammatory and anti-oxidant molecules, thereby contributing to improved wound healing. This suggests that the heme–HO system executes regulatory functions in a diverse range of cellular processes with a direct influence on a timely execution of the inflammatory phase, including resolution of inflammation, apoptosis, and proliferation. Differential protection against oxidative and pro-inflammatory insults may also explain clinical differences in fibrogenesis.

PHARMACOLOGICAL REGULATION OF HO-1 AND ITS EFFECTOR MOLECULES AS THERAPEUTIC TARGETS IN INFLAMMATION AND FIBROSIS

A rapidly growing body of experimental evidence shows that specific overexpression of HO-1 has protective effects in inflammation and fibrosis (Bauer et al., 2008). Accordingly, pharmacological induction of HO-1 or administration of its effector molecules may be a promising therapeutic approach for the treatment of inflammatory disorders associated with fibrosis in various organs. However, to apply HO-1 for specific therapeutic interventions a number of important issues have to be taken into consideration.

ANTI-INFLAMMATORY EFFECTS OF HO-1 ARE MEDIATED IN A CELL TYPE-SPECIFIC MANNER

HO-1 gene expression is up-regulated in a wide range of cell types and tissues. The specific anti-inflammatory effects of HO-1, however, seem to be dependent on its coordinate up-regulation in a cell type- and cell-context-specific manner. Notably, the regulation of HO-1 expression in mononuclear/myeloid and in endothelial cells appears to be of major importance for the immunomodulatory and anti-inflammatory functions of this enzyme.

The key immunomodulatory role of HO-1 in mononuclear cells has been shown in conditional knockout mice, in which the HO-1 gene has been specifically deleted in myeloid cells. In this report, various pathological immune reactions in experimental infectious and autoimmune conditions have been demonstrated *in vivo* (Tzima et al., 2009). HO-1 has also been demonstrated to play a major role in the function of dendritic cells, which are a bone marrow-derived myeloid cell population involved in mediating adaptive immune responses (Remy et al., 2009; Park et al., 2010). Moreover, HO-1 appears to be of major significance for modulating inflammatory responses in endothelial cells. This has been shown in the initial reports on HO-1 knockout mice (Poss and Tonegawa, 1997) and in the first human case of genetic HO-1 deficiency (Yachie et al., 1999). In either case, the endothelium revealed specific pathological alterations, which have been ascribed to an increased endothelial sensitivity to oxidative stress. Accordingly, it was reported that genetic HO-1 deficiency exhibited phenotypic alterations of the vascular endothelium, causing accelerated formation of arterial thrombosis (True et al., 2007).

REGULATION OF HO-1 GENE EXPRESSION

To apply HO-1 induction by specific pharmacological compounds for therapeutic interventions, it is not only important to understand the physiological functions of HO-1 and its products.

It is also important to understand the specific regulatory pathways that up-regulate HO-1. Although primarily known to be induced by oxidative stress, HO-1 is also up-regulated by a variety of stress-independent stimuli including various well-known pharmacologic compounds (Immenschuh and Ramadori, 2000; Li et al., 2007). Regulation of HO-1 is mainly, but not exclusively governed on the transcriptional level. Accordingly, a large variety of transcription factors has been shown to be involved in HO-1 induction. In particular, redox-dependent transcription factors such as Nrf2, which is a master regulator of the anti-oxidant response, activator protein-1 (AP-1) and nuclear factor (NF)-κB, but also other nuclear factors such as Egr-1, upstream regulatory factor-2 (USF-2), and specificity protein (SP)-1 have been shown to mediate HO-1 induction (Ryter et al., 2006; Paine et al., 2010). A key role in governing HO-1 expression has also been recognized for the transcriptional repressor Bach1. Bach1 is a heme-binding protein, the activity of which is primarily regulated via intracellular levels of free heme (Ogawa et al., 2001; Igarashi and Sun, 2006). As both, Bach1 and Nrf2, interact with the anti-oxidant response element (ARE), which is the major *cis*-acting regulatory DNA element in the HO-1 gene promoter, the interplay of these two nuclear factors appears to be of major importance for inducible HO-1 gene expression. Whether and how Bach1 mediates the specific regulation of HO-1 if compared to other ARE-dependent stress response genes such as (NADPH):oxidoreductase-1 (NQO1) or glutathione-*S*-transferase (GST)-1 is a subject for currently ongoing investigations (Dhakshinamoorthy et al., 2005; MacLeod et al., 2009; Okada et al., 2010). The complexity of HO-1 gene regulation is further illustrated by the array of signaling cascades including protein kinase Cs (PKCs) and mitogen-activated protein kinases (MAPKs) that are involved in the regulation of this gene (for a review, see Paine et al., 2010). In addition, some of these pathways such as p38 MAPK appear to have contradictory cell-specific regulatory effects on HO-1 gene expression (Naidu et al., 2009) indicating that HO-1 is governed by a highly complex interplay of various signaling modules. Macrophage-specific up-regulation of HO-1 gene expression in inflammation has been

demonstrated in various experimental models (Paine et al., 2010). As an example, HO-1 in liver tissue macrophages (Kupffer cells) is up-regulated in response to the cytokine adiponectin via an IL-10 dependent pathway (Mandal et al., 2010). Moreover, macrophage-specific induction of HO-1 by various toll-like receptors (TLR) ligands, including lipopolysaccharides (LPS), requires activation of the Tec kinase Bruton's tyrosine kinase (Vijayan et al., 2011).

TARGETED PHARMACOLOGICAL HO-1 INDUCTION AS THERAPY FOR INFLAMMATION AND FIBROSIS

Numerous compounds have been shown to provide therapeutic effects via HO-1 induction. However, rather than giving an exhaustive list of compounds that may up-regulate HO-1 for therapeutic purposes in inflammatory disorders, we refer to more specific overviews on this topic (Li et al., 2007; Abraham and Kappas, 2008; Vijayan et al., 2010).

Briefly, cobalt-protoporphyrin (CoPPIX), which has been extensively applied in various experimental models to induce HO-1, does not seem an ideal compound for targeted therapeutic HO-1 up-regulation due to possible toxicity. Interestingly, systemic administration of heme (e.g., heme arginate) to healthy individuals has been shown to increase HO-1 levels in serum (Doberer et al., 2010), and has been used in the clinics for years in the treatment of porphyria (Tenhunen and Mustajoki, 1998; Ma et al., 2011). More research is warranted to explore the potential induction of systemic HO activity in the human setting by heme arginate.

Natural products such as quercetin or theaflavin (Loke et al., 2008, 2010) are less toxic and may be more appropriate for potential therapeutic applications in inflammatory disorders. Interestingly, Loke et al. (2010) recently demonstrated increased expression of endothelial HO-1 in aortic lesions and subsequently attenuation of aortic lesion formation in quercetin-fed ApoE knockout mice, underscoring the potential of targeted HO-1 induction in clinical therapies.

Curcumin is another naturally occurring compound with a broad range of pharmacological activities, including anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-diabetic, and anti-viral effects (Motterlini et al., 2000; McNally et al., 2007), mediating cellular protection against ROS (Barzegar and Moosavi-Movahedi, 2011; Wang et al., 2012; Yin et al., 2012) by up-regulating HO-1 through Nrf2 (Balogun et al., 2003). Recent studies have shown beneficial effects of curcumin in a liver injury model (Cerny et al., 2011), and curcumin treatment reduced radiation-induced lung fibrosis (Lee et al., 2010).

Moreover, macrophage-specific (Wijayanti et al., 2005) HO-1 induction by defined compounds such as 4-(2-aminoethyl)-benezensulfonyl fluoride (AEBSF) and endothelial cell-specific HO-1 induction by statins (Grosser et al., 2004; Lee et al., 2004), might be useful for such therapeutic applications.

Another key issue that needs attention when applying HO-1 induction for therapeutic applications deals with the question at what stage of the inflammatory phase HO-1 should be induced to afford its salutary protective effects. Importantly, HO-1 induction after the onset of an inflammatory disorder or in full-blown inflammation seems to be not effective in certain circumstances. This has been shown in an experimental model of dextran-sulfate-induced colitis, in which HO-1 failed to have anti-inflammatory effects if induced after the onset of this disease (Paul et al., 2005) or in an experimental model of acute pancreatitis (Nakamichi et al., 2005). Therefore, the time point of targeted HO-1 induction for therapeutic applications needs further attention. Alternatively, administration of HO-effector molecules may mediate more direct anti-inflammatory effects.

EXPOSURE TO HO-EFFECTOR MOLECULES CO AND BILIRUBIN: POSSIBLE AMELIORATING EFFECTS ON INFLAMMATION AND FIBROSIS

A wide array of preclinical and epidemiological evidence suggests that the protective properties of HO can also be mediated via its effector molecules bilirubin, CO, and co-induced ferritin. In inflammatory and fibrotic models both CO and bilirubin demonstrated significant protection (recently reviewed in Grochot-Przeczek et al., 2012).

CO exerts its cytoprotective effects through different mechanisms, including anti-flammatory (Neto et al., 2006; Chora et al., 2007), vasodilatory (Sammut et al., 1998), anti-coagulative (Chlopicki et al., 2006), anti-apoptotic (Wang et al., 2007), and anti-fibrotic pathways (Neto et al., 2006). CO deliverance is achieved through inhalation (Mayr et al., 2005; Moore et al., 2005), CO saturated physiological solutions (Nakao et al., 2006), and CO-releasing molecules (CO-RMs; Motterlini et al., 2002).

Interestingly, there are recent attempts to translate the promising findings with CO inhalation and administration of CO-RMs toward the human setting (Motterlini and Otterbein, 2010). The CO-RMs have been demonstrated to provide cytoprotective effects in different *in vitro* (Clark et al., 2003; Sandouka et al., 2005; Sawle et al., 2005; Srisook et al., 2006; Zobi et al., 2010) and *in vivo* (Vera et al., 2005; Sandouka et al., 2006; Zhou et al., 2009; Wei et al., 2010) models, which urge for translation toward the clinic.

However, the potential safety issues with these compounds must be thoroughly addressed. CO-RMs are complexes of heavy metal, like nickel, cobalt, iron, or ruthenium surrounded by carbonyl groups that are released as CO under the appropriate conditions (Motterlini et al., 2002, 2003). Ruthenium-based CO-RMs have recently been suggested to generate cytotoxic, CO-depleted by-products *in vitro* (Winburn et al., 2012). However, the development of water-soluble CO-RMs (Clark et al., 2003; Foresti et al., 2004), the use of other transition metals, e.g., manganese, in the chemical structure of CO-RMs (Motterlini et al., 2002; Crook et al., 2011), and even the use of CO-RMs devoid of transition metals (Motterlini et al., 2005) may promote the development of clinically safe CO-RMs due to less confounding effects.

Bilirubin is another generated HO-effector molecule with promising clinical applications. Besides being a powerful anti-oxidant (Stocker et al., 1987), several studies have shown that biliverdin/bilirubin administration has cytoprotective effects during ischemia–reperfusion injury and graft rejection after transplantation (Clark et al., 2000; Fondevila et al., 2004). Also, bilirubin exerts immunomodulatory and anti-inflammatory effects (Willis et al., 1996) by down-regulating expression of inflammatory cytokines and adhesion molecules (Nakao et al., 2004), as well as by reducing immune cell infiltration (Hayashi et al., 1999; Nakao et al., 2004).

Individuals with Gilbert's syndrome have a polymorphism in the UGT1A1 promoter and are protected against cardiovascular complications (Schwertner and Vitek, 2008). This polymorphism results in slower glucuronidation and therefore diminished excretion of bilirubin, leading to elevated bilirubin levels. Unfortunately, bilirubin is not available for human use. We and others postulated that experimentally induced mild hyperbilirubinemia would mimic the positive effects seen in Gilbert's syndrome (McCarty, 2007; Dekker et al., 2011). In a double-blind, placebo-controlled crossover design, we demonstrated that elevating bilirubin levels using the HIV protease inhibitor atazanavir indeed ameliorates vascular function in type 2 diabetes mellitus patients (Dekker et al., 2011). In addition, the redox status in these patients was improved following induction of mild hyperbilirubinemia (Dekker et al., 2011). This demonstrates for the first time the potential beneficial power of bilirubin as a novel mechanism in humans. We expect that these clinically significant protective effects of HO-effector molecules in humans strongly encourage more translational research toward the protective properties of HO and its effector molecules in inflammatory and fibrotic conditions.

Finally, three examples of inflammatory disorders, which are linked to organ fibrosis and in which HO-1 and its effector molecules may be of therapeutic use, are discussed in more detail.

The heme–HO system in hypertrophic scarring

Except for fast healing wounds, such as shallow scratches, most wounds will result in visible scar formation, which is a natural part of the wound healing process. However, in some individuals – depending on geographic and ethnographic distribution as well as the character of the injury – excessive deposition of ECM occurs, resulting in the formation of a raised, discolored hypertrophic scar.

The involvement of the HO system is evident from knockout mouse models being devoid of HO-1 or HO-2, respectively. Both models demonstrate delayed wound healing due to poor response toward inflammatory and oxidative insults (Braun et al., 2002; Seta et al., 2006; Deshane et al., 2007; Bellner et al., 2008, 2009, 2011; Patil et al., 2008; Kovtunovych et al., 2010; Halilovic et al., 2011). Also, pharmacological inhibition of HO activity has a negative impact on dermal wound healing (Grochot-Przeczek et al., 2009).

Also in dermal wound healing, the duality of heme is evident. Local accumulation of free heme liberated by spontaneous degradation of hemoglobin at the wound site may promote free radical formation and oxidative damage (Balla et al., 1991, 2000; Jeney et al., 2002). We have postulated that this injury-derived heme may be the trigger that initiates the inflammatory and the innate immunity response (Wagener et al., 2003a). To rapidly cope with oxidative stress, dermal fibroblasts are capable to rapidly induce HO-1 expression as a feedback response, and keratinocytes contain high levels of HO-2 (Applegate et al., 1995). When HO-1 levels are increased at the wound site resolution of inflammation will take place and the wound healing cascade can enter the next phase.

Pre-induction of HO by daily heme administration (30 mg/kg/day i.p.) in a mouse excisional wound healing model enhances wound contraction by increasing cellular proliferation and collagen synthesis (Ahanger et al., 2010). HO-1 induction also resulted in reduced transcription of pro-inflammatory cytokines, e.g., TNFα, and an up-regulated transcription of the anti-inflammatory cytokine interleukin-10 (Ahanger et al., 2010). This demonstrates that low levels of heme may promote wound healing via induction of HO-1, whereas high levels of heme may prolong oxidative and inflammatory stress and result in a pro-fibrotic environment. Indeed, up-regulation of HO-1 expression in keratinocytes in transgenic mice bearing the HO-1 gene under the control of keratin 14 promoter resulted in improved neovascularization and accelerated wound healing (Grochot-Przeczek et al., 2009). Also, adenoviral mediated HO-1 gene delivery significantly improved dermal wound healing (Grochot-Przeczek et al., 2009).

Together, these data suggest that increased levels of HO-1 may improve wound healing and reduce dermal scarring.

Also, (myo)fibroblast apoptosis is essential in normal and hypertrophic scarring, as fibroblasts in pathological wound healing remain in the wound area and deposit excessive amounts of ECM components, contributing to fibrogenesis. At low concentrations, curcumin has been shown to induce HO expression, whereas HO-1 expression and activity was negligible at high curcumin doses (Scharstuhl et al., 2009). Despite this, HO-1 expression has been demonstrated to protect against fibroblast apoptosis induced by high concentrations of curcumin through the actions of effector molecules biliverdin/bilirubin (Scharstuhl et al., 2009). Importantly, pre-induction of HO-1 with low doses of curcumin also protected fibroblasts against curcumin-induced apoptosis (Scharstuhl et al., 2009). This suggest that curcumin in high doses may affect pathological scar formation through affecting fibroblast apoptosis, while HO-1 and its effector molecules can fine-tune this response.

The heme–HO system as therapeutic target in hepatic and pancreatic fibrosis

The current therapeutic options in specific treatment of both hepatic (Popov and Schuppan, 2009) as well as pancreatic fibrosis (Braganza et al., 2011) are only limited and novel therapeutic options are urgently needed. Interestingly, independent groups have demonstrated that specific overexpression of HO-1 had an inhibitory effect on chronic viral hepatitis (Zhu et al., 2008; Hou et al., 2010) as one of the major causes of hepatic fibrosis. Exposure to high levels of heme has been demonstrated to result in inflammatory leukocyte influx and fibrosis into the liver (Wagener et al., 2001b; Tolosano et al., 2002). Moreover, in a more recent study pharmacological HO-1 induction via CoPPIX has been shown to prevent liver fibrosis in a Mdr2 knockout mice (Barikbin et al., 2012). Importantly, in this report up-regulation of HO-1 not only reduced activation of HSCs, but also reversed established hepatic fibrosis in this mouse model.

Heme exposure has also shown to strongly promote inflammation in the pancreas, as illustrated by increased vascular permeabilization (Wagener et al., 2001b). HO-1 might also serve as a therapeutic target in pancreatic fibrosis. Schwer et al. (2008) have shown that up-regulation of HO-1 by curcumin inhibited PSC proliferation, which plays a crucial role in the progression of pancreatic fibrosis. These findings have been extended in a more recent report, in which PSC proliferation was inhibited via activation of the p38 MAPK/HO-1 pathway (Schwer et al., 2010). Moreover, these authors also indicated that the protective effect on PSC may be mediated via HO-1-dependent up-regulation of the cell cycle inhibitor p21/CIP-1 (Schwer et al., 2010).

The heme–HO system in CTR

A late but severe clinical complication after solid organ transplantation is CTR, which is currently considered the major limiting factor for long-term graft survival in particular in kidney and heart transplantation (Mitchell, 2009). A hallmark of CTR is TV, also termed graft vascular disease or allograft arteriopathy, in which the success rate of current therapeutic regimens including treatment with immunosuppressive drugs is very poor (Cornell et al., 2008). Pathologic features of TV include diffuse concentric intimal hyperplasia and adventitial sclerosis due to proliferation of various cell types of the vascular wall.

Survival of cardiac xenografts has been shown to be critically dependent on the expression of endothelial HO-1 (Soares et al., 1998). Accordingly, targeted overexpression of HO-1 via a gene therapy approach has been demonstrated to protect against TV and to cause a prolonged survival of chronic allogenic rejection of aortic vascular transplants in a rat model (Chauveau et al., 2002). Additionally, HO-1 activity has been shown to reduce atherosclerosis (Bouche et al., 2002; Ferran, 2006; Du et al., 2007), which could further contribute to a higher transplantation success rate.

Although the underlying mechanisms of how HO-1 mediates its protective effects are not well understood, one major beneficial effect of HO-1 may be its anti-proliferative effects in the vasculature (Valenzuela and Reed, 2011), which has previously also been shown in an experimental angioplasty rat model (Duckers et al., 2001). In corroboration, an increasing number of studies have proven HO-1 activity to be important for transplant graft survival (Ollinger and Pratschke, 2010), as HO-1 expression induction improves recipient immune tolerance (see reviews of Soares and Bach, 2007; Blancou et al., 2011) and donor graft survival after ischemia/reperfusion injury (for recent reviews, see Ferenbach et al., 2010; Sass et al., 2012).

The beneficial effects of HO-1 activity have been linked to the induction level of HO-1, as HO-1 gene promoter polymorphisms affect transplantation outcome (Immenschuh and Ramadori, 2000; Baan et al., 2004; Geuken et al., 2005; Holweg et al., 2005; Courtney et al., 2007; Gerbitz et al., 2008). However, it is not only the direct effect of HO-1 on limiting heme levels in injury sites that mediates the beneficial outcome of HO-1 activity. Also, the cytoprotective and anti-inflammatory effects of the end products generated by heme degradation, CO and biliverdin/bilirubin, have proven pivotal in improving the success of graft transplantation (Soares et al., 1998; Immenschuh and Ramadori, 2000; Kato et al., 2003; Nakao et al., 2004; Lee et al., 2007; Brugger et al., 2010).

The implication of heme-mediated regulation of the expression of the transcription factor and inflammatory mediator Egr-1 in atherosclerosis may have a significant impact on the success rate of graft survival after transplantation, as atherosclerosis is one of the causative agents for graft rejection and transplantation failure. Interestingly, HO-1 effects have been associated with its functional interaction with the cyclin-dependent kinase inhibitor p21/CIP-1, which belongs to the Cip/Kip protein family of cell proliferation inhibitors (Duckers et al., 2001). In addition, the function of p21/CIP-1 has been demonstrated to up-regulate the cellular anti-oxidant response via direct binding to the master HO-1 gene transcriptional regulator Nrf2 (Chen et al., 2009). These findings are in keeping with a recent report showing a major regulatory role for p21/CIP-1 in endothelial cell proliferation in atherosclerosis (Obikane et al., 2010), and may suggest that the interplay of p21/CIP-1 with HO-1 and/or Nrf2 is involved in the pathogenesis of TV.

Together, this exemplifies that the HO system is of major interest for the development of future therapeutic approaches that take advantage of pharmacological induction of HO-1 in endothelial cells. As a precedent, it has recently been demonstrated in a rodent atherosclerosis model that HO-1 induction by the pharmacological compound probucol had salutary effects via inhibition of vascular smooth muscle cell proliferation (Wu et al., 2006). Also, the importance of transcriptional regulation of HO-1 further underscores the clinical significance of the HO-1 promoter polymorphisms on therapy success, and the need for personalized medicine in the future.

FUTURE PERSPECTIVES

In this review we showed examples clearly demonstrating that excess levels of heme may promote pro-oxidant, pro-inflammatory, and subsequently fibrotic processes, in which the HO system may have counter-regulatory effects. These effects may occur via degradation of pro-inflammatory free heme and by the cytoprotective and anti-inflammatory effects of the HO-effector molecules CO and biliverdin/bilirubin. Promising (pre)clinical data support the utilization of HO and its effector molecules as novel therapeutic targets to reduce tissue inflammation, oxidative stress, and fibrosis. However, in order to translate this toward the clinical setting, safe and potent inducers of HO-1 are needed.

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Heme oxygenase-1, oxidation, inflammation, and atherosclerosis

Jesus A. Araujo*, Min Zhang and Fen Yin

Division of Cardiology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

Edited by:
Mahin D. Maines, University of Rochester School of Medicine, USA

Reviewed by:
Anderi Adrican Tica, University of Medicine Craiova Romania, Romania
Jozsef Balla, University of Debrecen, Hungary
Jawed Alam, Ochsner Clinic Foundation, USA

****Correspondence:***
Jesus A. Araujo, UCLA Division of Cardiology, Department of Medicine, David Geffen School of Medicine, 10833 Le Conte Avenue, CHS 43-264, Los Angeles, CA 90095, USA.
e-mail: jaraujo@mednet.ucla.edu

Atherosclerosis is an inflammatory process of the vascular wall characterized by the infiltration of lipids and inflammatory cells. Oxidative modifications of infiltrating low-density lipoproteins and induction of oxidative stress play a major role in lipid retention in the vascular wall, uptake by macrophages and generation of foam cells, a hallmark of this disorder. The vasculature has a plethora of protective resources against oxidation and inflammation, many of them regulated by the Nrf2 transcription factor. Heme oxygenase-1 (HO-1) is a Nrf2-regulated gene that plays a critical role in the prevention of vascular inflammation. It is the inducible isoform of HO, responsible for the oxidative cleavage of heme groups leading to the generation of biliverdin, carbon monoxide, and release of ferrous iron. HO-1 has important antioxidant, antiinflammatory, antiapoptotic, antiproliferative, and immunomodulatory effects in vascular cells, most of which play a significant role in the protection against atherogenesis. HO-1 may also be an important feature in macrophage differentiation and polarization to certain subtypes. The biological effects of HO-1 are largely attributable to its enzymatic activity, which can be conceived as a system with three arms of action, corresponding to its three enzymatic byproducts. HO-1 mediated vascular protection may be due to a combination of systemic and vascular local effects. It is usually expressed at low levels but can be highly upregulated in the presence of several proatherogenic stimuli. The HO-1 system is amenable for use in the development of new therapies, some of them currently under experimental and clinical trials. Interestingly, in contrast to the HO-1 antiatherogenic actions, the expression of its transcriptional regulator Nrf2 leads to proatherogenic effects instead. This suggests that a potential intervention on HO-1 or its byproducts may need to take into account any potential alteration in the status of Nrf2 activation. This article reviews the available evidence that supports the antiatherogenic role of HO-1 as well as the potential pathways and mechanisms mediating vascular protection.

Keywords: heme oxygenase, bilirubin, carbon monoxide, iron, oxidative stress, inflammation, atherosclerosis

HEME OXYGENASE-1 AND VASCULAR INFLAMMATION: IMPORTANCE OF BASAL LEVELS

Heme oxygenase (HO) is a rate-limiting enzyme in the catabolism of heme. In association with cytochrome P450 reductase and in the presence of NADPH and three molecules of molecular oxygen (O_2) per heme molecule, it catalyzes the oxidative cleavage of heme (Fe-protoporphyrin-IX) to render equimolar amounts of biliverdin, ferrous iron (Fe^{2+}), and carbon monoxide (CO; Maines, 1997; **Figure 1**). Biliverdin can then be converted to bilirubin by the cytosolic enzyme biliverdin reductase (BVR). There are three HO isoforms (HO-1, HO-2, HO-3) that have been described (Siow et al., 1999), although HO-3 may be a pseudogene derived from HO-2 transcripts (Hayashi et al., 2004). While HO-2 is constitutively expressed, HO-1 is normally expressed at low levels in most tissues but it is highly inducible by a variety of stimuli. Indeed, HO-1 may be among the most critical cytoprotective mechanisms that are activated during times of cellular stress such as inflammation, ischemia, hypoxia, hyperoxia, hyperthermia, or radiation (Choi and Alam, 1996). It is thought to play a key role in maintaining antioxidant/oxidant homeostasis and in the prevention against vascular injury (Abraham and Kappas, 2008).

Cumulative evidence has shown that expression of HO-1 in the vasculature exerts protective effects against vascular inflammatory processes, thanks to its antioxidant, antiinflammatory, antiapoptotic, and possibly immunomodulatory properties. Thus, HO-1 antioxidant protection is particularly evident against the vascular inflammation occurring in models of antigen-independent ischemia reperfusion injury (IRI), where the reintroduction of oxygen after a period of ischemia and generation of reactive oxygen species (ROS) constitutes the central pathogenic event. Pharmacological competitive inhibitors of HO activity such as the heme analogs SnPPIX or ZnPPIX (Kato et al., 2001) or decreased HO-1 expression in genetically engineered HO-$1^{+/-}$ mice lead to significant worsening of IRI outcomes (Tsuchihashi et al., 2006). On the other hand, HO-1 overexpression by either pharmacological means or via genetic engineering has been reported to exert potent cytoprotective effects in hepatic IRI transplant models, with profoundly diminished proinflammatory and apoptotic responses (Amersi et al., 1999; Kato et al., 2001; Coito et al., 2002).

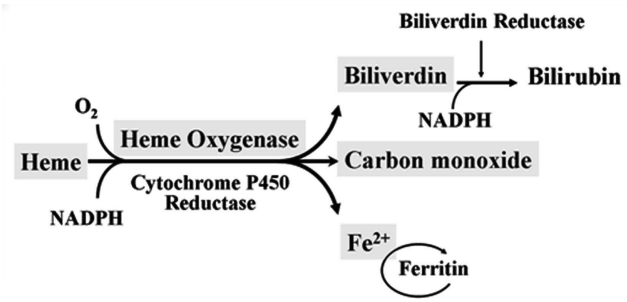

FIGURE 1 | Heme oxygenase enzymatic activity. HO enzymatic leads to the generation of biliverdin, release of carbon monoxide (CO) and Fe^{2+}. Biliverdin is transformed into bilirubin by the biliverdin reductase (BVR) enzyme. Fe^{2+} can be bound by the iron storage protein ferritin.

Of importance, basal HO-1 levels, rather than the degree of HO-1 upregulation, appear to be crucial in the antioxidant cytoprotection as indicated by data obtained from HO-$1^{+/-}$ and HO-$1^{+/+}$ mice subjected to partial liver warm ischemia for 90 min followed by 6 h of reperfusion (Tsuchihashi et al., 2006; **Figure 2**). Liver IRI injury is characterized by hepatocellular damage consisting in neutrophil infiltration, sinusoidal congestion, hepatocyte apoptosis/necrosis, and ballooning degeneration as well as impaired hepatocyte function that can be determined by elevation of serum transaminases such as the serum glutamic-oxaloacetic transaminase (sGOT) and the glutamic-pyruvic transaminase (sGPT). Treatment with CoPP (Cobalt Protoporhyrin), a synthetic analog of heme that induces HO-1 without competing for its catalytic site, led to upregulation in both basal (**Figure 2A**) and post-IRI HO-1 mRNA levels (**Figure 2B**) that led to decreased IRI overall, evidenced by decreased post-IRI serum transaminases. Interestingly, both basal (**Figure 2C**) and post-IRI HO-1 mRNA levels (Tsuchihashi et al., 2006) correlated negatively with sGPT levels but the degree of correlation was bigger with the basal ($R^2 = 0.87$, $p = 0.0002$) than with the post-IRI HO-1 levels ($R^2 = 0.55$, $p = 0.0002$). In addition, sGPT levels correlated positively with the degree of HO-1 fold induction (**Figure 2D**), suggesting that the basal HO-1 levels (**Figure 2A**) may be more important than the degree of upregulation. However, this could also be due to the HO-1 behavior as a stress-responsive gene since it can be rapidly and highly upregulated by a large variety of acute stressors. Therefore, the degree of HO-1 fold induction might reflect the level of stress induced in the liver by the ischemia reperfusion rather than an ability to protect against the injury.

Heme oxygenase-1 antiinflammatory properties are clearly evident in models of organ transplantation, not only as a consequence of the protection against IRI but also by protecting against antigen-dependent immune responses. Thus, pharmacological inhibition (Hancock et al., 1998) or decreased HO-1 expression in mouse cardiac allografts lead to accelerated acute rejection, in parallel with similar results in mouse to rat xenografts (Soares et al., 1998). In addition, HO-1 overexpression in the cardiac allograft results in prolongation of the graft survival, which is much more dramatically improved when HO-1 is overexpressed in the recipient host rather than the donor organ (Araujo et al., 2003). It is possible then that HO-1 antiinflammatory properties involve specific immunomodulatory actions that could affect antigen presentation and/or shifting of the type of cellular immune response (Araujo et al., 2003; Chauveau et al., 2005). Interestingly, in a mouse model of heterotopic cardiac allograft transplantation, where C57BL/6J hearts were transplanted into BALB/cByJ recipients, hearts from HO-$1^{\pm}$ mice, with HO-1 mRNA levels ∼60% of wild-type (WT) controls displayed shorter survival, despite a much larger degree of HO-1 upregulation after the transplant (unpublished), suggesting that basal HO-1 could be key in anti-inflammatory protection. However, the different degree of HO-1 fold induction in HO-$1^{\pm}$ mice could also reflect here, as it does in the case of the IRI, a greater level of stress induced in the transplanted hearts by various inflammatory stressors rather than a different ability to protect against the rejection response.

This notion was further confirmed in human aortic endothelial cells (HAECs) from 149 individual donors treated with oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (oxPAPC) as mentioned in more details below (Romanoski et al., 2011; **Figure 3**). In brief, baseline HO-1 mRNA levels, as judged by HAECs treated with media alone, exhibited a significant variation among different donors whereas treatment with oxPAPC resulted in significant upregulation of HO-1 levels that unlike baseline levels vary little in between donor cells (**Figure 3B**). oxPAPC treatment also led to the upregulation of several proinflammatory cytokines such as Interleukin (IL)-6. There was a negative correlation between basal HO-1 and levels of proinflammatory cytokines (**Figure 3C**). In addition, basal HO-1 levels were highly correlated with individual responsiveness to oxPAPC treatment at thresholds above 2-fold and 7.5-fold ($R = -0.57$) when responsiveness was defined as the number of genes that were regulated above a given threshold per individual. This suggests that basal HO-1 levels are important in the protection against inflammation and in mitigating multiple cellular responses to oxPAPC (Romanoski et al., 2011).

The greater importance of basal HO-1 levels as compared to the level of upregulation has important implications, especially since these observations have been made in human primary cells that did not undergo any genetic manipulation. First, if basal HO-1 expression is an important protective factor against vascular inflammation, why are the higher levels following induction unable to abrogate the inflammatory process? Second, how functional is the induced HO-1 protein? Third, what would be the ideal timing for potential therapeutic interventions and how should HO-1 responses be assessed, by changes in the level of mRNA, protein, functional activity, or levels of its enzymatic byproducts? While it appears to be clear that HO-1 functional activity is important for its vascular protection as will be discussed in Section "HO System, Enzymatic Byproducts and Protection Against Atherosclerosis," there is a possibility that various stimuli or microenvironments could lead to changes in the specific enzymatic activity, which is the HO-1 enzymatic activity/HO-1 protein. Thus, Romanoski et al. (2011) showed that while oxPAPC treatment led to increased HO-1 mRNA, protein, and enzymatic activity in HAECs, the HO activity increased in a lesser degree than the HO-1 protein, suggesting a lower HO specific activity after treatment with oxPAPC as compared with basal levels. This is consistent with another study that showed a disparity between HO-1 protein and HO activity in a rat model of metabolic syndrome

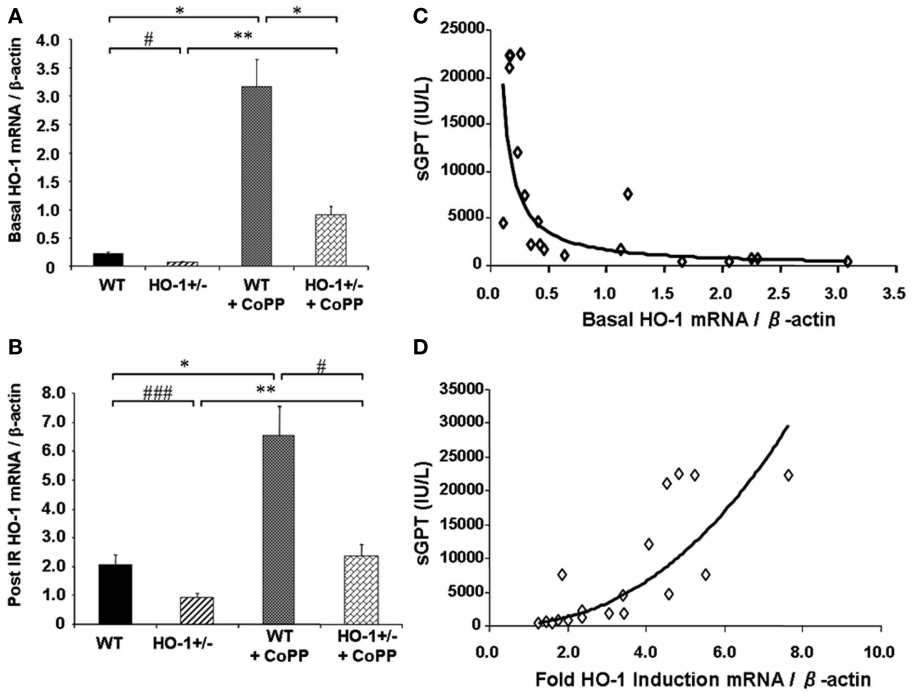

FIGURE 2 | Basal HO-1 protects against oxidation and inflammation. HO-$1^{+/-}$ and $HO^{+/+}$ (WT) mice were subjected to partial liver warm ischemia for 90 min followed by 6 h of reperfusion. Two groups of mice were treated with CoPP. **(A)** Basal HO-1 mRNA levels, **(B)** Post-ischemia reperfusion (IR) HO-1 mRNA levels, **(C)** Basal HO-1 levels correlated negatively with post-IR serum GPT levels ($R^2 = 0.69$, $p < 0.0001$) **(D)** HO-1 fold induction had a positive correlation with sGPT levels instead ($R^2 = 0.75$, $p = 0.0001$). Taken from Tsuchihashi et al. (2006). Copyright 2006. The American Association of Immunologists, Inc.

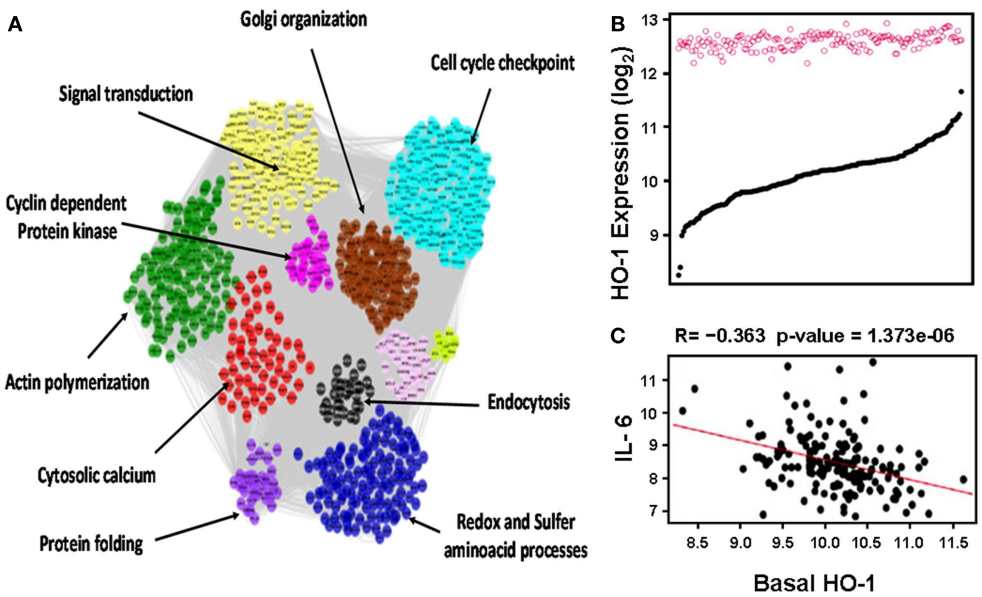

FIGURE 3 | Heme oxygenase-1 is a critical gene in the activation of endothelial cells by oxPAPC. (A) Visualization of the whole network consisting of 2000 transcripts, organized into 11 modules, shown by distinct colors. Network was constructed based on genomic expression data of HACEs from 149 donors, treated with oxPAPC. Examples of module enrichment in specific pathways are shown. HO-1 is a "hub" of gene-gene interactions in the blue module, enriched in redox and sulfur aminoacid processes. **(B)** HO-1 mRNA expression. Basal levels varied approximately ninefold at baseline (black curve) but only approximately twofold after treatment with oxPAPC (red circles). **(C)** Basal HO-1 mRNA levels correlated negatively with IL-6 mRNA expression. Taken from Romanoski et al. (2011).

where HO-1 protein was likely modified by nitrosylation resulting in decreased functionality (Kruger et al., 2006). While our current understanding of the biology of HO-1 may not be sufficient to answer all these questions at the present time, the following sections attempt to provide information that we hope will help to address them.

ROLE OF HO-1 AND OTHER ANTIOXIDANT GENES IN ATHEROSCLEROSIS

Atherosclerosis is an inflammatory process of the vascular wall characterized by the accumulation of lipids and fibrous elements in large and medium-sized elastic and muscular arteries (Lusis, 2000). Infiltrating lipids come from circulating low-density lipoprotein (LDL) particles that are retained in the vascular wall. The retention of LDL is favored by its oxidative modification which leads to activation of endothelial cells, monocyte recruitment with internalization into the vasculature, differentiation into macrophages and generation of foam cells by increased lipid uptake. Vascular infiltration of lipids and inflammatory cells further enhances oxidative stress and a vicious cycle of inflammation (Araujo et al., 2002). While the pathogenic role of vascular oxidative stress has been challenged by the argument that oxidative modifications present in the plaques could be consequential rather than causal to lesion formation (Stocker and Keaney, 2004), it does appear that the interplay between prooxidant and antioxidant factors in the vasculature may determine the degree of ROS generation in a way that can affect lesion formation. Thus, established risk factors such as diabetes and cigarette smoking or novel risk factors such as exposure to air pollution enhance ROS generation in the vasculature and promote atherogenesis (Araujo, 2011). Part of these actions may be mediated by activation of NADPH oxidase, which has been shown to be an important source of vascular ROS and deficiency of its p47phox subunit results in decreased atherosclerotic lesion formation in ApoE null mice (Barry-Lane et al., 2001). On the other hand, organisms have numerous antioxidant resources that may protect against increased ROS formation in the vasculature. Some of them are in the circulating blood such as albumin, bilirubin, or plasma high-density lipoproteins (HDL) that can exert antioxidant protection. Others are within vascular cells and include a large number of antioxidant genes and phase-2 detoxifying enzymes regulated by the transcription factor Nrf2 (NFE2 related factor 2). HO-1 is among those Nrf2-target genes, which is significantly expressed in all main cell types present in mouse and human atherosclerotic lesions, such as endothelial cells (EC), macrophages, and smooth muscle cells (SMCs; Wang et al., 1998; Ishikawa et al., 2001b).

HEME OXYGENASE-1 AND OTHER ANTIOXIDANT GENES PROTECT AGAINST ATHEROSCLEROSIS

The importance of HO-1 expression in the protection against human atherosclerotic lesions has been emphasized by various genetic population studies, which have shown that a GT length polymorphism in the promoter region of the human HO-1 gene is related to susceptibility for atherosclerosis. Thus, a shorter number of repeats has been associated with decreased susceptibility to coronary artery disease (CAD) in diabetic Japanese (Kaneda et al., 2002) and Chinese (Chen et al., 2002) populations, abdominal aortic aneurysms (Schillinger et al., 2002) and post-angioplasty restenosis, in both coronary (Chen et al., 2004) and peripheral arteries (Exner et al., 2001) in comparison with a longer number of repeats. Short GT variants (S) are likely to result in increased HO-1 expression in comparison with long variants (L) as judged by reporter transfection assays (Yamada et al., 2000), even though the cutoff to differentiate between S and L variants have been different among various studies. This was confirmed in human umbilical vein endothelial cells (HUVECs). Cells carrying the S allele exhibited higher levels of HO-1 mRNA, protein, and HO activity than cells carrying the L allele (Taha et al., 2010). While HO-1 deficiency is very rare in humans, additional confirmation of the importance of HO-1 against vascular inflammation derives from the first autopsy report of a HO-1 deficient 6-year-old boy who exhibited hyperlipidemia (Yachie et al., 1999), foamy macrophages in the liver as well as fatty streaks and fibrous plaques in the aorta (Kawashima et al., 2002). In addition, HO-1 deficiency led to very high concentrations of circulating heme and damage to vascular endothelium that was probably mediated through the generation of oxidized forms of LDL (Jeney et al., 2002) which could have contributed to atherosclerotic lesion formation. It is unclear however whether these changes can be solely attributed to HO-1 deficiency since this boy had undergone a long-term steroid treatment for a presumptive diagnosis of juvenile rheumatoid arthritis that was not confirmed upon pathological examination (Kawashima et al., 2002).

We and others have shown that HO-1 is an antiatherogenic gene in animal models (Ishikawa et al., 2001a,b; Juan et al., 2001; Yet et al., 2003; Orozco et al., 2007) as summarized in **Table 1**. Systemic lack of HO-1 has been reported to promote atherosclerosis in ApoE null mice (Yet et al., 2003) as well as aortitis in chow-fed old C57BL/6 mice (Ishikawa et al., 2012), while its overexpression by adenoviral means results in decreased atherosclerotic lesions (Juan et al., 2001). Likewise, pharmacological manipulation of the gene results in similar effects. Thus, administration of hemin results in significant HO-1 upregulation and decreased atherogenesis in LDLR$^{-/-}$ mice (Ishikawa et al., 2001b), ApoE$^{-/-}$ mice (Cheng et al., 2009), and rabbits fed a high fat diet (Li et al., 2011; Liu et al., 2012). Since increased expression of HO-1 carries the danger of increased release of ferrous iron (Fe^{2+}) and exacerbation of iron-mediated ROS formation, Ishikawa et al. (2001b) administered iron-chelating deferoxamine to LDLR$^{-/-}$ mice that were treated with hemin to avoid this potential problem. However, subsequent studies in ApoE$^{-/-}$ mice and rabbits showed that this was not necessary (Cheng et al., 2009; Li et al., 2011; Liu et al., 2012). This is likely due to the fact that HO-1 upregulation colocalizes with increased expression of ferritin in atherosclerotic lesions, which may ensure safe disposal of the released Fe^{2+}.

On the other hand, inhibition of HO enzymatic activity by SnPPIX leads to enhanced atherosclerotic lesions in LDL-R$^{-/-}$ mice (Ishikawa et al., 2001b), Watanabe hyperlipidemic rabbits (Ishikawa et al., 2001a) and rabbits fed a high fat diet (Li et al., 2011; Liu et al., 2012; **Table 1**). In addition, inhibition of HO enzymatic activity by ZnPPIX augmented plaque vulnerability in ApoE null mice by decreasing the relative cap thickness and intimal surface area of SMC, at the same time of increasing the lipid content and the degree of core necrosis (Cheng et al., 2009). It is clear then that modulation of HO-1 expression significantly alters atherogenesis in various animal models. The different degrees how atherogenesis

Table 1 | Animal studies supporting HO-1 antiatherogenic role.

Study/References	Experimental model	HO-1 modulation	Findings
Ishikawa et al. (2001a)	Watanabe rabbits on a chow diet	SnPPIX (−)	I.P. SnPPIX 5×/week for 5 weeks inhibited aortic HO activity, increased plasma, aortic, and liver lipoperoxides and enhanced aortic atherosclerotic plaques (en face) by 155% as compared with controls.
Ishikawa et al. (2001b)	LDL-R$^{-/-}$ mice fed a chow diet or HFD	Hemin (+) SnPPIX (−)	I.P. hemin or hemin + deferoxamine 4×/week for 6 weeks induced aortic HO-1 and decreased aortic root lesions. I.P. SnPPIX decreased aortic HO activity and enhanced lesions as compared with controls.
Juan et al. (2001)	ApoE$^{-/-}$ mice fed a chow diet	I.V. Adv HO-1 vs. L.V. Adv HO-1	I.V. Adv HO-1 for 1 week increased liver and aortic HO-1 and enhanced aortic root lesions while L.V. Adv HO-1 failed to increase aortic HO-1 and did not affect lesion formation as compared with controls. Similar findings in 14-week and 20-week old mice.
Yet et al. (2003)	HO-1$^{-/-}$ApoE$^{-/-}$ vs. HO-1$^{+/+}$ ApoE$^{-/-}$ mice on a HFD for 8 weeks	Genetic deletion of HO-1	12-week-old HO-1$^{-/-}$ApoE$^{-/-}$ mice, fed 8 weeks on HFD developed increased atherosclerotic lesions in the right brachiocephalic arteries by ∼260% as compared with HO-1$^{+/+}$, ApoE$^{-/-}$ controls.
Orozco et al. (2007)	Bone marrow transplantation of HO-1$^{-/-}$ vs. HO-1$^{+/+}$ into LDL-R$^{-/-}$ mice fed a HFD	Genetic deletion of HO-1 in bone marrow-derived cells	Sublethally irradiated LDL-R$^{-/-}$ mice reconstituted with HO-1$^{-/-}$ bone marrow developed aortic root plaques with greater macrophage content than control mice reconstituted with HO-1$^{+/+}$ bone marrow. HO-1 expression decreased generation of foam cells in macrophages.
Cheng et al. (2009)	ApoE$^{-/-}$ mice fed a HFD diet, with a cast around right carotid artery for 9 weeks	CoPPIX (+) ZnPPIX (−)	I.P. CoPPIX for 3 weeks increased relative cap thickness and decreased necrotic core/intima ratio while ZnPPIX resulted in the opposite despite no effects on plaque burden, suggesting that HO-1 promotes plaque stability.
Li et al. (2011)	New Zealand White Rabbits fed a HFD, subjected to balloon-induced aortic injury	hemin (+) SnPPIX (−)	I.P. hemin every other day for 12 weeks decreased intimal area positive for macrophages, lipids by 35 and 43% respectively, but increased intimal SMCs and collagen by 100 and 42% respectively. SnPPIX resulted in opposite results. Hemin decreased apoptosis and MMP-9 expression while SnPPIX resulted in the opposite.
Liu et al. (2012)	New Zealand White Rabbits, fed a chow diet and HFD	hemin (+) ZnPPIX (−)	I.P. ZnPPIX everyday for 12 weeks increased plaque area by 19% while hemin led to a three-fold decrease in plaque area vs. controls fed a HFD. Hemin increased CO generation and decreased eNOS activity and NO tissue levels.

HFD, high fat diet, I.V., intravenous, L.V., left ventricular, I.P., intraperitoneal, Adv, adenoviral.

was affected in all these studies may have to do with differences in the experimental designs and the inherent characteristics of the animal models employed (**Table 1**).

As ROS generation and tissue oxidative stress have been implicated in all stages of atherosclerosis, it appears that HO-1 expression is protective against the development of both early and advanced atherosclerotic plaques. HO-1 antioxidant and antiinflammatory properties may be crucial against the development of early plaques (Ishikawa et al., 2001b, 2012; Orozco et al., 2007), while its antiapoptotic activities can be important in lesion progression and/or plaque vulnerability for rupture (Cheng et al., 2009; Li et al., 2011). Interestingly, HO-1 expression could inhibit maturation of dendritic cells (Chauveau et al., 2005) and may modulate antigen presentation and participate in macrophage differentiation/polarization, phenomena that could be particularly important in the development of early stage lesions. On the other hand, as intraplaque hemorrhage and accumulation of heme/iron are an important component in lesion progression, HO-1 expression could be important to ensure catabolism of the heme groups and recycling of iron, which could affect the development of advanced plaques as well.

Heme oxygenase-1 antiatherogenic properties parallel its vascular protective effects in other models of vascular inflammation such as post-angioplasty restenosis (Duckers et al., 2001; Tulis et al., 2001), allograft rejection (Araujo et al., 2003), and ischemia reperfusion (Tsuchihashi et al., 2006). Like HO-1, other Nrf2-regulated antioxidant genes such as glutamate-cysteine ligase, modifier subunit (Gclm), glutamate-cysteine ligase, catalytic subunit (Gclc), peroxiredoxin 2, glutathione peroxidase, have been shown to protect against atherosclerosis. Thus, while deficiency of Gclm accelerates advanced atherosclerosis in ApoE null mice, overexpression of Gclc results in the opposite (Callegari et al., 2011). Systemic deficiency of peroxiredoxin or in bone marrow-derived cells enhances atherosclerotic lesions in ApoE null mice (Park et al., 2011). Similarly, deficiency of glutathione peroxidase exacerbates diabetes mellitus-associated atherogenesis in ApoE null mice (Lewis et al., 2007). On the other hand, adenoviral administration of endothelial cell Superoxide dismutase (EC-SOD; Laukkanen et al., 2002; Brasen et al., 2007), CuZnSOD (Durand et al., 2005), and catalase (Durand et al., 2005) inhibit post-angioplasty restenosis instead. Overall, HO-1 and several antioxidant genes, all regulated by Nrf2, exert protection against atherosclerosis.

Nrf2 TRANSCRIPTION FACTOR PARADOXICALLY PROMOTE ATHEROSCLEROSIS

The substantial evidence in support of the antiatherogenic role of several Nrf2-regulated antioxidant genes led to the hypothesis that this transcription factor should play an inhibitory role of atherogenesis as well. Surprisingly, we and others have reported three separate studies showing that Nrf2 deficiency in gene-targeted mice leads to decreased atherosclerotic lesions in ApoE null mice (Sussan et al., 2008; Barajas et al., 2011; Freigang et al., 2011), suggesting that its expression may play a proatherogenic role despite its antioxidant actions. $Nrf2^{-/-}ApoE^{-/-}$ mice developed reduced aortic atherosclerosis as compared to $Nrf2^{+/-}ApoE^{-/-}$ (Barajas et al., 2011; Freigang et al., 2011) or $Nrf2^{+/+}ApoE^{-/-}$ controls (Sussan et al., 2008; Barajas et al., 2011). The type of diet appears to be an important modulating factor as these effects were only seen in males when mice were fed a chow diet (Barajas et al., 2011).

Various putative mechanisms, as shown in **Figure 4**, could mediate Nrf2 proatherogenic effects as recently discussed in an editorial article (Araujo, 2012): (1) Nrf2 expression leads to increased levels of plasma non-HDL cholesterol in Chow-fed ApoE null mice, likely via enhancing liver lipogenesis (Huang et al., 2010; Barajas et al., 2011), (2) Nrf2 promotes foam cell formation, partially due to upregulation of the CD36 scavenger receptor (Ishii et al., 2004; Barajas et al., 2011), (3) Nrf2 enhances increased expression of IL-1α in macrophages which may promote greater monocyte migration to the lesions (Burns and Furie, 1998; Freigang et al., 2011), and (4) Nrf2 may regulate the differentiation of macrophages to subtypes different to the classical M1 and M2, named Mox (Kadl et al., 2010) and possibly Mhem (Boyle et al., 2011, 2012), with functions in atherosclerotic lesion formation still to be determined.

The opposite effects of HO-1 and Nrf2 in atherogenesis shown in **Figure 4**, underline the high degree of complexity of pathways involved in the response to prooxidative and proatherogenic stimuli. In addition, it appears that the level of regulation or intervention upon these responses is quite important as downstream interventions such as deletion of antioxidant genes like HO-1 result in exacerbation of atherosclerosis but a more upstream level of intervention, such as deletion of Nrf2 transcription factor, results in the opposite. It is therefore quite important to understand how HO-1, as well as other antioxidant genes, exert their antiatherogenic effects.

HEME OXYGENASE-1 ANTIATHEROGENIC EFFECTS: SYSTEMIC VS. VASCULAR MECHANISMS

Heme oxygenase-1 is ubiquitously expressed and highly upregulated in all the main cell types present in human (Wang et al., 1998) and murine atherosclerotic lesions (Ishikawa et al., 2001b), including endothelial cells, macrophages, and SMCs, but it is very low in neighboring unaffected vascular tissue. Such upregulation is particularly noticeable in macrophages, foam cells, and endothelial cells (Ishikawa et al., 2001b). The overall protective effects of HO-1 have been well documented and the subject of recent reviews (Morita, 2005; Abraham and Kappas, 2008; Soares and Bach, 2009; Durante, 2011) but the importance of the tissue and cellular localization as well as precise mechanisms responsible for these effects remain unclear. Several studies have addressed the role of HO-1 in atherogenesis as mentioned in the previous section (**Table 1**). For the most part, these studies have used systemic modulation of either HO-1 expression and/or HO activity, which occurs in both vascular and non-vascular cells, with subsequent alteration of oxidant and inflammatory parameters in the circulating blood. It is possible that HO-1 antiatherogenic actions are due to a combination of systemic and local vascular mechanisms.

SYSTEMIC EFFECTS

Systemic modulation of HO-1 expression levels or activity can lead to changes in the systemic levels of its enzymatic byproducts that could mediate some of the effects in the vasculature. Thus, systemic transgenic mice that overexpressed HO-1 by only ∼30–50%

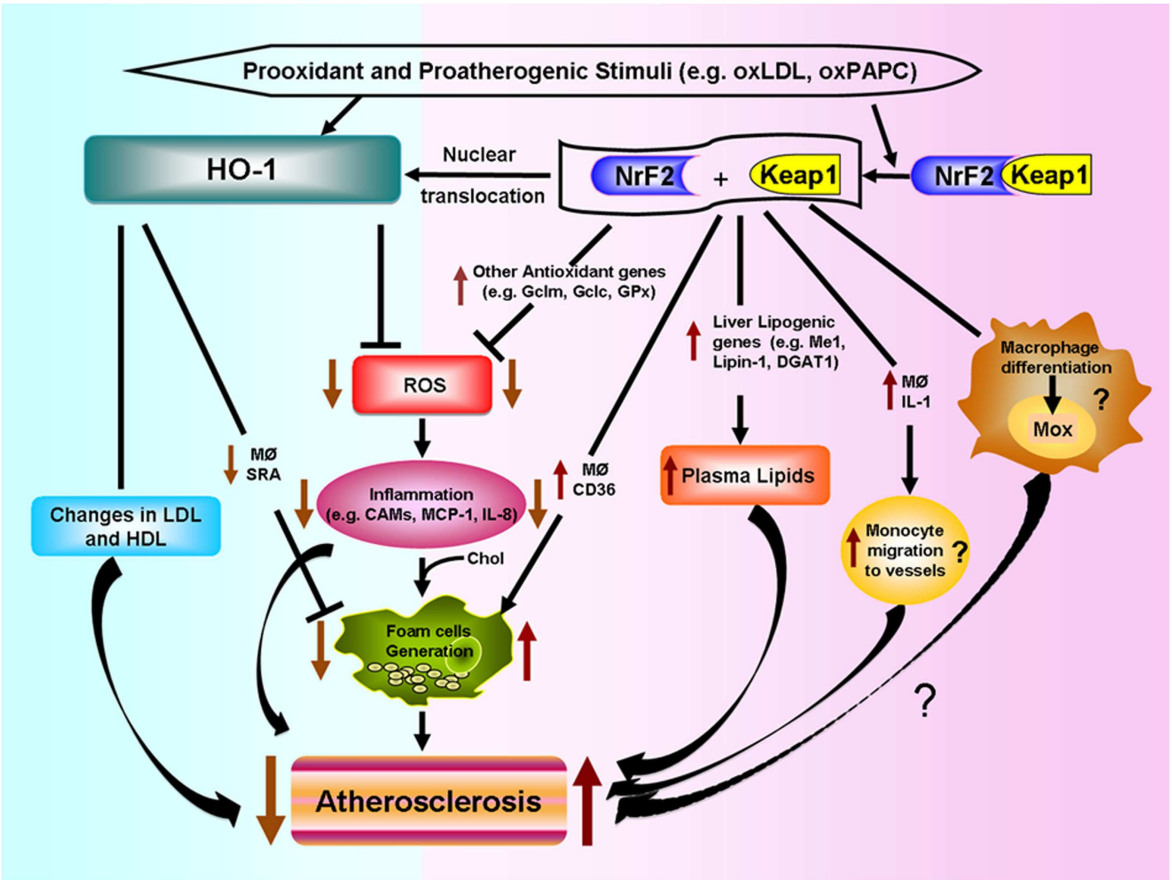

FIGURE 4 | Differential effects of HO-1 and NRF2 in atherosclerosis. Prooxidant and proatherogenic stimuli such as oxLDL and oxPAPC can induce HO-1 expression in vascular cells via Nrf2 activation or other pathways. HO-1 expression leads to decreased ROS generation, decreased inflammatory events such as lower expression of cell adhesion molecules (CAMs) and decreased secretion of inflammatory factors such as monocyte chemotactic protein (MCP)-1 and IL-8. HO-1 also leads to decreased foam cell formation and changes in LDL and HDL lipoproteins, all of which lead to decreased atherogenesis. Nrf2 is bound by chaperone Keap1 in the cytosol. Electrophilic agents lead to dissociation of the Nrf2-Keap1 complex, with release of Nrf2 and nuclear translocation that leads to the induction of HO-1 and several other antioxidant genes that result in decreased ROS formation. However, Nrf2 expression promotes atherosclerotic lesion development. Possible mechanisms include: (1) increased macrophage lipid uptake and foam cell formation, (2) increased lipogenesis and greater levels of non-HDL plasma cholesterol, (3) greater macrophage secretion of IL-1 and monocyte migration to the vessels, and (4) possible macrophage differentiation into a proatherosclerotic phenotype.

in the liver over WT controls, exhibited a modest increase in blood unconjugated bilirubin (Araujo et al., 2003). Likewise, I.P. treatment of rabbits with hemin for 12 weeks led to increased HbCo levels while I.P. treatment with inhibitor SnPPIX for similar time led to the opposite (Li et al., 2011). In humans, small variations in bilirubin levels translate into clinical effects.

Changes in blood or vascular concentrations of HO byproducts could lead to alterations in plasma lipoproteins that may affect susceptibility to atherogenesis. Indeed, HO-1 null mice exhibit increased levels of plasma lipid peroxides (Ishikawa et al., 2012) that are parallel to their increased levels of lipid peroxides and protein carbonyls in the liver and kidneys (Poss and Tonegawa, 1997). Likewise, inhibition of HO activity by ZnPPIX leads to increased serum levels of oxLDL (Liu et al., 2012). It is possible that HO-1 deficiency or inhibition of HO enzymatic activity could lead to increased levels of circulating heme which may mediate lipid peroxidation in plasma lipoproteins, resulting in oxidized LDL (Balla et al., 1991) and lipid oxidation in atherosclerotic plaques (Nagy et al., 2010). Indeed, the 6-year-old boy, deficient in HO-1, was reported to have chronic intravascular hemolysis (Yachie et al., 1999) resulting in very high concentrations of circulating heme and a high proportion of methemoglobin (Jeney et al., 2002). The latter is a form of the oxygen-carrying hemoglobin that can not bind oxygen and releases iron in a greater degree than the normal oxy-hemoglobin with the subsequent potential of promoting oxidative injury and endothelial cell toxicity (Jeney et al., 2002). Therefore, HO-1 mediated disposal of heme groups may help to decrease systemic lipid peroxidation, generation of oxidized LDL and cellular injury (**Figure 4**).

Heme oxygenase-1 systemic effects also appear to involve HDL lipoproteins as HO-1 null mice exhibit decreased levels of paraoxonase activity and altered ratio of apoAI to apoAII proteins, which are likely to alter HDL function (Ishikawa et al., 2012). Therefore, HO-1 expression may protect against LDL oxidation or decrease

its susceptibility to oxidation as it alters HDL protective qualities (**Figure 4**). In addition, HO-1 expression may also affect inflammatory mediators at a systemic level, as HO-1 null mice have been reported to exhibit greater plasma levels of monocyte chemotactic protein (MCP)-1 protein and increased levels of MCP-1 mRNA in circulating leukocytes as compared with wild-type mice (Pittock et al., 2005).

Heme oxygenase-1 and BVR, the latter responsible for the conversion of biliverdin into bilirubin, have been reported to exert beneficial effects in metabolic pathways. For instance, BVR has been shown to be a member of the insulin receptor substrate family (Lerner-Marmarosh et al., 2005) with pleiotropic functions that include effects on metabolism and cytoprotection (Kapitulnik and Maines, 2009). In addition, HO-1 has been shown to be induced by apoAI mimetic peptides and mediate some of their beneficial effects on decreasing endothelial cell sloughing and improving vascular reactivity in a rat model of diabetes (Kruger et al., 2005). Whether these effects are due to a systemic or a vascular level of action is not clear. HO-1 null mice do not exhibit alteration of plasma glucose and/or lipid levels indicative of overt diabetes, glucose intolerance, or other frank metabolic disorders. It might be possible that HO-1 deficient mice may experience upregulation of other compensatory mechanisms that could inhibit the development of obvious metabolic abnormalities.

In addition to these systemic effects, it also appears that vascular local expression is important. Indeed, adenoviral HO-1 overexpression via intracardiac administration, resulting in both aortic and liver overexpression, confers protection against atherosclerosis whereas tail vein administration, only resulting in hepatic but not aortic overexpression, does not (Juan et al., 2001). Although the contribution of systemic effects can not be ruled out by this approach since adenoviral administration via tail vein may have elicited a degree of systemic inflammation that could have masked the effects of sole hepatic HO-1 overexpression, the study does underline the importance of HO-1 expression in the vasculature.

ENDOTHELIAL CELLS

The vessels contain a monolayer of endothelial cells which represent the first cellular component that any infiltrating lipid or inflammatory cell encounter as they enter the vascular wall. The activation of endothelial cells leading to the expression of cell adhesion molecules (CAMs) and proinflammatory factors may be key in atherogenesis. Therefore, HO-1 expression in endothelial cells can be an important factor in the prevention against atherosclerosis. Indeed, Romanoski et al. (2011) recently reported that HO-1 expression in endothelial cells constitutes a critical gene in the global response of endothelial cells against oxidized phospholipids. In this study, a "systems-level" approach was used to understand the multiplicity of pathways that are elicited in HAECs, from 149 donors, in response to a treatment with oxPAPC, products of the oxidation of phospholipid PAPC (1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine), which are generated during the oxidation of LDL particles and known to induce HO-1. The approach took advantage of naturally occurring genetic variations in the human population that perturbed individual gene expression patterns with and without oxPAPC treatment, which enabled the construction of a gene co-expression network, consisting of 11 modules (groups) of tightly connected genes. Some genes exhibited many more connections than others ("hubs"), which in general, appear to be much more important in the functioning of the cell/organism as a whole. Notably, HO-1 emerged as a "hub" in one of the most interesting modules (blue module, **Figure 3A**), with various degrees of connection with other antioxidant genes (e.g. NQO1, Gclm), unfolded protein response (UPR) as well as transcription factors (e.g. Nrf2, MAFF).

This approach not only helped to identify novel factors that could be involved in the regulation of human HO-1 such as G-protein coupled receptor (GPR)-39 but also unraveled clues about the dynamics of the HO-1 response to oxPAPC in human subjects. While HAECs from the 149 donors exhibited a ninefold difference between extremes of HO-1 expression, they only exhibited a twofold difference after treatment with oxPAPC, suggesting that there was a ceiling to oxPAPC-induced HO-1 expression in this system. Importantly, basal HO-1 expression levels, rather than HO-1 levels after oxPAPC treatment, strongly correlated negatively with the expression of proinflammatory cytokines such as IL-6 ($r = -0.36$, $p = 1.37\ e-06$), IL-8 ($r = -0.28$, $p = 2.41\ e-04$), CAMs such as VCAM1 ($r = -0.22$, $p = 3.31\ e-03$), UPR such as Activating factor (ATF)3 ($r = -0.40$, $p = 5.01e-08$). Silencing of HO-1 by siRNA resulted in upregulation of many of the same genes (Romanoski et al., 2011). Altogether, this suggests that basal HO-1 levels are major determinants in the protection against inflammation and possibly atherogenesis, in parallel with our similar findings in the models of liver ischemia reperfusion (Tsuchihashi et al., 2006) and cardiac allograft rejection (see Heme Oxygenase-1 and Vascular Inflammation: Importance of Basal Levels). In addition, it will be important to determine whether GPR39 or other genetic factors could help explain the ninefold difference observed in basal HO-1 in HAECs, especially since the (GT)n polymorphisms data could not explain that variation (Romanoski et al., 2011).

Heme oxygenase-1 expression in endothelial cells leads to decreased expression of vascular cell adhesion molecule-1 (VCAM-1) as well as the expression and release of chemokines and proinflammatory cytokines such as CC-chemokine ligand (CCL)2, also known as MCP-1 (Sacerdoti et al., 2005). Taha et al. also reported that HUVECs collected from individuals exhibited differences in their basal (unstimulated) HO-1 levels that were dependent on their genotype for the (GT)n microsatellite DNA. In this study, alleles were classified as Short (S), Medium (M), and Long (L) based on the number of GT repeats, $\leq$23, 24–28, and $\geq$29 repeats, respectively. Cells carrying the S allele exhibited higher HO-1 basal levels than those carrying the M and/or L alleles. Likewise, similar differences in expression levels ensued after treatment with H_2O_2, CoPP, LPS, 15d-PGJ_{2b}, but not after treatment with hemin or in the presence of hypoxia where there were no differences. Here also, cells with the S allele, with higher basal HO-1 levels exhibited lower basal levels of IL1β, IL-6, and ICAM-1 (Taha et al., 2010). Despite the potential differences in behavior between endothelial cells in culture and those in the vascular wall *in vivo*, these studies support a protective role for HO-1 expression in endothelial cells that could represent a significant portion of its protection against atherogenesis.

Heme oxygenase-1 expression in endothelial cells could also play a role in the protection against atherosclerosis induced by environmental factors such as smoking and exposure to air pollution, which has been shown to lead to increased atherosclerosis in several animal models (Araujo, 2011). Indeed, it is upregulated in systemic tissues in response to the exposure to ambient ultrafine particles and its upregulation in a cell line of human microvascular endothelial cells, in response to oxPAPC and diesel exhaust particle (DEP) chemicals, occurs in a synergistic manner (Gong et al., 2007). It remains to be determined whether HO-1 plays indeed a protective role in this context.

MACROPHAGES, DENDRITIC CELLS, AND LYMPHOCYTES

Macrophages are the main inflammatory cells that infiltrate the vascular wall in atherogenesis, involved in the initiation as well as progression of atherosclerotic lesions. Upon activation of endothelial cells, blood monocytes are recruited to the site of injury and/or EC activation where cytokines and/or chemokines are released, adhere to the endothelium and transmigrate to the subendothelial space where they further differentiate into macrophages. HO-1 is highly upregulated in macrophages in atherosclerotic lesions and several lines of evidence support its protective antiatherogenic role in these cells. First, HO-1 has been shown to exert antioxidant and antiinflammatory effects in macrophages (Lee et al., 2003; Philippidis et al., 2004; Levonen et al., 2007; Orozco et al., 2007). Indeed, we have reported that decreased or absent HO-1 expression in peritoneal macrophages results in enhanced ROS formation and increased inflammatory cytokines such as MCP-1, interleukin 6 (IL-6), and the murine interleukin 8 homolog (KC; Orozco et al., 2007; **Figure 5A**). Second, HO-1 expression influences lipid loading and foam cell formation as both partial and total HO-1 deficiency results in enhanced lipid loading and foam cell formation in peritoneal macrophages treated with oxLDL, at least partially mediated by increased expression of the scavenger receptor A (SR-A; Orozco et al., 2007). Third, lack of HO-1 expression in bone marrow-derived cells resulted in atherosclerotic plaques with a larger inflammatory component, suggestive of greater vulnerability for rupture (Orozco et al., 2007). Irradiated LDL-R$^{-/-}$ mice with their bone marrow reconstituted with HO-1$^{-/-}$ donor cells resulted in plaques with increased macrophage area as compared with control mice reconstituted with WT bone marrow (Orozco et al., 2007). Fourth, HO-1 expression may play a role in macrophage differentiation and polarization (**Figure 5B**).

Heme oxygenase-1 antiatherogenic actions may also be due to its expression in other myelocytic or lymphocytic cells. Thus, it has been shown that HO-1 is expressed in immature dendritic cells and its overexpression inhibits dentritic cell maturation (Chauveau et al., 2005) which may play a role in atherogenesis. Several studies have highlighted a prominent role of dendritic cells in atherosclerotic lesions (Llodra et al., 2004; Yilmaz et al., 2004; Bobryshev, 2005) as they are present especially in regions of atherosclerotic plaques that are prone to rupture (Yilmaz et al., 2004) and its emigration from the lesions to lymph nodes associate with reduction in the size of atherosclerotic plaques (Llodra et al., 2004). Antigen presentation by dendritic cells as well as lymphocyte infiltration and the triggering of Th1 immune responses appear to play a role in atherosclerosis (Bobryshev, 2010; Manthey and Zernecke, 2011). Atherosclerotic lesion development is favored by Th1 responses while it is inhibited by conditions that promote a Th2 response (Minamino et al., 2001). HO-1 overexpression may lead to a switch from a Th1 to a Th2 response (Ke et al., 2000, 2001). These immunomodulatory actions parallel the effects of HO-1 expression against acute rejection response in models of allograft transplantation (Araujo et al., 2003). As macrophages can also function as antigen-presenting cells, they can play a role in the modulation of Th immune responses. Therefore, macrophage HO-1 expression could not only affect their function as effectors but also as presenting cells.

Macrophage differentiation, polarization, and function could indeed be affected by HO-1 expression. Upon differentiation, macrophages may polarize into distinct subsets with characteristic phenotypes and likely to exhibit different behaviors in atherosclerotic lesion formation, as schematized in **Figure 5B**. The first subtypes of polarized macrophages that were described were named as M1 and M2. Macrophages stimulated by lipopolysaccharide (LPS) or interferon gamma (IFN-γ) polarize into M1 macrophages, characterized by increased expression of proinflammatory molecules such as IL-1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α) as well as inducible nitric oxide synthase (iNOS), which typically trigger Th1 T cell responses (Wuttge et al., 2001; Yan and Hansson, 2007; Krausgruber et al., 2011). On the other hand, M2 macrophages stimulated by IL-4 (M2a), immune complexes (M2b), or IL-10/IL-13 (M2c) are characterized by increased expression of antiinflammatory molecules including IL-10, arginase-1 (Arg1), mannose receptor, tumor growth factor beta (TGF-β), which are likely to play an antiatherosclerotic role (Wilson, 2010). While both types of macrophages have been identified in atherosclerotic lesions (Bouhlel et al., 2007) and exhibit low expression of HO-1, it has been reported that increased HO-1 expression may contribute to a M2 macrophage activation profile, which is involved in the resolution of inflammation (Weis et al., 2009). Furthermore, different macrophage subtypes have been recently described where expression of HO-1 may play a characteristic role, either as a part of their phenotypic traits or in the modulation of their potential role in lesion formation.

Treatment of macrophages with oxPAPC led to a phenotypic switch of both M1 and M2 phenotypes into a new subtype named as Mox, characterized by exclusive upregulation of redox-regulated genes such as HO-1, sulforedoxin-1 (Srxn1), glutamate-cysteine ligase, modifier subunit (Gclm), glutamate-cysteine ligase, catalytic subunit (Gclc), thioredoxin reductase (Txnrd) 1, but also vascular endothelial growth factor (VEGF), nuclear receptor Nr4A2 (Nurr1) and Trb3 (Kadl et al., 2010). Approximately 30% of macrophages present in atherosclerotic lesions developed by LDLR$^{-/-}$ mice had this phenotype. Interestingly, polarization into a Mox phenotype was Nrf2 dependent and also characterized by a decreased phagocytic activity (Kadl et al., 2010), which raises the question whether this macrophage could exert proatherosclerotic actions, despite its high expression of HO-1 and other antioxidant genes (Araujo, 2012). More recently, another subtype of macrophages has been described in association with intraplaque hemorrhage named as Mhem, characterized

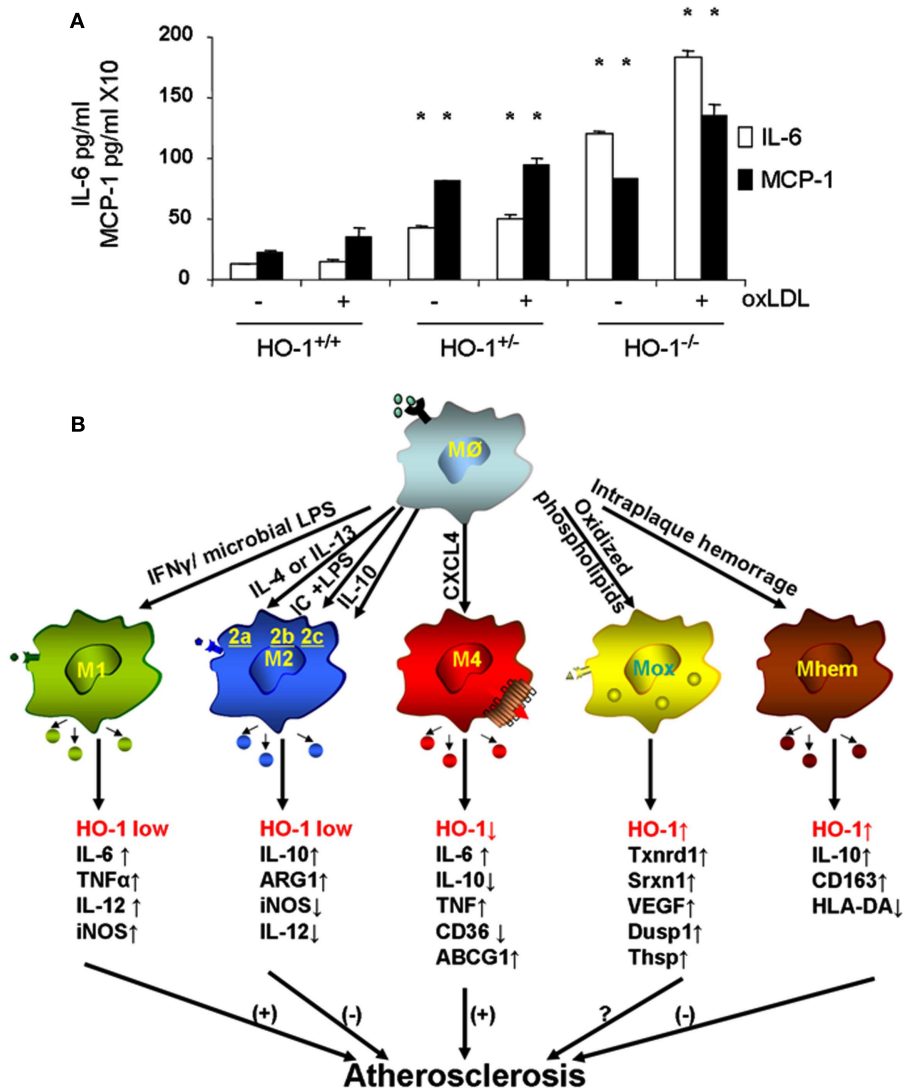

FIGURE 5 | Heme oxygenase-1 inhibits macrophage proinflammatory activity. (A) Peritoneal macrophages from HO-1$^{+/+}$, HO-1$^{+/-}$, and HO-1$^{+/+}$ mice were cultured in the presence or absence of oxLDL 50 μg/ml for 6 h. IL-6 and MCP-1 were determined by ELISA. *$p < 0.001$ as compared with controls. Data taken from Orozco et al. (2007). **(B)** HO-1 expression varies in different subtypes of macrophages. Upon monocyte differentiation into macrophages, they can polarize into one of the five proposed subtypes, M1, M2, M4, Mox, and Mhem. Each subtype is induced by specific stimuli as shown along the arrows that derive from the macrophage Mø atop, and characterized by a set of phenotypic markers and/or gene expression shown below each one of them. While some of macrophage subtypes are proposed to simulate atherogenesis such as M1 and M4, others are proposed to inhibit lesion development such as M2 and Mhem. Mox macrophages, generated after treatment with oxPAPC, are dependent on Nrf2 with a role in atherogenesis still to be determined.

by increased expression of IL-10 and CD163, macrophage scavenge receptor for hemoglobin-haptoglobin complex (Hb-Hp), low expression of human leukocyte antigen-DR (HLA-DR; Boyle et al., 2009). Mhem macrophages also exhibit increased expression of HO-1, dependent on Nrf2 (Boyle et al., 2011) in addition to activating transcription factor 1 (Boyle et al., 2012). These macrophages could play an antiatherogenic role by virtue of its participation in Hb-Hp clearance, with subsequent reduction in ROS formation and antiinflammatory effects. One last macrophage subtype named M4 is induced by CXCL4 and characterized by a gene expression profile that contained both M1 and M2 genes (Gleissner et al., 2010). Unlike Mhem macrophages which have increased expression of CD163 (also known as ED2) and HO-1, M4 macrophages do not express Hb-Hp scavenger receptor CD163 and do not upregulate HO-1 expression (Gleissner, 2012). M4 macrophages appear to play a proatherogenic role, which is consistent with the ∼60% decrease in aortic atherosclerotic lesions exhibited by CXCL-4$^{-/-}$ApoE$^{-/-}$ mice (Sachais et al., 2007). It is clear then that macrophage differentiation and polarization is quite complex as they may respond to the very specific microenvironments that monocytes and macrophages encounter. These various macrophage subtypes exhibit different patterns of

HO-1 expression (**Figure 5B**) and it is unknown whether HO-1 could play a role in the polarization into any of these subtypes or in the function that these macrophages could have in atherogenesis. For instance, Nrf2 expression appears to be essential in the generation of Mox macrophages and participate in the generation of Mhem macrophages as well, both of which express high levels of HO-1 but perhaps with different roles in the development of atherosclerotic lesions. Thus, the role for HO-1 expression in these various macrophage subtypes still need to be determined (**Figure 5B**).

SMOOTH MUSCLE CELLS

Heme oxygenase-1 is also expressed in vascular SMCs and could play an important role in the protection against atherogenesis. This is supported by evidence accrued in the model of post-angioplasty restenosis where the proliferative response of SMCs constitutes a fundamental aspect of the disease (Duckers et al., 2001; Tulis et al., 2001). SMC proliferative response has been shown to be important in the progression of atherosclerotic plaques as well. On the other hand, SMC and their synthesis of collagen are important contributors to plaque composition and stability as thicker fibrous caps are associated with lesser vulnerability for rupture. As mentioned above, inhibition of HO activity by ZnPPIX decreased relative cap thickness and intimal surface of SMC in ApoE null mice (Cheng et al., 2009). Likewise, administration of SnPPIX to rabbits resulted in plaques with decreased content of SMC while hemin led to the opposite (Li et al., 2011). Whether these effects are due to HO-1 expression in SMC *per se* or due its expression in neighboring cells is not clear.

In summary, the overall antiatherogenic role of HO-1 expression may be due to a combination of complex systemic and vascular local effects that converge in the inhibition of lipid peroxidation with effects on circulating lipoproteins, decrease in the activation of endothelial cells, inhibition of macrophage proinflammatory activity and possible participation in its differentiation/polarization, inhibition of dendritic cell maturation as well as modulation of immune-mediated responses and regulation of the proliferative response of SMCs.

HEME OXYGENASE SYSTEM, ENZYMATIC BYPRODUCTS, AND PROTECTION AGAINST ATHEROSCLEROSIS

Heme oxygenase-1 is not only expressed ubiquitously in the body but it is also located in several compartments within the cell. It is a 32-kDa protein most abundantly located in the microsomal fraction, with a short transmembrane segment of ∼ 2 kDa in the endoplasmic reticulum membrane and ∼30 kDa cytosolic portion. However, HO-1 can also be found in the plasma membrane, associated with caveolae (Kim et al., 2004), in the mitochondria (Converso et al., 2006) and in the nucleus (Lin et al., 2007, 2008). Interestingly, while nuclear HO-1 was shown to be catalytically inactive (Lin et al., 2008), it appeared to modulate the expression of itself (Lin et al., 2008) and that of activating protein (AP)-1 (Lin et al., 2007). Although HO-1 does not have DNA binding domains in its sequence and no definite protein-protein interactions have been demonstrated, additional work is required to elucidate whether HO-1 may play a role in transcriptional regulation. It appears that the biological effects due to HO-1 expression are mostly due to its enzymatic activity, since its pharmacological inhibition results in almost complete abolishment of those biological effects. Therefore, the various byproducts of HO enzymatic activity could be considered as its "branches" or "arms of action."

BILIVERDIN/BILIRUBIN

There is evidence that biliverdin/bilirubin can mediate some of HO-1 antiatherogenic effects. Exogenous administration of biliverdin, which is promptly converted to bilirubin, appears to be effective in inhibiting atherogenesis in ApoE null mice (personal communication from Miguel Soares, Gulbenkian Institute of Sciences, Portugal). This parallels the protective effects of biliverdin and/or bilirubin in other models of vascular inflammation, such as liver ischemia reperfusion injury (Fondevila et al., 2004), cardiac allograft graft rejection (Yamashita et al., 2004), and post-angioplasty restenosis (Ollinger et al., 2005, 2007). In addition, epidemiological studies show an inverse relationship of plasma or serum bilirubin concentrations and the risk of CAD (Schwertner et al., 1994; Schwertner and Vitek, 2008), at a strength that was found to be similar to that of smoking, elevated systolic blood pressure, and low HDL cholesterol (Schwertner et al., 1994; Hopkins et al., 1996; Schwertner, 1998). Serum bilirubin concentrations are inversely correlated with the severity of atherosclerosis in men (Novotny and Vitek, 2003). Thus, individuals with Gilbert syndrome, characterized by increased levels of circulating unconjugated bilirubin due to a low reactivity of the bilirubin-uridine diphosphate glucuronyl transferase (bilirubin-UGT) enzyme, exhibit a marked reduction in CHD risk (Schwertner and Vitek, 2008).

Biliverdin/Biliribin could inhibit atherogenesis through to its antioxidant properties. Biliverdin's antioxidant properties appear to depend on the expression of BVR (Singleton and Laster, 1965) and its conversion to bilirubin. Indeed, bilirubin is a powerful antioxidant (Stocker et al., 1987; Stocker and Keaney, 2004) that could inhibit the oxidation of LDL and other lipids (Neuzil and Stocker, 1994; Wu et al., 1996), scavenge oxygen radicals (Stocker et al., 1987) and counteract oxidative stress overall (Schwertner, 1998). The production of bilirubin could participate in an amplification cycle by getting oxidized into biliverdin and then reduced back into bilirubin by BVR (Baranano et al., 2002).

Bilirubin also exerts antiinflammatory and antiproliferative actions that can account for its antiatherogenic effects. Indeed, congenitally hperbilirubinemic Gunn rats exhibit almost no neointimal proliferation after balloon injury in comparison with significant proliferation observed in WT rat controls (Ollinger et al., 2005). Likewise, 1-h local treatment of a rat carotid artery with biliverdin significantly reduces neointima formation after balloon injury (Ollinger et al., 2007). These effects were mediated by inhibition of cell cycle progression and proliferation of SMC (Ollinger et al., 2005, 2007), which could protect against atherosclerosis (Sriram and Patterson, 2001). Various mechanisms may explain antiproliferative effects of Biliverdin/Bilirubin such as: (1) Overexpression of cdk inhibitor p53 resulting in increased apoptosis of rat SMCs (Liu et al., 2002), (2) Inhibition of cyclin A, D1 and E, YY1 and cdk 2 expression, leading to hypophosphorylation of the retinoblastoma tumor suppressor protein (Rb) and

cell cycle arrest (Ollinger et al., 2005), (3) Inhibition of JNK activation (Ollinger et al., 2007), and (4) Modulation of p38 MAPK activation (Ollinger et al., 2005). Interestingly, bilirubin/biliverdin have been shown to be ligands of the aryl hydrocarbon receptor (Ahr) in mouse hepatoma hepa 1c1c7 cells (Sinal and Bend, 1997; Phelan et al., 1998) and SMCs from Ahr KO mouse aortas exhibit a prolonged cell cycle time as compared with controls (Ollinger et al., 2007), which suggest that Ahr activation could participate in the regulation of proliferation of SMCs. In support of this possibility, Ahr null mice exhibit decreased atherosclerosis in the ApoE null background (Wu et al., 2011).

All this evidence, together with the findings that relate the participation of BVR in insulin-induced pathways (Lerner-Marmarosh et al., 2005; Kapitulnik and Maines, 2009), make this arm attractive candidate for pharmacological intervention. Thus, development of compounds that either increase the endogenous levels of biliverdin/bilirubin by targeting genes involved in the metabolism or resemble their biological activity can lead to promising new therapies.

CARBON MONOXIDE

The generation of CO constitutes the second arm how HO-1 may prevent atherosclerosis. In fact, administration of CO by inhalation results in suppression of intimal hyperplasia associated with chronic graft rejection and balloon injury (Otterbein et al., 2003). Although there are no reports yet about CO inhibitory effects on atherosclerotic lesions due to hyperlipidemia, there are several lines of evidence and potential mechanisms that make it very likely. First, CO exhibits cytoprotective properties in vascular endothelial cells by inhibiting apoptosis, likely through activation of p38 mitogen-activated protein kinase (MAPK) signaling pathway (Brouard et al., 2000). Second, CO exerts antiinflammatory effects. Indeed, CO has been shown to modulate the response of monocytes/macrophages to bacterial LPS (Otterbein et al., 2000), decrease macrophage toll-like receptor (TLR) signaling with downstream activation of NF-kB (Wang et al., 2009) and inhibit expression of GM-CSF with probable alteration of macrophage differentiation (Sarady et al., 2002). Inhalation of CO leads to decreased leukocyte infiltration of rat aortic allografts (Otterbein et al., 2003), accompanied by decreased expression of proinflammatory genes associated with macrophage activation (Otterbein et al., 2000). Interestingly, antiinflammatory effects are also mediated by activation of the MAPK signaling pathway, but they appear to require the generation of ROS as inhibition of ROS in macrophages leads to loss of CO antiinflammatory effects (Bilban et al., 2008). Third, HO-1 and CO may crosstalk with Nitric Oxide Synthases (NOS) and NO. Administration of hemin to rabbits resulted in greater CO levels in the aorta, accompanied by decreased levels of aortic NO and inducible Nitric oxide synthase (iNOS; Li et al., 2011), while HO inhibition by SnPPIX or ZnPPIX resulted in the opposite (Li et al., 2011; Liu et al., 2012). CO might bind and activate guanylate cyclase, leading to increased intracellular levels of cyclic guanine monophosphate (cGMP; Morita et al., 1995; Hartsfield, 2002). However, the end result in this crosstalk between CO and NO may depend on their relative concentrations as both gaseous molecules compete for the same enzyme but with different affinities. In addition, the biological effects due to this interplay between CO and NO may be characteristic of each cell type. Fourth, CO displays antiproliferative actions in SMCs (Morita et al., 1997), which could account for a good portion of its beneficial effects in the model of balloon injury (Otterbein et al., 2003). These CO effects require activation of p38 MAPK pathway, which appears to occur via a cGMP-dependent mechanism and require activation of guanylate cyclase as well (Otterbein et al., 2003). Last, there is a possibility that CO could exert antioxidant effects via binding of the Fe^{2+} in heme groups and preventing the oxidation of hemoproteins, subsequently inhibiting the release of free heme (Balla et al., 1993). This mechanism appears to be important in the suppression of experimental cerebral malaria by CO (Pamplona et al., 2007).

HEME CATABOLISM AND RELEASE OF IRON

A third arm of action of the HO-1 system with antioxidant effects consists in the catabolic processing of heme groups, including the release and safe disposal of ferrous iron (Fe^{2+}). Heme groups, derived from hemoglobin and other hemoproteins, can exacerbate the generation of ROS and membrane lipid peroxidation via the participation of Fe^{2+} in the Fenton reaction (Ryter and Tyrrell, 2000). Heme groups consist of a central iron atom in a coordinated binding to a protoporphyrin, composed of four pyrrole rings. The oxidative cleavage of this tetrapyrrolic ring leads to the release of its central Fe^{2+} with the potential of facilitating its participation in redox chemistry. This is how in certain circumstances, overexpression of HO, either HO-1 or HO-2 have been shown to exert prooxidant effects instead (Lamb et al., 1999; Ryter and Tyrrell, 2000). However, the simultaneous expression of ferritin, an important multimeric protein complex with a high capacity for iron storage, helps to channel the safe disposal for Fe^{2+}. In addition, it appears that several of the same stimuli that trigger HO-1 upregulation also increase ferritin expression, at the same time that labile Fe *per se* can upregulate the expression of heavy-chain (H-) ferritin, which in association with light-chain (L-) ferritin subunits lead to the assembly of ferritin protein (Harrison and Arosio, 1996). Indeed, ferritin expression colocalizes with HO-1 expression in atherosclerotic lesions and in common with HO-1, ferritin is markedly increased in various cell types present in atherosclerotic plaques such as myofibroblasts, macrophages, and endothelial cells, where it may play a protective role against oxidized lipoproteins (Juckett et al., 1995). Coordinate expression of HO-1 and ferritin can lead to antioxidant effects by different mechanisms. First, it decreases the bioavailability of Fe^{2+} to participate in redox chemistry. Second, the ferroxidase activity of the H-chain subunit, leads to the conversion of Fe^{2+} to Fe^{3+} and therefore, decrease in iron prooxidant potential (Balla et al., 1992; Berberat et al., 2003). Third, multimeric ferritin can bind free heme with subsequent reduction in its bioavailability (Kadir and Moore, 1990). The prooxidative effects of HO-1 overexpression could be seen when increases in HO-1 levels overwhelm concurrent expression of ferritin. It seems, however, that these potentially deleterious effects that are noted in experimental studies are less likely to occur in normal physiological conditions. Instead, co-expression of both HO and ferritin appears to play a significant antioxidant role.

Overall, it appears that all HO byproducts could contribute to the HO-1 antiatherogenic effects. It remains to be elucidated whether these actions are complementary or merely overlapping and what is the degree of contribution of each one of these arms of action in the HO-1 global effects.

SUMMARY AND PERSPECTIVES

The overall importance of HO is underlined by its large degree of evolutionary conservation in all kingdoms of life as it is expressed in prokaryotic and eukaryotic cells, bacteria, yeasts, protozoa, plants, invertebrate and vertebrate animals. In all these various living organisms, HO contributes to the catabolic processing of hemoproteins and avoidance of the potential toxicity that could be associated to the release of Fe atoms present on them.

Heme oxygenase appears to exert most of its actions thanks to its enzymatic activity and the generation of various enzymatic byproducts that act on a multiplicity of pathways. Therefore, HO activity can be visualized as a HO system with three branches or arms of action that consist in: (1) generation of Biliverdin and subsequent reduction to bilirubin, (2) release of CO, and (3) release and safe disposal of Fe^{2+}. These arms of action modulate various pathways with a high degree of overlap and/or cross-over, which may help to ensure the induction of the desired biological effects. HO-1 is most abundant in the cytosol, attached to the smooth endoplasmic reticulum (SER) membrane. However, its presence in other subcellular localizations such as the plasma membrane, mitochondria, or nucleus raises the question whether HO could exert some protein-protein or protein-DNA interactions that could play other roles, such as transcriptional regulation, which requires further exploration. HO-1 and the HO system in general are thought to play a critical role in cellular homeostasis where the presence of complementarity or overlap between the arms of action could result in some degree of redundancy that can help to ensure the induction of HO mediated critical effects. These principles are also seen in the protection against vascular inflammation and atherosclerosis. Thus, HO three arms of action have various degrees of antioxidant, antiinflammatory, antiapoptotic, antiproliferative, and immunomodulatory effects, most of which may play a significant role in the protection against atherosclerotic lesion formation.

Heme oxygenase-1 has been shown to be a critical gene in the cellular response against prooxidative stimuli such as oxidized phospholipids. When considering the cellular response as a whole, HO-1 acts as a hub of gene-gene interactions that modulate the triggering and activation of several protective molecular pathways. This has been demonstrated in endothelial cells and is likely to be the case in other vascular cells such as monoctyes/macrophages. In addition, HO-1 may be a protective gene against proatherogenic effects caused by environmental factors such as cigarette smoking or exposure to air pollution.

Basal HO-1 mRNA expression levels in response to injurious stimuli appear to be more important than the degree of upregulation or fold induction for the protection against those stimuli in the vasculature. In some circumstances, the levels of HO-1 protein and HO functional activity may not be concordant which could be due to posttranslational modifications. Therefore, a better understanding of their kinetics in vascular processes such as atherosclerosis is required to shed light on the reasons why very high levels of upregulated HO-1 in vascular cells may be overwhelmed by the pathogenic process and fail to abrogate the progression of atherosclerotic lesions. Importantly, HO-1 can be targeted pharmacologically by interventions that seek its upregulation or enhancement of its various arms of action, via: (1) induction of HO-1 expression with drugs such as heme arginate, (2) administration of CO by inhalation or use of CO releasing molecules (CORM), and (3) administration of biliverdin/bilirubin or use of inhibitors of bilirubin conjugation that would result in increased bilirubin levels.

One potential caveat, however, is that modulation of HO-1 could result in alteration of the activation status of its transcription factor Nrf2, which has been shown to exert proatherosclerotic effects. The discordant effects between Nrf2 and HO-1 underline the high degree of complexity that therapeutic modulation of vascular oxidative stress must address. This is in addition to studies that have shown that while some oxidation products promote inflammation in the vascular wall, others could have antiinflammatory activity instead. Indeed, electrophilic nitro-fatty acids which are formed via nitric oxide or nitrite-dependent redox reactions have been shown to exert antiinflammatory actions (Khoo and Freeman, 2010). Therefore, any pharmacological modulation of HO-1 should take into consideration any potential alteration of the Nrf2 activation status.

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Metalloporphyrins – an update

Stephanie Schulz, Ronald J. Wong*, Hendrik J. Vreman and David K. Stevenson

Division of Neonatal and Developmental Medicine, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, USA

Edited by:
Mahin D. Maines, University of Rochester School of Medicine, USA

Reviewed by:
Thor Willy Ruud Hansen, Oslo University Hospital, Norway
Jon Watchko, University of Pittsburgh School of Medicine, USA

****Correspondence:***
Ronald J. Wong, Division of Neonatal and Developmental Medicine, Department of Pediatrics, Stanford University School of Medicine, 300 Pasteur Drive, Room S230, Stanford, CA 94305-5208, USA.
e-mail: rjwong@stanford.edu

Metalloporphyrins are structural analogs of heme and their potential use in the management of neonatal hyperbilirubinemia has been the subject of considerable research for more than three decades. The pharmacological basis for using this class of compounds to control bilirubin levels is the targeted blockade of bilirubin production through the competitive inhibition of heme oxygenase (HO), the rate-limiting enzyme in the bilirubin production pathway. Ongoing research continues in the pursuit of identifying ideal metalloporphyrins, which are safe and effective, by defining therapeutic windows and targeted interventions for the treatment of excessive neonatal hyperbilirubinemia.

Keywords: bilirubin, heme oxygenase, hemolysis, neonatal hyperbilirubinemia

INTRODUCTION

Metalloporphyrins (Mps) and their potential use in the management of neonatal hyperbilirubinemia has been the subject of considerable research for more than three decades. The therapeutic approach for using this class of anti-hyperbilirubinemia drugs is the targeted blockade of bilirubin production through competitive inhibition of heme oxygenase (HO), the key enzyme in the heme degradative pathway (Tenhunen et al., 1968).

Neonatal jaundice is one of the most common problems for newborn infants during the first weeks of life, affecting approximately 60–70% of term babies and almost all premature babies (American Academy of Pediatrics, 2004). Hyperbilirubinemia is due to a transitional imbalance between bilirubin production and elimination processes. To date, the most commonly used treatments of pathologic bilirubin levels only remove bilirubin that has already accumulated in the body, by initiating phototherapy or, in the extreme cases, performing an exchange transfusion (American Academy of Pediatrics, 2004; Stevenson and Wong, 2010). However, the total serum or plasma bilirubin (TB) concentration at which to begin phototherapy is still controversial and difficult to define to a precise number that can be applied universally to all newborn infants. Instead, it differs according to age (term or preterm), genetic, and ethnic backgrounds, hepatic conjugation capacity, albumin binding, blood/tissue distribution of bilirubin, physiological homeostasis, presence of pre-existing hemolytic conditions, and also individual susceptibility to bilirubin toxicity. In addition, the use of intravenous immunoglobulin (IVIG) has been shown to be effective in reducing TB levels in infants with ABO hemolytic disease, reducing the degree of hemolysis by stabilizing red blood cells (RBC; American Academy of Pediatrics, 2004). Adverse effects related to IVIG therapy include fever, allergic reactions, rebound hemolysis, and fluid overload.

The pharmacologic use of Mps for controlling bilirubin production rates may be strategically a more effective approach (Drummond and Kappas, 1981, 1982a; Stevenson et al., 1989). Its efficacy as a therapeutic and preventive treatment strategy in the management of neonatal hyperbilirubinemia has been confirmed in a large number of animal and clinical studies. In spite of this, Mps still have not left the clinical study stage for their actual application in human neonates, mainly due to the photosensitizing potential of these compounds. This property becomes particularly problematic in preterm infants, a very vulnerable patient group, with thin, transparent skin, reduced antioxidant capacity, a high surface to volume ratio, and frequent potential exposure to phototherapy (Morris et al., 2008). Moreover, a selective review of only available randomized, controlled clinical trials, comparing Mp treatment with placebo or conventional treatments, shows that the combined number of subjects studied is actually relatively small and the authors conclude that more studies are still needed to evaluate the reduction of bilirubin-induced neurological dysfunction (BIND) compared to other treatments (Suresh et al., 2003). Also the short- and long-term effects of Mps, such as the possible release, accumulation, and toxicity of the metal moiety (Hiles, 1974; Maines, 1992), and effects on oxygen radical diseases of prematurity (e.g., bronchopulmonary dysplasia, intraventricular hemorrhage, patent ductus arteriosus, retinopathy of prematurity, and necrotizing enterocolitis) need to be further elucidated (Suresh et al., 2003).

Ongoing research continues in the pursuit of identifying ideal Mps, and, most importantly, of allaying concerns about toxicity, through defining therapeutic windows, and safe treatment strategies of potential candidate compounds.

NEONATAL HYPERBILIRUBINEMIA

When bilirubin levels in circulation become excessive, it may lead to bilirubin deposition in the brain, and if left untreated, cause severe and permanent neurological damage (or BIND; Penn et al., 1994; Gourley, 1997; Govaert et al., 2003; Stevenson et al., 2011). Bilirubin derives from the degradation of heme, the prosthetic group of hemoglobin, and other hemoproteins, which occurs

primarily in the spleen and liver. In this enzymatic pathway, HO catalyzes the rate-limiting oxidation of heme to release equimolar amounts of free iron (Fe^{2+}), carbon monoxide (CO), and biliverdin. The latter is subsequently and rapidly reduced to bilirubin by biliverdin reductase. Both reactions require NADPH as a reducing agent (Tenhunen et al., 1968, 1970; **Figure 1**).

Once in circulation, bilirubin becomes bound to albumin and is then transported to the liver, where it is conjugated to mono- and diglucuronic acids by uridine diphosphoglucuronate glucuronosyltransferase (UGT). Being water-soluble, the conjugated bilirubin is excreted in the bile and finally eliminated from the body through the bowel. However, the glucuronides are relatively unstable and can be hydrolyzed to unconjugated bilirubin, which can be absorbed by the intestinal mucosa, and re-enters the circulation (enterohepatic circulation). Besides increased enterohepatic reabsorption of bilirubin, decreased hepatic uptake, and conjugation, are the major factors contributing to the impaired elimination of bilirubin observed after birth (Kaplan et al., 2011).

THE RATIONALE FOR THE USE OF METALLOPORPHYRINS

The bilirubin production rate of newborn infants is normally two to three times higher than that of adults, which is mainly due to an increased circulating RBC mass and a shortened RBC lifespan, and hence an increase in RBC turnover (Stevenson et al., 1994). Since all newborn infants have impaired bilirubin clearance, any condition causing an increased production rate, such as hemolysis, represents a serious risk. This unconjugated hyperbilirubinemia occurs primarily in infants with isoimmune hemolytic diseases caused by blood group incompatibilities between mother and fetus, such as Rh isoimmunization and ABO incompatibility, or by glucose-6-phosphate dehydrogenase (G6PD) deficiency. When uncontrolled, it can lead to the development BIND. Normally, peak TB concentrations in term infants range from 5 to 6 mg/dL (86–103 μmol/L) at 48–120 h after birth in Caucasian and African-American infants and from 10 to 14 mg/dL (171–239 μmol/L) at 72–120 h after birth in Asian-American infants. In premature infants, TB levels peak by the fifth day of life, reaching 10–12 mg/dL (171–205 μmol/L; Kaplan et al., 2011).

In addition to an immature and temporally insufficient bilirubin clearance and a physiological increased production in newborn infants, genetic vulnerabilities, such as polymorphisms in the UGT1A1 promoter (low bilirubin eliminator) and/or in the HO-1 promoter (less GT repeats equals high bilirubin producer) and G6PD deficiency (high bilirubin producer) can place the infant at

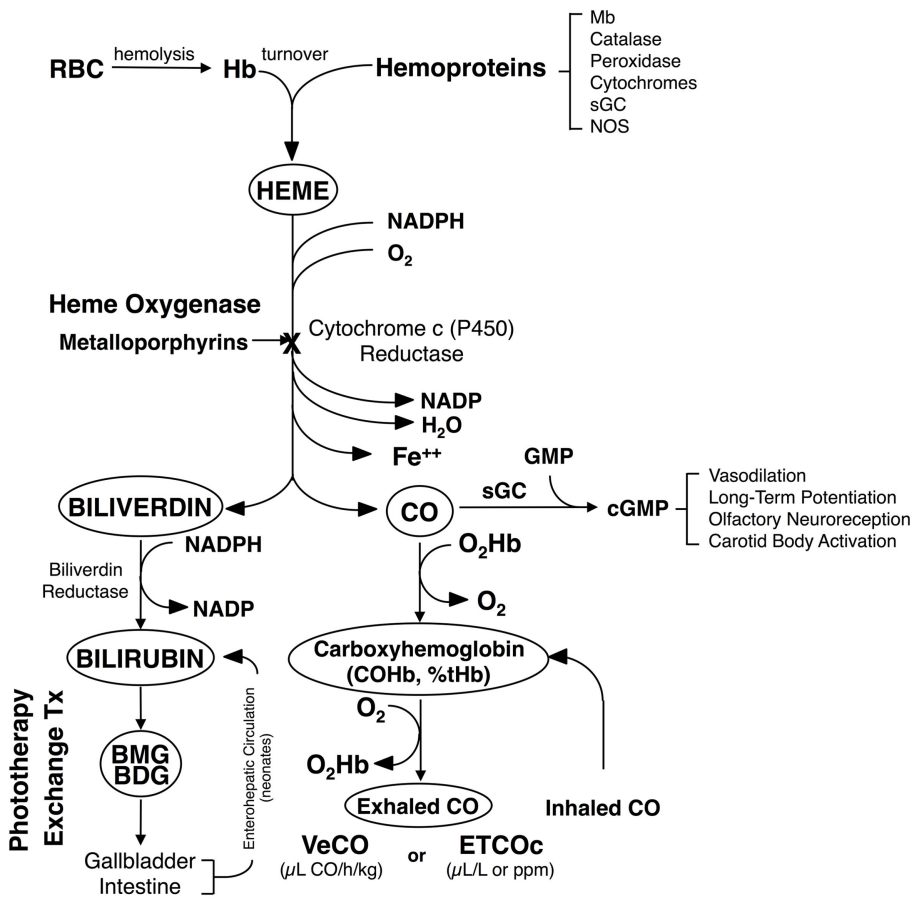


FIGURE 1 | Heme degradation pathway. The turnover of hemoglobin (Hb) and other hemoproteins yields heme. This heme is metabolized to equimolar quantities of carbon monoxide (CO), iron (Fe^{2+}), and biliverdin. Biliverdin is subsequently reduced to form bilirubin. CO is bound to circulating red blood cells (RBC) and is excreted through the lungs, where it can be measured as the rate of total body CO excretion (VeCO) or as a concentration in end-tidal breath, corrected for ambient CO (ETCOc). Modified from Vreman et al. (2001).

high risk for developing hyperbilirubinemia (Cohen et al., 2010). The use of CO detection technologies, e.g., end-tidal breath CO measurements, corrected for ambient CO (ETCOc; Vreman et al., 1994, 1996, 1999), or total body excretion rates of CO (VeCO), can provide estimates of total CO production, which is a direct index of bilirubin production (Stevenson et al., 1979) under steady state conditions, where the CO produced from other sources (15–20%), such as lipid peroxidation or photo-oxidation, are controlled for (Dercho et al., 2006). Thus, the antenatal diagnoses of genetic predispositions and the use of ETCOc could allow the identification of high bilirubin producers, who could be targeted for treatment with Mps before TB levels become excessive. No clinical device is presently commercially available. However, a prototype instrument (Co-Sense, Capnia, Inc., Palo Alto, CA, USA) is currently evaluated for use in clinical studies.

Although phototherapy can be regarded as a "drug" for the treatment of hyperbilirubinemia, its therapeutic use not only differs from "classic" pharmaceuticals, but also has several characteristic limitations. Ideally, phototherapy devices should deliver light with: an emission spectrum between 400 and 520 nm (blue–green; Vreman et al., 2004); an irradiance footprint which exposes at least one entire horizontal body surface plane; an irradiance (intensity) level of $\geq$30 μW/cm^2/nm; and an optimized duration of exposure (American Academy of Pediatrics, 2004; Maisels and McDonagh, 2008). Compared to a traditional drug, phototherapy is a non-specific, instead of a targeted, treatment strategy and it only removes bilirubin, which already has been formed. Moreover, its therapeutic dose is not a fixed number, but a still debated light intensity range, which is dependent on an accurate measurement of the irradiance of a given light source, often problematic itself (Vreman et al., 2008). Also, the spectral characteristics of phototherapy devices are quite different and may account for variations in efficacy and safety (Vreman et al., 2008).

Nonetheless, phototherapy is generally considered safe, effective, and simple to administer and therefore used routinely in the clinical setting. Recently, however, concerns have been raised about its safe use in extremely low birth weight (ELBW) infants (501–750 g). Because their antioxidant capacity is often limited, phototherapy has been shown to promote oxidative stress in this patient group (Gathwala and Sharma, 2002). Evidence of injurious effects of phototherapy has been found in a National Institute of Child Health and Development trial comparing the use of aggressive vs. conservative phototherapy. Although there was a significant decrease in neurodevelopmental impairment in ELBW infants, *post hoc* analyses revealed an increased mortality in this cohort, which did not reach statistical significance (Morris et al., 2008). Moreover, some studies have reported that re-opening of the ductus arteriosus has been associated with phototherapy use for premature infants (Barefield et al., 1993; Benders et al., 1999);whereas, others failed to show this correlation (Scheidt et al., 1987; Travadi et al., 2006).

A more strategic approach may be through the direct inhibition of bilirubin production using Mps. Targeting high bilirubin producers (such as infants with hemolytic diseases, the most common cause of pathological unconjugated hyperbilirubinemia) would be the most beneficial application for Mps, and therefore may reduce or eliminate the need for exchange transfusion in this infant population. The effectiveness in reducing severe hemolytic hyperbilirubinemia and thereby preventing the need for an exchange transfusion has been described in a case report using SnMP (Reddy et al., 2003). Additionally, phototherapy has been shown to have limited effect in modulating elevated TB levels due to Coombs-positive hemolytic disease and cannot be considered as a substitute for exchange transfusion (Maurer et al., 1985). It is also conceivable that hyperbilirubinemia treatment with Mps could be beneficial for premature infants, which have very thin skin, thus light can penetrate deeper into tissue and cause photo-oxidative injury (Vreman et al., 2004; Hintz et al., 2011). This effect might be reduced with Mps treatment, if they are used alone and not in combination with phototherapy. A clinical study by Valaes et al. (1994), using SnMP to control TB in premature babies described no adverse effects of SnMP treatment alone (without phototherapy). However, to state unequivocally that the use of Mps is advantageous over phototherapy for these ELBW infants, who appear to be more sensitive to the adverse effects of phototherapy, is complex and mostly speculative.

PHARMACODYNAMICS OF METALLOPORPHYRINS

Porphyrins (Greek for "purple") are a class of tetrapyrrole macrocycles with a skeleton of 16-atom rings containing four nitrogen atoms. The porphine free base has 11 double bounds and can easily be transformed into an Mp by replacing the inner two pyrrole protons with a metal ion. The porphyrin ring itself has a planar structure due to the high number of double bonds (Fleischer, 1970). Depending on the side chains and central metal ion, a large number and variety of Mps are possible (**Figure 2**).

The inhibition of HO by Mps was initially reported in 1981 by Maines (1981) and Drummond and Kappas (1981). Zinc (Drummond and Kappas, 1981; Maines, 1981), tin (Drummond and Kappas, 1981; Maines, 1981), and manganese protoporphyrin (Drummond and Kappas, 1981; ZnPP, SnPP, MnPP, respectively) were the first Mps observed to be competitive inhibitors for HO in the liver (Drummond and Kappas, 1981; Maines, 1981),

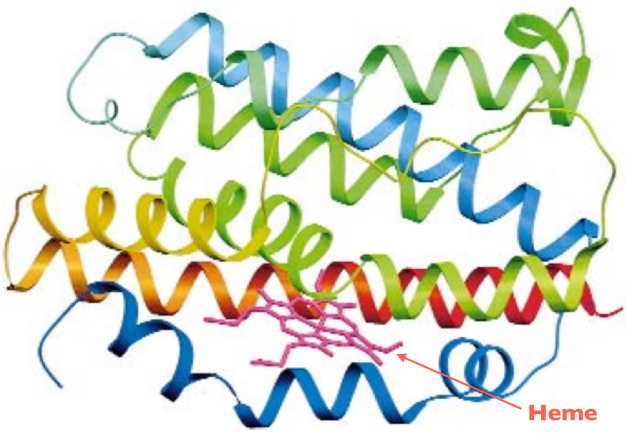


FIGURE 2 | Ribbon diagram of HO-1. The N-terminus is blue and the C-terminus is red, with green in the middle. Heme is shown by the arrow. Adapted from Schuller et al. (1999).

spleen (Drummond and Kappas, 1981; Maines, 1981), kidney (Drummond and Kappas, 1981; Maines, 1981), and skin tissue (Drummond and Kappas, 1981) *in vitro* and *in vivo*. These compounds have a much higher binding affinity (e.g., SnPP: $K_i = 0.011\,\mu M$ in rat spleen tissue; Drummond and Kappas, 1981) than heme to HO-1 and HO-2 ($K_m = 0.24$ and $0.67\,\mu M$, respectively; Ryter et al., 2006). They are not oxidatively degraded because they have no oxygen-binding capacity. Chromium protoporphyrin (CrPP) has also been shown to inhibit HO activity *in vitro* (rat and human spleen) and *in vivo* (rat liver and spleen) and thus prevent hyperbilirubinemia in neonatal rats (Drummond and Kappas, 1982b). Protoporphyrins with cobalt (Co; Drummond and Kappas, 1981; Maines, 1981), iron (Fe; Drummond and Kappas, 1981; Maines, 1981), or cadmium (Cd; Drummond and Kappas, 1981) as central metals have been found to induce HO; but only iron containing Mps, such as heme (FePP), act as actual substrates. CoPP is a unique Mp exhibiting a dualism: significantly inhibiting HO activity *in vitro* (Maines, 1981; Yoshinaga et al., 1982) and enhancing HO activity *in vivo* (Drummond and Kappas, 1981; Maines, 1981) due to its strong activation of HO-1 gene expression (Maines, 1981; Kappas and Drummond, 1986; Shan et al., 2006). Subsequent studies showed that iron deuteroporphyrin is also significantly metabolized by liver tissue homogenates in an HO-like mechanism (Vreman et al., 1993). In contrast, HO activity is largely unaffected by protoporphyrins with nickel (Ni), copper (Cu), and magnesium (Mg) as central atoms (Drummond and Kappas, 1981).

SnPP has been shown to be effective toward inhibiting HO activity *in vivo* and *in vitro*, preventing the development of neonatal hyperbilirubinemia shortly after birth in the rat (Drummond and Kappas, 1981, 1982a) and rhesus neonate (Cornelius and Rodgers, 1984). A decrease in TB has also been demonstrated in adult mice with congenital forms of hemolytic anemia (Sassa et al., 1983), in the postnatal suckling rat with heme- or δ-aminolevulinic acid-induced hyperbilirubinemia (Drummond and Kappas, 1984), in the bile-duct ligated rat (Kappas et al., 1984; McMillan et al., 1987), and in a number of clinical studies with human adults (Anderson et al., 1986; Berglund et al., 1988, 1990) or newborns (Kappas et al., 1988). However, studies showing that SnPP is a photosensitizer of bilirubin destruction *in vitro* (McDonagh and Palma, 1985), and phototoxic *in vivo* (Hintz et al., 1990) led to its abandonment for use in human infants. Nonetheless, it should be noted that the photosensitizing properties of SnPP can be advantageous, such as in the photodynamic treatment of psoriasis (Emtestam et al., 1989).

The naturally occurring ZnPP appeared to be especially attractive as it is relatively inert to light activation and thus has no photosensitizing/phototoxic effects *in vivo* (Hintz et al., 1990; Labbe et al., 1999). Early studies by Maines showed that the subcutaneous (s.c.) application of ZnPP at a dose of 40 μmol/kg body weight (BW) was effective in inhibiting HO activity in neonatal rats and neonatal rhesus monkeys (Maines, 1981; Qato and Maines, 1985). The same ZnPP dose given intravenously (i.v.) also significantly reduced total body VeCO in rhesus neonates (Rodgers et al., 1990), and, in the newborn rhesus with iatrogenic hemolysis, VeCO, carboxyhemoglobin, TB, and spleen HO activity (Vreman et al., 1990b).

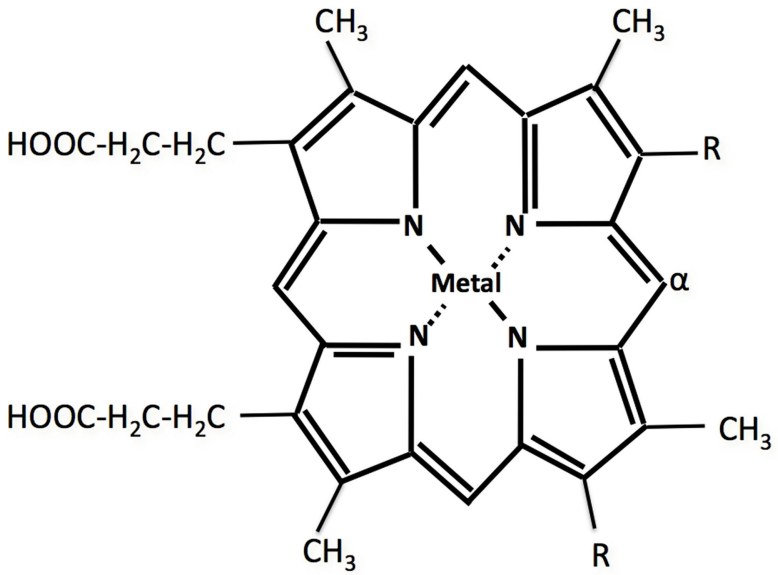
Further research demonstrated that tin mesoporphyrin (SnMP; Drummond et al., 1987), chromium mesoporphyrin (CrMP; Vreman et al., 1993), and zinc deuteroporphyrin IX bis glycol (ZnBG; Martasek et al., 1988; Chernick et al., 1989; Vreman et al., 1991) are also attractive candidates for use in the treatment of neonatal jaundice primarily due to their high potency.

Three HO isoenzymes have been identified to date (Maines et al., 1986; Cruse and Maines, 1988; McCoubrey and Maines, 1994). Whereas HO-1 and HO-2 actively catalyze heme to biliverdin and CO, HO-3 is regarded as a pseudogene of HO-2 and its functional activity is still uncertain (McCoubrey et al., 1997; Ryter et al., 2006).

The HO-2 isoform (∼36 kDa) is constitutively expressed in all tissues, primarily expressed in the brain and highest in the testes (Trakshel et al., 1986, 1988). Conversely, under homeostatic conditions, most tissues express HO-1 at relatively low levels, but can respond to stress with rapid transcriptional activation of the HO-1 gene. The spleen and reticuloendothelial cells in the liver and bone marrow degrade senescent RBCs, and thus highly express HO-1 under basal conditions (Ryter et al., 2006). The catalytic pocket of the HO-1 enzyme with its substrate heme is shown in **Figure 3**.

Different enzyme kinetics and heme K_m values are known for HO-1 and HO-2 ($K_m = 0.24$ and $0.67\,\mu M$, respectively; Maines et al., 1986; Ryter et al., 2006) and make varying interactions of Mps with HO-1 and HO-2 very plausible. A recent study by Wong et al. (2011) characterized the *in vitro* potency of a variety of Mps toward inhibiting HO-1 and HO-2 isoenzymes, using rat and mouse spleen and brain tissue, respectively, as sources of the isoenzymes. SnMP, CrMP, and ZnBG were shown to have the highest potency toward suppression of HO-1 and HO-2 activities. Interestingly, all Mps more selectively inhibit the HO-2 isoenzyme over HO-1. However, CrMP had the highest selectivity toward HO-1 inhibition of all Mps tested, followed by ZnBG and ZnPP. SnPP appeared to be most selective for HO-2. It is conceivable that inhibition of the inducible HO-1 is preferable in a clinical setting because its activity increases in response to hemolytic conditions. Moreover, a strong and a prolonged inhibition of HO-2 may be detrimental as HO-2 is the predominant form in most organs under homeostatic conditions. An early report using rats also describes a selectivity of SnPP toward HO-2 inhibition in addition to a dramatic disruption of the integrity of the HO-2 protein, which may add to the significant suppression of TB formation by SnPP (Maines and Trakshel, 1992a). Similar results regarding the potency of various Mps have also been described by Vreman et al. (1993) comparing their efficacy to inhibit rat liver HO activity, with HO-1 and HO-2 equally contributing to the total HO activity under non-stimulated conditions. Whereas CrMP was most effective in inhibiting total liver HO activity *in vitro*, SnPP, SnMP, ZnPP, and ZnMP appeared nearly equally potent.

In vivo, the efficacy of Mps is dependent on several factors: route of administration, plasma and tissue distribution, and also underlies species differences. Although ZnPP appeared less potent than SnPP, both Mps effectively suppressed HO activity in liver, spleen, kidney (ZnPP only rat tissue), and TB levels with a long duration of action (ZnPP up to 12 days in rhesus neonates; SnPP up to 42 days in rats; Drummond and Kappas, 1981, 1982a; Maines, 1981; Cornelius and Rodgers, 1984; Qato and Maines, 1985; Rodgers et al.,



Porphyrin Type Based on Ring Substituent and Chelated Metal

Metal	Deuteroporphyrin (R = –H)	Mesoporphyrin (R = $–CH_2–CH_3$)	Protoporphyrin (R = $–CH=CH_2$)	Bis Glycol Porphyrin (R = $–CHOH–CH_2OH$)
Metal-Free	MfDP	MfMP	MfPP	MfBG
Iron (Fe^{2+})	FeDP	FeMP	**FePP (Hemin)**	FeBG
Zinc (Zn^{2+})	ZnDP	ZnMP	ZnPP	ZnBG
Tin (Sn^{4+})	SnDP	SnMP	SnPP	SnBG
Chromium (Cr^{2+})	CrDP	CrMP	CrPP	CrBG
Manganese (Mn^{2+})	MnDP	MnMP	MnPP	MnBG
Copper (Cu^{2+})	CuDP	CuMP	CuPP	CuBG
Nickel (Ni^{2+})	NiDP	NiMP	NiPP	NiBG
Magnesium (Mg^{2+})	MgDP	MgMP	MgPP	MgBG

FIGURE 3 | Basic porphyrin IX structure with central metal and two ring substitution sites (R). Oxidation of susceptible porphyrins, catalyzed by HO, occurs at the α-position to yield a tetrapyrrole. Modified from Vreman et al. (2001).

1990). Variations of the porphyrin side chain enhanced the effectiveness toward HO inhibition 10-fold for SnMP compared to SnPP (Drummond et al., 1987). However, ZnBG seems to be one of the most potent inhibitors *in vivo*, but with a short duration of action (Vreman et al., 1991; He et al., 2011).

Besides having HO isoform selectivity, Mps also differ in tissue distribution, potency, photosensitivity, and other side effects. Although the bioavailability and photosensitivity of Mps are dependent on certain aspects of the Mps structure, as the hydrophilicity of the side chains, and the electronic configuration of the metal atom, in general, there is no set pattern, which can allow one to predict the behavior of a given Mp *in vivo*.

CLINICAL STUDIES

To date, clinical efficacy studies have only been performed with SnPP and later with SnMP. An early human trial with a limited number of adult subjects ($n = 6$) given SnPP (0.25–2.0 μmol/kg BW i.v.) demonstrated a decrease in TB from 7 to 23% in patients with cholestasis secondary to primary biliary cirrhosis, and 29–43% in patients with Gilbert syndrome (Anderson et al., 1986). However, some treated subjects (4/28 normal subjects, and 3 with hyperbilirubinemia) developed mild to moderate transient erythema and conjunctival irritation after sunlight exposure. In term newborns with hyperbilirubinemia due to direct Coombs-positive ABO incompatibility, SnPP diminished TB compared to control infants with significant differences in the incremental changes in TB concentration after two or three intramuscular (i.m.) doses of 0.75 μmol/kg BW. The effect of a single dose of 0.5 μmol SnPP/kg BW did not reach statistical significance. The need for phototherapy was reduced in the SnPP-treatment group, but following light treatment, 2 of 24 infants treated with SnPP developed transient erythema (Kappas et al., 1988). In several other studies with healthy human subjects or patients with hepatic dysfunction affecting heme metabolism or bilirubin conjugation, SnPP administration was described as being relatively innocuous, despite causing transient photosensitizing effects after single or multiple applications (Kappas et al., 1984; Berglund et al., 1988, 1990; Galbraith et al., 1992).

Since SnMP has been shown to be at least 10-fold more potent than SnPP in inhibiting HO activity (Drummond et al., 1987), clinical studies were pursued with the expectation that its high potency would allow for its use at much lower doses, and therefore its photosensitizing effects would be minimized or maybe even eliminated. In spite of this rationale, SnMP was used in a

dose of 1–6 μmol/kg BW, which was equal to or higher than the dose of SnPP used in earlier studies. Clinical studies in human preterm (Valaes et al., 1994), full-term (Martinez et al., 1999), and near-term newborns (Kappas et al., 1995) showed that SnMP substantially moderated the course of hyperbilirubinemia, significantly decreasing the mean peak incremental TB concentration (Valaes et al., 1994), phototherapy use (Valaes et al., 1994; Kappas et al., 1995; Martinez et al., 1999), and length of hospital stay (Kappas et al., 1995; Martinez et al., 1999) compared to controls. However, no significant difference in the TB concentrations was shown between control groups, who mostly received phototherapy vs. SnMP groups (Kappas et al., 1995). Several infants who needed phototherapy in addition to SnMP treatment developed transient erythema similar to that observed in SnPP-treated newborns (Valaes et al., 1994; Kappas et al., 1995). These studies used "special blue" Philips F20T12/BB fluorescent tubes, with an emission spectrum (maximum intensity 440–460 nm), which does not extend into the Soret peak as full spectrum white light does (Valaes et al., 1994). Delaney et al. (1988) demonstrated that the triplet lifetime of SnPP decreases ∼95% when excited at 450 nm, which presumably also decreases its phototoxicity in the emission range of the special blue lamp compared to full spectrum light from any source. Moreover, the irradiance of these earlier studies was relatively low (12–14 $\mu W/cm^2/nm$) compared to that recommended in the 2004 American Academy of Pediatrics (AAP) practice guideline ($\geq$30 $\mu W/cm^2/nm$; American Academy of Pediatrics, 2004). The phototoxicity of Mps appears to be strongly dependent on the irradiance and spectral quality of the light source (Schulz et al., 2012), and therefore the occurrence of more worrisome photosensitizing side effects should not be excluded. In G6PD-deficient newborns, a preventive or therapeutic SnMP administration supplanted the need for phototherapy, but SnMP showed no advantages over phototherapy in its effectiveness in controlling TB levels (Valaes et al., 1998).

Suresh et al. (2003) reviewed the available data from clinical studies with Mps in order to determine the efficacy of Mps in reducing TB levels, the need for phototherapy or exchange transfusion, and the incidence of BIND in neonates with unconjugated hyperbilirubinemia. We have summarized the clinical studies described to date in **Table 1**. A multicenter clinical trial conducted by InfaCare Pharmaceutical Corporation evaluating the long-term effects of SnMP (Stannsoporfin) was begun in 2008 and the results from this study are still pending.

SIDE EFFECTS

A question that surfaced early was the fate of the potential cytotoxicity of heme after blockade of its metabolism. Studies performed using bile-cannulated rats have demonstrated that after administration of exogenous heme or heat-damaged RBCs together with SnPP, the amount of heme excreted into the bile markedly increased; whereas, the biliary output of bilirubin diminished (Kappas et al., 1985; Hintz et al., 1987). Therefore, it appears that no accumulation of the cytotoxic and irritant heme occurs after HO inhibition. An enhanced excretion of heme in the bile after SnPP-mediated HO inhibition has also been shown in a study with 10 healthy adults, using duodenal intubation (Berglund et al., 1988).

Mps have been found to also interact with other heme-containing enzyme systems, such as nitric oxide synthase (NOS; Luo and Vincent, 1994; Meffert et al., 1994), soluble guanylyl cyclase (sGC; Ignarro et al., 1984; Grundemar and Ny, 1997), and cytochrome P450 (CYP_{450}; Drummond et al., 1989; Trakshel et al., 1992). They also affect hematopoiesis (Maines and Trakshel, 1992b; Lutton et al., 1997), steroidogenesis (Maines and Trakshel, 1992b; Drummond et al., 1996), and the iron status of the body (Kappas et al., 1993; Berglund et al., 1999). However, the most prominent and concerning side effect is the photosensitizing property of the majority of Mps.

Photosensitivity

It is understood that the Mp-sensitized photodynamic damage is mainly caused by the absorption of light at wavelengths of 400 (Soret band), 540, and 580 nm, the peak absorptions of Mps. This subsequently causes the formation of triplet excited states, long triplet lifetimes, and high quantum yields for sensitizing the formation of singlet oxygen, which reacts with biological substrates (e.g., amino acids, guanine bases of DNA and RNA, and unsaturated lipids, including cholesterol and fatty acids; Tonz et al., 1975; Land et al., 1988). *In vitro* studies with SnPP demonstrated that its photophysical parameters (high quantum yield and long triplet lifetime) and singlet oxygen-sensitizing ability are similar to metal-free porphyrins, and it was thus expected to have phototoxic effects *in vivo* (Land et al., 1988). The triplet lifetime of SnMP has been found to be much higher than SnPP; the addition of quenching groups, like iodine, to the macrocycle reduced the triplet lifetime [tin diiododeuteroporphyrin (SnI_2DP)] (Fort and Gold, 1989). As expected, also the excitation wavelengths influenced the triplet lifetimes of these Mps (Delaney et al., 1988). *In vivo*, all three compounds caused photosensitization in guinea pigs, with SnPP being the strongest photosensitizer and, SnI_2DP and SnMP having less photoreactivity probably due to the higher potency and thus use at lower doses for SnMP and the quenching iodine for SnI_2DP (Fort and Gold, 1989). In general, it appears that the photophysical properties found *in vitro* do not completely translate to *in vivo* conditions. In a different study, mortality was detected in rats treated with SnPP and SnMP and simultaneous exposure to cool white fluorescent light, with an LD_{50} of 11.7 μmol/kg BW for SnPP and 40% mortality for SnMP at a dose of 20 μmol/kg BW (Hintz et al., 1990). No mortality was observed in rats exposed to similar light conditions after treatment with ZnPP and ZnMP. In human subjects, transient erythema have been reported following treatment with SnPP and SnMP (Kappas et al., 1988, 1995; Berglund et al., 1990; Galbraith et al., 1992; Valaes et al., 1994). The underlying mechanisms, which lead to lethality in the rats after SnPP or SnMP treatment and light exposure are not known. Interestingly, toxicity has also been reported in a study with rhesus monkeys given 25 and 100 μmol SnPP/kg BW. The study was not designed to investigate photosensitizing effects, and information about the quality of light exposure is not given. Nonetheless, death associated with light exposure is conceivable at these high doses, especially, since biopsies revealed cutaneous bullae and dermal inflammation. Moreover, gross histology of livers, spleens, and kidneys showed evidence of infarction (Cornelius and Rodgers, 1984).

Table 1 | Clinical studies using metalloporphyrins.

Subjects	Mp(s)	Doses	Outcomes	Side effects	Reference
28 Healthy adults (men from 23 to 62 years-old)	SnPP	0.001–1.0 μmol/kg BW i.v., i.m., p.o.	Pharmacokinetics Log-linear clearance $t_{1/2} \approx 4$ h Excretion: rapidly in the urine (0.1–5.6%), more gradually in feces (3.7–11.3%) i.m. administration similar to i.v. Not orally absorbable	Mild to moderate erythema after sunlight and long-wave ultraviolet light exposure in four subjects Discomfort at injection side after i.m. administration	Anderson et al. (1986)
Patients with primary biliary cirrhosis ($n = 4$) and Gilbert Syndrome ($n = 2$)	SnPP	0.025–2.0 μmol/kg BW i.v.	Plasma bilirubin declined Biliary cirrhosis → 7–25% Gilbert syndrome → 29–43%	Mild to moderate erythema in three subjects	Anderson et al. (1986)
10 Healthy adults (men and women 21–48 years-old)	SnPP	1–2 μmol/kg BW i.v. in two doses	Plasma bilirubin declined 38% (mean) for at least 4 days $t_{1/2} \approx 3.4$ h Heme excreted in the bile	None mentioned	Berglund et al. (1988)
Term newborns with direct Coombs-positive ABO incompatibility (69 controls, 53 treated)	SnPP	0.5–2.25 μmol/kg BW i.m. in one to three doses	Moderated postnatal plasma bilirubin increase Diminished intensity of hyperbilirubinemia Decreased phototherapy use $t_{1/2}$ of SnPP in term babies different from adults: $t_{1/2} \approx 1.6$ h	Transient erythema in two babies who also received phototherapy	Kappas et al. (1988)
Six patients with biliary cirrhosis, four patients with idiopathic hemochromatosis	SnPP	1–2 μmol/kg BW i.v. in two doses	Plasma bilirubin declined Biliary cirrhosis → 20% Hemochromatosis → 32% Decrease in biliary bilirubin $t_{1/2} \approx 3.4$ h Heme excreted in the bile	Transient photosensitizing effects	Berglund et al. (1990)
24 Healthy adult subjects (men)	SnMP	1 μmol/kg BW i.v., i.m., p.o.	Pharmacokinetics Log-linear clearance $t_{1/2} = 3.8$ h (i.v.) Excretion: urinary and fecal <1% Not orally absorbable Significant decreased plasma bilirubin after 24–48 h	Transient photosensitizing effects after sunlight exposure in three out of four subjects	Galbraith and Kappas (1989)
Three patients with porphyria	SnPP	4 μmol/kg BW;	SnPP significantly reduced excretion of ALA, porphobilinogen, and porphyrins	All three displayed photosensitivity after exposure to fluorescent and sunlight	Galbraith and Kappas (1989)
	SnMP	2 μmol/kg BW; each in four doses	SnMP significantly reduced ALA and porphyrins (two patients)		

(Continued)

Table 1 | Continued

Subjects	Mp(s)	Doses	Outcomes	Side effects	Reference
20 Healthy adults; seven with primary biliary cirrhosis; four with idiopathic hemochromatosis	SnPP SnMP	1–2 μmol/kg BW i.v. in two doses; 1.0 μmol/kg BW	Study designed to look at side effects of SnPP and SnMP treatment	Substantial, but transiently increased serum ferritin levels	Berglund et al. (1999)
Two boys with Crigler–Najjar type I	SnMP	40 doses of 0.5 μmol/kg BW and 70 doses of 1.0 μmol/kg BW i.v. during a period of 425 days	Decreased plasma bilirubin levels and rebound hyperbilirubinemia, which occurred after plasmapheresis Prolonged treatment was well-tolerated	Episodic mild reversible cutaneous photosensitivity after sun exposure Hemoglobin, hematocrit, mean corpuscular volume, changed similar to an iron deficiency Plasma iron-binding proteins increased	Galbraith et al. (1992)
517 Preterm newborns (30– ≤ 36 weeks of gestation)	SnMP	1–6 μmol/kg BW i.m.	Reduced mean peak incremental plasma bilirubin levels by 41% Reduction was equal for control (receiving phototherapy if needed) and SnMP groups Phototherapy requirement decreased by 76% compared to control subjects given 6-μmol/kg BW	Mild, transient erythema, which disappeared without sequelae, in 13 newborns of 127 who required phototherapy together with SnMP treatment	Valaes et al. (1994)
Male term infants, near-term infants of both genders (42 pairs of SnMP and phototherapy treatment)	SnMP	6 μmol/kg BW i.m.	Effectively controlled hyperbilirubinemia and was superior to phototherapy in the majority of cases (time interval between enrollment and closure of case was reduced by >24 h with SnMP treatment)	Slight erythema in one infant after sun exposure Two control infants who just received phototherapy developed erythema No neurodevelopmental adverse effects after 18-month follow-up	Kappas et al. (1995)
G6PD-deficient neonates ($n = 42$ preventive use; $n = 44$ therapeutic use)	SnMP	6 μmol/kg BW i.m.	Effectively controlled hyperbilirubinemia None of the 86 infants needed phototherapy Preventive use of SnMP was superior to therapeutic use	None of the 86 neonates developed photosensitivity erythema	Valaes et al. (1998)
84 Full-term breastfed newborns ($n = 40$ SnMP-treated; $n = 44$ controls)	SnMP	6 μmol/kg BW i.m.	Effectively controlled hyperbilirubinemia No supplemental phototherapy needed 27% of controls needed phototherapy Reduced use of medical resources	No erythema observed in control or SnMP-treated groups No differences in liver function tests	Martinez et al. (1999)

Two Jehovah Witness newborns with hemolytic disease	SnMP	6 μmol/kg BW i.m. at the time when exchange transfusion would have been initiated	Effectively terminated the progression of hyperbilirubinemia	Mild, short-lasting erythema in one case	Kappas et al. (2001a)
230 G6PD-deficient newborns ($n = 172$ SnMP-treated; $n = 58$ treated with phototherapy as required); $n = 168$ G6PD-normal	SnMP	6 μmol/kg BW i.m.	Treatment on the first day of life significantly lowered plasma bilirubin compared to G6PD-deficient controls Decreased plasma bilirubin even in relation to G6PD-normal infants No need for phototherapy in the SnMP-treated group	No systemic or local reactions at injection site No evidence of untoward effects in physical, neuromotor, and mental development, at 18-month follow-up	Kappas et al. (2001b)
Very-low-birth-weight infant	SnMP	4.5 mg/kg BW (or 6 μmol/kg BW) i.m.	Plasma bilirubin declined within 10 h after administration and avoided the need of exchange transfusion	No erythema reported	Reddy et al. (2003)

ALA, δ-aminolevulinic acid; BW, body weight; G6PD, glucose-6-phosphate dehydrogenase; i.m., intramuscular; i.v., intravenous; Mps, metalloporphyrins; p.o., oral; $t_{1/2}$ drug plasma elimination half-life.
Dose conversion factor: μmol/kg BW = 1.33 × mg/kg BW.

In recent studies by our laboratory, we observed phototoxic effects of ZnBG in neonatal mice and found a significant increase in lipid peroxidation in liver and heart tissues after intraperitoneal (i.p.) administration of 30 μmol/kg BW and light exposure. This was accompanied by elevations in aspartate aminotransferase (AST) and creatine kinase activities, inferring the possibility of heart and liver damage (Schulz et al., 2012). We also established that the LD_{50} for ZnBG was 19.5 μmol/kg BW, which is similar to an LD_{50} of 23 μmol/kg BW shown in earlier studies in rats (Vreman et al., 1991). In general, ZnPP and ZnMP appear to be far less photoreactive than the tin derivatives *in vitro* (Vreman and Stevenson, 1990; Vreman et al., 1990a, 1993) and with no phototoxicity *in vivo* at concentrations up to 60 and 45 μmol/kg BW, respectively (Hintz et al., 1990). Also, the chromium derivatives are not photoreactive *in vitro* (Vreman and Stevenson, 1990; Vreman et al., 1990a, 1993), and we have recently found that CrMP showed no phototoxicity *in vivo*. However, we did observe a chemical toxicity with CrMP (Schulz et al., 2012), which is in agreement with a previous study by Lutton et al. (1997), who showed that CrMP given at a dose of 10 μmol/kg was lethal in rabbits. In summary, the *in vivo* phototoxicity potential of the studied Mps appears to follow this pattern: SnPP > SnMP ≥ ZnBG > ZnMP > ZnPP. Moreover, these studies indicate that the degree of photodamage caused by Mps can be influenced by several factors, including the dose, route of administration, state of Mp aggregation, the time between administration and light exposure, and the spectral quality of the light.

Other side effects

Due to the blockade of heme metabolism, Mps subsequently reduce the CO and free iron status of cells. This, as well as their heme analog structure, may affect hemoproteins and other enzymes. Several studies demonstrated that SnPP diminishes CYP_{450} capacity and, thus reduces corticosterone levels, CYP_{450}-related drug metabolism, and the CYP_{450} content in testes (Stout and Becker, 1988; Maines and Trakshel, 1992b; Trakshel et al., 1992). Others showed that hepatic CYP_{450} content is only transiently altered after administration of SnPP or SnMP to neonatal rats and does not persist into adulthood. The studies used several different doses and application routes to adult or neonatal rats (Drummond et al., 1989, 1996). Overall, SnPP and SnMP decrease CYP_{450} activity and thus affect CYP_{450}-dependent enzymes of adrenal synthesis and drug metabolism in animal models. However, clinical studies with SnPP and SnMP lack information about these parameters (see Clinical Studies). Although the zinc derivatives appear to not affect the hepatic CYP_{450} system (Trakshel et al., 1992), inhibition of hematopoiesis by ZnPP and ZnMP was found *in vitro* in animal and human bone marrow (Lutton et al., 1997). ZnMP, but not SnMP, also displayed inhibitory action on hematopoiesis and on mobilization of progenitor cells *in vivo* (Lutton et al., 1999). The underlying mechanisms are still unclear, and might not exclusively be attributed to the type of central metal (Zn), but also to the side chains of the porphyrin ring, because ZnBG did not affect bone marrow cell growth (Lutton et al., 1991). Most Mps seem to interact with NOS and sGC, but to different degrees. CrMP and ZnBG have been shown to marginally impair the activity of NOS and sGC at concentrations that

effectively inhibit HO activity, and thus seem to be more selective toward HO than ZnPP and SnPP (Appleton et al., 1999). In a different study, SnMP was also found to have minimal effects on hippocampal NOS activity similar to that of ZnBG (Meffert et al., 1994). Recently, it has been shown that CrMP (also SnPP and SnMP) negatively affects systemic macro hemodynamics and the hepatic microcirculation. Intravenous administration of 40 μmol CrMP/kg BW to rats decreased mean arterial pressure, sinusoidal diameter, and hepatic blood flow, and induced hemolysis, marked inflammatory responses, and increased AST levels. SnMP displayed the least effects on those parameters in this study compared to SnPP and CrMP (Scheingraber et al., 2009). The described side effects of CrMP could be responsible, at least in part, for its toxicity seen in certain animal models (see Photosensitivity) and thus its use in human neonates should be discouraged.

Iron deficiency anemia has been reported following long-term treatment with SnMP in rats (Boni et al., 1993) and patients with Crigler–Najjar Syndrome Type I (110 doses of SnMP 0.5 or 1.0 μmol/kg BW during a 400-day study; Boni et al., 1993; Kappas et al., 1993). Because SnMP inhibits intestinal HO (Vreman et al., 1989) and decreases intestinal heme–iron absorption (Boni et al., 1993), this may account for the iron deficiency-like anemia that results after long-term SnMP exposure. Kappas et al. (1993) reported that the deficiency was easily reversed by supplementation with iron. It is also interesting to speculate that Mps may be useful clinically in the treatment of iron overload.

Administration of SnPP or SnMP transiently increased the acute phase protein ferritin, in healthy volunteers as well as in patients with primary biliary cirrhosis or idiopathic hemochromatosis (Berglund et al., 1999). The underlying mechanism is unclear, particularly, since it would be expected that, due to the release of free iron, ferritin levels would increase in response to HO activation, but not due to inhibition (**Figure 1**).

Of interest is also the possible passage of Mps through the blood–brain barrier and the subsequent effects in this tissue. A study by Drummond and Kappas (1986) showed that SnPP given s.c. crossed placenta and blood–brain barrier of neonatal rats and subsequently inhibited brain HO activity. The blood–brain barrier is most permeable immediately after birth up to a period between 20 and 28 days of postnatal life, suggesting that the ability of SnPP to enter the brain is age-dependent. However, its clearance $t_{1/2}$ was 1.7 days and therefore relatively rapid compared to other tissues (Drummond and Kappas, 1986). Studies with adult rats also observed low but detectable levels of SnPP in the brain, which are cleared relatively rapidly (Anderson et al., 1984). Intravenous administration of SnPP to adult rats markedly decreased HO and NADPH–CYP450 reductase activity in the brain (Mark and Maines, 1992). In contrast ZnPP, SnMP, CrMP, ZnMP did not appear to affect brain HO activity after i.v. (ZnPP) or s.c. administration to adult rats (Mark and Maines, 1992; Bundock et al., 1996). In the adult brain, HO-2 is the isoenzyme predominantly expressed. In contrast, HO-1 expression in the brain is developmentally regulated, being highest in the early gestational ages and progressively decreasing during the perinatal period to adulthood (Zhao et al., 2006). The constitutive HO-2 isoform is important in the maintenance of neuronal function, whereas HO-1 is believed to play a protective role (Snyder et al., 1998; Maines, 2000). Therefore, it is conceivable that HO inhibition in the brain is not desired, although some have speculated that it may be advantageous in premature infants with intracranial bleeding, where a possibility of enhanced local bilirubin formation exists (Drummond and Kappas, 1986). In studies using hippocampal slices, CrMP, SnMP, ZnPP, and ZnBG all inhibited HO, however only CrMP and ZnPP reduced long-term potentiation (LTP) and also inhibited NOS, which is speculated to be the underlying mechanism for LTP reduction (Meffert et al., 1994).

HO-1 promoter activation

HO-1 gene expression is induced by its substrate heme and a variety of stimuli, e.g., heat shock, oxidative stress, hyperoxia, hypoxia, heavy metals, ultraviolet A radiation, pro-inflammatory mediators, Mps, and many others (Ryter et al., 2006).

Using our HO-1-*luc* transgenic mouse model where the transgene contains the full-length HO-1 promoter driving expression of the reporter gene luciferase (*luc*), we found increased reporter gene expression after SnMP and ZnPP treatment, which depended on the route of application, differing from 3-fold to 10-fold (Zhang et al., 2002). Further studies in mice confirmed that the SnMP-mediated induction of the HO-1 gene subsequently leads to a significant increase in HO-1 protein (Morioka et al., 2006). Clinical studies do not report about induced HO-1 protein expression, but describe sufficient reductions of TB levels without a rebound. Therefore it is conceivable that the induction of HO-1 is negligible in the doses used in human studies, which are at least one to two orders of magnitude less than those used animal studies (see Clinical Studies), reinforcing the observation that care must be taken when extrapolating animal studies to the human circumstance.

Several regulatory elements have been shown to be crucial for the activation of the HO-1 gene in response to different stimuli. Bach1, a leucine zipper protein, is a transcriptional repressor. Upon exposure to heme, Bach1 dissociates from its heterodimerization partners within the distal enhancer of the HO-1 promoter and is exported out of the nucleus. Displacement of Bach1 leads to recruitment of the activating NF-E2-related factor 2 (Nrf2) and thus, stimulates HO-1 gene expression (Abate et al., 2007). Bonkovsky and co-workers have demonstrated that CoPP, and ZnMP upregulate HO-1 expression through the repression of Bach1 and upregulation of the Nrf2 protein (Shan et al., 2006; Hou et al., 2008). Our laboratory has shown that SnMP not only induces HO-1 expression by binding to Bach1, but also by increasing Bach1 protein degradation, and thereby affecting the HO-1 promoter directly and indirectly, respectively (Abate et al., 2007).

ZnBG appears to be less effective in HO-1 upregulation, only producing small changes in HO-1 transcription and protein in newborn and adult mice given a heme load (Morioka et al., 2006; He et al., 2011). This induction of HO-1 by ZnBG might be an indirect effect due to the accumulation of heme after inhibition of HO enzyme activity and not a direct interaction with Bach1 (unpublished data).

PHARMACOKINETICS OF SELECTED METALLOPORPHYRINS

Due to effects of the central metal ion and especially to the lipophilicity or hydrophilicity of the side chains, Mps differ in their pharmacokinetic properties, stability, and solubility. Because the

protoporphyrin derivatives are the most lipophilic, their solubility in aqueous solution is minimal. The meso derivatives share similar chemical properties. However, the incorporation of the two bis glycol side chains to the porphyrin ring renders the molecule more polar, thus increasing its solubility in aqueous solutions. In general, all Mps are highly soluble and stable in alkaline aqueous solutions or basic organic solvents, such as pyridine and ethanolamine (Labbe et al., 1999). Although the pharmacokinetics of SnPP are well-studied, its use was abandoned due to the described side effects (especially phototoxicity). Thus, we will focus on the most promising Mps to date: SnMP, ZnPP, ZnMP, and ZnBG.

SnMP

In general, successful oral administration of Mps would be clinically most desirable. However, the chemical characteristics of most Mps preclude this route of administration. Interestingly, absorptivity appears also to be species-specific. For example, SnMP has been shown to be not orally absorbed by rats (Vreman et al., 1988) and by human subjects (Galbraith and Kappas, 1989), but oral administration of SnMP to adult mice significantly decreased VeCO levels, demonstrating an absorption by the intestine and subsequent systemic effects (Morioka et al., 2006). Others have also shown that SnMP inhibits intestinal HO activity after oral administration (Drummond et al., 1992). Moreover, differences in tissue distribution have been observed between adult and neonatal rats. Tissue concentrations of SnMP given s.c. peaked later in neonatal rats than in adults. In general, SnMP is rapidly cleared from the circulation, but appears to have high tissue "stickiness" (up to 27 days in rats), especially in the liver and spleen, and is also found in the kidney and brain. HO activity was reduced in liver, spleen, kidney (neonates only), and brain (not significant) up to 27 days (spleen), but at different time points after administration and to a greater extent in neonates than in adults (Bundock et al., 1996). A fast plasma clearance following i.v. administration with a plasma half-life of 3.8 h and a log-linear decline (similar to SnPP), was also found in adult healthy volunteers (Galbraith and Kappas, 1989). Moreover, SnMP showed a very low excretion rate in feces and urine, suggesting a rapid uptake into intra- or extravascular spaces and tissue binding (Galbraith and Kappas, 1989). Effective doses in human adults and neonates ranged from 1 to 6 μmol/kg BW (Valaes et al., 1994, 1998; Kappas et al., 1995; Martinez et al., 1999) and in animal studies from 1 to 30 μmol/kg BW (Drummond et al., 1987; Morioka et al., 2006).

ZnPP

ZnPP also needs to be administered parenterally (Vreman et al., 1988). Administration by s.c., i.m., or i.p. have been used frequently in animal studies (Maines, 1981; Qato and Maines, 1985; Rodgers et al., 1996). ZnPP at a dose of 40 μmol/kg BW given s.c. to rhesus neonates reduced TB levels within 24 h and lasted up to 12 days. HO inhibition occurred in the liver and spleen, but not in the kidney or brain (Qato and Maines, 1985; Rodgers et al., 1990). Biliary and urinary excretion also was very low. However, ZnPP is extensively incorporated into RBCs (≈45% of the administered dose; Qato and Maines, 1985). Furthermore, it is endogenously generated in cases of iron deficiency and found located primarily in the RBCs (Labbe et al., 1999). Studies in rats showed that ZnPP is relatively fast-acting (∼4 h after s.c. administration), with a duration of action of 1–4 days after the administration of 40 μmol/kg i.p for hepatic HO inhibition (Hamori et al., 1989). In contrast to the rhesus neonate, concentrations of ZnPP found in the spleen tissues of rats were low, and thus splenic HO inhibition was marginal (Rodgers et al., 1996). The spleen is the site of greatest heme catabolism, and therefore targeted inhibition of splenic HO inhibition could increase the *in vivo* effectiveness of Mps in reducing TB levels. This approach has been attempted through incorporating Mps into liposomes. This strategy significantly increased Mp delivery to the spleen and thus enhanced their efficacy (Landaw et al., 1989; Cannon et al., 1993; Hamori et al., 1993).

ZnMP

Interestingly, ZnMP binds very tightly to human serum albumin (Greenbaum and Kappas, 1991), thus its tissue accessibility is actually very low (27%; Bundock et al., 1996). Therefore, it did not significantly inhibit HO activity in any tissue after s.c. injection (rat) with 1–10 μmol/kg BW. A similar dose range of SnMP significantly reduced HO activity in liver and spleen rat tissue up to 4 and 27 days, respectively. In contrast, when 15-μmol ZnMP/kg BW, bound to albumin in a 1:1 ratio, was administered i.v., it was rapidly cleared from plasma (half-life = 3.6 h), with uptake occurring primarily in liver and spleen (less in the kidney), but was not detected in brain (rats). Inhibition of liver HO activity was still 50% 1 week after administration (Russo et al., 1995).

ZnBG

ZnBG has a higher hydrophilicity due to the bis glycol side chains. It is, besides CrMP, the only Mp, proven to be orally absorbed by mice and rats (Vallier et al., 1991a,b; Morioka et al., 2006). ZnBG is absorbed relatively quickly (within 15 min; Vallier et al., 1991a), highly effective toward inhibiting spleen and liver HO activities, has a rapid onset of action (≈70% inhibition after 1–3 h of administration) and is cleared by the kidneys in 2-week-old suckling rats (Vallier et al., 1991b). In adult rats, HO inhibition after an oral dose of 30 μmol ZnBG/kg BW was approximately 20% (liver), 50% (spleen), and 0% (intestine) after 48 h compared to 60% (liver), 80% (spleen), and 40% (intestine) inhibition with the same dose of SnMP, demonstrating a short duration of action for ZnBG compared to other Mps (Morioka et al., 2006). Supporting data were conducted in a hemolytic mouse model with 1-week-old mice, measuring the bilirubin production as VeCO. The bilirubin production returned back to baseline 6 h after oral gavage of 15 μmol ZnBG/kg BW (He et al., 2011). After i.p. injection to 3-day-old mice of very low doses of ZnBG (0.325 μmol/kg/BW) HO inhibition was 50% after 3 h and returned to baseline after 24 h (Katayama et al., 2012). Negligible amounts of ZnBG (<0.001% of the administered dose) have been found in the brain after oral administration to neonatal rats (Vallier et al., 1991b). No inhibition of HO in the brain of mouse neonates was found after oral gavage of up to 30 μmol ZnBG/kg/BW 3 h after administration, which let us conclude that ZnBG does not pass the blood–brain barrier (He et al., 2011). However, after i.p. administration of 3.75–15 μmol ZnBG/kg BW to 3-day-old mice we observed 30–45% HO inhibition in the brain 3 h after administration (unpublished

Table 2 | Major advantages and disadvantages of promising metalloporphyrins.

Mps	Advantages	Reference	Disadvantages	Reference
SnMP	Highly potent	Drummond et al. (1987), Wong et al. (2011)	Photosensitizer (animal/human studies)	Galbraith and Kappas (1989), Hintz et al. (1990), Kappas et al. (1995), Valaes et al. (1994)
	Well-studied	Kappas (2004), Wong et al. (2007)	Phototoxic (animal studies)	Hintz et al. (1990)
	Clinical efficacy shown	Kappas et al. (1995), Martinez et al. (1999), Reddy et al. (2003), Valaes et al. (1998), Valaes et al. (1994)	Affects NOS, sGC, CYP_{450}	Appleton et al. (1999), Maines and Trakshel (1992b), Trakshel et al. (1992)
			Activates HO-1 gene transcription	Abate et al. (2007), Morioka et al. (2006)
			Not orally absorbable in rat and human studies	Galbraith and Kappas (1989), Vreman et al. (1988)
			Crosses the blood–brain barrier (controversial)	Boni et al. (1993), Bundock et al. (1996), Galbraith et al. (1992)
			Long-term treatment possibly leads to iron deficiency	Boni et al. (1993), Galbraith et al. (1992)
			Long-term tissue deposition.	Bundock et al. (1996), Galbraith and Kappas (1989)
			Long duration of HO inhibitory action (could also be advantageous under certain circumstances)	
ZnPP	Contains an essential metal atom		Least potent in this group	Morioka et al. (2006), Wong et al. (2011)
	Naturally occurring		Affects NOS, hematopoiesis	Appleton et al. (1999), Lutton et al. (1997)
	Not phototoxic at doses $\leq$60 μmol/kg BW	Hintz et al. (1990)	Incorporates into RBCs	Labbe et al. (1999), Qato and Maines (1985)
	Effective in rhesus monkey	Maines (1981), Qato and Maines (1985), Rodgers et al. (1990), Vreman et al. (1990b)	Activates HO-1 gene transcription	Zhang et al. (2002)
	May not cross the blood–brain barrier	Qato and Maines (1985), Rodgers et al. (1990)	Not orally absorbed	Vreman et al. (1988)
			Long-term deposition in tissue (see above)	Qato and Maines (1985), Rodgers et al. (1990)
ZnMP	Contains an essential metal atom		Binds tightly to human serum albumin	Bundock et al. (1996), Greenbaum and Kappas (1991)
	Not phototoxic at doses $\leq$45 μmol/kg BW	Hintz et al. (1990)	Long-term deposition in tissue (see above)	Russo et al. (1995)
	May not cross the blood–brain barrier	Russo et al. (1995)	Affects hematopoiesis	Lutton et al. (1997), Lutton et al. (1999)
			Activates HO-1 gene transcription	Hou et al. (2008)
			Less well-studied	
ZnBG	Highly potent		Photosensitizer (animal studies)	Schulz et al. (2012)
	Contains an essential metal atom		Phototoxic (animal studies)	Schulz et al. (2012)
	Only minimally affects NOS, sGC	Appleton et al. (1999)	Rapid onset with a short duration of action (may require multiple dosing, can also be advantageous in cases of infants with protracted hemolysis)	Katayama et al. (2012), Katayama et al. (unpublished data)

(Continued)

Table 2 | Continued

Mps	Advantages	Reference	Disadvantages	Reference
	Only minimally affects HO-1 transcription	He et al. (2011), Morioka et al. (2006), Zhang et al. (2002)	Less well-studied	
	Orally absorbed	Vallier et al. (1991a,b)		
	Short duration of action	He et al. (2011), Katayama et al. (2012), Morioka et al. (2006)		
	No known long-term tissue deposition	Katayama et al. (2012), Katayama et al. (unpublished data)		
	May not or minimally cross the blood–brain barrier	He et al. (2011), Vallier et al. (1991a,b)		

Mps, metalloporphyrin; NOS, nitric oxide synthase; sGC, soluble guanylyl cyclase; CYP_{450}, cytochrome P450; RBCs, red blood cells.

data). If this discrepancy between both studies may be due to the route of administration, or the fact that the blood–brain barrier is more permeable to many chemicals in the immediate postnatal period (Drummond and Kappas, 1986), but possible not permeable to ZnBG anymore in the 1-week-old mice, needs further investigation.

SUMMARY AND CONCLUSION

Although Mps have been studied extensively in animal models and some human trials, their safety has not been unequivocally proven yet. Ideally, a desirable Mp should have high potency and selectivity toward inhibiting HO without affecting other enzymes, not be photosensitizing, not alter HO-1 gene expression and protein levels, be short-acting, be easily eliminated without the subsequent release of the sequestered metal or preferably contain an essential metal atom, and be orally absorbable (Vreman et al., 2001; **Table 2**).

ZnBG appears to have many of these desirable pharmacologic and pharmacokinetic properties, and thus appears to be a promising anti-hyperbilirubinemia drug. Its advantages due to *in vitro* and *in vivo* animal studies include: its extremely high potency, oral absorptivity, short duration of action, no long-term deposition in tissues, minimal interference with hemoproteins, and minimal effects on HO-1 gene expression and subsequent protein synthesis. Even though ZnBG is photoreactive and shows phototoxicity after i.p. administration, those effects appear negligible when administered orally and in therapeutic doses ($\leq$7.5 μmol/kg BW established in newborn mice) due to its high potency and short duration of action, which minimizes the time neonates need to be protected from direct light exposure. Moreover, a short duration of action would allow the pediatrician in a clinical setting to better "titrate" more accurately the dose required to lower TB levels without the danger of its accumulation in certain tissues and thus minimizing long-term side effects.

Currently pediatricians are dependent upon one frontline treatment strategy: phototherapy, which is well established, successful, and generally safe, at least for larger infants. However, using CO detection technologies and antenatal analyses of genetic predispositions to identify infants at high risk for developing hyperbilirubinemia, could enable us to seek and treat high producers of the pigment, in particular those with hemolysis, who might benefit most from targeted Mp treatment. Introduced in this strategic way, Mps still represent a promising alternative in the management of neonatal jaundice, although more work is required to define safe preventive or therapeutic approaches.

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Heme and HO-1 inhibition of HCV, HBV, and HIV

Warren N. Schmidt[1,2]*, M. Meleah Mathahs[1,2] and Zhaowen Zhu[1,2]

[1] Department of Internal Medicine and Research Service, Veterans Affairs Medical Center, University of Iowa, Iowa City, IA, USA
[2] Department of Internal Medicine, Roy G. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Edited by:
Mahin D. Maines, University of Rochester School of Medicine, USA

Reviewed by:
Andrea Branch, Mount Sinai, USA
Curt Hagedorn, University of Utah School of Medicine, USA

***Correspondence:**
Warren N. Schmidt, Department of Internal Medicine, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, 4553 JCP, Iowa City, IA 52242, USA.
e-mail: warren-schmidt@uiowa.edu

Hepatitis C virus, human immunodeficiency virus, and hepatitis B virus are chronic viral infections that cause considerable morbidity and mortality throughout the world. In the decades following the identification and sequencing of these viruses, *in vitro* experiments demonstrated that heme oxygenase-1, its oxidative products, and related compounds of the heme oxygenase system inhibit replication of all 3 viruses. The purpose of this review is to critically evaluate and summarize the seminal studies that described and characterized this remarkable behavior. It will also discuss more recent work that discovered the antiviral mechanisms and target sites of these unique antiviral agents. In spite of the fact that these viruses are diverse pathogens with quite profound differences in structure and life cycle, it is significant that heme and related compounds show striking similarity for viral target sites across all three species. Collectively, these findings strongly indicate that we should move forward and develop heme and related tetrapyrroles into versatile antiviral agents that could be used therapeutically in patients with single or multiple viral infections.

Keywords: Heme, viruses, HCV, HIV, HBV, metalloporphyrins, biliverdin, proteases

INTRODUCTION

Hepatitis C virus (HCV), Hepatitis B virus (HBV), and Human immunodeficiency virus (HIV) are three of the most common chronic viral infections worldwide. All of these viruses share common risk factors and modes of transmission including sexual, human blood product transfusion, and intravenous drug use (Koziel and Peters, 2007). In the decades following discovery and sequencing of all three viruses, there has been a constant medical need to update treatment regimens and employ new and more versatile antiviral agents. This review will focus on *in vitro* and molecular studies that have evaluated metalloporphyrins, specifically heme, and related derivatives, for their virucidal activity against the three viruses. What are emerging from these collective works are not only fascinating pictures of multiple viral targets to explain the antiviral activities of these metalloporphyrins, but there is also a promise that these compounds can be developed successfully into powerful, yet versatile antiviral agents.

THE HEME AND HEME OXYGENASE SYSTEMS

Heme (iron protoporphyrin IX) is the most common metalloporphyrin (MP) in eukaryotic cells and is essential for life. Heme is constructed from a highly conserved sequence of eight enzymatic reactions that sequentially link precursors into four pyrrole precursor rings to form the tetrapyrrole, protoporphyrin IX. In the final anabolic step, ferrous iron is inserted by the enzyme ferrochelatase to form heme or iron protoporphyrin IX (**Figure 1**; Heinemann et al., 2008). Heme is regularly complexed into respiratory proteins such as hemoglobin and myoglobin to form a vital oxygen carrying and delivery platform. As a prosthetic group heme also performs essential activities for electron transfer and oxidation (Gray and Winkler, 1996). Because of the critical nature of all these basic activities, it is not surprising that virtually all steps of heme synthesis and degradation are tightly regulated with oxidative balance and many signaling pathways (Mense and Zhang, 2006).

Since the 1970s, commercial preparations of heme (*Hemin* and *Panhematin*), have been approved by the FDA for use in patients with acute porphyria. In addition to iron, natural metalloprotoporphyrins (MPP) and a variety of synthetic MP can complex other metals such as zinc, copper, cobalt, magnesium, manganese, tin, nickel, or chromium. Naturally occurring MPs serve as necessary co-factors for numerous oxygenases, peroxidases, and catalases. ZnPP, formed naturally during times of iron deficiency, is an excellent serum marker for iron deficiency anemia (Labbe, 1992) and has generated therapeutic interest for the treatment of jaundice of the newborn.

Initial catalysis of heme occurs via oxidation with heme oxidase (HO; **Figure 2**). The major isoforms of HO are HO-1 and HO-2. The role of HO-3 is not clear and is considered a pseudo transcript of HO-2 (Cruse and Maines, 1988; McCoubrey et al., 1997; Hayashi et al., 2004). HO-1 and HO-2 perform similar net enzymatic functions, yet only HO-1 is usually induced in response to cellular stressors such as hypoxia, cytotoxic agents, and infection (Immenschuh and Ramadori, 2000). HO opens the porphyrin ring of heme which is rate limiting for heme catabolism, and liberates equimolar ratios of Fe^{+2}, carbon monoxide, and the linear tetrapyrrole biliverdin (BV). The reaction uses 3 mol of oxygen and reducing equivalents from NADPH: cytochrome P-450 (cytochrome *c*) reductase. BV is then rapidly converted to bilirubin (BR) by BV reductase (BVR). The entire sequence of heme oxidation and BV

Abbreviations: BR, bilirubin; BV, biliverdin; BVR, biliverdin reductase; FL, full length; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HO, heme oxygenase; MP, metalloporphyrin; MPP, metalloprotoporphyrin; NS, non-structural; RT-PCR, reverse transcriptase polymerase chain reaction.

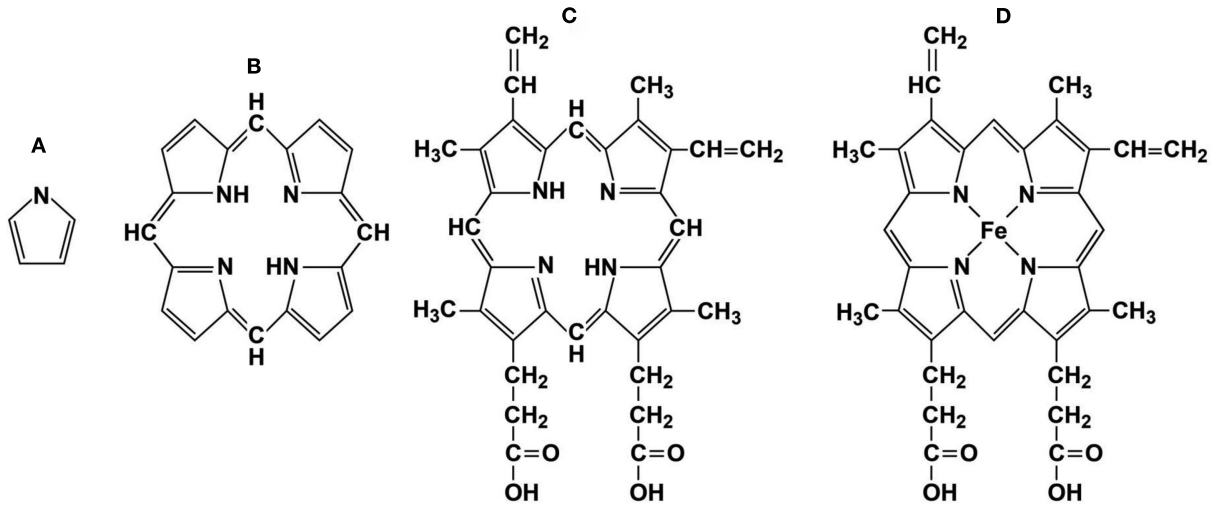

FIGURE 1 | Representative porphyrin structures. (A) pyrrole ring, **(B)** porphyrin ring, **(C)** protoporphyrin IX, and **(D)** Heme, iron protoporphyrin IX.

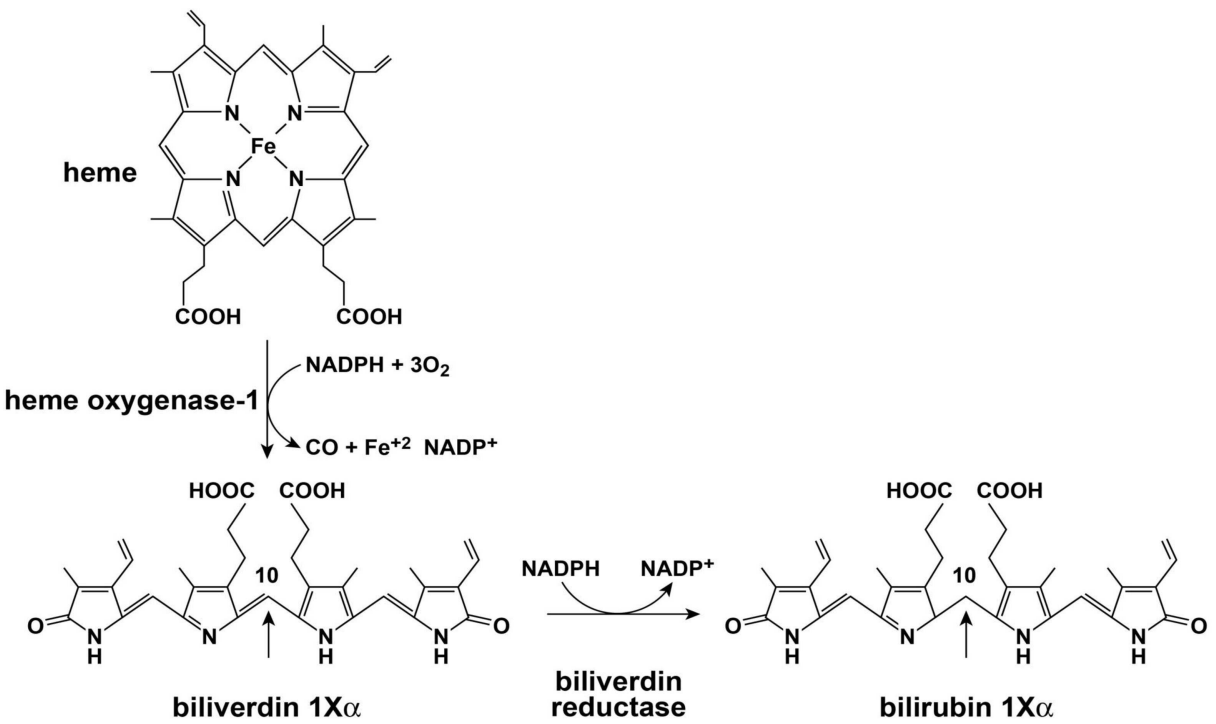

FIGURE 2 | Heme oxygenase and biliverdin reductase enzymes.

reduction likely takes place in synchrony upon a large complex of enzymes and co-factors that include HO, BVR, and NADPH (Ryter et al., 2006). BR and other tetrapyrrole derivatives are ubiquitous in nature and have a wide variety of functions in animals (McDonagh, 2001). Both HO-1 and BVR are highly inducible enzymes and respond to signaling and feedback in numerous cellular systems (Ryter et al., 2006; Kapitulnik and Maines, 2009). HO-1 induction provides important protection from oxidative stress and cellular damage (Abraham et al., 1995; Lee et al., 1996; Guo et al., 2001). Over the last two decades it is also becoming apparent that the precursors and catabolic products of the heme oxygenase system are capable of antimicrobial and antiviral activities. Of importance here, HO-1 induction or overexpression promotes a wide range of antiviral activities for the major viral pathogens discussed in this review: HIV, HBV, and HCV (Devadas and Dhawan, 2006; Protzer et al., 2007; Shan et al., 2007; Zhu et al., 2008).

GENERAL CONSIDERATIONS OF VIRUCIDAL ACTIVITIES OF HEME AND RELATED COMPOUNDS

Heme and structurally related MPs have actually generated interest as antimicrobials and antiviral agents for some time (Stojiljkovic et al., 2001). Porphyrins induce antiviral reactions through both non-specific and specific interactions with either host or virus which typically interfere with the viral life cycle. Typical non-specific interactions include activities such as hydrophobic binding of the porphyrin with host cell membranes or viral envelope that inhibit viral binding and cellular entry. Generalized antiviral activities reflect the diversity of porphyrin structure as well as chemical complexity and versatility. Photoactive MPs absorb photons and partition into membranes to where they can exert a virucidal effect, especially with enveloped viruses as noted below.

In contrast, specific interactions of natural MPs appear highly dependent on structure and reflect the fact that the primary function of heme and similar MPs usually behave as crucial co-factors in specific oxidase and peroxidase reactions. Consequently, an emerging pattern suggests that natural MPs show more affinity and specificity for viral inhibition because they are the preferred cofactor for established cellular reactions that are metabolically protective for cellular stress which may also severely reduce viral fitness. This assertion is clearly tentative, however, and has not been formally studied.

Recently, a group of alkylated porphyrins was reported to inhibit hepadnaviruses, flaviviruses, filoviruses, and arena viruses (Guo et al., 2011). The antiviral effects of these compounds, or chlorophyllides (an alkylated porphyrin containing copper charged at neutral ph), were discovered by screening over 2,000 compounds for their ability to decrease HBV DNA in cultured cells. A derivative of chlorophyllide, the metal free chlorin e6a,was the most active compound of this group. Antiviral activity for other viruses such as HCV, HIV-1, Dengue (DENV-2), Marburg virus (MARV), Junin viruses (JUNV), and herpes simplex (HSV-1) was also shown. Activity had high EC_{50}s in the low micromolar and nanomolar range for some viruses such as DENV and was selective for enveloped viruses. Non-enveloped viruses such as encephalomyocarditis (EMCV) and adenovirus (Ad) were highly resistant. The active porphyrin compounds caused disruption and dissociation of viral envelope proteins as assessed in a particle gel assay. These studies suggest use for these compounds as generalized virucidal agents for enveloped viruses.

Alternatively, an interesting antiviral application of some photoactive MPs has been studied for non-enveloped viruses such as Hepatitis A virus (HAV). HAV is a small picornavirus that is orally transmitted from fecal contaminated water and food. Like all picornaviruses (the family includes notable human pathogens such as polioviruses, enteroviruses, and rhinoviruses) the non-envelope structure of HAV presents challenges for prevention of infection because of its small size and resistance to inactivation. These viruses are highly resistant to common bacterial filtration techniques and the protein capsid renders the virus more resistant to chemical disinfection (Lemon et al., 1994). Casteel et al. (2004) showed that synthetic porphyrins could photo-inactivate HAV in plasma and other body fluids. Consequently, synthetic porphyrins may offer an effective and relatively safe approach to disinfection of non-enveloped viruses in various types of aqueous media. Potentially, this remarkable property could widely influence the epidemiology and pathogenesis of a variety of common pathogens that cause considerable morbidity and mortality worldwide.

Song et al. (1997) assessed the anti-HIV properties of various water-soluble polysulfonated and polycarboxylated porphyrins and their metal (Mn, Fe, and Ni) derivatives against HIV-1, HIV-2, and simian immunodeficiency virus infection in MOLT4 target cells. Some of these compounds were potent inhibitors of HIV infection and they likely interfere with the binding of HIV protein gp120 to the CD4 cellular receptor. Specific antibody studies demonstrated that the porphyrins directly bound to the gp 120 protein to inhibit formation and uptake of gp 120 and CD4+ T cell receptor binding complex. Interestingly, the gp 120 protein was also the probable binding site of a group of boronated porphyrins and other related MPs that were reported to bind gp120 and inhibit infection of CD4+ T cell line MT-2 (Debnath et al., 1994, 1999).

The antiviral activities of some more specialized synthetic porphyrins and derivatives against HCV have also been studied recently (Cheng et al., 2010). These workers evaluated structure-activity relationships of synthetic tetraphenylporphyrins and their anti-HCV properties as a proof-of- concept model for the development of proteomimetics in HCV drug discovery. Nanomolar levels of a biphenyl porphyrin derivative were noted to be the most potent inhibitor of full length Con1 replicons (Blight et al., 2002) but were less active for genotype 2a (JFH-1) subgenomic replicons (Kato et al., 2003). Furthermore, the porphyrin showed synergistic antiviral activity when incubated with replicons together with a known HCV protease inhibitor BILN 2061 as well as the antiviral cytokine α-interferon-2a. While the cellular targets for the porphyrin derivatives are not yet clear, these studies demonstrate that the tetraphenylporphyrin backbone is a useful scaffold on which to hang a variety of chemical groups and define structure-function relationships for additional antiviral development.

DIRECT EFFECTS OF HEME AND RELATED COMPOUNDS

Direct interactions of heme and related MPs are important medically for the development of new and improved antiviral agents against HIV, HBV, and HCV in addition to other human pathogens that cause persistent infection and chronic disease. Viral targets might include any of the key steps in a virus' life cycle such as entry, replication, assembly, and exit from the host cell. Alternatively, interactions of MP that enhance pathogen recognition and immune response pathways would be expected to decrease viral fitness and replication mechanisms. In this regard, heme and related natural compounds have recently been shown to possess astonishingly specific antiviral protease and polymerase activities for both human hepatotrophic and HIV viruses. As discussed below, an emerging pattern demonstrates novel actions of heme that influence not only viral replication, but immune recognition, inflammation, primary interferon responses, apoptosis, and oxidative defense mechanisms.

ACTIONS ON SPECIFIC VIRUSES

HEPATITIS C VIRUS

Hepatitis C virus is a small enveloped RNA virus and occupies a single genus of Hepacivirus of the Flaviviridae family. This

family also includes such human pathogens as yellow fever and dengue. HCV has a plus-stranded RNA genome with a single long open-reading frame that is translated into a large polyprotein, then processed by host and viral proteases (Lindenbach and Rice, 2005). The non-structural (NS) proteins NS3/4A protease, NS5B RNA dependent RNA polymerase (RdRp) and the NS5A protein, necessary for replication, have been the targets of intense research efforts to develop new antiviral drugs (Soriano et al., 2009). The ability to study the HCV life cycle and develop anti-HCV agents was initially hampered for nearly a decade by the inability to grow and pass the virus in cell culture or small animal models. However, viral replicon models began to appear by 1999 (Lohmann et al., 1999; Pietschmann et al., 2001; Blight et al., 2002) and these constructs enabled high throughput *in vitro* testing for a wide variety of potential antiviral drugs. In addition to replicons, defined infectious strains (Cai et al., 2005; Wakita et al., 2005) and patient wild type viruses (Castet et al., 2002; Gondeau et al., 2009) have been passed in primary hepatocytes and other permissive cell lines. Recently, reliable chimeric mouse-human models for *in vivo* testing of antiviral activity have also been introduced (Bissig et al., 2007, 2010; de Jong et al., 2010; Washburn et al., 2011).

DIRECT ANTIVIRAL EFFECTS OF HEME AND DERIVATIVES ON HCV

Indications that heme and its enzymatic products may interact with HCV actually arose from assays of human liver slices collected from HCV infected patients. Abdalla et al. (2004) evaluated relative expression of oxidative defense enzymes in infected patient liver samples as compared to uninfected control liver. HO-1 was dramatically reduced in HCV infected liver, in contrast to other antioxidative enzymes, catalase, CuZn and Mn super oxide dismutases which all remained unchanged. Furthermore, immunohistochemistry confirmed that the reduction of HO-1 expression was limited to hepatocytes, the site of HCV replication, and not Kupffer cells. Since chronic HCV infection causes hepatic inflammation and progressive fibrosis, HO-1 downregulation was quite unexpected as it might be anticipated that the enzyme would be induced as in other liver diseases (Makino et al., 2001; Bauer et al., 2003). Moreover, in the same study, autoimmune hepatitis and chronic HBV showed marked HO-1 upregulation in affected hepatocytes (Abdalla et al., 2004). These findings suggested that the virus can specifically modulate HO-1 expression although reasons for this behavior were not apparent at the time. Nevertheless, reduction of this important oxidative defense enzyme in the hepatocyte may indirectly contribute to progressive oxidative injury and facilitate fibrosis. In fact, later experiments demonstrated that overexpression of HO-1 in replicon cells promoted increased resistance to oxidant-induced cytotoxicity (Zhu et al., 2008).

Ensuing work by a number of groups has revealed dramatic innate anti-hepatitis C activities for heme and the oxidative products of the heme oxygenase system. It was first demonstrated that iron can inhibit the HCV NS5B RdRp by high affinity (Kd = −6 μM) binding to the divalent cation binding pocket of the polymerase (Fillebeen et al., 2005). The Kd for ferrous ion was found to be 500 times greater than the preferred Mg^{+} ion. Either divalent Mg or Mn is absolutely required for NS5B enzymatic activity (Ferrari et al., 1999; Bougie et al., 2003; Benzaghou et al., 2004). Antiviral activity of free iron has been confirmed by others (Yuasa et al., 2006; Zhu et al., 2008). However, free iron also induces HO-1 suggesting that the virucidal activity of Fe is likely more complicated than just binding and inactivating the polymerase (Hou et al., 2009). Iron would also be an unlikely choice as a therapeutic agent for HCV since it is usually considered to be a hepatotoxin (Ryter and Tyrrell, 2000) and it seems doubtful whether sufficient intracellular levels of free iron might be achieved *in vivo* to be useful therapeutically without causing cellular injury. Mild iron accumulation in HCV infected human liver samples has been correlated with more severe liver disease in some but not all studies (Beinker et al., 1996; Kayali et al., 2005, 2007). Some but not all earlier clinical studies with α-interferon treatment regimens suggested that phlebotomy could improve viral clearance and increase sustained virological responses to α-interferon (Di Bisceglie et al., 2000; Fontana et al., 2000; Desai et al., 2008). Finally, some of the experiments looking at the effects of iron on replication *in vitro* were actually performed using hemin to load cells with iron instead of iron salts (Fillebeen et al., 2005, 2007). Consequently the potency of free iron may have been overestimated since more recent studies have shown that heme as well as its initial oxidation product biliverdin can also directly inhibit the HCV NS3/4A protease (Zhu et al., 2010a).

As noted above, earlier work demonstrated that BV and bilirubin (BR) were able to inhibit the aspartyl protease of HIV (McPhee et al., 1996). In contrast, HCV NS3/4A protease is serine activated with a classical common catalytic mechanism like other members of this large class of proteolytic enzymes (Love et al., 1996; Yan et al., 1998). Inhibition of serine activated proteases with a tetrapyrrole appears common as a recent study demonstrated that both BV and BR were capable of inactivating intestinal trypsin and chymotrypsin (Qin, 2007). In this regard, Zhu et al. (2010a) reported that BV, heme, and to a much lesser extent BR were able to attenuate HCV replication in NS and full length replicons *in vitro*. BV was found to be a potent inhibitor of recombinant NS3/4A protease using Fluorescence Resonance Energy Transfer (FRET) inhibition assays in contrast to BR (**Figure 3**). After testing a number of biologically relevant linear tetrapyrroles, BV showed the lowest IC_{50} of all compounds tested (9.3 μM) and was considerably more potent than its reduction product, BR (**Figure 3A**). Assays conducted in the presence of both BV and *AnaSpec* #25346, a known commercially available inhibitor, showed an additive effect. Lineweaver–Burk plots indicated a mixed competitive and non-competitive inhibitory mechanism ($K_i = 0.6\,\mu M$ and $K_i' = 1.1\,\mu M$). Taken together, the kinetic experiments suggest there is associated non-competitive protease inhibition that probably occurs in an allosteric fashion and requires further study.

The marked difference in the avidity of BV and BR for the HCV NS3/4A protease may be explained by differences in structure and physical chemistries of the two linear tetrapyrroles. BV and BR have widely different secondary structures [see McDonagh, 2001 for review] in spite of the fact that they differ only by a double vs. single C–C bond at the 10 "hinge" position respectively (**Figure 2**). The hinge double bond restrains BV into a flat rather fixed limited plane. In contrast, the free rotation about the hinge single bond of BR allows internal hydrogen bonding of the COOH

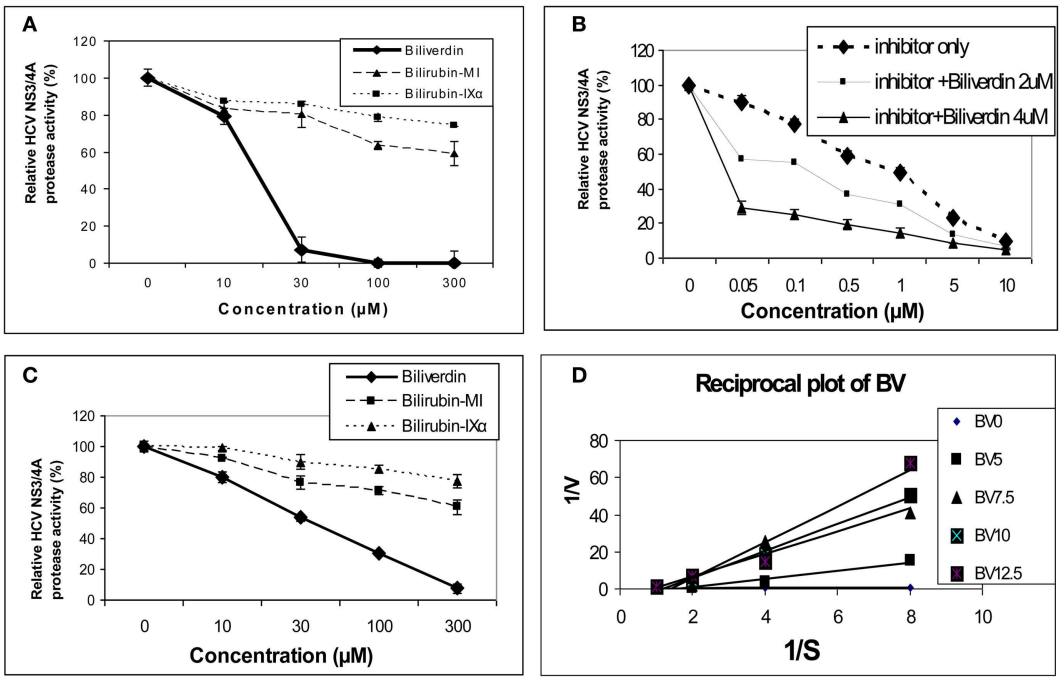

FIGURE 3 | Tetrapyrrole inhibition of HCV NS3/4A protease. (A,B) Protease activity was determined fluorometrically (FRET assay) using recombinant NS3/4A enzyme and various concentrations of inhibitors. **(C)** Endogenous NS3/4A protease activity in microsomes of replicons was measured using the same FRET assay but employing endogenous, partially purified NS3/4A protease from replicon cells (Zhu et al., 2010a). **(D)** Reciprocal (Lineweaver–Burk) plot of substrate concentration vs. enzyme activity. Recombinant protease activity was determined fluorometrically. Each point is the mean ± SEM of 3–5 determinations per point. Plot of [BV] vs. either 1/*V*ap or Km/V (not pictured) showed highly significant linearity ($r = 0.975$ and $r = 0.979$ respectively, $p < 0.005$) indicating mixed inhibition of NS3/4A protease by BV ($K_i' = 1.1$ mM and $K_i = 0.6$ mM, respectively). *0* to the commercial Inhibitor = NS3/4A protease competitive inhibitor, *AnaSpec* #25346. Biliverdin = >99% Biliverdin IX-α. Bilirubin-mixed isomers (MI) = 93% Bilirubin IX-α, and 6% associated Bilirubin isomers. Bilirubin IX-α = >99% bilirubin IX-α. (With permission Zhu et al., 2010a see original manuscript for further details.)

groups which folds the molecule into a hydrophobic "ridge–tile" structure (Nogales and Lightner, 1995). The NS3/4A protease has an unusually long and shallow active site groove that is quite atypical for serine activated proteases (Love et al., 1996). Presumably, the active site accommodates the flat BV molecule to allow stable occupation while the bulky hydrophobic BR fits poorly. Presently, it is unclear which functional groups interact with the serine active site triad and where potential allosteric sites may be located. However, these interactions appear important for future drug design of tetrapyrroles as antiviral agents.

At first we attributed the antiviral activity of heme to its induction of HO-1 and rapid oxidation with liberation of free iron and BV. However, direct testing of heme against the recombinant HCV NS3/4A protease in the FRET assay has revealed that heme, like BV, is also a direct protease inhibitor. Furthermore, some related metalloprotoporphyrins (MPP) such as ZnPP, also showed potent antiprotease and antiviral activities *in vitro* which occurred at quite similar inhibitor concentrations (**Figure 4**; Zhu et al., 2011). Further studies looking at relationships between tetrapyrrole structure, HCV protease binding affinity, and antiviral activity are ongoing in our laboratory. However, as noted above, planar compounds such as metal protoporphyrins appear to bind protease tightly in contrast to folded structures such as bilirubin derivatives, conjugated bilirubins, and mesoporphyrins (Zhu et al., 2010a).

Porphyrins with structures related to heme and ZnPP, such as the meso derivative ZnMP, have also been shown to have antiviral actions through HO-1 induction, however, with a more indirect mechanism. Transcriptional activation of HO-1 depends, in part, on Bach 1 a negative repressor of the HO-1 promoter region (Ogawa et al., 2001; Kitamuro et al., 2003). ZnMP was shown to induce the degradation of Bach 1 protein through increased proteasome degradation thus alleviating HO-1 promoter repression, facilitating HO-1 induction, and subsequent reduction of HCV RNA in NS replicons (Hou et al., 2008). Furthermore, a follow up study from the same group demonstrated that ZnMP could target HCV NS proteins such as NS5A for proteasomal degradation through ubiquitination (Hou et al., 2010).

INDIRECT ANTI-HCV EFFECTS OF HEME AND DERIVATIVES

Other reasons for the antiviral activity of heme and related molecules have also been investigated. Lehmann et al. (2010) reported that BV induced type I interferon expression in replicon cells which was accompanied by expression of interferon stimulated gene products. While this study was the first to describe direct interaction of an important natural tetrapyrrole with the innate immune system, the mechanism is not yet clear (Lehmann et al., 2010). Primary induction of interferon by BV *in vitro* has not been reported previously and appears contrary to the known anti-inflammatory

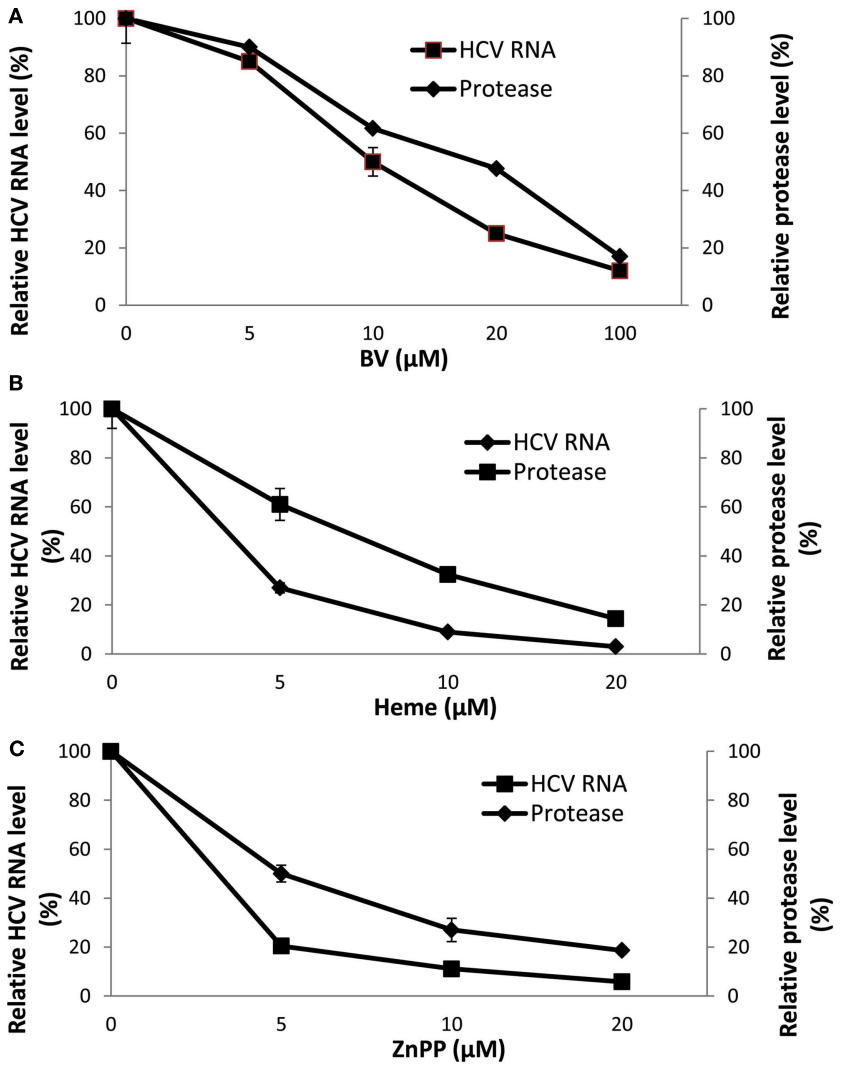

FIGURE 4 | Disappearance of HCV RNA and HCV NS3/4A inhibition with biliverdin, heme, or ZnPP. (A–C) HCV RNA was determined in total RNA extracted from replicon cells incubated with the indicated concentrations of tetrapyrrole for 48 h. Independent determinations of the inhibitory activity of each tetrapyrrole for recombinant HCV NS3/4A was determined by FRET analysis (Zhu et al., 2010a). Results are means ± SEM of six determinations per point. (With permission; Zhu et al., 2011.)

character of BV and HO-1 induction. BV has been shown to induce nuclear accumulation of biliverdin reductase which then decreases nuclear NFkB in Hek293 cells (Gibbs and Maines, 2007; Gibbs et al., 2010). The latter findings are consistent with an immunosuppressive role for BV (Ollinger et al., 2007) in contrast to the more pro-inflammatory properties of heme (Gozzelino et al., 2010). On the other hand, in other cell types such as human myeloid cells, HO-1 induction was noted to be essential for IRF3 and NFkB activation and nuclear accumulation which are prerequisites for type I interferon induction (Tzima et al., 2009; Kalliolias and Ivashkiv, 2010). HO-1 knockout mice demonstrated that HO-1 induction is necessary for early phase activation of the innate immune sensing pathways TRIF–IRF3 and RIG-I–IRF3 with Sendai virus for the production of β-interferon and other primary response cytokines (Tzima et al., 2009; Koliaraki and Kollias, 2011). It is not known whether an HO-1 enzymatic product or a secondary mediator is required for these activities or whether they occur in other cell types such as hepatocytes. Nevertheless, these findings expand the influences of HO-1 into innate immunity and potentially other related pathways.

BV AND HEME CAN REVERSE HCV NS3/4A INHIBITION OF TYPE I INTERFERON INDUCTION

As is typical of viral pathogens, the NS3/4A protease has extra viral proteolytic activities that serve to increase the efficiency of viral infection and evasion of host immune system. NS3/4A can cleave pattern recognition receptors (PRR) or their interface adapter proteins within type I interferon signaling pathways and thus disable transcriptional induction of α and β interferon (Li et al., 2005b; Meylan et al., 2005; Loo et al., 2006; Bode et al., 2007; Kalliolias and Ivashkiv, 2010). The two best studied induction pathways likely important for HCV responses are those that recognize intracellular

double stranded (ds) RNA and include the Toll like receptor 3 (TLR3) and the Retinoic acid inducible gene-1 (RIG1) pathways. HCV NS3/4A cleaves specific adapter proteins of both pathways including TRIF of the TLR3 system and Cardiff of the RIG1 system (**Figure 5**; Li et al., 2005b; Meylan et al., 2005; Bode et al., 2007). Knowing that heme and other tetrapyrroles inhibit the HCV protease, we recently investigated whether these compounds would restore type I interferon induction after disruption of interferon signaling with the protease.

Clonal cell lines of Huh 7 such as Huh7.5 and Huh 515 are known to have poor dsRNA and dsDNA innate immunity recognition which is a likely reason that they are permissive for HCV replication (Li et al., 2005a; Cheng et al., 2007). Consequently, to evaluate the effects of heme on type I interferon signaling, it was necessary to construct a reconstituted model system. Hek293 cells were chosen because they are known to have relatively intact innate immune recognition and high transfection efficiency (Khvalevsky et al., 2007). We transfected dsRNA antigen into Hek293 cells and 48 h later assessed interferon induction using luciferase reporter gene constructs containing promoter regions for β interferon. Transfection of dsRNA elicited robust induction of type I interferon, however, this was clearly attenuated when cells were co-transfected with NS3/4A expression vectors (**Figure 6**). In contrast, incubation with heme or BV significantly restored induction in the presence of protease and depending on protease and substrate concentrations, this was nearly complete in some cases (**Figures 6A,B**; Zhu et al., 2011). Additionally, restoration of interferon induction in the presence of protease was sharply accompanied by induction of interferon stimulated response gene (ISRG) products such as OAS-1 as shown here (**Figures 6C,D**; Zhu et al., 2011). Furthermore, we noted that BV or heme only controls without protease, showed little ability to augment type I interferon induction when incubated with cells either with or without dsRNA antigen. This suggests that in the present system the tetrapyrroles are unlikely to induce type I interferons as a primary antiviral mechanism. Nevertheless, these data expand the capabilities of heme and similar tetrapyrroles to promote beneficial effects for the innate immune system.

HUMAN IMMUNODEFICIENCY VIRUS

Human Immunodeficiency virus is a human pathogenic RNA retrovirus of the genus *Lentivirus* first identified in the early 1980s (Barresinoussi et al., 1983; Popovic et al., 1984) and found worldwide. HIV is not a hepatotrophic virus, however as noted

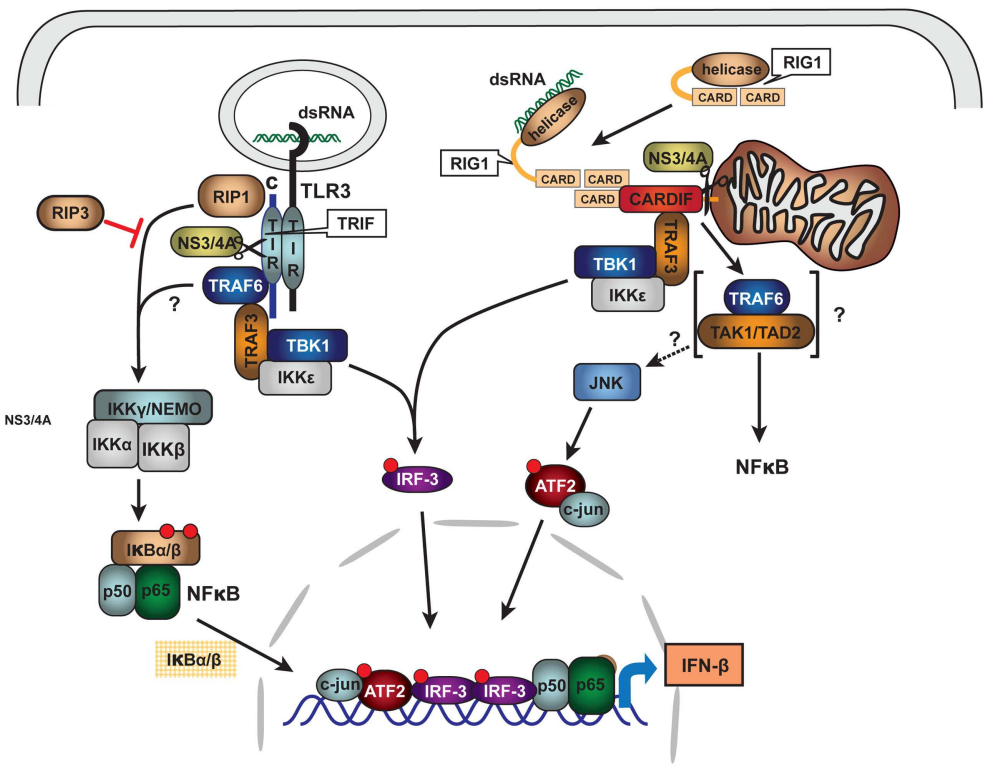

FIGURE 5 | Schematic of HCV NS3/4A protease inhibition of innate immune signaling pathways: TLR3 and RIG1. Double stranded RNA (dsRNA) viruses or viruses with dsRNA intermediate activate innate immune system signaling pathways for type I interferon induction through binding to Pattern Recognition Receptors (PRR). For HCV, PRR are associated with at least two major signaling pathways: TLR3 and RIG1. Upon intracellular binding of dsRNA to associated adapter proteins TRIF and CARDIFF, respectively, signaling is transmitted through complex intermediate steps of recognition, binding, and phosphorylation leading to activation of the transcriptional factors IRF3 and NFκB. The activated nuclear transcription factors bind to type I interferon promoters and induce α/β-interferon transcription. HCV NS3/4A protease is known to cleave both TRIF and CARDIFF adapters thus crippling innate immune antigen recognition and signaling for type I interferon induction. With permission (Bode et al., 2007), see original review for terminology and abbreviations.

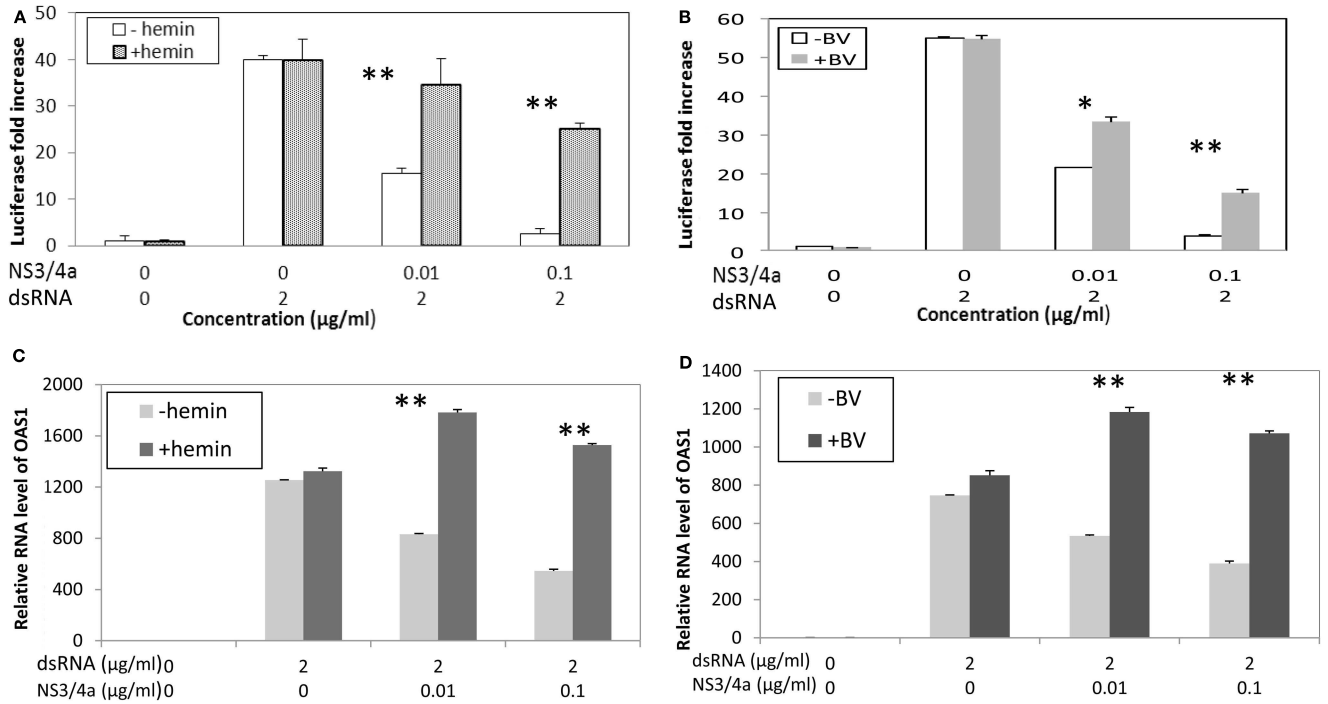

FIGURE 6 | Biliverdin and Heme can restore IFN promoter activation and Interferon Stimulated Response Genes (ISRG) after interference of IFN signaling with HCV NS3/4A. (A,B) HEK 293 cells were transfected with dsRNA and vectors containing Type I IFN promoter-luciferase construct and complete NS3/4A sequence plasmid construct or control plasmid. Cells were then incubated with heme (20 μM) or Biliverdin (50 μM) for 48 h. Cellular lysates were prepared and assayed for interferon promoter activation using luciferase assay (Zhu et al., 2010b). Each point is the mean of six determinations (two separate cell culture incubations, and then three replicates for luciferase assay per incubation). **(C,D)** Parallel experiments showing that heme and BV can restore interferon stimulated response genes (ISRG) after inhibition with NS3/4A protease. HEK 293 cells were transfected with dsRNA with or without HCV NS3/4A plasmid then incubated with heme or BV for same time and concentrations as **(A,B)** OAS-1 mRNA was measured using *Real Time* RT-PCR. Each point is the mean of six determinations as described for **(A,B)** $**p < 0.01$; $*p < 0.05$; NS, not significant. (With permission Zhu et al., 2011.)

previously, a high percentage of patients with HIV are co-infected with HCV and/or HBV because they share common risk factors for transmission (Quan et al., 1993; Zylberberg and Pol, 1996). HIV co-infected patients, especially those with markedly depressed CD4 T cell levels and AIDS, are known to have more aggressive liver disease with increased incidence of cirrhosis as compared to patients with controlled HIV or HCV/HBV monoinfection (Telfer et al., 1994; Darby et al., 1997). Because of the global impact of the AIDS pandemic in the 1980s a tremendous research effort has been invested in drug discovery for an ever expanding list of HIV targets. While mechanistic studies of HIV are not directly applicable to the hepatotrophic viruses *per se*, the large research effort that developed model systems for anti-HIV drug design forged a path that has facilitated drug discovery for hepatitis viruses.

The antiviral interactions of heme with HIV were investigated early after the virus was discovered. These experiments established that heme has the ability to inhibit reverse transcriptases (RT) such as Rauschermurine leukemia virus RT (Tsutsui and Mueller, 1987). Ensuing work (Levere et al., 1991) showed that heme administered alone or together with 3′-azido-3′-deoxythymidine (AZT) was able to repress replication of HIV in human peripheral blood lymphocytes and H9 cell lines. This activity was found for both AZT-resistant and drug-sensitive HIV strains. Dose response studies showed significant antiviral activity at physiological levels of heme as low as 1 μM and there was a clear, enhancing effect of heme in combination with AZT. Additional work from the same group demonstrated that heme was a non-competitive inhibitor of HIV-1 RT in contrast to classical competitive inhibition with deoxythymidine triphosphates (Staudinger et al., 1996). Some early structure-function relationships were also established from testing of heme analogs and other more diverse MP structures. Interestingly, the protoporphyrin structure was found to be crucial for anti-RT activity since metal meso derivatives were inactive.

Argyris et al. (1999) then identified a unique non-nucleoside inhibitory binding site for heme and putatively other MPs located in the connection domain (region 398–407 aa residues) of HIV-1 and 2 RT. The site was revealed through heme binding assays matched to a phage-displayed 12 mer peptide library. Analysis of the peptides that actively bound heme showed that, as a group, they tended to be enriched in the aromatic amino acids Trp and Tyr while Cys residues were completely absent. There was synergism for RT inhibition with the unique heme binding site and another well-characterized non-nucleoside binding site located in the p66 palm sub domain (BHAP; Argyris et al., 1999). The authors speculated that the Tyr and Trp residues in this site of the enzyme

facilitate heme binding through aromatic stacking with the planar porphyrin ring, while the propionyl and vinyl side chains anchor the holo heme.

The linear tetrapyrrole oxidation product of heme, biliverdin (BV), and its reduced derivative, bilirubin (BR) have also been reported to directly inhibit the HIV aspartic acid activated protease (McPhee et al., 1996). Using assays of recombinant HIV-1 protease, BV and BR showed nearly equal K_i values of about 1 μM for HIV-1, 2, and simian immunodeficiency virus proteases. Related tetrapyrroles showed some antiprotease activity but at much higher concentrations. It should be emphasized that the inhibitory activities of BR and BV were noted to take place at near physiological concentrations of BR (17 μM is 1.0 mg/dl). Additionally, several synthetic boronated porphyrins also showed competitive inhibition of the HIV protease with K_i's in the mid nanomolar range (Decamp et al., 1992).

ANTI-HIV EFFECTS OF HO-1 INDUCTION ON HIV

More recent work on the effects of heme on HIV replication demonstrated that heme also elicits antiviral activity through induction of HO-1 (Devadas and Dhawan, 2006). Heme was antiviral *in vitro* in a dosage dependent manner and elicited more than a 90% reduction of intracellular HIV in monocytes or T cells and was even active on drug resistant strains. While the heme antiviral targets that enabled these data were not specifically pursued, we can assume that heme likely worked through inhibition of viral uptake, RT inhibition, and/or inhibition of the HIV protease through heme oxidation products BV and BR as discussed above. An important point of this study is that it established that heme can attenuate viral levels *in vivo*. Intraperitoneal administration of heme attenuated HIV replication in humanized non-obese diabetic SCID mice carrying infected human PBMCs (Devadas and Dhawan, 2006).

HEPATITIS B VIRUS

Hepatitis B virus is the most common chronic viral pathogen worldwide. The virus is a member of the hepadnavirus family and is one of the smallest known pathogenic human DNA viruses of about 3.2 kb. The virus replicates through a unique pre-genomic (pg) mRNA intermediate made by host nuclear enzymes which is then reverse transcribed into genomic DNA using a unique hepadnaviral reverse transcriptase. The reverse transcriptase of HBV shares a number of active site and substrate features with the HIV RT resulting in inhibitory drug overlap with HIV. However, hepadnaviral DNA synthesis employs a unique protein priming method for initiation of replication from pg RNA. The RT protein acts as both a protein primer and polymerase through interaction with a viral RNA structure (60 nucleotides) called ∈ at the 5′ end of pg RNA. This complex facilitates origin of reverse transcription, packaging, and replication fidelity (Pollack and Ganem, 1994; Tavis et al., 1994; Wang et al., 1994). In contrast to both HIV and HCV, replication and assembly of HBV occurs without a viral protease.

As compared to HIV and HCV, interactions of heme and heme oxygenase-1 with HBV have not been extensively evaluated. Nevertheless, HO-1 appears to be upregulated in response to chronic HBV infection as shown by immunohistochemical labeling of human liver samples (Abdalla et al., 2004). In a transgenic mouse model of acute and chronic HBV infection, Protzer et al. (2007) showed that induction of HO-1 attenuated viral replication as determined by reduced levels of HBV core protein. In addition to virucidal activity, HO-1 induction also led to reduced liver injury and hepato protective effects. The authors suggested that HO-1 induction worked posttranscriptionally to reduce the stability of HBV core protein (Protzer et al., 2007). Furthermore the combination of hepato protective effects from cellular injury and virucidal capabilities of HO-1 induction suggest additional benefits for potential antiviral drugs that could be designed using this important pathway.

Because heme was shown to have a specific inhibitory binding site on the HIV RT, we might predict that a similar interaction would occur with HBV. While recent evidence indicates that HBV replication is indeed inhibited by heme *in vitro*, at least one mechanism is decidedly different (Lin and Hu, 2008). Heme blocked the interaction of the RT protein with the ∈ segment of pg RNA, thus inhibiting the protein priming step of HBV replication. The heme binding site was localized to the N-terminal domain of the RT protein which is unique to hepadnaviral RT. In addition to heme, protoporphyrin free base and biliverdin were also active. While further studies of viral resistance, ligand specificity, and mechanism of these interactions need to be explored, these data suggest that similar compounds could be employed as either primary or adjunct therapy with nucleoside analogs for patients with chronic HBV infection.

FINAL CONSIDERATIONS

In spite of development of new and improved antiviral agents for HIV, HCV and HBV, there is a persistent and urgent need for new treatment modalities as well as combination therapies. Nearly all compounds developed against these pathogens have associated problems with toxicity, viral resistance, and selectivity (De Clercq, 2007; Firpi and Nelson, 2007; Zoulim, 2011) which makes ongoing discovery of new antivirals with improved clinical spectrum and versatility necessary.

Table 1 summarizes the various sites of antiviral activity of the precursors and products of the heme oxygenase-1 system for all three viruses. It is apparent that the viral targets for heme are quite similar among the viruses in spite of viral diversity. All three viruses are inhibited by HO-1 induction and heme targets both HBV and HIV RT. Interestingly, however, heme RT inhibition occurs at different relative sites on the respective enzymes. Furthermore, heme, BV and BR inhibit both HIV and HCV proteases, in spite of the fact that the proteases differ markedly in primary structure, catalytic sites, and reaction mechanisms (Wlodawer et al., 1989; Love et al., 1996; Yan et al., 1998; Brik and Wong, 2003). Finally, HO-1 induction offers protection from oxidative injury in HBV and HCV infection thus suggesting additional benefits against oxidative induced tissue injury that is considered to be a primary chronic disease mechanism leading to hepatic injury and fibrosis (Lieber, 1997; Lee et al., 2004; Qadri et al., 2004; Seronello et al., 2007).

Collectively, these findings indicate that heme and related porphyrins clearly should be considered for further *in vivo* translational studies, especially in suitable animal models and patient pilot trials, at least for proof-of-concept. The ability to inhibit

Table 1 | Activities of heme and related agents for HCV, HBV, and HIV.

Virus	Agent	Mechanism studied	Reference
HCV	BV	Anti-NS3/4A protease	Zhu et al. (2010a)
	BV	Type I interferon induction	Lehmann et al. (2010)
	Heme	Anti-NS3/4A protease	Zhu et al. (2010a)
	Heme	HO-1 induction	Shan et al. (2007), Zhu et al. (2008)
	ZnMP	HO-1 induction and Bach 1 inhibition	Hou et al. (2008)
	ZnMP	Ubiquitination of NS5A	Hou et al. (2010)
	Fe	Antipolymerase	Fillebeen et al. (2005)
	Fe	Decreased HCV replication	Yuasa et al. (2006), Zhu et al. (2010a)
	Fe	HO-1 induction	Hou et al. (2009)
	Zn	Decreased viral replication	Yuasa et al. (2006)
	HO-1 enzyme	Enzyme overexpression	Zhu et al. (2008)
HBV	Heme	Anti-reverse transcriptase	Lin and Hu (2008)
		HO-1 induction	Protzer et al. (2007)
HIV	BV/BR	Anti-HIV protease	McPhee et al. (1996)
	Synthetic porphyrins	Anti-HIV protease	Decamp et al. (1992)
	Heme/MPs	Anti-reverse transcriptase	Levere et al. (1991), Staudinger et al. (1996), Argyris et al. (1999)
	HO-1 induction	HO-1 induction	Devadas and Dhawan (2006)
	MPs	Gp120 inhibition	Song et al. (1997)

multiple viral target sites on different viruses indicates that these compounds would be also useful for patients with dual or even triple infections. Co-infected patients are a serious problem worldwide and invariably present with more severe medical disease, aggressive hepatitis, and a number of treatment dilemmas (den Brinker et al., 2000; Koziel and Peters, 2007; Zhou et al., 2011). Considering the antiviral fidelity of heme and products of the heme oxygenase system it is an open question as to the why these agents have not been developed further for antiviral therapy. Heme is proven to be efficacious for use *in vivo* for acute porphyria and is usually well tolerated. Further *in vivo* studies should focus on evaluation of porphyrin based antivirals with oral bioavailability, safety, and attractive pharmacodynamic capabilities to expand their use for HCV, HIV, and HBV infections.

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Bilirubin: an endogenous molecule with antiviral activity *in vitro*

Rosaria Santangelo[1†], Cesare Mancuso[2*†], Simona Marchetti[1], Enrico Di Stasio[3], Giovambattista Pani[4] and Giovanni Fadda[1]

[1] *Institute of Microbiology, Catholic University School of Medicine, Roma, Italy*
[2] *Institute of Pharmacology, Catholic University School of Medicine, Roma, Italy*
[3] *Institute of Biochemistry and Clinical Biochemistry, Catholic University School of Medicine, Roma, Italy*
[4] *Institute of General Pathology, Catholic University School of Medicine, Roma, Italy*

Edited by:
Mahin D. Maines, University of Rochester School of Medicine, USA

Reviewed by:
Paavo Honkakoski, University of Eastern Finland, Finland
Pavel Anzenbacher, Palacky University, Czech Republic
Peter Gibbs, University of Rochester School of Medicine and Dentistry, USA

****Correspondence:***
Cesare Mancuso, Institute of Pharmacology, Catholic University School of Medicine, Largo Francesco Vito, 1, 00168 Roma, Italy.
e-mail: cmancuso@rm.unicatt.it

[†]Rosaria Santangelo and Cesare Mancuso have contributed equally to this work.

Bilirubin-IX-alpha (BR) is the final product of heme metabolism through the heme oxygenase/biliverdin reductase (HO/BVR) system. Previous papers reported on the microbicidal effects of the HO by-products biliverdin-IX-alpha, carbon monoxide and iron, through either direct or indirect mechanisms. In this paper the evidence of a virucidal effect of BR against human herpes simplex virus type 1 (HSV-1) and the enterovirus EV71 was provided. Bilirubin-IX-alpha, at concentrations 1–10 μM, close to those found in blood and tissues, significantly reduced HSV-1 and EV71 replication in Hep-2 and Vero cell lines, respectively. Bilirubin-IX-alpha inhibited viral infection of Hep-2 and Vero cells when given 2 h before, concomitantly and 2 h after viral infection. Furthermore, BR retained its antiviral activity even complexed with a saturating concentration of human serum-albumin. Moreover, 10 μM BR increased the formation of nitric oxide and the phosphorylation of c-Jun N-terminal kinase in Vero and Hep-2 cell lines, respectively, thus implying a role of these two pathways in the mechanism of antiviral activity of the bile pigment. In conclusion, these results support the antiviral effect of BR against HSV-1 and enterovirus *in vitro*, and put the basis for further basic and clinical studies to understand the real role of BR as an endogenous antiviral molecule.

Keywords: bilirubin, biliverdin reductase, enterovirus, heme oxygenase, herpes simplex virus, nitric oxide

INTRODUCTION

The heme oxygenase/biliverdin reductase (HO/BVR) axis is the main metabolic pathway by which heme is degraded. The combined action of these enzymes converts heme into ferrous iron (FeII), carbon monoxide (CO), and biliverdin-IX-alpha (BV) which is the precursor of bilirubin-IX-alpha (BR; Maines, 1997; Mancuso and Barone, 2009). For several years, the by-products of the HO/BVR axis were considered mere waste products, but over the past two decades, a number of investigators have focused their attention on both HO/BVR and their products in an attempt to elucidate their true biological functions. Stocker et al. (1987b) described the antioxidant properties of BR, and 6 years later Verma et al. (1993) proposed a role for CO as an endogenous neuromodulator. These observations were followed by numerous papers demonstrating CO's important role as a regulator of synaptic transmission, cardiac function, and vessel tone (Wu and Wang, 2005). Bilirubin-IX-alpha helps maintain the cell's redox equilibrium by activating important pro-survival signaling pathways, such as those involving the proto-oncogene Akt or the mitogen-activated protein kinase family (MAPK), and by scavenging reactive oxygen and nitrogen species (ROS and RNS, respectively; Stocker et al., 1987b; Minetti et al., 1998; Kaur et al., 2003; Mancuso et al., 2006b, 2008). These findings suggest that up-regulation of the HO/BVR axis is a useful mechanism for improving cells' responses to stress, and substances known to increase HO activity *in vitro* are being explored as potential drugs for the treatment of free radical-induced diseases (Mancuso and Barone, 2009). In addition, the up-regulation of HO was shown to play an important role against bacterial and viral infections. The overexpression of the inducible isoform of HO (HO-1) displayed a significant antiviral activity against enterovirus and hepatitis viruses (Zhu et al., 2010; Tung et al., 2011). With regard to the by-products of the HO system involved in this antiviral activity, BV was shown to interfere with human hepatitis C virus (HCV) and human herpes virus (HHV)-6 replication as well as to reduce the cytopathic effect of human immunodeficiency type 1 virus (HIV) in Huh or MT-4 cells (Mori et al., 1991; Nakagami et al., 1992; Lehmann et al., 2010; Zhu et al., 2010). In addition, CO inhibited the growth of enterovirus, *E. coli*, *P. aeruginosa* as well as *S. aureus* (Nobre et al., 2007; Tung et al., 2011; Desmard et al., 2012) and iron almost completely decreased HCV core mRNA and protein (Hou et al., 2009). Taken together these results underlined a major role for HO in antiviral and antibacterial defense. That said, it is noteworthy to mention that no data are available to date about the potential antiviral activity of BR produced by BVR. As mentioned before, BR was shown to serve as an efficient scavenger for ROS which, in turn, are currently viewed as effectors of the antimicrobial activity of the immune response (Bogdan et al., 2000; Fang, 2004; Fialkow et al., 2007), therefore a direct antiviral role for BR is unlikely. On the other hand, recent findings demonstrated alternative mechanisms

for the cytoprotective activity of BR, including the stimulation of nitric oxide (NO) release and the modulation of the MAPK system in pheochromocytoma cells and primary cultures of rat cerebellar granules (Mancuso et al., 2008). NO plays an important role in the antiviral defense (Croen, 1993; Akaike and Maeda, 2000) and it is also an intermediate in the bactericidal activity of certain drugs (Timmins et al., 2004; Koide et al., 2009). Moreover, the up-regulation of specific MAPK, such as the c-Jun N-terminal kinase (JNK), was shown to increase the cytoprotective defense against viruses (Hrincius et al., 2010).

The aim of this work was to explore the antiviral activity of BR *in vitro* and the plausible intracellular systems involved in this protective effect. Since intracellular BR exists in the free form whereas in the bloodstream the bile pigment is bound to human serum-albumin (HSA), the antiviral activity of both BR and BR–HSA were studied. The viral species against which BR and BR–HSA were tested are the human herpes simplex type 1 virus (HSV-1), a DNA virus which belong to the Herpesviridae family, and enterovirus 71 (EV71) which belong to the Picornaviridae family (RNA viruses). Both these viruses are responsible for important acute and chronic diseases in humans.

MATERIALS AND METHODS

CHEMICALS AND ANTIBODIES

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Bilirubin-IX-alpha (Frontier Scientific, Carnforth, Lancashire, UK) was dissolved in sodium hydroxide (0.1 M) at a concentration of 10 mM and further diluted in double-distilled water. Bilirubin-IX-alpha solution was freshly prepared before each experiment and protected from light. The formation of the BR–HSA complex, at saturating concentrations of the protein, was allowed by incubating BR and HSA for 10 min at 37°C in the dark at a ratio up to 1:2 (Mancuso et al., 2006a). The fluorescent cell-permeant dye 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate was purchased from Molecular Probes (Invitrogen Carlsbad, CA, USA) and the JNK inhibitor SP600125 was from Assay Design (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). Anti-pJNK Thr183/Tyr185, antibody was purchased from Cell Signaling, (Danvers, MA, USA) and the anti-α-tubulin rabbit monoclonal antibody was from Thermo Scientific (Rockford, IL, USA).

CELL CULTURE AND VIRUS PROPAGATION

The human laryngeal carcinoma cell line (Hep-2) and African green monkey kidney epithelial cells line (Vero) were maintained in Minimum-Essential-Medium (MEM) additioned with 10% fetal calf serum (FCS), glutamine (200 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Human herpes simplex virus type 1 (KOS strain) and EV71 were purchased from the ATCC (Milan, Italy) and propagated and titrated in Hep-2 and Vero cells, respectively. The virus titre for both HSV-1 and EV71 was calculated by the plaque assay (Saijets et al., 2003; Hung et al., 2010).

The day before the experiment, 1×10^5 Hep-2 or Vero cells were seeded in 24-multiwell plates at a density of 400 cells/mm^2. After overnight incubation, cells were treated with HSV-1 or EV71 alone or in the presence of test substances as described in Protocols 1–4.

STUDY DESIGN

In order to mimic the different conditions during which BR could interfere with viral infectivity, four different protocols were designed.

Protocol 1

Hep-2 and Vero cells were pre-incubated with BR (1–10 μM) for 2 h. At the end of incubation, the BR-containing medium was discarded, replaced with fresh medium and the cells infected with HSV-1 and EV71. After 2 h, the inocula were removed and the cells incubated in fresh culture medium for 24–48 h. These time points were selected considering the replication cycles of both HSV-1 and enterovirus which are 10–20 h.

Protocol 2

Herpes simplex virus type 1 and EV71 were pre-incubated with BR (1–10 μM) alone or complexed with saturating HSA (20 μM) for 1 h and then transferred to Hep-2 and Vero monolayers for the infection. After 2 h, the inocula were removed and the cells incubated in fresh culture medium for 24–48 h. The effect of HSA *per se* on viral replication was also evaluated by incubating either HSV-1 or EV71 with 20 μM HSA and then transferring the inocula to Hep-2 and Vero cells as described above.

Protocol 3

Hep-2 and Vero cells were exposed to BR (1–10 μM) alone or complexed with saturating HSA (20 μM) and concomitantly infected with HSV-1 and EV71 for 2 h. At the end of incubation, the inocula were removed and the cells incubated in fresh culture medium for 24–48 h. The antiviral effect of HSA *per se* was also evaluated by infecting Hep-2 and Vero cells with either HSV-1 or EV71 in the presence of 20 μM HSA as described above.

Protocol 4

After 2 h of infection with HSV-1 and EV71, the inocula were removed and Hep-2 and Vero monolayers treated with BR (1–10 μM) for further 2 h. At the end of incubation the BR-containing medium was discarded and the cells incubated in fresh culture medium for 24–48 h.

In preliminary experiments, both Hep-2 and Vero cells were incubated with 10–100 μM NaOH for 2 h and then incubated with fresh medium for 24–48 h to evaluate whether or not this substance affected viral infectivity.

In selected experiments, performed following protocols 1 and 3, Hep-2 and Vero cells were pre-incubated with the NO synthase (NOS) inhibitor L-NG-monomethyl arginine citrate (L-NMMA, 1 mM) for 1 h and further for 2 h in the presence of BR (10 μM) or BR (10 μM)-HSA (20 μM).

The number of infected cells were calculated by immunofluorescence as described below.

QUANTIFICATION OF INDIVIDUAL INFECTED CELLS

At the end of the 24 or 48-h incubation, cells were washed with PBS to remove residual culture medium, gently scraped and counted. Twenty-five thousand infected cells were seeded on single slide (four slides for each well) and incubated with anti HSV-1 monoclonal antibody (Imagen Herpes simplex virus direct IF test,

Dako Cytomation, UK) or Pan-enterovirus antibody blend (PAN ENTERO BLEND, Light Diagnostics, Temecula, CA, USA) according to the manufacturers' instructions. Immunofluorescence is a simple and reliable method to assess viral infection and is routinely used to estimate either herpesvirus or enterovirus infectivity (Klespies et al., 1996; Saijets et al., 2003; Copeland et al., 2009; Prichard et al., 2009). Both total cells and immunofluorescence-positive cells in each sample were counted in at least five microscopy fields (120–160 total cells and ∼5–140 positive cells per field) by two independent blinded investigators and this latter expressed as percentage respect to total cells. The inter-investigator variability rate of cell count was less than 10%.

MEASUREMENT OF NO

Intracellular NO was detected in Vero cells treated for 2 h with BR (1–10 μM) in 0% FCS MEM by using the fluorescent probe DAF-FM-DA as previously described (Mancuso et al., 2008).

WESTERN BLOT ASSAY

Confluent Hep-2 and Vero cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate) containing 1% Triton X-100 and 0.1% SDS; lysates were cleared by centrifugation, and equal amounts of protein (40 μg) for each sample were subjected to SDS-PAGE on 10% gel. Western blot analysis was performed as previously described (Mancuso et al., 2008) by using primary antibodies (see above) diluted 1:1000 and HRP-conjugated secondary antibodies as appropriate.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM of (N) individual samples per group. Statistical analysis was performed using ANOVA combined with Dunnet's or two-tailed Student's *t*-tests for comparison within the same group or two groups, respectively. Differences were considered significant at $P < 0.05$. The maximal inhibitory activity values were calculated and confirmed by non-linear regression analysis using a Prism 4.0 software (GraphPad Software).

RESULTS

EFFECTS OF BR OR BR–HSA ON HSV-1 AND EV71 INFECTIVITY

The first step was to titrate both HSV-1 and EV71 by the plaque assay. The titer for HSV-1 was calculated to be 1.3×10^7 plaque forming unit (PFU)/ml whereas for EV71 was 1×10^9 PFU/ml. At these titers both HSV-1 and EV71 completely detached the Hep-2 and Vero cell monolayers as early as 24 from infection and this did not allow us to carry out any experiment to prove the possible protective effect of 1–10 μM BR. For this reason, in all the experimental protocols described below, cells were infected with either HSV-1 or EV71 at the titer 1×10^5 PFU.

In preliminary experiments, the unspecific effects of NaOH, the vehicle for BR, on viral replication was studied. Sodium hydroxide in the range 10–100 μM (i.e., the concentrations in 1–10 μM BR) did not have any significant effect on HSV-1 or EV71 infectivity at 24–48 h of incubation (data not shown).

Protocol 1

As shown in **Figure 1**, Hep-2 and Vero cells pre-incubated with BR (1–10 μM) for 2 h and then infected with HSV-1 and EV71, were markedly resistant to the lethal effect of both the viruses and the percentage of infected cells were significantly reduced both at 24 and 48 h after infection (**Figure 1**). However, in this experimental setting BR was slightly more efficacious against HSV-1 than EV71 at 48 h from infection (mean maximal inhibitory activity 95 and 83%, respectively) whereas the efficacy at 24 h was comparable. It is noteworthy to mention that BR displayed a greater potency in inhibiting both HSV-1 and EV71 infectivity at 24 h than at 48 h from the infection.

Protocol 2

When HSV-1 and EV71 were pre-incubated with BR (1–10 μM) alone or complexed with HSA (20 μM) for 2 h and then transferred

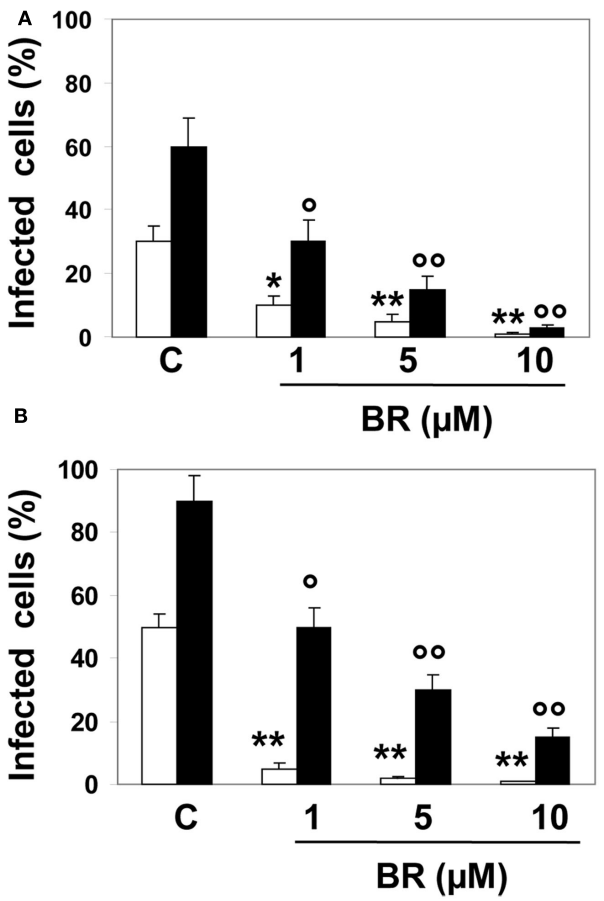

FIGURE 1 | Effect of bilirubin (BR) on herpes simplex virus type 1 (HSV-1) and enterovirus EV71 replication. Hep-2 **(A)** and Vero **(B)** cells were pre-incubated with BR (1–10 μM) for 2 h. At the end of incubation, BR was removed and Hep-2 and Vero cells infected with HSV-1 and EV71, respectively, both at the titer 1×10^5 PFU. After 2 h, the inocula were removed and the cells incubated in culture medium for 24 (white columns) or 48 h (black columns). Data are expressed as the percentage of infected cells, mean ± SEM of six individual samples per group. $^*P < 0.05$ and $^{**}P < 0.01$ versus control (C) after 24 h from infection; $^\circ P < 0.05$ and $^{\circ\circ}P < 0.01$ versus control after 48 h from infection.

onto Hep-2 and Vero monolayers, the bile pigment significantly inhibited viral infectivity (**Figure 2**). In particular, BR alone exhibited lower efficacy against HSV-1 than EV71 at 24 h from infection (mean maximal inhibitory activity 98 versus 82%, respectively) whereas at 48 h the efficacy was comparable. Furthermore, the complexation with HSA did not alter the efficacy of BR against HSV-1 and EV71 at shorter time from infection whereas reduced the efficacy after 48 h (mean maximal inhibitory activity 54 versus 82%, respectively). Moreover, the complexation with HSA reduced the potency of BR to block HSV-1 replication after 24 h from infection.

Protocol 3

In Hep-2 and Vero cells treated with BR (1–10 μM) alone or complexed with HSA (20 μM) and concomitantly infected with HSV-1 and EV71, the linear tetrapyrrole significantly modified viral infectivity (**Figure 3**). In details, BR alone showed a comparable efficacy against HSV-1 and EV71 at both 24 and 48 h. The complexation with HSA only partially confirmed this trend, since BR exhibited similar efficacy against HSV-1 and EV71 at 24 h, but a lower efficacy at 48 h of incubation (mean maximal inhibitory activity 62 and 82%, respectively). Similarly to Protocol 2, the complexation with HSA reduced the potency of BR to block HSV-1 replication after 24 h from infection.

Protocol 4

When given after viral infection, BR (1–10 μM) significantly reduced HSV-1 virulence only within the first 24 h from the infection, whereas after 48 h only the higher concentration was

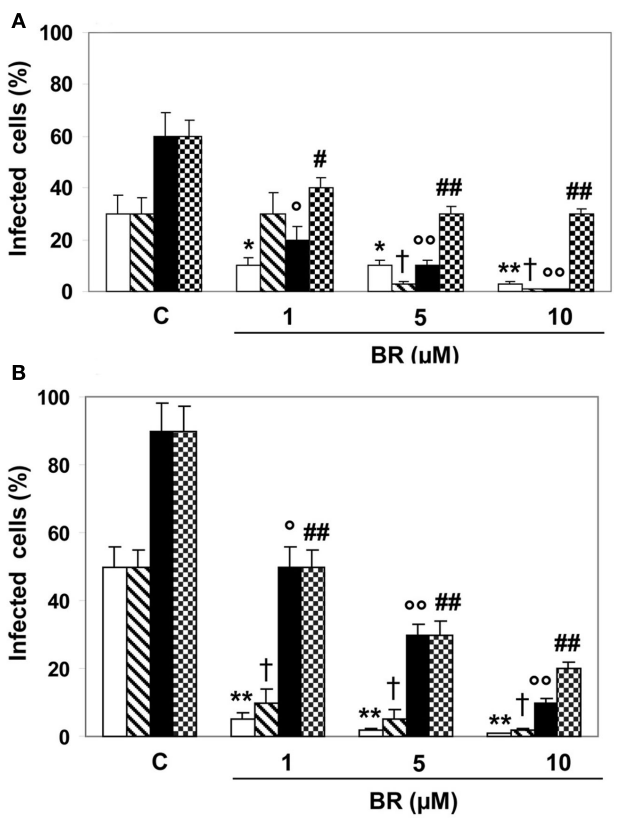

FIGURE 2 | Direct effect of bilirubin (BR) and albumin-bound bilirubin (BR–HSA) on herpes simplex virus type 1 (HSV-1) and enterovirus EV71 and how this affects viral replication. Both HSV-1 and EV71 (1×10^5 PFU) were pre-incubated with BR (1–10 μM) alone or complexed with saturating HSA (20 μM) for 1 h and then transferred to Hep-2 **(A)** and Vero **(B)** cell monolayers for the infection. After 2 h, the inocula were removed and the cells incubated in culture medium for 24 h (white and dashed columns) or 48 h (black and dotted columns). Both dashed and dotted columns refer to HSA treatment as described in the Section "Materials and Methods." Data are expressed as the percentage of infected cells, mean ± SEM of six individual samples per group. $^{*}P < 0.05$ versus control (C) after 24 h from infection; ** or $^{\dagger}P < 0.01$ versus respective controls after 24 h from infection; ° or $^{\#}P < 0.05$ and °° or $^{\#\#}P < 0.01$ versus respective controls after 48 h from infection.

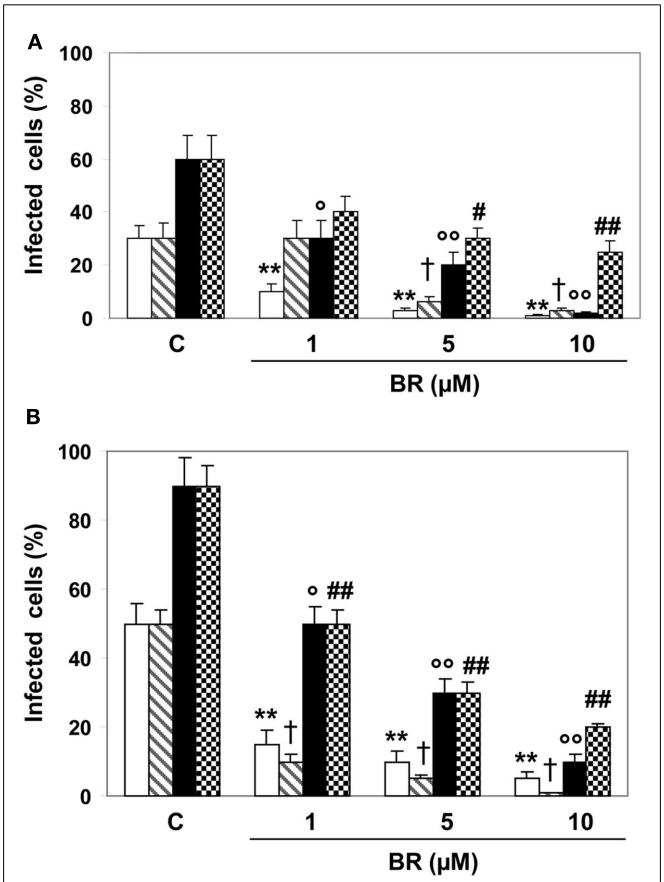

FIGURE 3 | Effect of bilirubin (BR) and albumin-bound bilirubin (BR–HSA) given concurrently with herpes simplex virus type 1 (HSV-1) and enterovirus EV71 and how this affects viral replication. Hep-2 **(A)** and Vero **(B)** cells were exposed to BR (1–10 μM) alone or complexed with saturating HSA (20 μM) and concomitantly infected with HSV-1 and EV71 (1×10^5 PFU) for 2 h. At the end of incubation the inocula were removed and the cells incubated in culture medium for 24 (white and dashed columns) or 48 h (black and dotted columns). Both dashed and dotted columns refer to HSA treatment as described in the Section "Materials and Methods." Data are expressed as the percentage of infected cells, mean ± SEM of six individual samples per group. $^{*}P < 0.05$ versus control (C) after 24 h from infection; ** or $^{\dagger}P < 0.01$ versus respective controls after 24 h from infection; ° or $^{\#}P < 0.05$ and °° or $^{\#\#}P < 0.01$ versus respective controls after 48 h from infection.

active (**Figure 4**). Conversely, BR maintained its antiviral action against EV71 at both 24 and 48 h from the infection (**Figure 4**).

INTRACELLULAR MECHANISMS THROUGH WHICH BR COULD EXERT ITS ANTIVIRAL ACTIVITY

Bilirubin-IX-alpha (1–10 μM) stimulated the phosphorylation of the member of the MAPK family JNK in Hep-2 and Vero cell lines (**Figure 5A** and data not shown), but a positive correlation between BR concentrations and JNK phosphorylation was not found. The JNK inhibitor SP600125 (10 μM) partially reversed the BR (10 μM)-induced block of HSV-1 and EV71 replication in Hep-2 and Vero cells at both 24 and 48 h from infection (protocols 1 and 3), but this result only approached statistical significance (data not shown).

In addition, BR (10 μM) increased the production of NO in Vero cells in a time-dependent manner reaching statistical significance as early as 2 h after the administration (**Figure 5B**). The involvement of BR-dependent generation of NO in the inhibition of viral replication was confirmed in specific experiments performed by incubating Hep-2 and Vero cells with L-NMMA (1 mM) 1 h before and then in the presence of BR (10 μM, 2 h) as described in protocol 1. These experiments demonstrated that the inhibition of NOS activity reversed the BR-induced block of viral replication in HSV-1 and EV71 after 24 h and 48 h from infection (**Figure 6**). A similar degree of inhibition of viral replication was found when HSV-1 and Vero cells were pre-incubated with L-NMMA for 1 h and then exposed simultaneously to BR alone or complexed with HSA plus HSV-1 or EV71 according to protocol 3 (data not shown). This effect of BR was comparable at both 24 and 48 h from infection (data not shown).

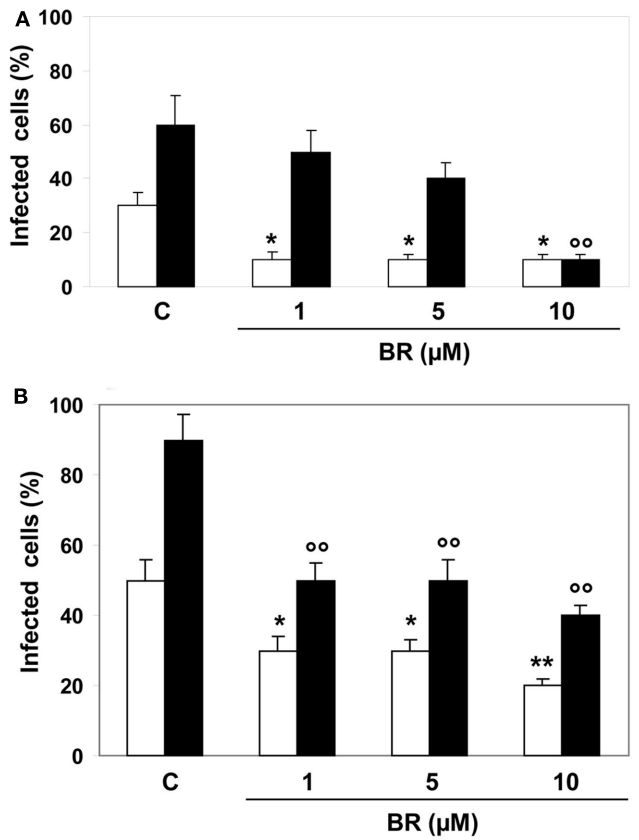

FIGURE 4 | Effects of bilirubin (BR) on cell monolayers infected with herpes simplex virus type 1 (HSV-1) and enterovirus EV71. After 2 h of infection with HSV-1 and enterovirus (1×10^5 PFU), Hep-2 **(A)** and Vero **(B)** cell monolayers were treated with BR (1–10 μM) for further 2 h. At the end of incubation the inocula were removed and the cells incubated in culture medium for 24 (white columns) or 48 h (black columns). Data are expressed as the percentage of infected cells, mean ± SEM of six individual samples per group. $^*P < 0.05$ and $^{**}P < 0.01$ versus control (C) after 24 h from infection; $^{°°}P < 0.01$ versus control after 48 h from infection.

DISCUSSION

Over the last years, several studies reported on the microbicidal activity of HO-1 and its by-products. Biliverdin-IX-alpha was shown to inhibit the replication of HCV, HHV-6, and HIV (Mori et al., 1991; Nakagami et al., 1992; Lehmann et al., 2010; Zhu et al., 2010), CO reduced the growth of *E. coli*, *P. aeruginosa*, and *S. aureus* (Nobre et al., 2007, 2009; Davidge et al., 2009; Desmard et al., 2012) and iron almost completely decreased HCV core mRNA and protein (Hou et al., 2009). The results shown in this paper provided original evidence about the antiviral role of BR, the formation of which requires the concerted action of both HO and BVR,

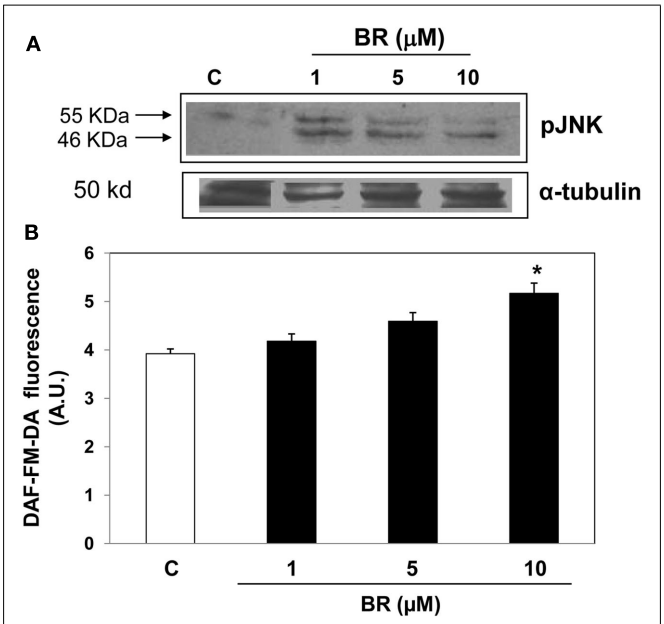

FIGURE 5 | Effect of bilirubin (BR) on cellular expression of the c-Jun N-terminal kinase (JNK) and nitric oxide (NO) production. Hep-2 and Vero cells were pre-incubated with BR (1–10 μM) for 2 h. At the end of incubation, BR was removed, the Hep-2 cells lysed and assayed for JNK phosphorylation by Western Blot **(A)** as described under the Section "Materials and Methods." α-tubulin was used as housekeeping gene to verify protein equal loading. On the contrary, Vero cells were assayed for NO production by using the fluorescent probe DAF-FM-diacetate [DAF-FM-DA, **(B)**] as described under the Section "Materials and Methods." **(A)** Shows a representative gel. In **(B)**, data are expressed as DAF-FM-DA fluorescence arbitrary units (A.U), mean ± SEM of six individual samples per group. $^*P < 0.05$ versus control (C).

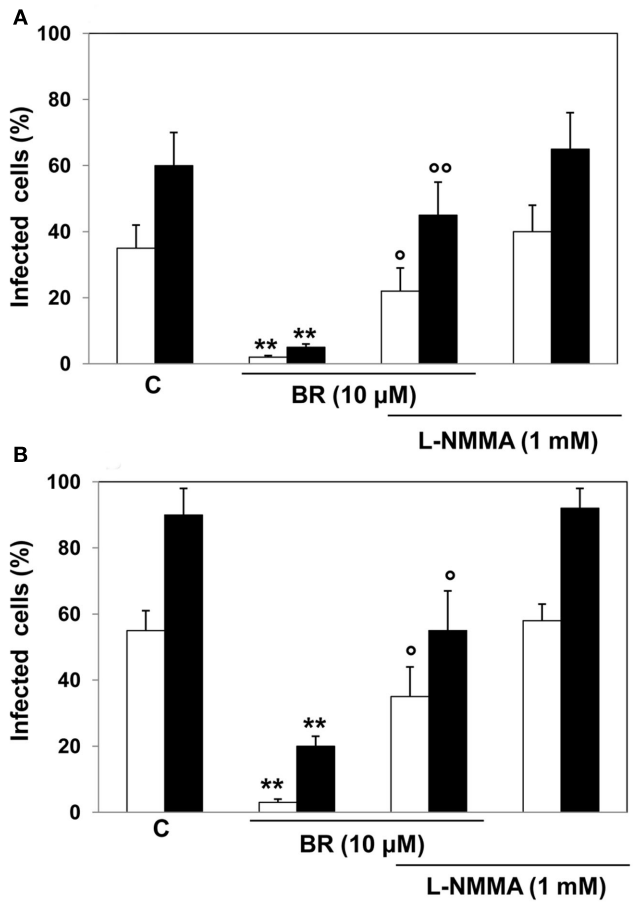

FIGURE 6 | Effect of bilirubin (BR) plus the nitric oxide synthase inhibitor L-NG-monomethyl arginine citrate (L-NMMA) on herpes simplex virus type 1 (HSV-1) and enterovirus EV71 replication. Hep-2 **(A)** and Vero **(B)** cells were pre-incubated with 1 mM L-NMMA for 1 h and then with L-NMMA plus 10 μM BR for further 2 h. At the end of incubation, the medium containing L-NMMA plus BR was removed and Hep-2 and Vero cells infected with HSV-1 and EV71 (1×10^5 PFU), respectively. After 2 h, the inocula were removed and the cells incubated in culture medium for 24 (white columns) or 48 h (black columns). Data are expressed as the percentage of infected cells, mean ± SEM of six individual samples per group. $^{**}P < 0.01$ versus respective controls (C) and $^{\circ}P < 0.05$ and $^{\circ\circ}P < 0.01$ versus 10 μM BR after 24 h (white columns) and 48 h (black columns) from viral infection.

thus expanding the cytoprotective role of this linear tetrapyrrole. However, in order to appreciate the novelty of our study, some considerations about the pathophysiology of the HO/BVR system are needed.

Although HO-1 is considered ubiquitous, this isoform is abundantly expressed in liver, spleen, heart, kidney, and selected brain regions and its activity increases greatly under stressful conditions (Maines, 1997). On the contrary, the constitutive HO-2 isoform prevails in neurons and testicular germ cells (Maines, 1997). Almost all organs retain BVR activity which transforms BV into BR (Maines, 1997, 2005). That said, it is plausible to wonder whether or not the antiviral activity of BV against HHV-6, HIV, or HCV (Mori et al., 1991; Nakagami et al., 1992; Lehmann et al., 2010; Zhu et al., 2010) should be attributable to its conversion into BR by the MT-4 or Huh cells used in those studies rather than a distinct effect of BV. The lack of inactivation of HHV-6 virions by BV and the consequent reduced antiviral effect lend support to this hypothesis. Another concern raised from the studies by Nakagami et al. (1992) and Mori et al. (1991) is related to the concentrations of BV used to inhibit HHV-6 and HIV replication. In these studies the antiviral effect of BV was observed at concentrations ranging from 10 to 66.7 μg/ml, equivalent to ∼17 and 113 μM, respectively, two values much higher than those expected to be produced by the HO activity in humans (Coburn, 1970). These potential biases were overcome in our study which demonstrated a direct antiviral effect for BR, the stable end product of heme metabolism, at concentrations from 1 to 10 μM which are very close to those found in human plasma (Berk et al., 1969; Bloomer et al., 1971) and about 1 order of magnitude lower than those previously used to demonstrate the antiviral effect of BV. From the analysis of the pharmacodynamic data, it emerges a different sensitivity of HSV-1 and EV71 to both free and albumin-bound BR under the various experimental conditions. A careful comparative evaluation of the mechanisms which underlie the ability of BR to inhibit HSV-1 and EV71 infectivity under the conditions which characterized Protocols 1–4, is out of the scope of this paper. However, it is important to highlight that, in this study, both BR and BR–HSA significantly inhibited the infectivity of both HSV-1 and EV71 in all the experimental settings well within the range of physiologic concentrations. A preliminary conclusion drawn from our results was that BR was much more efficacious to inhibit viral infectivity after 24 h from infection and, in particular, when the bile pigment was complexed to HSA.

The results shown in this study suggest that BR reduce viral replication by acting at several stages. The ability of BR to decrease HSV-1 and EV71 infectivity if pre-incubated with Hep-2 and Vero cells (**Figure 1**) resembles the pathophysiologic condition in which a virus infects cells endowed with high concentration of BR (e.g., hepatocytes, neurons). This finding is clinically relevant because nervous system and liver are both target organs for HSV-1 and enterovirus. Taking into consideration that there is not a direct clinical evidence of a reduced incidence of viral diseases in subjects affected by conditions characterized by unconjugated hyperbilirubinemia, such as Gilbert's syndrome (Hirschfield and Alexander, 2006), we hypothesize a protective role for BR in viral diseases in analogy with the well known protective effect of the bile pigment against respiratory diseases and cardiovascular accidents (Vitek et al., 2002; Horsfall et al., 2011). With regard to the inhibition of viral infectivity by both BR and BR–HSA pre-incubated directly with virions (**Figure 2**), it recalls the pathophysiologic condition in which a virus comes in contact with BR or BR–HSA in the blood, as during the viremic phase, and then infects tissues. Similarly, both BR and BR–HSA reduced viral replication if given cells concomitantly with HSV-1 and EV71 (**Figure 3**) and this mimics the pathophysiologic condition in which a virus comes in contact with BR/BR–HSA and cells at the blood/tissue interface. However, it is well known that both herpesviruses and enteroviruses have a short viremic phase since they colonize target organs very fast and, therefore, the potential clinical importance of both these mechanisms is limited to severe diseases characterized by long-lasting veremic

phase such as in immunocompromised patients (Stanberry et al., 1994). Finally, BR was efficacious in reducing viral infection even when administered to Hep-2 and Vero cells already infected by HSV-1 and EV71, respectively (**Figure 4**). This experimental condition reminds of the clinical situation in which jaundice is secondary to a viral infection, such as hepatitis. As a matter of fact, in viral hepatitis unconjugated BR increases when hepatic function decreases under a critical threshold and it is very often accompanied by hypoalbuminemia and hypofibrinogenemia and these alterations represent a life-threatening condition. The possible protective effect of BR at this stage is, therefore, unlikely.

Overall speaking, the mechanisms involved in the antiviral activity of BR within the cell or at the interface blood/tissue could be related either to the stimulation of intracellular pro-survival pathways, such as the MAPK system, or the production of microbicidal molecules such as NO. The BR-induced stimulation of NO release in Vero cells (**Figure 5**) should be considered as one of the main mechanisms involved in the antiviral effect of the linear tetrapyrrole against both HSV-1 and EV71. The importance of NO to decrease HSV-1 and EV71 infectivity was also corroborated by the evidence that the blockade of NOS, by the inhibitor L-NMMA, reverted the reduction of viral infectivity due to 10 μM BR (**Figure 6**). This result is not surprising and is in good agreement with previous studies which demonstrated the virucidal effect of NO (Croen, 1993; Akaike and Maeda, 2000). The JNK signaling cascade is also a crucial effector of the antiviral immune response in influenza virus (Hrincius et al., 2010), but there are no data in favor of a cytoprotective effect against HSV-1 or EV71. The lack of a positive correlation between BR concentrations and JNK phosphorylation (**Figure 5A**, in particular the 55-KDa isoform) could be explained keeping in mind that the bile pigment was used within the range 1–10 μM. It is interesting to recall that higher concentrations of BR (50 μM) were necessary to clearly stimulate the phosphorylation of MAPK members including JNK (Fernandes et al., 2007). In addition, the lack of any significant effect of the JNK inhibitor SP600125 (data not shown) on HSV-1 and EV71 infectivity lends support to the hypothesis that this pathway is not so efficacious to prevent viral infection as NO production. The parallel antiviral activity of BR against HSV-1 and EV71, characterized by important differences in terms of virion structure, a DNA-containing core surrounded by an envelope in HSV-1 and a RNA-containing core within a capsid without envelope in EV71, suggests that BR acts directly on the virion particles or on nucleic acids. In this light, some studies reported on a toxic effect of BR, in the presence of transition metals, on nucleic acids (Asad et al., 1999). Furthermore, the lack of direct antiviral activity of BV on HHV-6 and HSV-1 (Nakagami et al., 1992) *vis-à-vis* with the efficient antiviral effect of BR against HSV-1 and EV71, implies an important role of the C-10, in analogy with previous results. The absence of double bonds at the γ-bridge (which includes C-10) stabilizes the structure of BR and increases its liposolubility with respect to BV. Moreover, the C-10 can undergo redox modifications under conditions of oxidative stress (Stocker et al., 1987a; Baranano et al., 2002). According to this hypothesis, free radicals could oxidize protein-bound BR at the C-10 generating BV which, in turn, is quickly reduced back to BR by the ubiquitous BVR (Baranano et al., 2002). However, this hypothesis is still debated in the scientific community (Maghzal et al., 2009), but it cannot be excluded to explain the antiviral effect of BR toward both HSV-1 and EV71.

In conclusion, the results in this paper provide the first evidence, to our knowledge, of a virucidal effect of BR against HSV-1 and EV71. The implication of these results for the development of new antiviral drugs designed to modulate the HO/BVR system are profound and research on these subjects is ongoing in our laboratories.

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Biliverdin reductase: more than a namesake – the reductase, its peptide fragments, and biliverdin regulate activity of the three classes of protein kinase C

Peter E. M. Gibbs[1], Cicerone Tudor[2] and Mahin. D. Maines[1]*

[1] *Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA*
[2] *Max Planck Institute for Immunobiology and Epigenetics, Freiburg, Germany*

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel
Cesare Mancuso, Catholic University School of Medicine, Italy

****Correspondence:***
Mahin. D. Maines, Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14624, USA.
e-mail: mahin_maines@urmc.rochester.edu

The expanse of human biliverdin reductase (hBVR) functions in the cells is arguably unmatched by any single protein. hBVR is a Ser/Thr/Tyr-kinase, a scaffold protein, a transcription factor, and an intracellular transporter of gene regulators. hBVR is an upstream activator of the insulin/IGF-1 signaling pathway and of protein kinase C (PKC) kinases in the two major arms of the pathway. In addition, it is the sole means for generating the antioxidant bilirubin-IXα. hBVR is essential for activation of ERK1/2 kinases by upstream MAPKK-MEK and by PKCδ, as well as the nuclear import and export of ERK1/2. Small fragments of hBVR are potent activators and inhibitors of the ERK kinases and PKCs: as such, they suggest the potential application of BVR-based technology in therapeutic settings. Presently, we have reviewed the function of hBVR in cell signaling with an emphasis on regulation of PKCδ activity.

Keywords: biliverdin reductase, protein kinase C, signaling pathways, peptides, biliverdin

STRUCTURE OF HUMAN BILIVERDIN REDUCTASE AND ITS FUNCTIONS IN CELL SIGNALING PATHWAYS

Biliverdin reductase (BVR), purified to homogeneity from rat liver, was characterized as an enzyme that was capable of reducing biliverdin-IXα to bilirubin-IXα and was shown to exhibit a unique, dual cofactor/pH optimum profile (Kutty and Maines, 1981). In the course of elucidating the underlying mechanism for this unprecedented activity profile, an extensive array of functions of the human BVR (hBVR) protein was uncovered (Kapitulnik and Maines, 2009; Gibbs et al., 2012). Examination of the sequence has revealed the presence of three domains, as illustrated in **Figure 1A**. The N-terminal domain contains the catalytic site, while the extreme C-terminus is cysteine-rich and binds metal ions, notably Zn^{2+}. The remainder of the molecule, as discussed below, harbors numerous regulatory motifs. An early observation made with the rat enzyme was that BVR was extensively modified posttranslationally (Huang et al., 1989), and it was subsequently demonstrated that this was a consequence of phosphorylation (Salim et al., 2001). The reductase activity of BVR required that the protein be phosphorylated. Rat or human BVR purified after expression in *E. coli* was found to be phosphorylated. Because *E. coli* has minimal Ser/Thr- or Tyr-kinase activity, the likely source of post-translational modification was autophosphorylation, indicating that BVR is a protein kinase. This kinase activity was characterized further using the human enzyme, and it was shown that BVR is a rare, soluble dual-specificity (Ser/Thr/Tyr) kinase (Lerner-Marmarosh et al., 2005). In its capacity as a Tyr-kinase, hBVR was shown to phosphorylate the insulin receptor substrate, effectively mimicking the insulin receptor kinase (IRK; Lerner-Marmarosh et al., 2005). This phosphorylation event initiates the down-regulation of glucose uptake (Tanti et al., 1994). hBVR itself was a substrate for tyrosine phosphorylation by IRK.

The extensive array of motifs in hBVR is illustrated further in **Figure 1B**. Each of the sequences shown in the figure was proposed as a functional site in the molecule, and has subsequently been demonstrated to be active. Sequence motifs in the C-terminal regulatory domain of BVR are, to a great extent, involved in interaction with other proteins. As an example, hBVR was found to bind AP-1 sites in DNA by means of its bZip motif (Ahmad et al., 2002). The reaction of hBVR with DNA required two binding sequences in the DNA target, suggesting that hBVR binds as a dimer; site-directed mutagenesis of residues in the leucine zipper segment of the bZip domain prevented DNA binding, presumably by preventing hBVR dimerization. The bZip domain probably also allows heterodimer formation with other bZip transcription factors (Ahmad et al., 2002; Kravets et al., 2004). Two motifs, the nuclear localization (NLS) and nuclear export (NES) sequences presumably allow hBVR to interact with components of the nuclear pore. Critically for signaling activity, hBVR also interacts with several protein kinases; this interaction aids in the activation of these kinases, as well as playing a role in their translocation to the site of activity. The experiments used to characterize these sequences relied on site-directed mutagenesis and siRNA studies to disrupt each function (Ahmad et al., 2002; Lerner-Marmarosh et al., 2007, 2008; Maines, 2007; Tudor et al., 2008; Miralem et al., 2010; Gibbs et al., 2012). More recent studies have focused on the use of peptides based on the hBVR sequence as a means of disrupting protein: protein interactions, and/or modulating the enzyme activities of hBVR or its binding partners. These experiments will be discussed below. We have

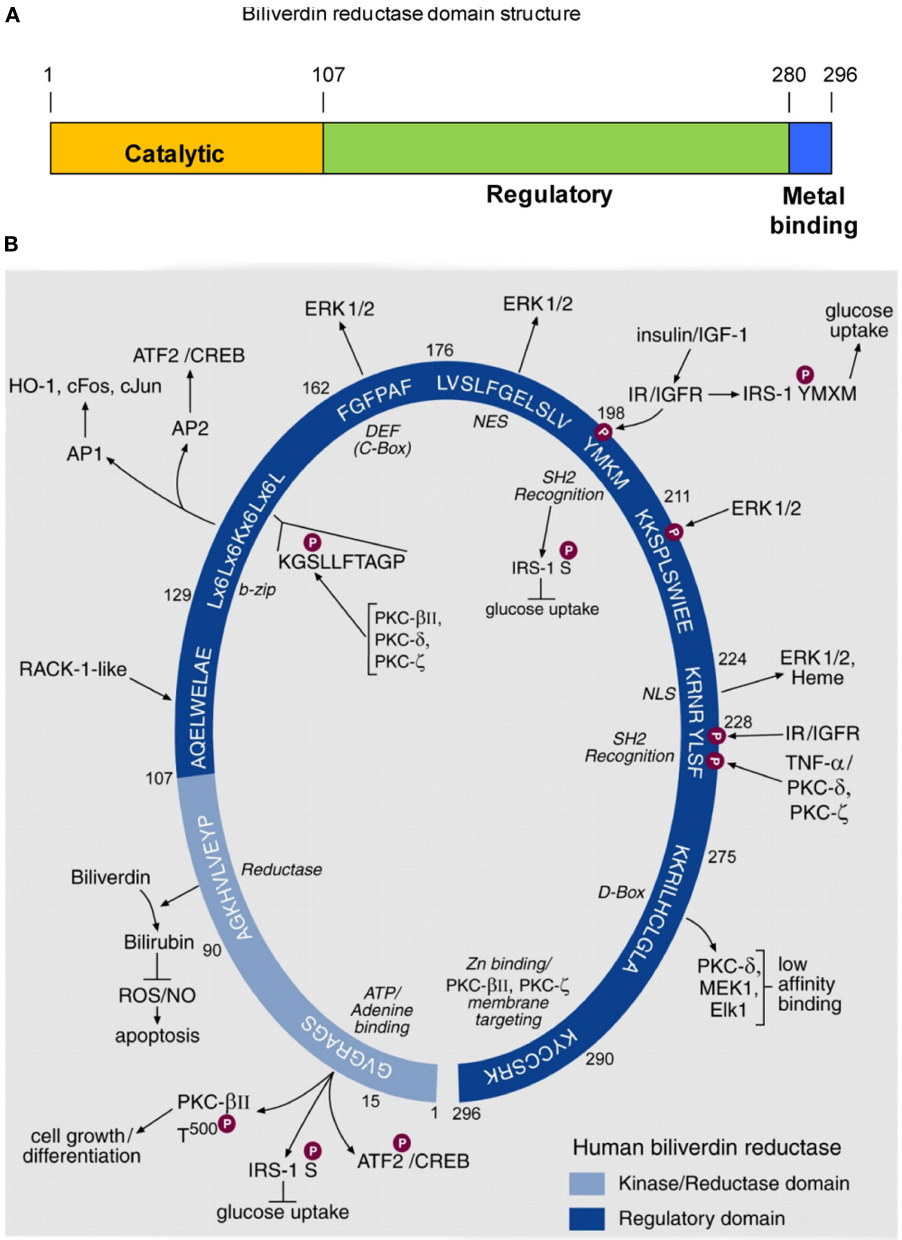

FIGURE 1 | Domain structure of hBVR, and its functional specific sequence motifs. (A) Three domains of hBVR. The N-terminal domain includes the sequences required for catalytic activity. The regulatory domain comprises residues beyond position 107, and there is some overlap with the C-terminal metal-binding sequence. **(B)** Schematic presentation of consensus sequences of hBVR for which functions have been ascribed. The numbers indicated for each consensus sequences are those of the hBVR primary structure. The N-terminal segment of 99 residues is the catalytic domain of hBVR; it houses a sequence of four valines followed by the consensus for the ATP/adenine ring-binding site. The kinase activity of hBVR is responsible for its autophosphorylation (Salim et al., 2001; Lerner-Marmarosh et al., 2005). hBVR is a kinase for serine phosphorylation of IRS-1, the phosphorylation of which halts glucose uptake (Tanti et al., 1994). hBVR is also a likely kinase for T^{500} in the activating loop of PKCβII (Maines et al., 2007); the PKC is a key component of cell growth and differentiation. The reductase domain catalyzes reduction of biliverdin to bilirubin, a component of cellular defense mechanisms protecting against ROS (Sedlak et al., 2009) and apoptosis (Miralem et al., 2005). The sequences starting at aa 107 and 211 closely resemble sites in the primary sequence of repeats V (QAMLWDLNE) and VI (SIKIWDLE) of the Receptor for Activated C-Kinase-1 (RACK1). RACK1 is a 36-kDa protein that is similar in size to hBVR (Ron et al., 1994). Activation of PKCs, including the β, δ and ε isoforms (Ron et al., 1994), is associated with conformational change that exposes their RACK-binding sites. We predict that the presence of RACK1-like sequences in hBVR may allow its binding to PKCs. The binding would not require kinase activity of hBVR. The bZip motif binds to 7 and 8 bp AP-1 and AP-2 sites. Stress response genes are activated by AP-1, and cAMP-responsive genes are regulated by AP-2 regulatory elements. hBVR regulates expression of stress-responsive HO-1, c-Fos, c-Jun, and ATF2/CREB (Kravets et al., 2004; Miralem et al., 2005; Maines et al., 2007). Within this sequence is a motif that strongly resembles a conserved protein kinase motif (Hanks and Hunter, 1995). The high affinity ERK binding site, known either as C-Box or DEF (Jacobs et al., 1999), is the site of interaction of ERK1/2 and hBVR, positioning ERK in proximity to its

(Continued)

FIGURE 1 | Continued
kinase (Lerner-Marmarosh et al., 2008). Nuclear localization of hBVR is also critical for transport of the transcriptional regulators ERK1/2 and heme into the nucleus (Lerner-Marmarosh et al., 2008; Tudor et al., 2008). Reentry of ERK into the cytoplasm requires the intact hBVR NES (Lerner-Marmarosh et al., 2008). hBVR is directly phosphorylated by IRK upon activation by insulin or IGF-1 (Lerner-Marmarosh et al., 2005). The tyrosine in the SH2 recognition motif of hBVR, as with other SH2 recognition motif-containing proteins, is predicted to form a platform for formation of signaling complexes (Pawson and Scott, 1997). hBVR is phosphorylated by ERK, and MotifScan predicts serine in the SP sequence as the phosphorylation target site of ERK1/2. A second SH2 recognition motif follows the nuclear localization signal and is involved in activation of PKCζ by TNF-α (Lerner-Marmarosh et al., 2007). The low affinity D-Box-like sequence is the binding site for kinases and substrates in the MAPK signaling cascade. The C-terminal six residues are the Zn-binding domain of hBVR (Maines et al., 1996). Based on the reported role of Zn for plasma membrane translocation of PKCs and nuclear translocation of NF-κB (Kabu et al., 2006) we predict that the function of hBVR in translocation of PKCs β and ζ to the cell membrane may involve its associated Zn. Notably, hBVR under resting conditions is found in the cytoplasm and membrane caveolae (Kim et al., 2004). The C-terminal lysine 296 is critical for hBVR's catalytic activity (unpublished); although it lies in a disordered region of the BVR molecule (Whitby et al., 2002), this does not preclude a catalytic function. The figure is adapted from a previously published version (Gibbs et al., 2012), and description is taken directly from the same publication.

examined in some detail the role played by hBVR in modulating the activity of five different kinases: three members of the protein kinase C family (PKCβII, PKCζ, and PKCδ, the extracellular signal responsive kinase 2 (ERK2) and the highly atypical but clinically relevant Goodpasture antigen binding protein (GPBP). The function of BVR differs somewhat for each of these kinases.

REGULATION OF PROTEIN KINASE C ENZYME ACTIVITIES BY hBVR

Studies in our laboratory have indicated that hBVR activates members of the three protein kinase C (PKC) classes, and is in turn, activated by the PKCs. It is apparent, however, that the mechanism by which activation occurs differs according to the class of PKC associating with hBVR. A brief description of the structure and activation of the PKCs is presented, followed by discussion of the function of hBVR in the regulation of each class.

STRUCTURE AND MATURATION OF PKC ENZYMES

The PKC family of signaling proteins functions at the intersection of a wide variety of signal transduction pathways, and the kinases are therefore considered as key regulators of responses to extracellular stimuli. The PKC family consists of three subgroups, i.e., the conventional, novel, and atypical PKCs (cPKC, nPKC, aPKC, respectively), which are characterized by differences in protein structure, mechanism of activation and function.

The cPKCs, nPKCs, and aPKCs have homologous catalytic kinase domains that encompass the C-terminal half of the molecules (**Figure 2**; Steinberg, 2008; Newton, 2010). The primary structure of this domain is well conserved between the proteins, and it includes essential sequence motifs that are common to all members as well as being required for maturation of the kinase. These motifs include the activation loop, which contains a threonine residue that generally must be phosphorylated for catalytic activity; in the case of PKCδ, phosphorylation of this site is

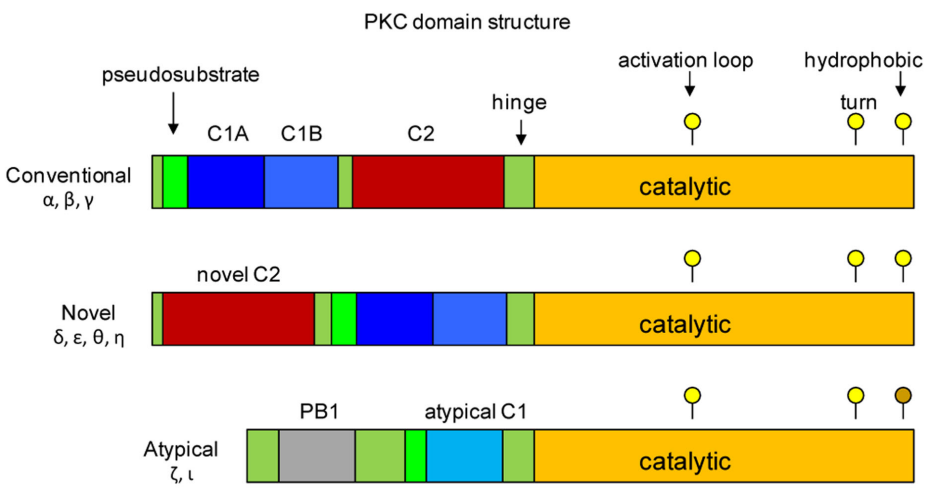

FIGURE 2 | Structural domains of protein kinase C. The three classes of PKCs are shown. Members of each class have a catalytic domain that encompasses the C-terminus of the protein (shown in orange). Positions of residues in the catalytic domain that are phosphorylated during activation of the kinases are shown as yellow circles above each map. The threonine residue in the activation loop is the first to be phosphorylated, which allows phosphorylation of the threonine/serine in the turn motif and the serine/threonine in the hydrophobic motif, resulting in full activity of the kinase. There is no phosphorylation target in the atypical kinase hydrophobic motif; negative charge is supplied by the glutamic acid residue (indicated by the brown circle). The regulatory domains are located in the N-terminal half, and consist of C1 (blue), C2 (red) and pseudosubstrate domains (green). The positions of the C1 and C2 domains of conventional PKCs are reversed in the novel PKCs. Atypical PKCs lack a C2 domain, and have only a partial C1 domain. Redrawn from Steinberg (2008), Newton (2010).

observed, but it is not essential. Phosphorylation of the activation loop threonine is otherwise the essential first step in maturation of the newly synthesized protein. All members have both a turn motif and a C-terminal hydrophobic motif; each motif contains serine or threonine targets for autophosphorylation, a requirement for full maturation of the enzyme. One exception is noted for the atypical kinases, which have glutamic acid, an amino acid known to mimic phosphoSer/Thr, at the hydrophobic motif phosphoacceptor site. Phosphate incorporated during the maturation process remains an integral part of the protein, with little or no turnover.

The PKC regulatory domains are located in the N-terminal half of the molecule, and it is these domains that differentiate the different classes from each other, and enable the PKCs to mount specific responses to different stimuli. Each PKC protein contains one or two conserved regulatory domains (C1, C2) and a pseudosubstrate sequence which is located N-terminal to the C1 domain. In the resting state, the pseudosubstrate is bound by the active site of the catalytic subunit, effectively preventing substrate binding. The C2 domain is found only in the conventional and novel PKCs – in the conventional PKCs, the C2 domain binds Ca^{2+}, which is critical for their activation. Ca^{2+} binding initiates a conformational change in the kinase exposing lipid biding sites in the C1 domain. The novel PKCs lack the Ca^{2+}-binding site, and are therefore Ca^{2+}-independent; however, they too must undergo a conformational change in response to stimuli that results in exposure of the C1 domain. The C1 region of cPKCs and nPKCs contains two sequences (C1A, C1B) that share a pattern of conserved His and Cys residues that coordinate Zn^{2+}. These regions serve as binding sites for membrane lipids, notably phosphatidyl serine and diacylglycerol (DAG), which is a product of phospholipase C activity. Interaction with the plasma membrane is a critical step in the activation of the conventional and novel PKCs; for the cPKCs the C1 domain is inserted into the membrane to bind DAG. In resting cells the lipid binding sites of the cPKC C1 domain are masked by the C2 domain – binding of Ca^{2+} triggers a conformational change that unblocks the C1 domain. The atypical kinases have only a single C1-type sequence that binds phosphatidylinositoltrisphosphate or ceramide, but not DAG.

REGULATION OF INDIVIDUAL PKC ENZYME ACTIVITIES BY hBVR

Protein kinase C-βII

The conventional PKCβII phosphorylates hBVR *in vitro*, under conditions that are unfavorable to hBVR kinase activity (and hence autophosphorylation). The presence of hBVR in kinase reactions increased the Vmax of the PKC without affecting the Km for its substrate. However, both wild-type and kinase-inactive PKC could serve as substrates for the hBVR kinase activity. Peptides, based on the three motifs of PKCβII that are required for maturation of the kinase, were used as hBVR kinase substrates and the data suggested that the hBVR phosphorylation target may be in the PKCβII activation loop (Maines et al., 2007). As noted above, phosphorylation at that site is the first step in maturation of PKCβII. Peptides that included the PKCβII turn and hydrophobic motif phosphorylation sites were not substrates for the hBVR kinase activity. These experiments do not preclude the possibility that hBVR phosphorylation of other sites in PKCβII could provide an additional activation mechanism.

Protein kinase C-βII and hBVR were found to co-immunoprecipitate from extracts of cells in which both proteins were over-expressed and stimulated with phorbol myristate acetate (PMA). Confocal microscopy of PMA-stimulated cells over-expressing PKCβII and hBVR indicated that both proteins colocalized to the cell membrane. Based on site-directed mutagenesis of its sequence, two regions of hBVR were required for the protein–protein interaction, notably the C-terminal cysteine-rich metal-binding sequence (**Figure 1B**) and an intact ATP binding site. hBVR was shown to be a Zn^{2+}-binding protein (Maines et al., 1996), and the hBVR metal-binding sequence closely resembles the Zn^{2+}-binding sequences in the PKC C1 domain. It is possible that hBVR may be binding to the C1 domain via a mechanism that involves the cysteine residues of both motifs and/or Zn^{2+} chelation. The hBVR Gly^{17} residue in the nucleotide binding site was essential for activation of PKCβII, as was the sequence of four consecutive valine residues ($Val^{11–14}$) located immediately upstream. The valines are unlikely to interact directly with PKCβII as they are buried within the molecule (Kikuchi et al., 2001; Whitby et al., 2002), but their mutation to alanine may alter or destabilize the BVR secondary structure so as to prevent PKCβII binding.

Activation of PKCβII by hBVR therefore occurs by two mechanisms – phosphorylation as a consequence of the hBVR kinase activity, and protein: protein interaction.

Protein kinase C-ζ

Human biliverdin reductase activates the atypical PKCζ, by a mechanism that clearly differs from that observed with PKCβII. PKCζ was able to use hBVR as a substrate *in vitro* but the PKC was not a substrate for the hBVR kinase activity (Lerner-Marmarosh et al., 2007). However, the presence of hBVR in PKCζ-catalyzed reactions stimulated the activity of the PKC toward its substrates and in autophosphorylation; in such reactions, stimulation of activity by kinase-inactive hBVR was at least as effective as that of the wild-type protein. Mutant hBVR proteins were used as substrates for *in vitro* kinase reactions to identify candidate phosphorylation sites. Mutation of either hBVR Ser^{149} in the S/T kinase motif (**Figure 1**) or Ser^{230} in one of the SH2-binding motifs to Ala yielded substrates that were phosphorylated to a significantly lesser extent than the wild-type protein. A protein having both of these mutations was a very poor substrate, incorporating less than 20% of the phosphate taken up by the wild-type protein. These data indicate that PKCζ phosphorylates hBVR at Ser^{149} and Ser^{230}.

In response to stimulation of cells with tumor necrosis factor-α (TNF-α), hBVR, and PKCζ formed a complex in the cell, as indicated by their co-immunoprecipitation and by confocal microscopy (Lerner-Marmarosh et al., 2007). Enhancement of PKCζ activity in the cell was observed if hBVR was over-expressed; conversely, ablation of hBVR from cells with siRNA significantly inhibited the activation of PKCζ in response to TNF-α. A scrambled version of the siRNA was ineffective in inhibiting PKCζ. The inhibition of the TNF-α response by siRNA was as effective as that seen by introducing a competitive inhibitor peptide containing the PKCζ pseudosubstrate sequence. The data indicated dependence of PKCζ activation, and hence downstream signaling events, on

hBVR. It is probable that binding of hBVR to PKCζ either initiates a conformational change to activate the protein, or binds to and preferentially stabilizes the active PKCζ conformation.

Protein kinase C-δ

Protein kinase C-δ is a member of the novel class of PKCs and, as with the other PKCs described above, is both activated by the presence of hBVR in kinase reactions and utilizes hBVR as a substrate (Gibbs et al., 2012). PKCδ was not a substrate for hBVR kinase activity, however, as enhanced PKC activity was observed with kinase-inactive hBVR mutant. A constitutively active mutant of PKCδ, lacking part of the pseudosubstrate sequence (Zhao et al., 1998) was also activated by hBVR (Lerner-Marmarosh et al., in preparation). The increased activity of PKCδ is dependent on formation of a complex with hBVR, and as described above for the other PKCs, a complex that included both hBVR and PKCδ could be immunoprecipitated from extracts of IGF-1-treated cells (Gibbs et al., 2012). More direct evidence for association of the proteins was provided by an *in vivo* assay, using FRET-FLIM and IGF-1-treated cells. To examine whether BVR–PKCδ interaction extended to other stimuli that activate hBVR and PKCδ, cells were instead treated with PMA. Cells transfected with PKCδ alone showed a fluorescence lifetime of 2.65 ± 0.09 ns (**Figures 3A,B**). However, when the cells are co-transfected with pDsRed2-hBVR, the PKCδ fluorescence lifetime was reduced to 2.31 ± 0.13 ns; the diminished lifetime is indicative of close juxtaposition of the hBVR and PKCδ proteins in the FRET detection system. In PMA-treated cells, the fluorescence lifetime is 2.39 ± 0.15 ns, again indicating association of the proteins in the cell. Based on these observations, it is likely that the binding between hBVR and the PKC triggers a conformational change in the latter that facilitates its activation.

ROLE OF hBVR IN ACTIVATION OF ERK1/2 BY MEK1/2 AND PKCδ

The ERK proteins are of fundamental importance in the regulation of cell proliferation and differentiation, and of the stress responses (Jackson and Foster, 2004; Boutros et al., 2008; Kim and Choi, 2010). ERK1/2 is known to activate some 50 transcription factors, including Elk and NF-κB (Ranganathan et al., 2006; Yazicioglu et al., 2007). hBVR was shown to be both an activator of extracellular receptor kinase 1/2 (ERK1/2) kinase activity, and a substrate for the kinase *in vitro* (Lerner-Marmarosh et al., 2008); this activation did not require hBVR kinase activity, as the kinase-inactive Val^{11-14}, Gly^{17} mutant was able to activate ERK1/2. In cells stimulated with IGF-1, the ERK proteins associate with hBVR in co-immunoprecipitation experiments. A central role for hBVR in ERK-mediated signal transduction was proposed based on hBVR serving as a scaffold in the formation of a series of complexes that included ERK proteins. These included a complex containing the MAPK kinase MEK1/2 (Lerner-Marmarosh et al., 2008), the upstream activator of ERK1/2. Association of hBVR and ERK1/2 enabled translocation of ERK1/2 into and out of the nucleus, a function dependent on the hBVR NLS and NES motifs. Mutation of the NLS resulted in reduced transport of ERK2 into the nucleus, whereas expression of the NES mutant in cells resulted in significant nuclear accumulation of ERK2. Whereas the Gab1 protein is a known carrier for import to the nucleus of ERK1/2, it does not

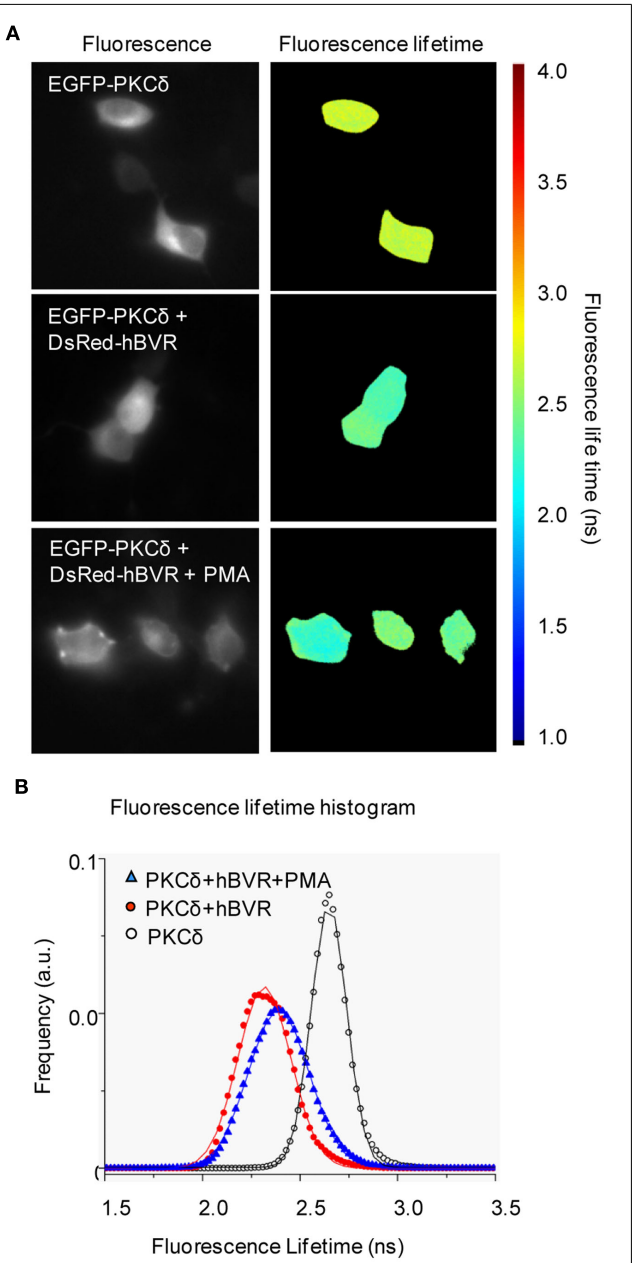

FIGURE 3 | FRET detection of association of BVR and PKCδ in living cells. (A) *FRET-FLIM images.* Representative fluorescence intensity and FLIM images of HeLa cells transfected with EGFP-PKCδ, EGFP-PKCδ + DsRed2-hBVR and EGFP-PKCδ + DsRed2-hBVR treated with PMA. The scale bar ranges from 1 to 4 ns. Frequency-domain FLIM (Schlachter et al., 2009) experiments were performed using a Zeiss Axiovert 200M inverted wide-field microscope and a Lambert Instruments Fluorescence lifetime Attachment (LIFA; Lambert Instruments, Roden, The Netherlands). FLIM images were taken in the presence or absence of 100 nM PMA added before image collection. Fluorescence lifetimes were calculated from several regions and included data from multiple cells. **(B)** Fluorescence lifetime data are represented as histograms for each of EGFP-PKCδ (○), EGFP-PKCδ + DsRed2-hBVR (●) and EGFP-PKCδ + DsRed2-hBVR + PMA (▲) transfected cells. Curves represent FLIM data recorded from ∼25 cells per condition; frequency of events is shown in arbitrary units (a.u.). Errors reported are one-half of the distribution width.

function to export the kinase (Osawa et al., 2004). On the other hand, hBVR functions as a bidirectional carrier protein for translocation of ERK1/2 into and out of the nucleus and, to date, it is the only protein known to fulfill this latter function. In the nucleus, hBVR brings ERK1/2 into proximity with the transcription factor Elk, enabling phosphorylation and activation of Elk. Ablation of hBVR with siRNA resulted in significantly reduced induction of ERK1/2 activation in response to IGF-1 treatment, and an attenuated response of Elk-dependent gene expression in response to IGF-1. The sites in hBVR required to form the complexes with ERK1/2 were determined by expressing mutant hBVR proteins in cells. Mutations in the ^{162}FGFPAF sequence, that resembles the high affinity C-Box binding motif found in ERK1/2 associated proteins (Jacobs et al., 1999) and in the low affinity ^{275}KKRILHCLGL D-Box-like sequence (Minden and Karin, 1997) prevented formation of the complex, and attenuated ERK activation and signaling in response to IGF-1.

Protein kinase C-δ is known to function in the ERK1/2 signaling pathway (Gorelik et al., 2007), and is believed to directly activate the ERK proteins. Examination of immunoprecipitates obtained from cells over-expressing both hBVR and PKCδ and stimulated with either IGF-1 or PMA revealed the presence of co-immunoprecipitated ERK2 (Gibbs et al., 2012). Formation of the ternary complex was required for downstream nuclear signaling mediated by ERK2. The activation of PKCδ by BVR in the absence of its kinase activity suggests that BVR may act as a scaffold to stabilize the active conformation of the PKC, while at the same time bringing the kinase into close association with its substrate. Site-directed mutagenesis implicated the hBVR C- and D-Boxes in the formation of the ternary complex. The C-Box was found to be critical for hBVR/ERK2 association, and the D-Box for hBVR/PKCδ interaction. These findings underscore the critical scaffolding function of hBVR as does the observation that siRNA-mediated reduction of hBVR protein in the cell resulted in a severe attenuation of PKCδ- and ERK2-dependent signaling, as determined from activation of Elk-dependent transcription. The siRNA-mediated inhibition was rescued by introduction of a plasmid to express hBVR into siRNA-treated cells. NF-κB is also activated in response to ERK2 (Ranganathan et al., 2006; Yazicioglu et al., 2007), and a transcription factor for expression of the HO-1 and iNOS genes. As with Elk, activation of the transcription factor, and expression of a downstream iNOS target, was shown to be blocked by siRNA treatment to ablate any of hBVR, PKCδ, ERK1/2, or MEK1/2. Restoration of protein expression by transfection with expression plasmids restored downstream NF-κB-dependent gene expression.

As described above, hBVR binds to ERK1/2 and to its upstream activating kinases MEK1/2 (Lerner-Marmarosh et al., 2008) and PKCδ (Gibbs et al., 2012). It was thought to be unlikely that two entirely distinct complexes, containing hBVR ERK2 and an upstream kinase would be formed in response to the same extracellular stimulus with IGF-1. Examination of the complex obtained by immunoprecipitation with anti-PKCδ antibodies revealed the presence of MEK1, in addition to the expected hBVR and ERK2 (Gibbs et al., 2012), suggesting the assembly of an elaborate, hBVR-anchored signal transduction complex. Because this function of hBVR is independent of its kinase activity, the role of hBVR in the complex is clearly that of a scaffold or bridge, and formation of the complex results in activation of both PKCδ and of ERK. Because unrestrained activation of the signaling complex is likely to be detrimental to the cell, it is noteworthy that the protein phosphatase, PP2A, that targets PKCδ (Srivastava et al., 2002), was also detected in the complex. This implies that the complex carries within it a means of self-regulation. PKCδ activity is commonly considered in the context of oxidant-dependent, mitochondrial-linked apoptosis (Buder-Hoffmann et al., 2009). Additionally, however, the enzyme is associated with functions that include cell cycle progression and proliferation, differentiation and tumorigenesis (Steinberg, 2008; Newton, 2010). Since hBVR has been observed to provide cytoprotective functions during oxidative stress (Miralem et al., 2005), it is possible that the association of hBVR, PKCδ and ERK1/2 in a ternary complex may provide a mechanism to protect the PKC from cleavage to its constitutively active, pro-apoptotic form, thereby favoring those functions involved in cell survival.

BVR AND BILIVERDIN-MEDIATED CYTOPROTECTION

Our studies with protein kinases had led to our proposing two hypotheses. The first predicted that BVR would affect signaling via Toll-like receptors (TLRs) and NF-κB (Maines, 2005), and this would also involve the PKCs and MAPKs, including ERK. The second prediction was that, by virtue of its interaction with the insulin receptor (Lerner-Marmarosh et al., 2005) and role in activation of PKCβII (Maines et al., 2007), BVR would be an integral component of the PI3K/Akt signaling pathway (Maines, 2007).

It has been reported that biliverdin is a protective agent in preventing damage due to ischemia and reperfusion (I/R) in cardiac and renal transplantation (Nakao et al., 2005). It has also been reported that the cytoprotective effects of Heme Oxygenase-1 (HO-1) in I/R injury, both in rat cardiomyoblasts (H9c2 cells) and in the intact mouse heart are mediated by rat and mouse BVR (rBVR, mBVR), respectively, and involve the PI3K/Akt signaling pathway (Pachori et al., 2007). Specifically, activation of PI3K and its downstream effector Akt, as measured by phosphorylation, was increased in the presence of elevated HO-1. Inhibition of PI3K or knockdown of rBVR with siRNA attenuated the HO-1-induced phosphorylation of Akt. As described above for the PKCs, rBVR was found to co-immunoprecipitate with the phosphorylated p85 subunit of PI3K. In mouse macrophages, activation of the PI3K/Akt pathway was shown to be initiated by conversion of biliverdin to bilirubin by membrane-bound mBVR, which led to tyrosine phosphorylation of the mBVR C-terminal region (Wegiel et al., 2009), and allowed subsequent binding of mBVR to p85, activating PI3K and then Akt. The cytoprotective effect of biliverdin was negated by siRNA-induced ablation of mBVR.

Biliverdin was also observed to enable tolerance of cardiac allografts (Yamashita et al., 2004), by a means of inhibiting the activation of the proinflammatory transcription factors, nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB). These transcription factors both function in production of inflammatory cytokines, and stimulation of T cell proliferation. We also examined the role of biliverdin in modulating the activity of NF-κB (Gibbs and Maines, 2007), and observed an inhibition of transcription factor activation in response to stimulation of the cells with TNF-α. This inhibition could be overcome by over-expression of

BVR, resulting in activation of NF-κB. Examination of NF-κB binding to its target sequence *in vitro* indicated that biliverdin was not an inhibitor of NF-κB binding to DNA, suggesting that biliverdin functions by quenching an upstream signaling event (Gibbs and Maines, 2007). Biliverdin was also effective in down-regulating the activity of the human BVR promoter (Gibbs et al., 2010).

Other studies have indicated that the endothelial nitric oxide synthase (eNOS) was phosphorylated by the calcium/calmodulin-dependent kinase in response to endotoxin, and that this was mediated by biliverdin binding to mBVR. This results in S-nitrosylation of mBVR, followed by its translocation to the nucleus and binding to AP-1 sites in the Toll-like receptor 4 (TLR4) promoter. This binding down-regulated TLR4 expression and subsequent resolution of the inflammatory response (Wegiel et al., 2011). The *in vivo* protection against acute liver damage was found to be dependent on the availability of NO. Moreover, macrophages from eNOS knockout mice displayed neither nitrosylation of BVR nor its translocation to the nucleus. Further, the expression of TLR4 was not inhibited in these cells. Recent studies also have revealed that biliverdin is a potent inhibitor of PKCδ *in vitro*. The mechanism of inhibition is presently unclear, although it does not appear to involve covalent modification of the protein (Lerner-Marmarosh et al., in preparation). These observations revealed a second mechanism by which BVR might activate PKCδ; in addition to the scaffolding function described above, BVR catalyzes the metabolism of a potent kinase inhibitor.

FUNCTION OF BVR AND ITS PEPTIDES IN THE IMMUNE SYSTEM

Goodpasture syndrome (GPS) is characterized by an autoimmune attack against the C-terminal non-collagenous-1 (NC1) domain of the α3 chain of the type IV collagen of basement membrane [α3(IV)NC1; Goodpasture antigen, GPA], resulting in deposits of autoantibodies along alveolar and glomerular basement membranes, which leads to hemorrhage in the lungs and a rapidly progressive glomerulonephritis (Saus, 1998; Hudson et al., 2003). The NC1 domain initiates the assembly of two individual triple helical protomers into a quaternary structure known as the hexamer; correct folding of the protein in the hexamer conceals the epitope for the autoantibody. The mechanism for its immunological exposure remains unknown; however GPBP targets the α3(IV)NC1 domain and regulates basement membrane collagen organization (Quinones et al., 1992; Raya et al., 1999; Granero et al., 2005; Revert et al., 2007); GPBP phosphorylates GPA at its N-terminus (Raya et al., 1999). Alterations in protein phosphorylation may lead to the autoimmune response by adversely modifying protein processing, resulting in epitope peptide presentation (Litersky and Johnson, 1992; Brown et al., 1995).

The association of GPBP with hBVR suggested by the yeast two-hybrid experiment was confirmed in the cell by co-immunoprecipitation and GST pull-down experiments (Miralem et al., 2010). hBVR down-regulated the TNF-α-stimulated kinase activity of GPBP without decreasing the level of the protein. In the same study, hBVR was found to stimulate GPBP gene expression by TNF-α-activated NF-κB. Ablation of hBVR with siRNA prevented GPBP gene expression, and led to a decrease in the cellular level of GPBP mRNA. Using a series of constructs expressing truncated forms of hBVR enabled the interacting domain to be mapped to the $C^{281}X_{10}CC$ motif in the hBVR C-terminal 24 residues (**Figure 2**). These findings suggest a role for hBVR in auto-immunity, since hBVR may modulate the activity of a key protein in presentation of an autoantigen.

REGULATION OF KINASE ACTIVITY BY hBVR-BASED PEPTIDES

Several sequence motifs within hBVR (**Figure 1**) were identified as possible protein–protein interaction sites: the cysteine-containing ^{275}KKRILHC and ^{290}KYCCSRK in the C-terminal α-helix, and the two SH2-binding motifs (^{198}YMKM and ^{228}YLSF). As noted above, ^{162}FGFPAF closely resembles the core of the high affinity C-Box for binding of ERK (Jacobs et al., 1999). The KKRILHC sequence resembles the core of the D-Box (KKRILHCLGL), a lower affinity binding site for MAPKs (Minden and Karin, 1997). Three peptides were initially examined for their effect on hBVR activity. The peptide KYCCSRK activated hBVR kinase activity, whereas KKRILHC and KRNRYLSF were both inhibitors (Lerner-Marmarosh et al., 2007).

The same peptides were also found to modulate the activity of PKCζ, with their effects on the PKC being similar to those observed with hBVR activity (Lerner-Marmarosh et al., 2007) – the BVR-activating peptide also activated PKCζ and allowed its translocation to the plasma membrane in response to TNF-α. Since the C-terminal lysine of the peptide was required for the PKC activation, it was proposed that the peptide enhances presentation of ATP to the kinase. The BVR inhibitor peptide KKRILHC clearly inactivated PKC activity, and also prevented transport to the membrane. The C-terminal cysteine residue of this peptide was found to be essential for kinase inactivation, and it is therefore possible that the peptide interacted with, and adversely affected the function of, the atypical C1 domain of PKCζ. Such binding might also be expected to inhibit the proposed hBVR association with the C1 domain. KRNRYLSF did not affect kinase activity of PKCζ *in vitro*, but did prevent translocation to the membrane in the intact cell. It is probable that this peptide prevented formation of the hBVR/PKCζ complex in response to TNF-α. Since translocation to the membrane is an integral part of PKC activation (Steinberg, 2008; Newton, 2010), it is clear that in the cell this peptide is also an inhibitor of the PKC.

The observation that, in response to IGF-1 or PMA, hBVR forms an essential complex for activation of PKCδ and ERK, which minimally includes PKCδ, MEK, and ERK1/2 (Lerner-Marmarosh et al., 2008; Gibbs et al., 2012) gave further scope to the use of BVR peptides as a means of disrupting intracellular signaling. As noted, mutation of either the C- or D-Box sequences of hBVR indicated that the interaction with ERK is dependent on the C-Box sequence, whereas the D-Box is required for interaction with PKCδ (Lerner-Marmarosh et al., 2008; Gibbs et al., 2012). Introduction into cells of peptides that include the C-Box core (FGFPAFSG) or the D-Box (KKRILHCLGLA) prevent complex formation in response to treatment with IGF-1, and thus prevent activation of ERK1/2 (Lerner-Marmarosh et al., 2008); The D-Box peptide is also sufficient to prevent association between PKCδ and ERK2, and blocks activation of PKCδ (Gibbs et al., 2012). Because the

hBVR/PKCδ/ERK2 complex is critical for downstream signaling by ERK2, the finding that treatment with peptides disrupts complex formation in the cell and leads to inhibition of ERK-dependent activation of Elk-mediated gene expression offers a novel approach to regulation of ERK signaling.

Peptides were also examined for their ability to modulate hBVR-dependent down-regulation of GPBP. The peptide KKRILHC, which inhibits hBVR and PKCζ activity, was found to be as effective as the intact 296 residue hBVR protein in inhibiting GPBP kinase activity in the cell. Two other peptides that were examined, KYCCSRK and KRNRYLSF, both of which affect the activities of hBVR and PKCζ, but showed no effect on GPBP. The specificity of the KKRILHC in inhibiting GPBP suggests that it might offer a new line of attack in prevention of auto-immunity.

CONCLUDING REMARKS

Altered activity of PKCδ has been implicated in a variety of disorders, including breast and lung cancer (Clark et al., 2003; McCracken et al., 2003), and in resistance to chemotherapeutic drugs. PKCδ is upregulated by estrogen (Cutler et al., 1994), and development of resistance to drugs such as tamoxifen that are used in treatment of hormone-responsive breast tumors is directly regulated by PKCδ. Resistance is enhanced by over-expression of the kinase (Nabha et al., 2005).

A major factor in the development of Parkinson's disease is PKCδ-mediated death of dopaminergic neurons (Anantharam et al., 2002). In this context, it is noteworthy that an early event in apoptosis is cleavage of PKCδ by caspase-3 that results in the separation of the catalytic and regulatory domains (Emoto et al., 1995). Nuclear transport of the constitutively active catalytic domain accelerates apoptosis by inhibition of DNA repair functions and phosphorylation of lamin-B, resulting in a loss of nuclear integrity (Bharti et al., 1998; Cross et al., 2000; Yoshida et al., 2003). Conversely, PKCδ may be cytoprotective in retinopathy associated with type-2 diabetes. On balance, it is apparent that PKCδ regulation is critical to health, and that over- or under-expression is likely to be detrimental. Restoration of normal levels of activity of this kinase is, therefore, likely to be importance in controlling disease.

The finding that kinases upstream of ERK1/2 are dependent on the hBVR scaffolding function defines hBVR as an essential partner in ERK1/2 signaling. hBVR therefore regulates one of the three major MAPK signal transduction pathways, a carefully orchestrated series of events that initiates at the cell membrane and ultimately results in gene activation. The significance of these findings is underscored by the essential role of ERK and PKCδ in a wide spectrum of cellular functions, including ERK1/2-dependent regulation of transcription factors and proteins involved in the cellular stress response. ERK kinases have major roles in regulation of cell growth, differentiation and division; uncontrolled ERK1/2 activation is a frequent event in cancer. Such dysregulation is frequently a consequence of mutation of upstream kinases and signaling molecules in the pathway. The search for inhibitors of the ERK1/2 pathway is a major thrust of drug development research (Chappell et al., 2011). Therefore, hBVR-based peptides targeting the PKCδ/ERK and MEK/ERK pathways may well be an effective approach for therapeutic intervention in drug resistant cancers that are linked to dysregulated signaling pathways. We envision that hBVR-based peptides that inhibit ERK1/2 activation, whether by disrupting the hBVR-scaffolded complexes, or that inhibit upstream kinases, would be antiproliferative if administered over the long term.

Examination of the role of hBVR in activation of kinases, such as PKCs and MEK1/2, that function upstream of ERK1/2 has led to development of hBVR-based peptides that have introduced a new dimension to the regulation of ERK signaling. These peptides hold the potential for development of novel therapeutic agents to regulate the ERK signaling pathway, and hence in ameliorating human disease.

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Go green: the anti-inflammatory effects of biliverdin reductase

Barbara Wegiel* and Leo E. Otterbein*

Department of Surgery, Transplant Institute, Beth Israel Deaconess Medical Center, Boston, MA, USA

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Cesare Mancuso, Catholic University School of Medicine, Italy
Andreas Daiber, University Medical Center Mainz, Germany

****Correspondence:***
Barbara Wegiel, Department of Surgery, Harvard Medical School, Beth Israel Deaconess Medical Center, 3 Blackfan Circle EC/CLS 601, 02215 MA, Boston, USA.
e-mail: bwegiel@bidmc.harvard.edu;
Leo E. Otterbein, Department of Surgery, Harvard Medical School, Beth Israel Deaconess Medical Center, 3 Blackfan Circle EC/CLS 603, 02215 MA, Boston, USA.
e-mail: lotterbe@bidmc.harvard.edu

Biliverdin (BV) has emerged as a cytoprotective and important anti-inflammatory molecule. Conversion of BV to bilirubin (BR) is catalyzed by biliverdin reductase (BVR) and is required for the downstream signaling and nuclear localization of BVR. Recent data by others and us make clear that BVR is a critical regulator of innate immune responses resulting from acute insult and injury and moreover, that a lack of BVR results in an enhanced proinflammatory phenotype. In macrophages, BVR is regulated by its substrate BV which leads to activation of the PI3K–Akt-IL-10 axis and inhibition of TLR4 expression via direct binding of BVR to the TLR4 promoter. In this review, we will summarize recent findings on the role of BVR and the bile pigments in inflammation in context with its activity as an enzyme, receptor, and transcriptional regulator.

Keywords: heme oxygenase, oxidative stress, innate immunity, nitric oxide, toll-like receptor

HISTORY AND CHARACTERIZATION OF THE BILE PIGMENTS

Bilirubin has been accepted for centuries, if not longer, as a major clinical manifestation of jaundice, a yellow hue of the skin. In fact bile pigments can be observed in individuals everyday who have the misfortune of sustaining a contusion or bruise. Indeed the colorimetric nature of the pigments with black heme, green biliverdin, and yellow bilirubin allow one to observe in action the enzymes responsible for heme degradation. Hippocrates was believed to have crafted the notion that "the body of man has in itself blood, phlegm, yellow bile, and black bile and that these four humors make up the nature of his body, and through these man feels pain or enjoys health. The most perfect health is enjoyed when these elements are duly proportioned to one another in respect of compounding, power and bulk, and when they are perfectly mingled." One might speculate whether Hippocrates was referring to heme or biliverdin as the black bile with BR as the yellow bile. BR is the most potent antioxidant in the serum of mammals, whereas BV is almost undetectable being readily converted to BR by biliverdin reductase (BVR) in most cells. In contrast, in lower vertebrates (avian or fish), BV is a critical pigment of the blood, bile, and egg shells of large bird species such as the Emu (Zhao et al., 2006). Cell energy expenditure for the reduction of BV to BR shows not only the importance for generation of a strong antioxidant, but also may underscore the mechanism for activation of critical BVR signaling cascades. Recent data describe kinase and transcriptional activities of BVR, which suggest important additional roles for BV as an intermediate in the catalysis of heme.

PHYSIOLOGY OF BILE PIGMENTS: WHY IS BV AN INTERMEDIATE OF HEME CATALYSIS? IS IT NEEDED AT ALL?

Biliverdin is formed in a single reaction during catalysis of heme by heme oxygenases (HO-1 and HO-2). Carbon monoxide and iron are also released at the same time. HO-1 is one of two HO isoforms and is inducible and designated as a protective gene mediating its effects through generation of one or more of its products (Otterbein et al., 2003). The rate of hemoglobin degradation in the reticulo-endothelial organs (spleen and liver) is slow, therefore all BV to BR is readily reduced particularly in the spleen, liver, kidney, and brain. Biliverdin, in contrast to BR, is soluble and readily excreted into bile. Bilirubin has to be glucuronidated to be excreted, which occurs in the liver. One of the major benefits in generating BR is its antioxidant power in the circulation. The normal concentrations of BR in humans varies between: 0.5 and 1 mg/dL (8.6–17.1 μM) which is sufficient to alleviate oxidative stress (McDonagh, 2001). *In vitro*, nanomolar concentrations of bilirubin inhibit ROS generation (Baranano et al., 2002). Bilirubin is toxic however, primarily in neonates in which the direct bilirubin may cause brain injury termed kernicterus if elevated prior to formation of the blood brain barrier. Bile pigments have a strong ability to absorb light and therefore phototherapy is used as a treatment option for newborns with hyperbilirubinemia. Under certain wavelengths of light, BR is converted to lumirubin and photobilirubin as isomers of BR which can readily be excreted in the urine (Pratesi et al., 1985). In contrast to the dangers of elevated BR in neonate, high normal concentrations of bilirubin

(>1 mg/dL; >34.3 μM) in adults correlates with better cardiovascular function and less inflammation (Schwertner et al., 1994; Mayer, 2000; Novotnây and Vâitek, 2003).

ROLE OF BR – MILD HYPERBILIRUBINEMIA

Approximately 5% of the human population carries a mutation in the glucuronyl transferase gene and therefore these individuals present with increased levels of direct bilirubin in the serum due to a low level of glucuronidation and secretion as bile. Gilbert's syndrome is associated with high normal bilirubin levels (∼2 mg/dL; ∼34.3 μM), which correlates with decreased risk of coronary heart disease and arteriosclerosis (Bulmer et al., 2008) as well as systemic lupus erythematosus (Vitek et al., 2010). Gilbert's syndrome is caused by a genomic homozygous polymorphism, A(TA)7TAA, in the promoter of the gene for UDP-glucuronosyltransferase 1A1 (UGT1A1), which leads to elevated serum levels of unconjugated bilirubin. Development and sustained presence of this mutation throughout evolution may indicate the importance of BR as a major cytoprotectant against ROS and RNS for mammals living in an oxidant environment. BR interacts with NO forming a *N*-nitroso derivative and therefore can be a scavenger of NO to counteract nitrosative stress (Minetti et al., 1998; Mancuso et al., 2003, 2006). BR is a more potent scavenger of superoxide radicals and peroxynitrite then BV, and therefore a lack of BVR may lead to accumulation of excessive oxidative stress in endothelial and other cells that leads to cell death and tissue injury (Jansen et al., 2010).

BVR-IDENTIFICATION AND CHARACTERIZATION

Biliverdin reductase has been known for many decades as the second enzyme in the heme degradation pathway necessary for conversion of BV to BR (Frydman et al., 1987; Bell and Maines, 1988) which serves as both a radical scavenger (Stocker et al., 1987), but is also critical in lipid emulsion during digestion. There are two isoforms of BVR. BVR-A, which catalyzes conversion of BV-a specifically and is expressed in the majority of adult tissues and inducible with stress, and BVR-B which is present during embryogenesis and is an isoform specific for the BV-d and b isomers. Both enzymes catalyze reduction of a double-bond between the pyrrole ring into a single-bond using NADH or NADPH as electron donors dependent on a pH optima (6.75 and 8.7, respectively; McCoubrey et al., 1995). BR and BV, through BVR have been shown to prevent cellular senescence (Kim et al., 2011) and apoptosis (Jansen et al., 2010). Regulation of HO-1 and BVR expression and their enzymatic activity is critical for function of the heme degradation pathway. BVR expression is regulated negatively by NF-κB activation and positively by hypoxia-mediated HIF1α stabilization and specific HRE binding sites in the BVR promoter (Gibbs et al., 2010). BVR is strongly induced by its substrate, biliverdin as well as other agents that induce oxidative stress including LPS, heavy metals, and toxins (Maines et al., 2001; Wegiel et al., 2009). BVR has also been demonstrated to be the target for statins with this interaction leading to significant cognitive benefits in a preclinical model of Alzheimer's disease (Barone et al., 2012). BVR is expressed ubiquitously in all tissues under basal conditions with high levels in the reticulo-macrophages in the spleen and liver. BVR can also be induced. We view this as placing BVR in the category of stress response genes, which is supported by the multiplicity of its functions within the cell, particularly in the context of inflammation. BVR is localized in different cellular compartments in response to stress. BVR has been detected in the membrane (Kim et al., 2004; Wegiel et al., 2009), cytoplasm/ER, mitochondria (Converso et al., 2006), and nucleus (Maines et al., 2001; Ahmad et al., 2002; Lerner-Marmarosh et al., 2008) and its translocation between different cellular compartments is regulated by nitrosylation, lipid modification, or phosphorylation (Wegiel et al., 2009, 2011). Importantly, BV induces NO generation allowing for the stabilization and translocation of BVR to the nucleus (Wegiel et al., 2011). We showed that BVR is strongly induced upon bacteria endotoxin treatment both in the nucleus and on the cell surface (BVR_{surf}). BVR_{surf} is newly characterized as an important isoform of BVR, which functions as a tyrosine kinase receptor-mediated signaling dimer in the membrane to bind and convert BV to BR. Simultaneously, with BV binding, BVR cross-phosphorylates Y ^{198}MKM motifs allowing for interaction with PI3K p85α (Lerner-Marmarosh et al., 2005; Maines, 2007; Wegiel et al., 2009; **Figure 1**).

Conversion of BV by BVR has been shown to occur in any cellular compartment, however the majority of activity is detected in the ER and cell membrane. Modification of BVR by phosphorylation (Pachori et al., 2007) or *S*-nitrosylation (Wegiel et al., 2011) are important regulatory mechanisms for its activity as an enzyme. Phosphorylation of BVR on tyrosine occurs after ligand binding and is necessary for transmission of the signal from BVR to PI3K and downstream activation of Akt (**Figure 1**). Additionally, phosphorylation of serine/threonine as well as *S*-nitrosylation of BVR increases enzymatic activity and are important for bilirubin generation (Salim et al., 2001; Pachori et al., 2007). Post-translational

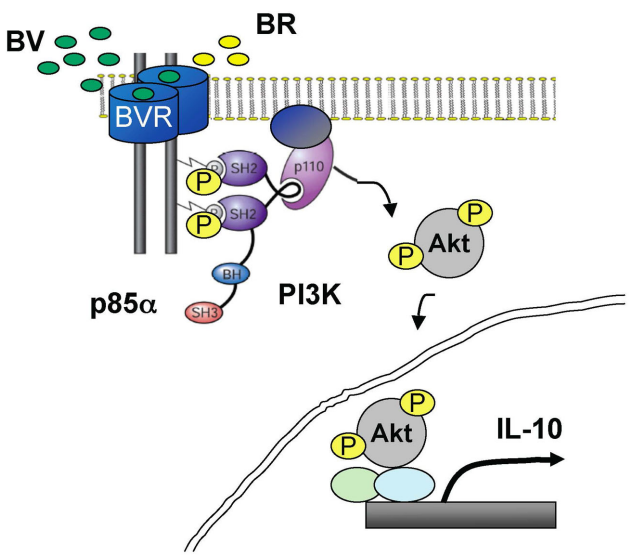

FIGURE 1 | A role of BV in activation of PI3K–Akt through cell surface BVR to elicit anti-inflammatory effects. BVR is expressed on the cell surface of macrophages and mediates the anti-inflammatory and cytoprotective signaling of BV. BV is converted to BR by BVR_{surf}, which drives the recruitment and activation of PI3K–p85α/p110α to BVR to increase the IL-10 levels.

modifications of BVR are evident in neurodegenerative models, where the presence of ROS and NO increases oxidative and nitrosative forms of BVR (Barone et al., 2011a,b). Nitrosylation of tyrosine residues in the brain of subjects with Alzheimer's disease and those with mild cognitive impairment regulates BVR activity (Barone et al., 2011b).

FUNCTION AND REGULATION OF BVR

ANTIOXIDANT BILIRUBIN REDOX CYCLE

The majority of the effects ascribed to BV are mimicked with BR, however the signaling pathways are vastly different including the effects on BVR. BR blocks iNOS expression as well as proinflammatory cytokine expression during endotoxemia (Wang et al., 2004; Lanone et al., 2005), improving the outcome in a lethal models of endotoxemia in rodents (Sarady-Andrews et al., 2005; Kadl et al., 2007; Wegiel et al., 2009). Some of the beneficial signaling and modification effects ascribed to BVR in response to BV, are also observed in response to BR (Baranano et al., 2002). In this cytoprotective amplification loop, biliverdin is able to be regenerated through oxidation of BR by ROS and then reduced back to BR by BVR (Baranano et al., 2002). Importantly, BR also interacts and neutralizes NO radicals forming NO-bilirubin (Barone et al., 2009). The cytoprotective function of BVR is therefore even more apparent with its dual ability to generate BV and BR interchangeably. However, this mechanism may not be fully functional as BR forms mainly non-specific oxidation products, which cannot be used by BVR in the amplification loop. Oxidation of BR by peroxyl radicals generates ∼30% BV as product, however when albumin-bound BR was used there was only a modest increase in BV (<10%; Maghzal et al., 2009). This amount however may be sufficient to drive activation of BVR and formation of additional BR in the amplification cycle described above due to exhaustion of the substrate. In this light, BVR can be labeled as a major protective enzyme. Sedlak et al. (2009) showed that knockdown of BVR *in vitro* leads to an increase in cell death in response to hydrogen peroxide. Similar antioxidant mechanisms of protection mediated by BVR have been reported in a model of experimental autoimmune encephalomyelitis (Liu et al., 2006).

KINASE ACTIVITY IGF/NF-κB

Biliverdin reductase exhibits theronine-serine and tyrosine kinase activity (Salim et al., 2001; Lerner-Marmarosh et al., 2005). While the enzymatic function of BVR was described in 1965, additional roles for BVR have just recently been described. BVR interacts and regulates the insulin receptor, extracellular regulated kinase (ERK), and phosphatidylinositol-3 kinase (PI3K), among other potential interacting proteins such as insulin receptor kinase-1 (IRK-1) or toll-like receptors (TLR). The unique Y^{198}MKM motif of BVR allows BVR the ability to mimic the function of a receptor-like protein by interaction with upstream signaling proteins similar to growth factor receptors such as PDGFR or EGFR. We showed that BVR interacts with PI3K–p85α to drive Akt signaling and IL-10 production, while others demonstrate that BVR regulates the activity of PKC, ERK, and IRK by direct interaction and phosphorylation. BVR can also autophosphorylate and in a hyperphosphorylated form functions better as a catalyst for generating BR (Salim et al., 2001). One of the critical targets for BVR in insulin signaling is IRS-1, which increases phosphorylation of BVR by IRK (Lerner-Marmarosh et al., 2005) and is a key element to insulin resistance. Indeed knockdown of BVR leads to an increase in glucose uptake after insulin treatment in HEK 293 cells.

While biliverdin treatment inhibits NF-κB activation in response to TNFα (Gibbs and Maines, 2007) or LPS (Wegiel et al., 2009) as well as activity of PKC, the role of BVR remains controversial. On the one hand, BVR inhibits NF-κB activation in response to LPS in macrophages and the effects are amplified with BV (Wegiel et al., 2011). Similarly, BV inhibited transcriptional activity of NF-κB in HEK293A cells (Gibbs and Maines, 2007). However, on the other hand, in the same cells it has been shown that overexpression of hBVR enhanced both basal and TNF-α-mediated activation of NF-κB (Gibbs and Maines, 2007). The differences between macrophages and HEK293A cells may be due to the presence or lack of receptors (TLRs or TNFR), which mediate the upstream signaling through NF-κB.

Both PKC-β II and ERK MAPK are important signaling targets for BVR (Lerner-Marmarosh et al., 2005, 2008). Interaction of BVR with PKC-β II as well as BVR-mediated activation of PKC-β II through phosphorylation is critical for its translocation to the membrane. Both BVR and PKC-β II colocalize in the cell membrane and signal from this localization. Similarly, BVR is critical in assuring the localization of ERK proteins to the nucleus (Lerner-Marmarosh et al., 2008). BR induces phosphorylation of Erk in astrocytes and primary rat cerebellar granule neurons and therefore implicates BVR in MAPK activation (Fernandes et al., 2007; Mancuso et al., 2008). BVR forms a ternary complex with PKCδ–ERK2 that is essential for ERK2 signal transduction and activation of genes linked to cell proliferation and cancer (Gibbs et al., 2012). In addition to the protein–protein interactions with BVR, hematin is transported to the nucleus in a complex with BVR to regulate HO-1 expression (Ahmad et al., 2002; Kravets et al., 2004).

SURFACE BVR AND PI3K–Akt SIGNALING

Biliverdin reductase is expressed in the membrane of endothelial cells as well as macrophages. It has not been formally evaluated in any other cell types. One of the reasons for the necessity of BVR to be located on the membrane of the cell is a need for rapid conversion of BV to BR as well as activation of signaling cascades initiated by BVR. Intravenous injection of BV leads to an immediate increase in serum BR and the process is completed 5–10 min after injection. BVR was first identified in caveolae together with HO-1 in EC (Kim et al., 2004). We further characterized expression on the external surface of the plasma membrane and, as such, colocalizes with surface markers such as TLR4 or F4.80 in macrophages (Wegiel et al., 2009). In this localization, BVR is a dimer and an active kinase, that upon encountering BV as its ligand, is cross-phosphorylated to transmit a signal to PI3K (**Figure 1**).

One of the major determinants of cellular fate is the Akt pathway. The broad number of targets of Akt kinase makes this cascade important not only for cell migration, survival, proliferation, growth, but also for metabolic control of the cell. BVR acts as a substrate for insulin receptor tyrosine kinase (IRK) as well as phosphorylation of IR substrate-1 (IRS-1) and therefore may regulate insulin signaling and glucose uptake (Lerner-Marmarosh

et al., 2005). Tyrosine 198 in the YMKM motif is a substrate for insulin-activated IRK (Lerner-Marmarosh et al., 2005) as well as the formation of the BVR dimer (Wegiel et al., 2009) and when phosphorylated binds PI3K p85α to drive Akt phosphorylation. We have shown that BVR–Akt signaling is active in response to exogenous treatment with biliverdin and drives expression of the anti-inflammatory cytokine IL-10 production in macrophages (**Figure 1**).

Biliverdin reductase mediates hypoxia induced epithelial to mesenchymal transition via PI3K (Zeng et al., 2008). The crosstalk between BVR and cellular signaling is very pleiotropic and not limited to PI3K–Akt. Further the signaling control by BVR is strongly correlated with its nuclear function. We posit that BVR is the principal receptor for BV and one of the reasons for its immediate conversion to BR is control of BVR functionality as a signaling molecule.

BVR AS A TRANSCRIPTIONAL MODULATOR

It is well-established that BVR dimerizes both in the nucleus and acts as a leucine zipper–like transcription factor (Ahmad et al., 2002; Wegiel et al., 2009). Binding of BVR to Ap-1 sites either activates transcription (HO-1 promoter; Ahmad et al., 2002) or blocks the expression of the gene (TLR4; Wegiel et al., 2011). BVR as a dimer binds to a 100-mer DNA fragment of the mouse HO-1 promoter region as well as in the ATF2 promoter (−612 to +33) encompassing two activator protein (AP-1) sites (Ahmad et al., 2002). Mutation of Lys(143), Leu(150), or Leu(157) blocks the interaction between hBVR DNA complex formation. Further, BVR is essential as a transporter of heme to the nucleus to regulate HO-1 gene expression in the cell (Tudor et al., 2008). The feed-forward activation of HO-1 via BVR may therefore allow for additional production of BV as the substrate for BVR and amplification of signal under situations of persistent cellular stress.

We recently described the ability of BV to trigger Ca^{2+}/CaMKK signaling that then leads to phosphorylation of eNOS and increased NO generation in macrophages with concomitant *S*-nitrosylation of BVR (Wegiel et al., 2011). This modification of BVR amplifies its enzymatic function and drives its nuclear translocation (**Figure 3**). In RAW264.7 macrophages, treatment with BV suppressed TLR4 expression and resulted in decreased proinflammatory cytokine release. Stable knockdown of BVR in macrophages resulted in elevated expression levels of TLR4 and TNFα. We further showed that inhibition of the TLR4 promoter occurs via a direct interaction of BVR with AP-1 sites on the TLR4 promoter. However due to the close proximity of the Ap-1 and GATA4 sites in the TLR4 promoter, BVR blocks TLR4 expression in contrast to the Ap-1-mediated effect on ATF2 where BVR activated the HO-1 promoter.

Knockdown of BVR results in a significantly heightened proinflammatory phenotype of macrophages with elevated levels of TNFα and activation of TLR4 expression basally reflecting the lack of BVR-regulated TLR4 expression. Further, we demonstrate that decreased NO bioavailability using an inhibitor of NOS in the liver prior to treatment with BV had similar effects as inhibition of BVR, suggesting that modification of BVR via *S*-nitrosylation is important for the protective effects of BV–BVR. Indeed, BV is required for the post-translational modifications of BVR that enable *S*-nitrosylation and phosphorylation (**Figure 2**). We speculate that inflammation or oxidative stress, which leads to induction of HO-1, generates BV to direct BVR to the nucleus to maintain the balance between the expression of cytoprotective genes (i.e., HO-1) and proinflammatory regulatory proteins (i.e., TLR4, TNFα) to prohibit unfettered inflammation.

BILIVERDIN/BILIRUBIN EFFECTS IN DISEASE MODELS

There are several preclinical models in which BV and BR have been shown to impart salutary effects in animals. Exogenous treatment with BV blocks inflammation including reducing infiltrating cells and proinflammatory cytokine expression in models of endotoxemia, ischemia reperfusion injury, or epithelial regeneration (Bellner et al., 2008, 2011). Further, biliverdin inhibits the complement cascade, especially at the C1 step in the classical pathway at low micromolar concentrations (Nakagami et al., 1993). A direct interaction between BV and C components of complement and its inhibition may be one of the cytoprotective mechanisms of BV during inflammation. Oral administration of BV before the antiserum injection protects against anaphylaxic shock induced-death (Nakagami et al., 1993).

Salutary effects of bile pigments have also been described in models of vascular injury and transplantation as will be described in detail below (see also **Figure 3**). The general hypothesis for the similarity in the action of BV and BR in these models stands from the fast conversion of BV to BR. Amplification of the BVR signal may be due to BV, which is generated from the oxidized BR in the cytoprotective cycle (Baranano et al., 2002). However, not

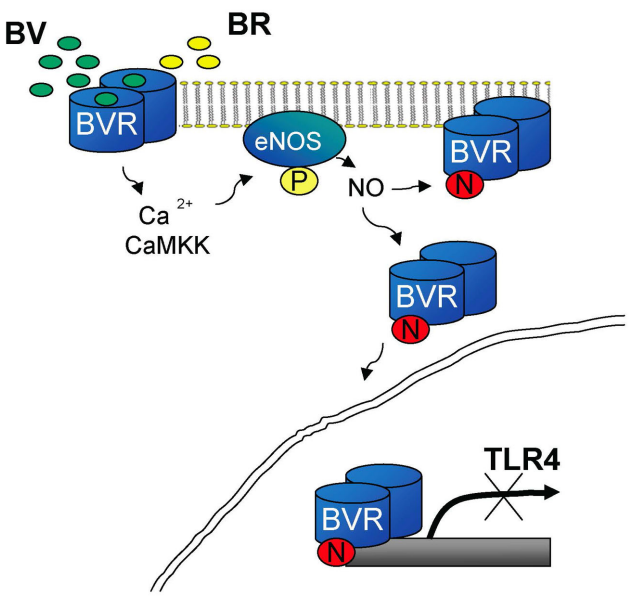

FIGURE 2 | BV-mediated inhibition of TLR4 is dependent on NO-driven BVR translocation to the nucleus. BV drives Ca^{2+}/CaMKK dependent activation of phosphorylation of eNOS and NO production. Presence of NO allows for *S*-nitrosylation of BVR and its translocation to the nucleus. Nuclear BVR as a transcriptional regulator binds to the TLR4 promoter to inhibit its expression. The mechanism is responsible for the preconditioning in the model of acute hepatitis.

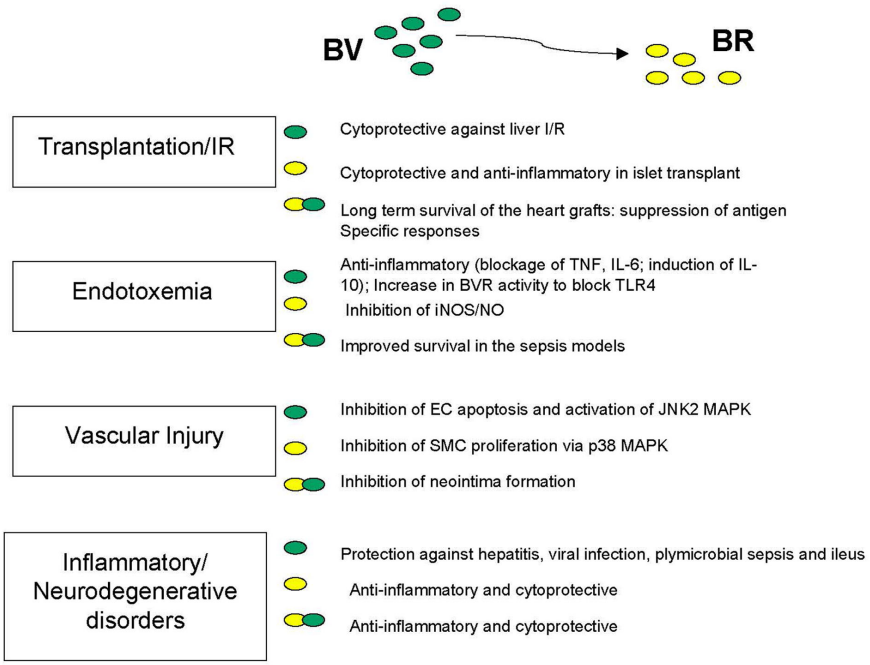

FIGURE 3 | The cytoprotective and anti-inflammatory role of BV and BR in the disease models.

all of the effects of BV are interchangeable with BR, which may be due to requirement for BV to activate BVR. We would argue that generation of even low amounts of BV in the BR → BV → BR amplification loop may be sufficient to activate BVR. This signaling from the oxidized BR (BV) may be responsible for overlapping effects of both molecules, However, the signal may be too low in some instances. The evolutionary conserved generation of BV but not BR (in lower vertebrates) may be due to its additional anti-inflammatory function of BV in regulating BVR–Akt and BVR–TLR4 signaling. Reduction of BV to BR, even energy consuming, maybe a critical regulatory event in activation of signaling function of BVR.

IRI/TRANSPLANTATION

Biliverdin/BR treatment can mimic that observed with HO-1 induction, exemplified by imparting strong anti-inflammatory and immunosuppressive effect in macrophages (Sarady-Andrews et al., 2005) and T cells (Yamashita et al., 2004). In models of heart or islet transplantation, BV/BR suppresses antigen-specific responses and leads to long-term allograft tolerance (Yamashita et al., 2004; Lee et al., 2007). BV/BR inhibits proliferation of recipient splenocytes in the mixed leukocyte reaction interfering with IL-2 production (Yamashita et al., 2004) as well as MHCII expression (Wu et al., 2005). BR administration blocks inflammatory cell infiltration into the graft and promotes anti-apoptotic gene expression while inhibiting proinflammatory cytokine expression in transplanted islets (Lee et al., 2007). BV alone improved liver function and decreased ALT levels after IRI associated with liver transplantation. Strong antioxidant and anti-inflammatory effects were observed when BV was applied before surgery in a model of small bowel transplantation in rats that concomitantly ameliorated postoperative ileus (POI), a complication from excessive bowel manipulation (Nakao et al., 2004). POI is a major clinical problem for any abdominal surgical procedures and results from a profound inflammatory response in the gut that can lead to sepsis, shock and death from lack of bowel motility. Clinically, this syndrome is very common and leads to extended hospital stays because physicians will not discharge a patient without the presence of bowel sounds. The protective effects of BV in ameliorating POI was associated with inhibition of proinflammatory cytokines (IL-6, IL-1β), decreased infiltration of neutrophils and suppression of intestinal circular muscle contractility (Nakao et al., 2004). Whether the beneficial effects observed with BV in the IRI and POI models are driven by BVR directly, or involve only BR exerting global anti-inflammatory effects remains to be elucidated.

ENDOTOXIC SHOCK AND HEPATITIS

Biliverdin has best been characterized as an anti-inflammatory molecule that can inhibit viral replication *in vitro* (Zhu et al., 2010) as well as protection against endotoxic shock (Sarady-Andrews et al., 2005; Wegiel et al., 2009) and microbial sepsis (Overhaus et al., 2006). Importantly, biliverdin has antiviral effects through inhibition of hepatitis C virus protease activity (Zhu et al., 2010). The effects of BV were mediated through BVR (Zhu et al., 2010). Further, BV ameliorates experimental dextran-sulfate-induced colitis (Berberat et al., 2005) inhibited IL-6 and MCP-1 while boosting IL-10 expression in a model of cecal ligation and puncture (CLP)-induced septic shock in rats. LPS induces BVR surface expression in macrophages. Expression of the anti-inflammatory cytokine IL-10 in response to BV–BVR activation is solely dependent on the Akt signaling pathway as BV-induced IL-10 is inhibited in the presence of dominant-negative Akt.

Depletion of BVR in the liver using small interfering RNA (siRNA) against BVR with an adenoviral construct completely inhibited the protective effects of BV against acute hepatitis (Wegiel et al., 2009) which partially involves NO-dependent BVR-regulated TLR4 inhibition (Wegiel et al., 2011).

VASCULAR INJURY

Biliverdin and BR have potent protective effects in models of vascular injury. Biliverdin prevents neointima formation in a model of vascular remodeling involving arterialization of vein grafts through inhibition of JNK1/2 MAPK in the graft as well as via inhibition of EC apoptosis (Nakao et al., 2005). Further, BV and BR blocked SMC proliferation through inhibition of p38 MAPK and blockade of cell cycle progression in G1-S phase, correlating with decreased hyperphosphorylation of Rb and YY1 expression (Ollinger et al., 2005). These *in vitro* studies were corroborated *in vivo* in rats in response to balloon trauma. Hyperbilirubinemic Gunn rats validated these observations as they were resistant to neointima formation in response to balloon trauma as compared to wild type controls (Ollinger et al., 2005). The role of BR remains to be determined. It has been well-described however with association studies that Gilbert's individuals show very low incidence of atherosclerosis and heart disease which suggest an important role for BR (Novotnây and Vâitek, 2003; Bulmer et al., 2008).

FUTURE STUDIES

BVR–fl/fl AND KNOCKOUT MICE

Small interfering RNA is the only strategy to date to inhibit BVR expression in order to genetically assess the role of BVR. The field is lacking critical knockout and conditional knockout mice that allow detailed mechanistic testing of BVR *in vitro* and *in vivo* and its contribution to bile pigment turnover and immune regulation. We have generated conditional *BVR–fl/fl* mice (unpublished data) and have specifically deleted BVR in macrophages. Preliminary data show that a lack of BVR in macrophages mimics that observed with siRNA studies; both resulting in a proinflammatory phenotype of myeloid cells, their expansion in the lung and spleen, and subsequent alterations in response to organ injury (data not shown). Deletion of BVR in conjunction with HO-1 knockout mice allows the opportunity to study individually the role of each of the components of the heme degradation pathway in various tissues and under differing kinetics and stimuli. Unlike typical knockout strategies, the conditional approach to tissue specific deletion of BVR and/or HO-1 allows the study of these genes without any compensatory mechanisms in place. Indeed the HO-1 knockout shows <5% fecundity, which begs the question as to what is truly being studied and what conclusions can be drawn from these mice. Have these 5% somehow adapted or compensated? And as such, are they truly allowing appropriate conclusions to be drawn as to the role of HO-1 in naïve and disease scenarios? The conditional knockout mice will certainly shed important light on the role of HO-1 and BVR in basal physiology and importantly, their role in pathophysiology.

SUMMARY AND CONCLUSION – POTENTIAL THERAPEUTIC MODALITIES AND APPLICATIONS

Biliverdin reductase, a pleiotropic signaling molecule and transcriptional regulator is a clear therapeutic target. The Asian community has been using bile salts and pigments for medicinal purposes for centuries if not millennia as a cure-all for everything from headaches to indigestion. In fact, it is firmly believed that bile from different species carries diverse biological effects. BV is an agonist of BVR and an activator of PI3K signaling, the activity of which is altered in various pathologies (insulin resistance, cancer). Recently BVR peptides have been designed to modulate insulin receptor signaling (Maines, 2010).

Importantly, a homozygous nonsense mutation in BVR (Cys214 → Ala) in humans has been described and results in accumulation of biliverdin during episodes of cholestasis (Nytofte et al., 2011). The functional consequences of this mutation and likely others are not yet known, however they may contribute to the development of proinflammatory syndromes, as we are starting to observe in the BVR-deficient mice. The complexity of BVR to both signal and modulate transcriptional activity in addition to enzymatic generation of BR, places it as a central control switch employed by the cell to respond appropriately to inflammation and stress. Exogenous delivery of BV is of potential therapeutic interest given its strong anti-inflammatory potential and defined mechanisms of action. That BV and BR are natural substances make clinical development perhaps more straightforward because the means by which the body metabolizes each pigment is known making pharmacokinetic and pharmacodynamic studies less complicated.

In conclusion, BVR is a truly remarkable molecule. It is triple-threat functionality places it in a category all its own with no other known molecule that has been defined that functions as an enzyme, a signaling kinase, and a regulator of transcription. Understanding what directs one activity over the other is the challenge for future research. Generating tools such as the cre–lox mice will no doubt open up knew avenues of research with exciting discoveries. Eventually, we envision BV and BR as powerful therapeutics that will prove useful in numerous disease pathologies.

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Direct antioxidant properties of bilirubin and biliverdin. Is there a role for biliverdin reductase?

Thomas Jansen and Andreas Daiber*

The 2nd Medical Clinic, Molecular Cardiology, Medical Center of the Johannes Gutenberg University, Mainz, Germany

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Mahin D. Maines, University of Rochester School of Medicine, USA
Ron Kohen, The Hebrew University of Jerusalem, Israel
Peter Gibbs, University of Rochester School of Medicine and Dentistry, USA

***Correspondence:**
Andreas Daiber, Universitätsmedizin der Johannes Gutenberg-Universität Mainz, II. Medizinische Klinik – Labor für Molekulare Kardiologie, Verfügungsgebäude für Forschung und Entwicklung – Raum 00349, Obere Zahlbacher Str. 63, 55101 Mainz, Germany.
e-mail: andreas.daiber@bioredox.com

Reactive oxygen species (ROS) and signaling events are involved in the pathogenesis of endothelial dysfunction and represent a major contribution to vascular regulation. Molecular signaling is highly dependent on ROS. But depending on the amount of ROS production it might have toxic or protective effects. Despite a large number of negative outcomes in large clinical trials (e.g., HOPE, HOPE-TOO), antioxidant molecules and agents are important players to influence the critical balance between production and elimination of reactive oxygen and nitrogen species. However, chronic systemic antioxidant therapy lacks clinical efficacy, probably by interfering with important physiological redox signaling pathways. Therefore, it may be a much more promising attempt to induce intrinsic antioxidant pathways in order to increase the antioxidants not systemically but at the place of oxidative stress and complications. Among others, heme oxygenase (HO) has been shown to be important for attenuating the overall production of ROS in a broad range of disease states through its ability to degrade heme and to produce carbon monoxide and biliverdin/bilirubin. With the present review we would like to highlight the important antioxidant role of the HO system and especially discuss the contribution of the biliverdin, bilirubin, and biliverdin reductase (BVR) to these beneficial effects. The BVR was reported to confer an antioxidant redox amplification cycle by which low, physiological bilirubin concentrations confer potent antioxidant protection via recycling of biliverdin from oxidized bilirubin by the BVR, linking this sink for oxidants to the NADPH pool. To date the existence and role of this antioxidant redox cycle is still under debate and we present and discuss the pros and cons as well as our own findings on this topic.

Keywords: heme oxygenase, bile pigments, biliverdin reductase, oxidative stress, vascular function

INTRODUCTION

The endothelial integrity is crucial for physiologic organ function and protects against vascular inflammation, sepsis, prothrombotic activity, and atherosclerosis (Bassenge et al., 2005). Disruption of this blood–tissue (endothelial) barrier results in endothelial dysfunction which is a hallmark of most cardiovascular complications (Cai and Harrison, 2000; Munzel et al., 2008). Reactive oxygen species (ROS) and signaling events are involved in the pathogenesis of endothelial dysfunction and represent a major contribution to vascular regulation. ROS are important regulators for cellular and metabolic conditions. Molecular signaling is highly dependent on ROS. But depending on the amount of ROS production it might have toxic or protective effects (Bachschmid et al., 2005; Daiber and Münzel, 2006; Ullrich and Kissner, 2006).

Despite a large number of negative outcomes in large clinical trials (HOPE, HOPE-TOO as well as a prospective study with vitamin C in postmenopausal women with diabetes mellitus; Yusuf et al., 2000; Muntwyler et al., 2002; Lee et al., 2004; Mann et al., 2004; Lonn et al., 2005), antioxidant molecules and agents are important players to influence the critical balance between production and elimination of reactive oxygen and nitrogen species (RONS). The latter assumption is supported by a great number of experimental animal studies (Watanabe et al., 1997; Bassenge et al., 1998; Crawford et al., 1998; Elhaimeur et al., 2002) as well as human studies with rather small numbers of patients and acute infusion of antioxidants (Heitzer et al., 1996; Gori et al., 2001) indicating that antioxidants may be highly

Abbreviations: 2-HE, 2-hydroxyethidium; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; ACE, angiotensin-converting enzyme; ApoE$^{-/-}$, apolipoprotein E deficiency (knock-out); ARB, angiotensin-receptor blocker; Aβ, amyloid-β peptide; BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin; BR, bilirubin; BSA, bovine serum albumin; BV, biliverdin; BVR, biliverdin reductase; CAD, coronary artery disease; CO, carbon monoxide; CORM-2, CO-releasing molecule-2; DHE, dihydroethidine (dihydroethidium); DHR, dihydrorhodamine 123; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; ECL, enhanced chemiluminescence; eNOS, endothelial nitric oxide synthase (NOS-3); GCH-1, GTP-cyclohydrolase-1; GTN, glyceryl trinitrate (nitroglycerin); HDF, human fibroblast; HPLC, high performance liquid chromatography; HO, heme oxygenase; HO-1, heme oxygenase-1 (inducible isoform); HO-2, heme oxygenase-2 (constitutive isoform); HRP, horseradish peroxidase; HUVECs, human umbilical vein endothelial cells; KO_2, potassium superoxide; L-012 luminol analog; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MnSOD, manganese superoxide dismutase (SOD2, mitochondrial isoform); mRNA, messenger ribonucleic acid; NO, nitric oxide (nitrogen monoxide); nNOS, neuronal nitric oxide synthase (NOS-1); PARP, poly(ADP-ribose)polymerase; PETN, pentaerithrityl tetranitrate; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; Sin-1,3-morpholino-sydnonimine (active metabolite of molsidomine); siBVR, small interfering RNA against BVR; siHO-1, small interfering RNA against HO-1; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; XO, xanthine oxidase.

beneficial in improving endothelial dysfunction. The main reasons for the failure of chronic oral antioxidant therapy could be as follows: vitamin E and C act as pro-oxidants (e.g., tocopheryl and ascorbyl radicals), the coronary artery disease (CAD) of included patients is already irreversible, the CAD patients are already treated with drugs displaying antioxidant properties [e.g., angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs)], chronic antioxidant therapy inhibits intrinsic ischemic preconditioning which relies on RONS formation or oral vitamin treatment does not result in high enough concentrations of the antioxidants at the place of oxidative stress (Chen et al., 2012).

Some of these reasons would favor the acute infusion of vitamin C in accordance with respective observations. However, infusion of antioxidants cannot be applied to a large number of patients over a long period and in addition may be affected by some of the draw backs listed above under chronic administration (e.g., interference with cellular redox signaling, suppression of oxidant-triggered ischemic preconditioning). Therefore, it may be a much more promising attempt to induce intrinsic antioxidant pathways in order to increase the antioxidants not systemically but at the place of oxidative stress and complications. A number of experimental animal studies have demonstrated that overexpression of ROS sources aggravates cardiovascular complications, whereas their suppression improves these adverse effects: the genetic deletion of NADPH oxidase subunits improved myocardial infarction damage and survival of mice (Doerries et al., 2007) and prevented angiotensin-II (Landmesser et al., 2002) as well as renovascular (clip model; Jung et al., 2004) induced hypertension, oxidative stress, and endothelial dysfunction. In contrast, overexpression of NADPH oxidase subunits further aggravated these complications (Dikalova et al., 2005). Vice versa, deletion of the mitochondrial manganese superoxide dismutase (MnSOD) increased age-dependent mitochondrial oxidative stress and endothelial dysfunction (Wenzel et al., 2008a) and deficiency in glutathione peroxidase-1 potentiated atherosclerosis and vascular complications in ApoE$^{-/-}$ mice (Torzewski et al., 2007). The recent observation that overexpression of mitochondrial superoxide dismutase improves angiotensin-II triggered hypertension and vascular dysfunction in mice shows that the different ROS sources can directly influence each other (Dikalova et al., 2010) and that especially the cross-talk between the mitochondrial and NADPH oxidase redox axis may play an important role for various diseases (Daiber, 2010).

Among others, heme oxygenase (HO) has been shown to be important for attenuating the overall production of ROS through its ability to degrade heme and to produce carbon monoxide (CO), biliverdin/bilirubin, and the release of free iron with subsequent ferritin induction (Vitek and Schwertner, 2007; Abraham and Kappas, 2008). It should be noted that free iron itself is toxic for the cell and may contribute to cellular oxidative damage (e.g., by reduction of free iron(III) to iron(II) by superoxide and subsequent reaction of ferrous iron with hydrogen peroxide to yield hydroxyl radicals. The latter step is termed "Fenton reaction," the overall process is the "Haber–Weiss-cycle" (Daiber and Münzel, 2006). Therefore, it is of great importance to keep the free iron levels low, which also includes that induction of HO is followed by subsequent induction of ferritin. Heme and P450 overload has been demonstrated to confer cytotoxic effects (Eipel et al., 2007), which provides a simple basis for the highly beneficial action of HO consisting of the direct detoxification of free heme. Besides the highly protective effects of heme oxygenase (HO-1) in various pathophysiological states, its down-stream targets bilirubin and biliverdin have reducing properties and are recognized as potent antioxidants (Stocker et al., 1987), but it is not fully understood how the bile pigments act in this molecular process and the role of biliverdin reductase (BVR) needs to be further explored. The localization of heme degradation enzymes (e.g., HO-1 and BVR) to plasma membrane caveolae and evidence on caveolin-1 interaction with HO-1 and modulation of its activity make the HO-1 system an attractive player in the vascular antioxidant system since also the redox-sensitive endothelial nitric oxide synthase (eNOS) as well as superoxide producing enzymes are located in the plasma membrane caveolae (Kim et al., 2004).

Heme oxygenase-1 degrades heme to biliverdin, which is then reduced by BVR to bilirubin. During the last decade BVR has attracted more attention on the background of an active antioxidant cycle providing a catalytic sink for RONS as well as cytoprotection (Baranano et al., 2002; Sedlak and Snyder, 2004). However, this concept is still under debate with a number of highly controversial reports (Maines, 2007; Pachori et al., 2007; Florczyk et al., 2008; Maghzal et al., 2009; Sedlak and Snyder, 2009; Young et al., 2009; Jansen et al., 2010). With the present review we provide an overview on our own results, the current literature and critically discuss the pros and cons of the antioxidant and cytoprotective concept underlying the action of BVR.

THE CONCEPT OF BILIVERDIN REDUCTASE AND OXIDANTS FORMING A CATALYTIC ANTIOXIDANT CYCLE

In 2002 an attractive concept was put forward by Snyder and colleagues consisting of the BVR mediated reduction of biliverdin (BV) to bilirubin (BR) being the much more potent antioxidant and subsequent oxidation of BR by hydrogen peroxide back to BV forming a catalytic antioxidant cycle that is driven by NADPH, the reducing cofactor of BVR (see **Figure 1**; Baranano et al., 2002; Greenberg, 2002; Sedlak and Snyder, 2004). Especially lipophilic ROS such as lipid hydroperoxides and peroxyl radicals were described to feed this antioxidant cycle and later, synergistic effects of glutathione have been described (Sedlak et al., 2009). In their initial work, Baranano et al. demonstrated that BR (10 nM) increased the viability of HeLa cells in the presence of toxic concentrations of hydrogen peroxide (100 μM). The fact that 10,000-fold lower BR concentrations prevented the toxicity of H_2O_2 implied that there was some kind of "recycling" of BR resulting in the proposed BV/BR redox cycle. In addition, small interfering RNA (siRNA) against BVR resulted in higher ROS levels in HeLa cells and neurons, decreased the viability of neurons upon treatment with H_2O_2, increased caspase activity and apoptosis [envisaged by poly(ADP-ribose)polymerase (PARP) activation] in these cells. In response to the review article of Sedlak and Snyder, Antony McDonagh (1990), a pioneer on the bile pigment field, questioned the importance of the oxidative conversion of BR to BV since BR radicals are also reduced in a non-enzymatic environment by

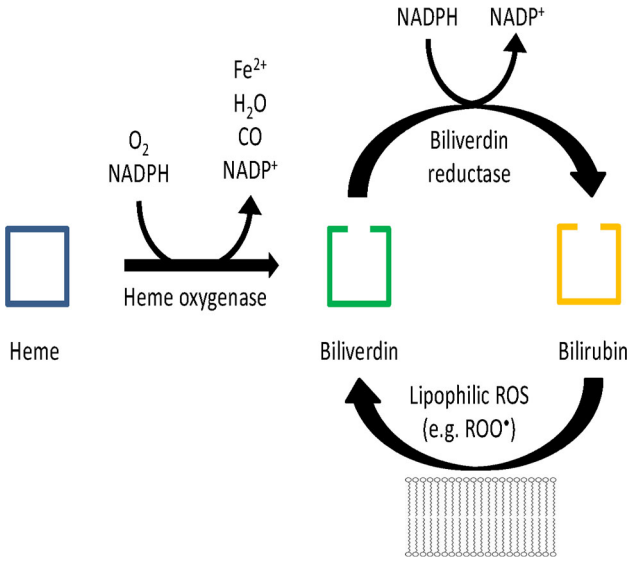

FIGURE 1 | Hypothetical scheme of the antioxidant redox cycle of the BR/BV/BVR system. Modified from Sedlak and Snyder (2004).

interaction with molecular oxygen – these considerations were published in a letter to the editor (McDonagh, 2004). In addition, Antony McDonagh pointed out that there is only marginal conversion of BR to BV under special conditions and generation of certain oxidants, which was supported by our observations using superoxide, peroxynitrite, hydrogen peroxide, and peroxidase systems (see chapter below). These reports stimulated us to investigate this potential sink for RONS *in vitro* (chemical models) and *ex vivo* (human endothelial cells).

OWN RESULTS

DIRECT ANTIOXIDANT PROPERTIES OF BILIRUBIN VERSUS BILIVERDIN *IN VITRO*

As shown by Jansen et al. (2010) bilirubin represents a superior antioxidant as compared to biliverdin when applied in high concentrations. Using cell-free systems (test tube chemistry) the direct antioxidant effects of bilirubin versus biliverdin were assessed. The peroxynitrite scavenging ability of both bile pigments was assessed by two different biochemical models: tyrosine residues in bovine serum albumin (BSA) were either nitrated by authentic peroxynitrite ($ONOO^-$) or *in situ* generated $ONOO^-$ from the thermal decomposition of 3-morpholino-sydnonimine (Sin-1), a more physiological model to assess peroxynitrite-mediated oxidations. Peroxynitrite-mediated tyrosine nitration involves tyrosyl-radical-intermediates as a consequence of homolytic bond cleavage in ONO-OH yielding hydroxyl ($HO^•$) and nitrogen dioxide ($^•NO_2$) radicals. Therefore, inhibition of peroxynitrite-mediated BSA nitration may be regarded as ability of BR and BV to scavenge peroxynitrite-derived free radicals and/or reduction of tyrosyl-radical-intermediates. According to our results, BR is at least threefold more potent than BV in inhibiting peroxynitrite-mediated protein tyrosine nitration. The used dot blot technique excludes any interference of the BR or BV color since the compounds are removed during transfer of the sample to the membrane.

Superoxide scavenging ability of BR and BV was determined in two different systems. In the first one superoxide was constantly generated by xanthine oxidase (XO) and hypoxanthine whereas in the second one we used authentic superoxide (KO_2) to exclude any inhibitory effects of the compounds on enzymatic activity (e.g., one of the compounds could be an inhibitor of XO). Since the color of BR and BV may lead to false-positive results using direct optical methods, we used HPLC-based detection of fluorescent 2-hydroxyethidium (2-HE), the specific oxidation product of dihydroethidium and superoxide. XO-derived superoxide was decreased in a concentration-dependent fashion by BR whereas BV had no inhibitory effect and even significantly increased the signal pointing toward pro-oxidative effects. It should be noted that the absolute increase in superoxide signal by BV was small (approximately 10%). Using KO_2 from a saturated stock solution in dimethyl sulfoxide (DMSO), the above described differences even became more pronounced: BR decreased the formation of 2-hydroxyethidium in a concentration-dependent fashion whereas BV, this time, dramatically increased its yield up to 2.5-fold over KO_2 control without BR or BV. This may be taken as evidence for redox-cycling of BV amplifying the superoxide formation rate. Again, the used HPLC technique excludes any interference of the BR or BV color since the compounds are separated from the product during their way through the column.

NO EVIDENCE FOR CONVERSION OF BILIRUBIN TO BILIVERDIN BY DIFFERENT OXIDANTS *IN VITRO*

Bilirubin or BV (each 50 μM) were incubated with authentic peroxynitrite, Sin-1, KO_2, hydrogen peroxide alone or together with horseradish peroxidase (HRP). The latter would generate peroxidase compound I, having hydroxyl radical-like, one-electron abstracting properties. There was a pronounced loss of BR in all systems used indicating that BR reacted with any reactive species formed. In contrast, BV was converted less efficiently (not at all by superoxide), only the peroxidase/H_2O_2 system completely consumed BV. Special emphasis was put on the conversion of BR to BV by the different oxidants, the process that was previously described by Snyder and coworkers (Baranano et al., 2002; Sedlak and Snyder, 2004). However, we were unable to detect any significant conversion of BR to BV questioning the role of BR/BV as catalysts in the break-down of RONS. We also observed no conversion of BR to BV by mitochondrial oxidative stress but also no significant conversion of BV to BR by intrinsic mitochondrial BVR. Also here, we strictly used an HPLC technique excluding any interference of the BR or BV color since the compounds are separated during their way through the column.

(IN)DIRECT ANTIOXIDANT PROPERTIES OF BILIRUBIN VERSUS BILIVERDIN *EX VIVO*

Efficacy of BR and BV as antioxidants was also tested in isolated heart mitochondria (see **Figure 2**). Mitochondrial respiratory chain was stimulated with succinate and blocked by the complex III inhibitor antimycin A, which directs ROS toward the intermembrane space. ROS and RNS formation was detected by the luminol analog L-012 enhanced chemiluminescence (ECL) detecting intra-

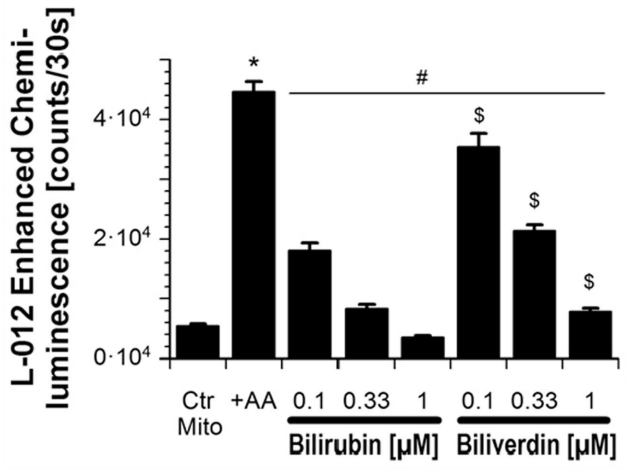

FIGURE 2 | Effects of BR and BV on mitochondrial oxidative stress. Adopted from Jansen et al. (2010). With permission of Rightslink® by Copyright Clearance Center (Elsevier).

and extra-mitochondrial reactive species. BR sensitively decreased the L-012 signal at submicromolar concentrations being threefold more efficient as compared to BV. In contrast, suppression of the intra-mitochondrial superoxide formation, that was measured by the yield of 2-hydroxyethidium, required 1000-fold higher concentrations of BR and the difference between BR and BV was less pronounced.

The inhibitory effects of BR and BV on oxidative burst in phorbol ester-stimulated neutrophils was detected by L-012 ECL indicating less pronounced differences between BR and BV, as compared to mitochondria. BR and BV decreased the signal in a concentration-dependent fashion but BR was only marginally more potent than BV. The less pronounced difference may be due to the involvement of myeloperoxidase/H_2O_2 in the signal intensity. The reactivity of BR and BV may be very similar for the reactive species derived from the peroxidase/H_2O_2 system in accordance with the almost identical break-down pattern of BR and BV caused by HRP/H_2O_2 (see third paragraph in the preceeding chapter). However, the superoxide-specific product 2-hydroxyethidium revealed a more pronounced antioxidative effect of BR as compared to BV, which even caused a marginal increase in the signal. Regarding the weak differences between BR and BV in the chemiluminescence assay it should be taken into account that the color of the bile pigments may have interfered with the ECL signal. Therefore, the HPLC data may be more reliable.

ANTIOXIDANT PROPERTIES OF THE HEME OXYGENASE SYSTEM AND EFFECTS OF BILIVERDIN REDUCTASE SILENCING IN HUMAN ENDOTHELIAL CELLS

Knock-out models by genetic gene deletion are useful tools to investigate enzyme and protein functions. Until now, BVR knock-out mice do not exist and inhibitors are not commercially available. Therefore, silencing of the BVR gene by siRNA is often used as molecular method in most experiments. We investigated BVR silencing in human umbilical vein endothelial cells (HUVECs) and observed significant effects in resting cells without oxidative stimulus characterized by an increase in intracellular oxidative stress (measured by intracellular 2-HE formation). With lipopolysaccharide (LPS)-elicited RONS formation we established a model for cellular oxidative stress in HUVECs and endothelial cell activation, which reflects endotoxin-triggered endothelial dysfunction and may be used as a clinical model for septic shock.

Treatment of HUVECs with siBVR or siHO-1 resulted in a >90% decrease at the messenger ribonucleic acid (mRNA) level which was measured by reverse transcription-polymerase chain reaction (RT-PCR) and envisaged on agarose gels. Basal superoxide levels in these cells were determined by 2-hydroxyethidium formation, which was significantly increased in BVR-silenced cells. After characterization of the ideal conditions for cellular oxidative stress and protection by HO-1 induction these findings were applied to BVR and HO-1-silenced HUVECs. Hemin decreased the LPS-triggered oxidative stress [dihydrorhodamine 123 (DHR) fluorescence] in control cells whereas this protective effect was almost lost in BVR-deficient cells and completely absent in HO-1-depleted cells. When the BVR substrate BV was added to lipopolysaccharide-stimulated HUVECs the decrease in DHR fluorescence in control cells was more pronounced at a percentage level as compared to BVR-silenced cells (20.1 versus 15.4%).

These findings were supported by a second parameter for oxidative stress, protein tyrosine nitration. 3-nitrotyrosine staining even revealed a more pronounced effect of BVR and HO-1 silencing. In LPS-stimulated HUVECs hemin caused a decrease in 3-nitrotyrosine staining at 3.3 and 10 μM whereas nitration was significantly increased in BVR- and HO-1-deficient cells and the protective effect of hemin was completely lost. In addition, the substrate for BR synthesis by BVR decreased the level of 3-nitrotyrosine- positive proteins in a concentration-dependent fashion in control cells whereas this protective effect of BV was lost in BVR-silenced cells. The highest concentration of BV (10 μM) even tended to increase the nitration instead of preventing it, supporting the observed redox-cycling by BV (see second paragraph in the preceeding chapter). Treatment of HUVECs with siBVR or siHO-1 resulted in a >80% or complete decrease at the protein level which was measured by Western blotting.

A last set of experiments aimed to demonstrate the importance of chronic indirect effects of the HO-1/BVR product BR. LPS treatment of an immortal endothelial cell line (EA.hy 926 cells) decreased the expression of the tetrahydrobiopterin (BH_4) synthase GTP-cyclohydrolase-1 (GCH-1). BV had no effect on endothelial expression of this important enzyme whereas BR as well as the CO-releasing molecule-2 (CORM-2) derived CO dramatically increased the GCH-1 protein levels. It should be noted that BH_4 levels are crucial for the function of eNOS and BH_4 deficiency leads to uncoupling of eNOS and subsequent formation of RONS. Therefore prevention of GCH-1 down-regulation or degradation may represent an important antioxidant pathway. Previous reports have demonstrated that oxidative stress may trigger the proteasomal degradation of GCH-1 (Xu et al., 2007, 2009). Especially for the endothelium it may be crucial to maintain the BH_4 levels under conditions of increased oxidative stress to prevent eNOS uncoupling and endothelial dysfunction. Interestingly, the levels of the dihydrobiopterin (BH_2) recycling enzyme

dihydrofolate reductase behave complementary to those of GCH-1 in accordance with our previous results in diabetic rats (Wenzel et al., 2008b; Oelze et al., 2011).

OTHER REPORTS IN FAVOR OF AN IMPORTANT ROLE OF BILIVERDIN REDUCTASE FOR ANTIOXIDANT AND CYTOPROTECTIVE EFFECTS OF THE HEME OXYGENASE SYSTEM

Previous reports suggested an efficient catalytic cycle between BV and BR (Baranano et al., 2002; Sedlak and Snyder, 2004; Liu et al., 2006) and others have observed conversion of BR to BV by peroxynitrite (Kaur et al., 2003), or peroxyl radicals (Baranano et al., 2002). In support of these previous findings and a role of BVR in cytoprotection, Young et al. (2009) demonstrated that angiotensin-II induced superoxide formation in renal tubular epithelial cells and inner medullary collecting duct (IMCD3) cells is aggravated by silencing of BVR. Superoxide formation was measured using dihydroethidine (DHE) fluorescence and lucigenin-derived chemiluminescence. BVR silencing resulted in a significantly decreased level of BVR protein (>30% of control cells) as well as diminished cellular bilirubin concentrations (50% of control cells). Co-treatment with BV could decrease the angiotensin-II induced superoxide formation in control cells, whereas it further aggravated the ROS levels in BVR-silenced cells. This is in good accordance with our observations on increased superoxide levels by biliverdin in the presence of xanthine oxidase or authentic superoxide, most probably via redox-cycling (see the preceeding chapter). However, it should be noted that Young et al. also observed BVR/BR independent antioxidant action of HO-1, since hemin co-treatment improved superoxide formation in BVR-silenced cells, indicating an important contribution of CO (and other so far not identified routes). The beneficial effect of hemin could of course also be related to increased break-down of superoxide in the presence of hemin leading to other reactive intermediates or non-toxic products that are not detected by DHE fluorescence. Of note, we have observed a more pronounced burden of oxidative stress in endothelial cells treated with HO-1 siRNA as compared to BVR siRNA suggesting on top protective pathways mediated by HO-1 activation (most probably CO formation) as compared with the BVR pathway alone (see the preceeding chapter).

Another previous report by Liu et al. (2006) demonstrated increased cellular oxidative stress in BVR-deficient cells using a model of experimental autoimmune encephalomyelitis. In detail, BVR treatment of encephalomyelitic rats improved the tissue levels of 8-isoprostane in the spinal cord but also the clinical score of the disease (50% for BVR and 66% for HO-1 of encephalomyelitic rats). In isolated cells, BVR silencing had much more pronounced effects on increase in ROS levels and decrease in cell viability as compared to HO-1 inhibition by Sn-pophyrin. The down-stream product of BVR, BR, showed a much more pronounced effect on erythrocyte hemolysis by the complement in the classical pathway and lymphocyte activity (antibody-dependent cell-mediated cytotoxic activities) as compared to the antioxidant glutathione. However, since HO-1 treatment or inhibition displayed weaker effects as compared to BVR treatment or silencing, the data of this study rather point to an effect of BVR that is independent of the antioxidant action of BR.

Panahian et al. (1999) reported on enhanced neuronal expression of BVR after permanent focal cerebral ischemia and speculated that this may be a new cellular mechanism for protecting neurons against ischemic injury and oxidative stress. Cerebral ischemia was induced by permanent middle cerebral artery occlusion and increased BVR expression obviously contributed to increased survival of neurons in the peri-ischemic region. Previous reports introduced an additional function for BVR. The protein contains a region with a leucine zipper deoxyribonucleic acid (DNA)-binding motif and binds as a homodimer to a region of the HO-1 promoter that contains two AP-1 sites (Ahmad et al., 2002). By this action BVR may prevent cellular damage not only by catalyzing the formation of the antioxidant bilirubin, but also by transcriptional activation of HO-1 triggering the efflux of potentially toxic iron from cells exposed to oxidative stress (Baranano and Snyder, 2001). The latter observation was also shared by us since BVR silencing also partially decreased HO-1 protein levels, which may be related to a role of BVR for transcriptional activation of HO-1 (Jansen and Daiber, unpublished).

Finally, it was shown by Maines and coworkers that BVR without any action of BR may change the cellular antioxidant profile by regulation of protein kinase C (PKC-βII) activity (Maines, 2007; Maines et al., 2007), which may represent an important BVR-dependent protective pathway based on PKC-ζ under inflammatory conditions [e.g., tumor necrosis factor-α (TNF-α) signaling; Lerner-Marmarosh et al., 2007]. This pathway involves insulin receptor 1/2 signaling, the insulin/growth factor signaling cascade for protein/ DNA synthesis and glucose transport down-stream of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). This kinase activity of BVR provides a great link between heme metabolism and cell signaling. Further downstream in this signaling cascade, BVR may also directly interact with the transcription factors ATF-2/CREB, c-fos, c-jun preventing apoptosis. Even protective actions of BVR on arsenite toxicity were described that were completely independent and not shared by HO-1 (Miralem et al., 2005).

There is also further wide-spread evidence for beneficial effects of BVR in various disease models. In a model of oxidative damage in maternal cholestasis during pregnancy, upregulation of BVR was suggested to confer protection of the placental–fetal unit since the viability of human choriocarcinoma JAr cells was significantly increased by bilirubin in a concentration-dependent fashion (Perez et al., 2008). Another report demonstrated that the antioxidants biliverdin/bilirubin might play an important role in the protection of the nitrergic neurotransmitter against oxidative stress in murine gastric fundus (De Backer and Lefebvre, 2008). The authors demonstrated, that neurotransmission (neuronal nitric oxide synthase, nNOS-dependent relaxation in murine jejunal circular smooth muscle strips) was impaired by the superoxide generator LY83583, a process that was prevented by BR and to a smaller extend by BV but not by CO. From these observations and the colocalization of HO-2 and BVR with nNOS they concluded that the BR pathway but not the CO pathway down-stream of heme oxygenase-2 (HO-2) confer antioxidant protection of the neurotransmission process in the gastric fundus although this

protective action was not observed under normal physiological conditions but only in response to oxidant challenges. In a model of cell senescence, Kim et al. (2011) found a striking decrease in BVR expression in senescent human fibroblasts (HDF cells) as compared to young HDF cells. Vice versa, senescent fibroblasts had significantly increased ROS levels in comparison with young HDF cells, a difference that was eliminated by BVR siRNA treatment suggesting a role of BVR in the young fibroblasts in the detoxification of ROS. Hydrogen peroxide caused an appreciable induction of HO-1 in the young fibroblasts (with high expression of BVR) whereas this antioxidant response was absent in the senescent HDF cells (with low BVR expression). Again, this differential stress response was eliminated when siRNA treatment against BVR was applied. As a functional read-out, the depletion of BVR isoform A could reduce the cell number by >60% in young HDFs even without additive exogenous oxidative stress, a process that was accompanied by increased expression of the expression of the senescence marker SA-β-gal. A recent study demonstrated that BV rescues the HO-2 knock-out mouse phenotype of unresolved chronic inflammation following corneal epithelial injury and the authors suggested a beneficial role of the upregulated BVR in this model (Bellner et al., 2011).

OTHER REPORTS NOT IN FAVOR OF AN IMPORTANT ROLE OF BILIVERDIN REDUCTASE FOR ANTIOXIDANT AND CYTOPROTECTIVE ACTIONS OF THE HEME OXYGENASE SYSTEM

According to a recent publication of Maghzal et al. (2009) revisiting the previous findings of Snyder and colleagues neither BVR overexpression nor silencing changed hydrogen peroxide toxicity in HeLa cells, an immortal human cancer cell line. In detail, Stocker and coworkers could show that lipid peroxidation-mediated oxidation of BR in chloroform, did not yield BV (see similar observations by us, preceeding chapters). Similarly, H_2O_2 did not oxidize albumin-bound BR to BV, and only modest yields of BV were observed upon *in vitro* oxidation of albumin- or ligandin-bound BR in the presence of peroxyl radicals. The viability of HeLa cells was not decreased in the presence of hydrogen peroxide after depletion of cellular BVR protein and activity *in vitro* using siRNA against BVR. Vice versa BVR overexpression failed to enhance protection of HeLa cells against H_2O_2-mediated damage, irregardless of whether BR or BV were added to the cells as substrate for the putative redox cycle. The authors also showed that overexpression of human BVR in HeLa cells did not decrease H_2O_2-induced ROS levels. Finally, they highlighted that HO-1 overexpression but not BVR overexpression rescued cell survival of HeLa cells in the presence of hydrogen peroxide. In a letter to the editor, Snyder and colleagues criticized the experimental setup in the work of Maghzal et al. with special emphasis on the need for providing BV by either exogenous addition or endogenous activation of the HO-1 system (Sedlak and Snyder, 2009), as done in our cell culture experiments (see preceeding chapters). In addition, these authors criticized the high hydrogen peroxide concentrations employed by Stocker and coworkers as well as the time point at which cell death and ROS measurements were detected (4–8 h) in contrast to the maximum protection observed by BVR at 24 h. In the direct reply letter to the editor, Stocker and Maghzal pointed out that previous work supporting a role for BVR in cytoprotection and antioxidant effects (e.g., Miralem et al., 2005; Maines, 2007) provided beneficial data that was largely independent of the presence of BR and the reductase activity of BVR and were therefore not in support of the antioxidant BV/BR/BVR redox cycle (Stocker and Maghzal, 2009). Even more important, these authors emphasized that BVR depletion also failed to affect the death of cells loaded with BV or BR prior to exposure to a 40-fold molar excess of H_2O_2 (Maghzal et al., 2009), whereas Snyder and associates reported BR to be able to defend against a 10,000-fold excess of H_2O_2 (Baranano et al., 2002).

In his recent study, Anthony McDonagh demonstrated that the peroxyl radical generator 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) did not result in the formation of BV from BR in air-saturated solution in the absence of serum albumin (McDonagh, 2010). He even observed consumption of BV under the same conditions arguing against an accumulation of BV under cellular conditions in the presence of peroxyl radical formation. In contrast, he described marginal BV formation from BR in the presence of serum albumin and air-saturated solution, which was even present in oxygen-free (argon flushed) solutions, whereas oxygen-saturation eliminated the conversion of BR to BV. This clearly pointed against a peroxyl-driven conversion of BR to BV since peroxyl radical generation from AAPH requires oxygen and most pronounced BR to BV conversion was observed in the absence of oxygen and required the presence of serum albumin. Therefore, Anthony McDonagh proposed that secondary protein radicals formed by the interaction of AAPH with serum albumin rather account for the conversion of BR to BV than peroxyl radicals, suggested to be the converting species by Snyder and colleagues.

OUTLOOK AND THERAPEUTIC IMPLICATIONS

ANTIOXIDANT AND CYTOPROTECTIVE ROLE OF BVR BUT EVIDENCE AGAINST THE BV/BR/BVR REDOX CYCLE

The data presented in our previous published work (Jansen et al., 2010) support the concept that BR is a more potent antioxidant than BV, but also provide evidence that direct and indirect antioxidant effects of the bile pigments contribute to the beneficial profile of the HO-1 pathway (e.g., GCH-1 in EA.hy cells; see **Figure 3** for summary). Despite our observations that at high concentrations of the bile pigments scavenging of RONS is more (peroxynitrite or superoxide) or less (H_2O_2 or H_2O_2/peroxidase) pronounced and that BV undergoes redox-cycling in the presence of superoxide, we could not establish an important role for an antioxidant cycle based on oxidative conversion of BR to BV and reductive reversal by BVR. The antioxidant effects of BV and BR are concentration-dependent. About 10 μM BR reflect the physiological situation of endothelial cells, which are in direct contact with the blood stream. The model used by Jansen et al. for cellular oxidative stress is based on LPS-elicited RONS formation in HUVECs, which reflects endotoxin-triggered endothelial dysfunction and may be used as a cellular model for septic shock. In this respect, identification of BVR as an important constituent of the antioxidant defense system in human endothelial cells has important implications for the prevention of endothelial dysfunction in cardiovascular diseases that are associated with increased oxidative

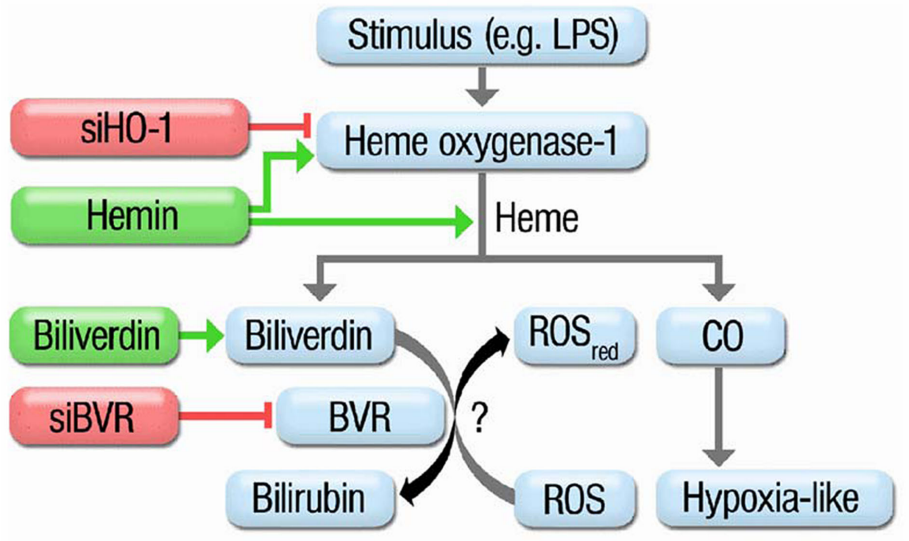

FIGURE 3 | Scheme summarizing the protective routes in the heme oxygenase-1 system. Treatment of HUVECs with oxidative stimuli (e.g., LPS) results in induction of HO-1 which catalyzes the break-down of metallo-porphyrins to CO (hypoxia-like pathway) or biliverdin. Biliverdin is further converted by biliverdin reductase (BVR) yielding the potent antioxidant bilirubin. siRNA against HO-1 blocks induction of HO-1. Hemin is a potent inducer of HO-1 but also a substrate for it resulting in increased CO and BR formation. Biliverdin is the substrate of BVR leading to an increase in BR. siRNA against BVR impairs the conversion of BV to BR. Previous reports speculated on a catalytic cycle of BV to BR conversion by BVR and subsequent oxidation of BR to BV by ROS. Adopted from Jansen et al. (2010). With permission of Rightslink® by Copyright Clearance Center (Elsevier).

stress or chronic inflammatory conditions. HO-1 silencing had more pronounced effects on oxidative stress than BVR deletion and accordingly HO-1 dependent protection involves other (CO- or ferritin-dependent) pathways besides the formation of BR or presence of BVR. Most probably, BR (and/or BVR) and CO act synergistically in conferring antioxidant protection of endothelial cells. The antioxidant effects of CO in this synergistic action are not based on direct RONS scavenging but rather on regulation of NADPH oxidase activity, P450 activity and induction, opening of potassium channels, angiogenesis, activation of soluble guanylyl cyclase, angiogenesis, interference with MAPK signaling, and many more (Abraham and Kappas, 2008). The antioxidant effects of BR in this synergistic action are partially based on direct RONS scavenging but also on regulation of NADPH oxidase activity, neutrophil adhesion and inflammatory pathways (Abraham and Kappas, 2008) as well as control of redox sensitive protein levels (e.g., GCH-1; Jansen et al., 2010; Schuhmacher et al., 2011). Since BV, in our hands, displayed redox-cycling under certain conditions, we speculate that removal of BV may be even more important than the formation of BR – or at least both processes contribute to the antioxidant role of BVR.

According to a recent publication of Maghzal et al. (2009) revisiting the previous findings of Snyder and colleagues neither BVR overexpression nor silencing changed hydrogen peroxide toxicity *in vitro*. Also these authors did not observe any significant oxidative conversion of BR to BV by hydrogen peroxide and only modest BV formation from BR in a chemical model of lipid peroxidation in chloroform or by peroxyl radicals. Whereas the latter observation is in good agreement with our present findings since we also did not observe significant conversion of BR by various oxidants or mitochondrial ROS, we disagree with the conclusion that BR and BVR have no crucial effect on antioxidant protection conferred by HO-1 under cellular conditions. We think that hydrogen peroxide is an unfortunate model to disproof a role for BVR/BR since we here show that hydrogen peroxide has only minor reactivity toward BR and BVR knock-down resulted in increased cellular oxidative damage in a septic shock model based on LPS-triggered peroxynitrite formation in HUVECs. These considerations are supported by findings of Young et al. (2009) demonstrating that angiotensin-II induced oxidative damage in renal tubular epithelial cells is aggravated by silencing of BVR. These authors also observed BVR/BR independent antioxidant action of HO-1 indicating an important contribution of CO and/or BV as well. In a letter to the editor, Snyder and colleagues criticized the experimental setup in the work of Maghzal et al. with special emphasis on the need for providing BV by either exogenous addition or endogenous activation of the HO-1 system (Sedlak and Snyder, 2009), as done in our cell culture experiments.

THERAPEUTIC POTENTIAL OF BVR IN ORGANIC NITRATE TOLERANCE AND OTHER NEUROLOGICAL/CARDIOVASCULAR COMPLICATIONS

Regarding the therapeutic use of modulating BVR activity, it would be interesting whether BVR deficiency would aggravate organic nitrate induced vascular damage characterized by endothelial dysfunction and nitrate tolerance. Vice versa, it would be of clinical importance whether BVR overexpression may improve the adverse effects of chronic organic nitrate therapy. During the past years we were able to demonstrate that HO-1 induction by hemin was able to prevent nitroglycerin (GTN) induced

vascular dysfunction and oxidative stress, whereas suppression of HO-1 activity by apigenin induced a tolerance-like effect in rats under pentaerithrityl tetranitrate (PETN) therapy (Wenzel et al., 2007). Previous reports demonstrated that PETN therapy is devoid of nitrate tolerance, endothelial dysfunction, and oxidative stress (Jurt et al., 2001; Gori et al., 2003; Schnorbus et al., 2010) and that this beneficial profile is at least partially based on the induction of HO-1 (Oberle et al., 1999, 2002, 2003) but also on activation of other cardio-protective genes (Pautz et al., 2009) as well as improvement of endothelial progenitor cell function (Thum et al., 2007). Recently, we have demonstrated that the beneficial profile of PETN is lost in HO-1 deficient mice and PETN therapy results in induction of nitrate tolerance as well as vascular oxidative stress (Schuhmacher et al., 2010). In a subsequent study we have identified the transcription factor Nrf2 as an important mediator of PETN-dependent HO-1 induction since cells with silenced Nrf2 did not show increased HO-1 promoter activity in response to PETN treatment anymore (Schuhmacher et al., 2011). Finally, BR caused a concentration-dependent decrease in nitroglycerin-induced mitochondrial RONS formation (Wenzel et al., 2007) suggesting that this powerful antioxidant may contribute to the prevention of adverse side-effects under chronic PETN therapy by eliminating toxic RONS species. Since our previous findings have revealed a dramatic increase in peroxynitrite (Daiber et al., 2005a,b), associated with enhanced protein tyrosine nitration (Warnholtz et al., 2002; Schuhmacher et al., 2009) and loss of function of prostacyclin synthase (Hink et al., 2003) under chronic nitroglycerin therapy, it may be speculated that the potent RONS (namely peroxynitrite) scavenging properties of bilirubin (Minetti et al., 1998; Kaur et al., 2003) contribute to the suppression of nitrate tolerance by HO-1 induction. Therefore, it would be of great clinical importance to test the effect of BVR silencing or pharmacological inhibition and vice versa investigate the effect of BVR overexpression or pharmacological induction/activation on chronic outcome of PETN therapy. This would help to clarify whether the conversion of BV to BR by BVR does significantly contribute to the beneficial profile of HO-1 induction under PETN therapy.

Recent reports propose a link between BVR and beneficial effects of statins in neurodegenerative disorders such as Alzheimer disease (Barone et al., 2012; Schneider and Simons, 2012). In detail, atorvastatin significantly increased BVR isoform A protein levels, phosphorylation and activity only in parietal cortex. Additionally, these authors found significant negative correlations between BVR isoform A and oxidative stress indices, as well as discrimination learning error scores. Finally, BVR isoform A up-regulation and post-translational modifications significantly correlated with β-secretase protein levels in the brain, suggesting a possible role for BVR isoform A in amyloid-β peptide (Aβ) formation. Preceding work has shown that nitrosative and oxidative stress-induced modifications on hippocampal BVR isoform A are an early event in the pathogenesis of Alzheimer disease (Barone et al., 2011). The Maines group reported on protection of cardiomyocytes by synergistic BVR and HO-2 upregulation as a potential strategy against cardiac dysfunction (Ding et al., 2011). In addition to these novel observations, a common BVR isoform A polymorphism was found to be associated with essential hypertension in Kazaks (Lin et al., 2011). A recent review by Maines highlights the beneficial role of BVR in insulin signaling suggesting a therapeutic potential of BVR in diabetes (Maines, 2011). The unique features and therapeutic potential of BVR were reviewed by Florczyk et al. (2008) and the same group also demonstrates that overexpression of BVR enhances resistance to chemotherapeutics (Florczyk et al., 2011).

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Bile pigments in pulmonary and vascular disease

Stefan W. Ryter [1,2] *

[1] Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA
[2] Adjunct Scientist, Lovelace Respiratory Research Institute, Albuquerque, NM, USA

Edited by:
Mahin D. Maines, University of Rochester School of Medicine, USA

Reviewed by:
William Durante, University of Missouri, USA
Leo Otterbein, Harvard Medical School, USA

***Correspondence:**
Stefan W. Ryter, Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.
e-mail: sryter@partners.org

The bile pigments, biliverdin, and bilirubin, are endogenously derived substances generated during enzymatic heme degradation. These compounds have been shown to act as chemical antioxidants *in vitro*. Bilirubin formed in tissues circulates in the serum, prior to undergoing hepatic conjugation and biliary excretion. The excess production of bilirubin has been associated with neurotoxicity, in particular to the newborn. Nevertheless, clinical evidence suggests that mild states of hyperbilirubinemia may be beneficial in protecting against cardiovascular disease in adults. Pharmacological application of either bilirubin and/or its biological precursor biliverdin, can provide therapeutic benefit in several animal models of cardiovascular and pulmonary disease. Furthermore, biliverdin and bilirubin can confer protection against ischemia/reperfusion injury and graft rejection secondary to organ transplantation in animal models. Several possible mechanisms for these effects have been proposed, including direct antioxidant and scavenging effects, and modulation of signaling pathways regulating inflammation, apoptosis, cell proliferation, and immune responses. The practicality and therapeutic-effectiveness of bile pigment application to humans remains unclear.

Keywords: antioxidant, biliverdin, bilirubin, cardiovascular disease, pulmonary disease

INTRODUCTION

Natural antioxidants constitute an important part of host defenses against environmental exposure to noxious agents (Davies, 1995). Pro-oxidant states arise when reactive oxygen species (ROS) formed during metabolism exceed cellular antioxidant capacity (Davies, 1995). Although ROS act as mediators of cellular homeostatic regulation and signaling (Forman et al., 2010), excessive ROS production may contribute to the pathogenesis of human diseases, including cancer, and cardiovascular diseases (CVD; Dröge, 2002; Valko et al., 2006; Sugamura and Keaney, 2011). Cells contain water and lipid soluble chemicals and antioxidant enzymes that function to limit harmful oxidative reactions and preserve tissue homeostasis (Halliwell and Gutteridge, 1999; Davies, 2000). Additionally, many dietary substances can be absorbed and serve as systemic antioxidants (Kaliora et al., 2006; García-Lafuente et al., 2009). Thus, much research has been directed toward the exploitation of naturally occurring antioxidant compounds as therapeutics in the prevention or treatment of human disease (Kaliora et al., 2006; García-Lafuente et al., 2009; Sugamura and Keaney, 2011).

The bile pigments biliverdin (BV) and bilirubin (BR) originate as the products of heme degradation (Roy-Chowdhury et al., 2008; **Figure 1**). These pigments have been regarded by the medical community as waste products of metabolism (Vitek and Ostrow, 2009). However, BV/BR exhibit antioxidant properties in model systems (Stocker et al., 1987a). Circulating BR acts as a potent serum antioxidant, and serves as a natural anti-atherogenic factor (Stocker et al., 1987b). Retrospective and prospective clinical studies indicate that mildly elevated levels of BR (mild hyperbilirubinemia) are associated with reduced CVD risk (Franchini et al., 2010). Beneficial effects of pharmacological BR/BV have been described in pre-clinical models of tissue injury and disease, including organ transplantation, lung disease, and CVD (Ollinger et al., 2007; Ryter et al., 2007). These effects involve antioxidant, anti-inflammatory, anti-apoptotic, and anti-proliferative mechanisms (Ollinger et al., 2007; Ryter et al., 2007). This review discusses the therapeutic application of the BR/BV in disease, as well as clinical data on the role of endogenous BR as an inverse risk factor for CVD.

ORIGIN AND METABOLIC FATE OF BILIRUBIN

Bilirubin formed *in vivo* originates from hemoglobin turnover (~80%) during the degradation of erythrocytes by reticuloendothelial macrophages. The remainder of BR formation results from hemoprotein turnover in systemic tissues. Biliverdin-IXα (BV-IXα), the precursor to BR, is a water soluble pigment that originates during heme degradation catalyzed by heme oxygenase activity (HO; E.C. 1:14:99:3), represented by constitutive (HO-2) and inducible (HO-1) isozymes (Tenhunen et al., 1969; Maines, 1997).

Heme oxygenase activity, the rate-limiting step in heme degradation, requires three moles O_2 per heme oxidized, and electrons from NADPH cytochrome-p450 reductase (Yoshida and Kikuchi, 1974; Noguchi et al., 1979; Yoshida et al., 1980). Each mole of BV-IXα formed yields one mole each of carbon monoxide (CO), derived from the heme α-methene carbon, and ferrous iron (Tenhunen et al., 1969). BV-IXα is reduced to bilirubin-IXα (BR-IXα), a lipid soluble pigment, by NADH/NADPH-dependent biliverdin reductase (BVR; E.C. 1.3.1.24; Tenhunen et al., 1970).

Bilirubin follows a sequence of biological transformation and elimination steps (**Figure 2**). BR formed *in situ* passes to the serum where it circulates in a complex with serum albumin (Roy-Chowdhury et al., 2008; Vitek and Ostrow, 2009). A fraction

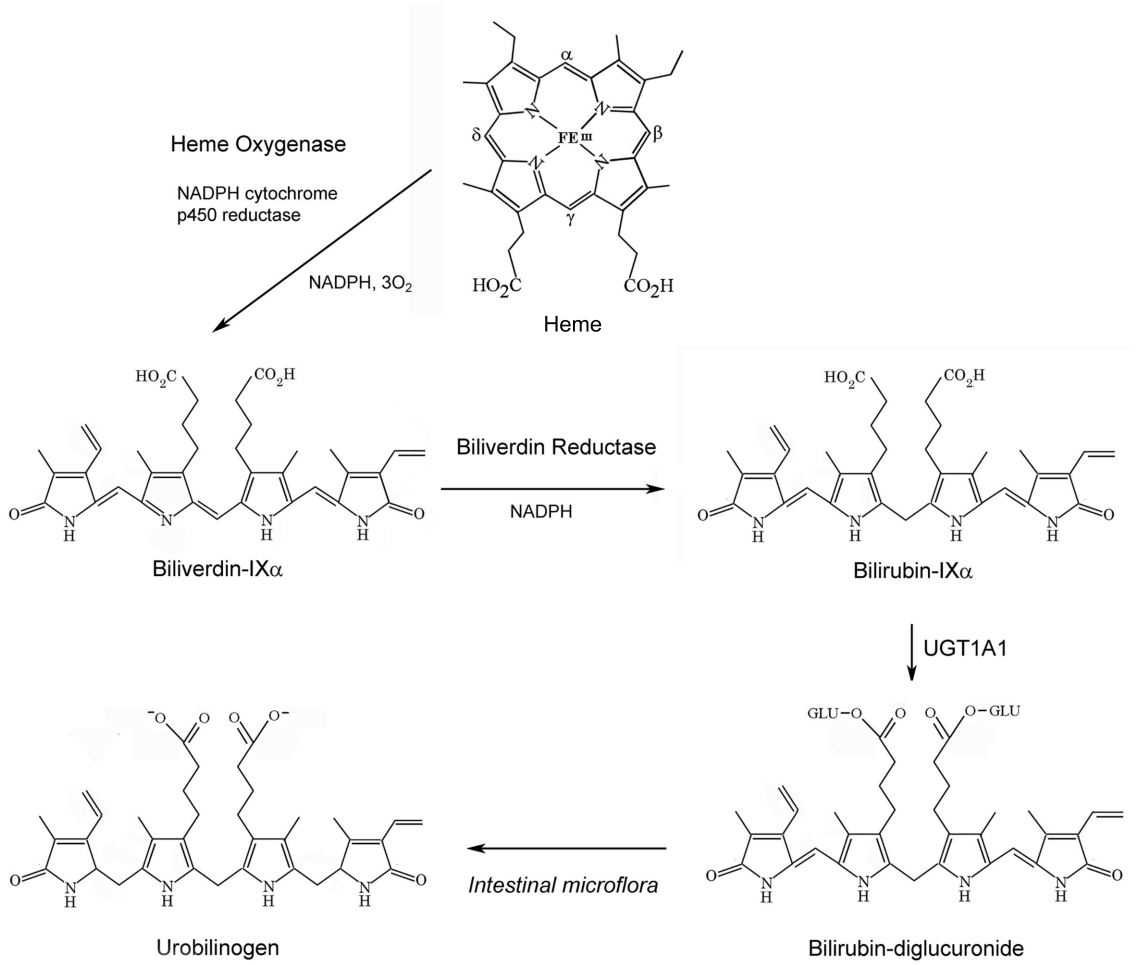

FIGURE 1 | Sequence of bile pigment formation and degradation. Heme Oxygenase (HO) degrades heme to biliverdin-IXα (BV), in a reaction generating carbon monoxide (CO) and ferrous iron, at the expense of NADPH and molecular oxygen. BV is reduced to bilirubin-IXα (BR) by NAD(P)H biliverdin reductase (BVR). BR is conjugated with glucuronic acid at its propionyl side chains by hepatic UDP-glucuronyltransferase-1A1 (UGT1A1), to form BR mono- and di-glucuronides. BR can be further metabolized to urobilinogen by intestinal microflora.

of serum unconjugated BR circulates freely (∼0.01%; Vitek and Ostrow, 2009). Unconjugated BR is taken up by the liver by facilitated diffusion, involving organic ion transporters (e.g., SLCO1B1; Kamisako et al., 2000; Cui et al., 2001). The intracellular hepatocyte transport of BR is assisted by ligandin, a complex of glutathione-*S*-transferase (GST) subunits, and by protein-Z (Litwack et al., 1971). BR is conjugated in hepatocytes by uridine diphosphate (UDP) glucuronyltransferase (UGT1A1; EC: 2.4.1.17; Chowdhury et al., 1979). Conjugated BR (mono- and di-glucuronides) is pumped from hepatocytes across the canalicular membrane by multidrug resistance protein-2 and excreted through the bile to the intestine (Chowdhury and Chowdhury, 1983; Wang et al., 2006a).

ANTIOXIDANT EFFECTS OF BILE PIGMENTS

The potential for the use of bile pigments as therapeutics originated with the discovery that these substances act as natural antioxidants. Stocker et al. demonstrated that BV, BR, and conjugated BR inhibit lipid peroxidation in liposomal preparations challenged with free radical initiating chemicals (Stocker and Ames, 1987; Stocker et al., 1987a; Stocker and Peterhans, 1989a). In these model systems, BR and BV exerted chain-breaking and peroxyl radical trapping activity (Stocker and Ames, 1987; Stocker et al., 1987a). BV and conjugated BR acted as co-antioxidants with α-tocopherol, and inhibited α-tocopherol consumption (Stocker and Peterhans, 1989a). BR also prevented oxidative damage to proteins, such as serum albumin exposed to ROS-generating systems (Stocker et al., 1987b; Neuzil and Stocker, 1993). Free and albumin-bound BR inhibited oxidation of LDL-bound lipids, by acting as co-antioxidants with the LDL-bound α-tocopherol (Neuzil and Stocker, 1994).

Bilirubin can react with superoxide anion radical, hypochlorous acid, and singlet molecular oxygen, inhibit the photo-oxidation of protein, and inhibit chemiluminescence in activated macrophages (Stevens and Small, 1976; Pedersen et al., 1977; Stocker and Peterhans, 1989b). BR can also react with NO or reactive nitrogen species (RNS; Kaur et al., 2003; Mancuso et al., 2003). Finally, conjugated BR forms a cupric complex that promotes decomposition

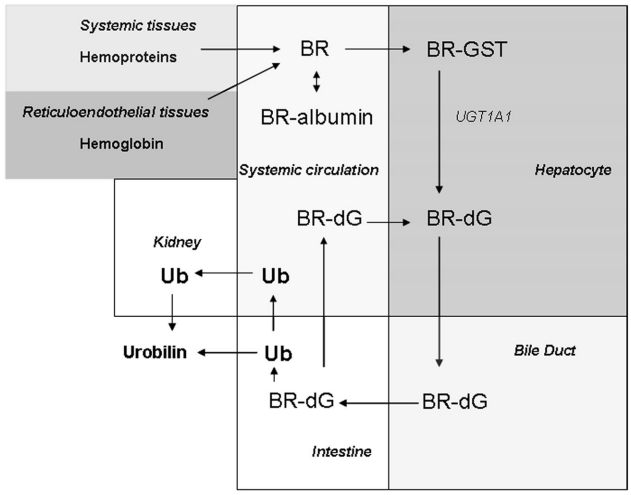

FIGURE 2 | Biodistribution of BR. BR is generated in systemic tissues as the product of hemoprotein (i.e., hemoglobin) degradation. BR formed in tissues passes freely to the circulation, where it exists mostly in a complex with serum albumin. BR is taken up by hepatocytes by facilitated diffusion. In the hepatocyte, BR is transported by glutathione-*S*-transferase (GST), and then conjugated by UDP-glucuronyltransferase-1A1 (UGT1A1) to form bilirubin di-glucuronide (BR-dG). Conjugated BR (BR-dG) is then pumped into the bile duct and reaches the intestine. Conjugated BR can be reabsorbed in the intestine and re-enter the circulation (Roy-Chowdhury et al., 2008; Vitek and Ostrow, 2009). The metabolism of BR in the intestine by bacterial action generates urobilinogen (UB) and its oxidation product urobilin, the latter which is eliminated in the feces. Intestinal urobilinogen can be reabsorbed by the intestine and eliminated in the urine as urobilin (Chowdhury and Chowdhury, 1983; Wang et al., 2006a).

of hydroperoxides, representing a pro-oxidant activity in the bile (Stocker and Ames, 1987).

Bilirubin serves as circulating antioxidant in human plasma. The treatment of plasma with oxidizing agents resulted in the depletion of endogenous antioxidants in the order ubiquinol-10, ascorbate, and bilirubin. The addition of BR to human plasma after depletion of naturally occurring antioxidants resulted in the inhibition of lipid peroxidation, and reduction of α-tocopherol consumption (Neuzil and Stocker, 1994). Serum samples from hyperbilirubinemic patients with Gilbert's syndrome were resistant to oxidation and displayed higher total antioxidant capacity than serum from subjects in the normal range of BR concentration (Bulmer et al., 2008).

Exogenous BR when applied pharmacologically to cultured cells can provide dose-dependent cytoprotection against oxidative stress (Clark et al., 2000a). For example, BR (1–5 μM) protected cultured vascular smooth muscle cells against cytotoxicity from enzyme-generated H_2O_2 (Clark et al., 2000a). BR when applied at nanomolar concentrations protected primary neurons against cytotoxicity caused by exogenous H_2O_2 (Doré et al., 1999).

Despite evidence suggesting that extracellular BR can act as a cytoprotectant, the role of BR as a cellular antioxidant remains unclear. BR produced *in situ* is secreted to the circulation, conjugated in the liver, and excreted (Roy-Chowdhury, 1996). However, the fraction of BR that is retained in cells to serve a membrane antioxidant function is unknown. A common argument against a cellular antioxidant role for BR is that the cellular milieu contains efficient and abundant endogenous antioxidant compounds, including millimolar quantities of reduced glutathione (GSH; Meister and Anderson, 1983), ascorbate, β-carotene, and α-tocopherol (Halliwell and Gutteridge, 1999). Given that BR is exported and eliminated, the relative contribution of BR to cellular antioxidant capacity in the presence of other endogenous antioxidants remains unclear.

Current evidence for the role of BR as a cellular antioxidant is based on evidence using siRNA studies targeting BVR, the enzyme responsible for BR formation. Knockdown of BVR sensitized cells to high concentrations of H_2O_2 (Baranano et al., 2002), and to arsenite-mediated apoptosis (Miralem et al., 2005). In the latter case, similar effects were not achieved with HO-1 knockdown, suggesting effects of BVR independent of BR generation (Miralem et al., 2005).

Snyder et al. observed that BV is formed during the oxidation of BR (Baranano et al., 2002). The authors proposed that BV formed by BR oxidation would act as a substrate for BVR, to regenerate BR, and thus represent a self-perpetuating antioxidant system, "the BVR antioxidant cycle." Evidence for and against this pathway has been debated elsewhere (Maghzal et al., 2009; Sedlak and Snyder, 2009; Stocker and Maghzal, 2009). It remains unclear if BV regenerated during the oxidation of BR would exceed but a minor fraction, and whether this would contribute significantly to cellular antioxidant capacity as proposed.

TOXICITY OF BILIRUBIN

Bilirubin accumulation can be harmful, especially in infants with neonatal hyperbilirubinemia. The selective toxicity of BR to the neonate is due to incomplete establishment of the blood–brain barrier. Neonatal unconjugated hyperbilirubinemia is associated with severe neurological side effects, which include neurological encephalopathy and kernicterus (Vitek and Ostrow, 2009). To avoid risk of neurotoxicity, phototherapy is applied to reduce the levels of unconjugated BR in jaundiced newborns (Blanckaert and Fevery, 1990). The mechanisms of BR toxicity in the brain have been reviewed elsewhere (Brito et al., 2008; Ghersi-Egea et al., 2009; Tell and Gustincich, 2009; Vitek and Ostrow, 2009).

PROTECTIVE EFFECTS OF BILE PIGMENTS IN ANIMAL MODELS OF TISSUE INJURY

Excluding neonatal toxicity, mild hyperbilirubinemia, due to elevated serum antioxidant capacity, may confer benefits to the host. In a mouse model of hyperbilirubinemia, jaundiced Gunn rats displayed lower indices of oxidative stress in the serum than wild-type mice when challenged with hyperoxia (Dennery et al., 1995).

Therapeutic application of BR preserved myocardial function during cardiac ischemia/reperfusion (I/R) injury (Clark et al., 2000b). In an isolated perfused heart model, heme preconditioning protected against myocardial infarction following I/R injury, associated with increased HO-1 expression and BR formation. Administration of BR at nanomolar concentrations improved cardiac performance and reduced infarct size and mitochondrial dysfunction following I/R injury (Clark et al., 2000b)

Injection of BV decreased pro-inflammatory cytokines production (i.e., IL-6), upregulated IL-10 levels, and reduced inflammatory lung injury in rats challenged with lipopolysaccharide (LPS). Thus, BV protected against systemic inflammation and lung injury after lethal exposure to LPS. The protective effects of BV against LPS-induced injury were observed in cultured lung endothelial cells and macrophages (Sarady-Andrews et al., 2005). Additional anti-inflammatory effects of BR have been reported in cell culture. For example, BR inhibited TNF-α dependent expression of adhesion molecules (i.e., E-selectin, VCAM-1, ICAM-1) in endothelial cells (Mazzone et al., 2009). Recently, enhanced nuclear translocation of BVR has been implicated in the anti-inflammatory effects of BV (Wegiel et al., 2011).

Bilirubin can act as an inhibitor of smooth muscle cell proliferation (Nakao et al., 2005; Ollinger et al., 2005). Exogenous BV administration inhibited neointimal hyperplasia associated with vascular injury in rats (Nakao et al., 2005; Ollinger et al., 2005). These effects were attributed to downregulation of JNK, and inhibition of endothelial cell apoptosis (Nakao et al., 2005). Similarly, hyperbilirubinemic animals were resistant to vascular injury (Ollinger et al., 2005). The anti-proliferative effects of BV and BR were demonstrated in vascular smooth muscle cell culture. Exogenous BV/BR arrested cells in G_1 phase after serum stimulation, associated with inhibition of p38 MAPK and retinoblastoma protein phosphorylation (Ollinger et al., 2005).

Experimental hyperbilirubinemia (3–10 mg/dl) induced by infusion protected against bleomycin-induced pulmonary fibrosis in rats (Wang et al., 2002). BR-infused rats displayed reduced lung injury in response to bleomycin challenge, including lowered lung hydroxyproline content, reduced polymorphonuclear lymphocyte and leukocyte counts, and reduced levels of transforming growth factor-β in bronchoalveolar lavage (Wang et al., 2002). Thus, the anti-fibrotic effects of BR may relate to both antioxidant and anti-proliferative effects of this pigment.

Recent studies have also implied a protective effect of BV/BR in diabetes. Application of BV to streptozotocin induced diabetic rats reduced urinary isoprostanes, and protected against endothelial cell sloughing (Rodella et al., 2006). BR ameliorated diabetic nephropathy, through the reduction of cytosolic ROS generation and anti-inflammatory effects (Fujii et al., 2010). Hyperbilirubinemic mice, or BV-treated diabetic mice, displayed reduced albuminuria, and urinary markers of oxidative stress relative to wild-type or untreated controls, respectively. Application of BV or BR inhibited ROS production in endothelial and mesangial cells induced by high glucose or angiotensin-II exposure (Fujii et al., 2010).

PROTECTIVE EFFECTS OF BILE PIGMENTS IN ORGAN TRANSPLANTATION

Therapeutic effects of exogenously applied bile pigments have been described in animal models of organ transplantation and acute graft rejection, including liver (Fondevila et al., 2004), kidney (Adin et al., 2005), and heart (Yamashita et al., 2004) transplantation. In the isolated perfused kidney, perfusion with BR protected against warm I/R-induced tissue injury and preserved renal function (Adin et al., 2005). BV provided tissue protection in an *ex vivo* model of cold hepatic I/R injury. Furthermore, inclusion of BV in the perfusate increased survival in rats subjected to orthotopic liver transplantation by preserving liver function (Fondevila et al., 2004). This protection conferred by BV was associated with decreased expression of pro-inflammatory indices, including neutrophil influx, pro-inflammatory cytokine expression, and iNOS activation (Fondevila et al., 2004). BV treatment improved survival of rat cardiac allografts, by reducing leukocyte infiltration and inhibiting T-cell proliferation (Yamashita et al., 2004). In transplant-associated cold I/R injury of heart and kidney grafts, the application of BV simultaneously with CO provided a synergistic tissue protection, whereas lesser effects were observed with either test agent alone (Nakao et al., 2000). BV also prevented rejection of lung grafts from brain-dead donors, which are more prone to rejection (Zhou et al., 2011). In allogeneic islet transplantation, treatment of the donor or the donor graft *ex vivo* with BR improved islet graft survival. Treatment of the recipient with BR also improved the survival of islet grafts (Wang et al., 2006b; Zhu et al., 2010). BR conferred transplant tolerance to islet grafts by up-regulating regulatory T cells (Lee et al., 2007). The anti-apoptotic and anti-inflammatory effects of BV/BR observed in I/R injury models may contribute to protection during I/R injury associated with transplantation. These experiments also suggest that pharmacological BR may have immunomodulatory functions that contribute to therapeutic effects in the setting of graft rejection (Ollinger et al., 2007).

BILIRUBIN IN CARDIOVASCULAR AND LUNG DISEASE

Recent clinical studies have reported inverse associations between serum BR levels and the risk factors associated with CVD. These studies collectively suggest that natural elevations in serum BR confer protection against CVD, including atherosclerosis, coronary artery disease (CAD)/ischemic heart disease (IHD), diabetes, and stroke (Novotný and Vítek, 2003; Franchini et al., 2010).

In one of the first studies reporting associations between BR and CVD, a study of 619 males adjusted for age-dependent risk factors, the level of serum BR was described as an inverse risk factor for CAD (Schwertner et al., 1994). BR was weakly predictive for CAD relative to lipoprotein markers (Levinson, 1997). Nevertheless, a strong correlation between BR levels and apolipoprotein B levels, a risk factor for atherosclerosis, was reported (Levinson, 1997). Serum BR levels represented an inverse risk factor for CAD in subjects with early familial CAD (Hopkins et al., 1996).

In a large-scale prospective study (7,685 men), subjects in the midrange of serum BR concentration displayed reduced risk of IHD, relative to subjects in the lowest quintile of serum BR distribution (Breimer et al., 1995). Individuals in the lowest BR quintile exhibited reduced high density lipoprotein (HDL) cholesterol, and reduced lung function. Although midlevel serum BR was associated with reduced CVD risk, the hyperbilirubinemic individuals (highest quintile) exhibited a similar risk of IHD as individuals in the lowest serum BR quintile (Breimer et al., 1995).

Serum BR levels have been indicated as an independent, inverse risk factor for peripheral vascular disease (Breimer et al., 1994). In large-scale cross-sectional studies, serum BR levels within the normal range were inversely associated with risk of peripheral vascular disease (Perlstein et al., 2008a) and stroke (Perlstein et al., 2008b). A 1.71 μM increase in BR level was associated with 6% reduction

in the odds of peripheral vascular disease (7,075 participants; Perlstein et al., 2008a), and 9% reduction in the odds of stroke (13,214 participants; Perlstein et al., 2008b).

In 72 healthy subjects, serum BR levels were inversely correlated with indicators for atherosclerosis (Erdogan et al., 2005). Low serum BR levels were associated with increased carotid artery intimal-medial thickness, and impaired flow-mediated vasodilation, indicative of endothelial dysfunction (Erdogan et al., 2005). Furthermore, BR levels were inversely correlated to carotid plaque formation in 1,774 subjects, with a reported odds ratio of 0.37 for an increase of 17.1 μM increase in BR (Ishizaka et al., 2001).

In a cross-sectional study of 2,307 Koreans, total and direct BR levels were inversely correlated to plasma levels of C-reactive protein (CRP), an indicator of vascular inflammation (Hwang et al., 2011). Similar inverse associations were reported between BR and levels of high sensitivity (hs)-CRP (Gullu et al., 2005; Yoshino et al., 2011). BR was inversely correlated with CVD in patients with hypercholesterolemia, and elevated in patients receiving statins (Nolting et al., 2011).

A prospective study (Framingham offspring study, 5,124 participants) concluded that a higher concentration of total serum BR was associated with lower risk of CVD in men, with unclear association for women (Djousse et al., 2001). This study revealed a higher risk of myocardial infarction for both men and women associated with low serum BR and low serum albumin (Djoussé et al., 2003).

Bilirubin levels have been examined as an independent predictor of CVD mortality. No association was found between serum BR and all-cause CVD mortality in a 10 year study of the Belgian population, although increased BR was associated with reduced cancer mortality in males (Temme et al., 2001). However, in a recently published study of 1,279 men, BR levels and cardiopulmonary fitness level was independently and negatively correlated to all-cause and CVD mortality (Ajja et al., 2011).

Additional studies have analyzed potential associations between BR levels and CVD-related diseases such as diabetes (Ko et al., 1996; Fukui et al., 2008, 2011; Cheriyath et al., 2010) and metabolic syndrome (Jo et al., 2011; Kwon et al., 2011). A large cross-sectional study (15,876 subjects) reported an inverse association between total BR and diabetes risk (Cheriyath et al., 2010). Patients with type II diabetes on hemodialysis displayed an increased risk of CVD associated with low serum BR, relative to diabetic patients not on hemodialysis (Fukui et al., 2011). Serum BR was inversely correlated with albuminuria in Type II diabetic patients (Fukui et al., 2008). In a Chinese cohort (1,508 subjects), low serum BR was associated with aberrations in glucose tolerance, and increases in CVD risk factors including triglycerides, very-low density lipoprotein, and glycated hemoglobin (Ko et al., 1996).

Additional studies have examined associations of BR with CVD in hyperbilirubinemia originating from metabolic disorders of BR metabolism. A TA repeat polymorphism of the UTG1A1 gene promoter (designated UGT1A1∗28, or TA7) results in reduced transcription of UTG1A and deficit in hepatic BR conjugation and clearance (Schwertner and Vítek, 2008). Individuals homozygous for UGT1A1∗28 (TA7/TA7) display a hyperbilirubinemia, referred to as Gilbert's syndrome, relative to wild-type subjects (TA6/TA6) or heterozygotes. A sixfold reduced risk of IHD and elevated HDL cholesterol was reported in a study of 50 Gilbert's patients relative to the general population (Vítek et al., 2002). In Gilbert's patients, unconjugated BR was negatively correlated with small dense low density lipoprotein cholesterol sd-LDL-C, oxidized LDL and hs-CRP (Tapan et al., 2011).

In the Framingham Heart study homozygote carriers for the UGT1A1∗28 allele with elevated serum BR concentrations displayed a strong association with lower CVD risk (Lin et al., 2006). These observations were further validated by conditional linkage and genome-wide association studies. These studies concluded that UTG1A is a major gene linked to CVD, and that the TA repeat polymorphism is strongly associated with reduced CVD risk (Lin et al., 2009). In a study of peripheral arterial disease, no association of UGT1A1 polymorphisms was observed, despite inverse correlation of CVD risk with BR levels (Rantner et al., 2008). Recent post-mortem studies also reported that UGT1A1 polymorphisms were not correlated with severity of CAD (Papez et al., 2009).

Finally, BR was inversely associated with risk of lung disease. In a cohort study (504,206 subjects) each 0.1-mg/dL increase in BR in males was associated with 9%, and 6% decreases in the risk of lung cancer, and chronic obstructive pulmonary disease, respectively (Horsfall et al., 2011).

CONCLUSION

Biliverdin and BR are naturally occurring substances derived from heme catabolism that possess antioxidant properties. Furthermore, circulating BR can contribute to serum antioxidant capacity. In pre-clinical studies, pharmacological application of BR confers protection against I/R injury, acute lung injury, pulmonary fibrosis, renal injury, and graft rejection. Several limitations of the therapeutic applications of bile pigments must be considered. Although BV is soluble in aqueous media, BR is lipophilic and soluble only in organic solvents, thereby posing challenges for therapeutic delivery.

Bilirubin is the by-product of the heme degradation pathway, and thereby may mediate the cytoprotective properties of HO-1 (Foresti et al., 2004). Pharmacological or gene therapy approaches involving the targeted expression of HO-1 are under development (Abraham et al., 2007). However, these approaches are not specific for BV/BR generation, since modulation of HO-1 has pleiotropic effects that impact systemic iron metabolism and CO generation. Similarly, targeted expression of BVR may affect cellular signaling pathways independently of its role in bile pigment generation (Kapitulnik and Maines, 2009). Clinical studies have suggested inverse associations between serum BR and CVD risk. Mild hyperbilirubinemia may reduce CVD risk, but this awaits further validation. To date, there remain no practical methodologies for inducing hyperbilirubinemia for clinical benefit. Despite therapeutic benefit in animal tissue injury models, the therapeutic potential of BR remains untested in humans.

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Bilirubin inhibits neointima formation and vascular smooth muscle cell proliferation and migration

Kelly J. Peyton[1], Ahmad R. Shebib[1], Mohammad A. Azam[1], Xiao-ming Liu[1], David A. Tulis[2] and William Durante[1]*

[1] *Department of Medical Pharmacology and Physiology, University of Missouri-Columbia School of Medicine, Columbia, MO, USA*
[2] *Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC, USA*

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Giovanni E. Mann, King's College London, UK
Adrian Manea, Institute of Cellular Biology and Pathology "Nicolae Simionescu," Romania

****Correspondence:***
William Durante, Department of Medical Pharmacology and Physiology, University of Missouri-Columbia School of Medicine, M409 Medical Sciences Building, One Hospital Drive, Columbia, MO 65212, USA.
e-mail: durantew@health.missouri.edu

Bilirubin is a heme metabolite generated by the concerted action of the enzymes heme oxygenase and biliverdin reductase. Although long considered a toxic byproduct of heme catabolism, recent preclinical, and clinical studies indicate the bilirubin exerts beneficial effects in the circulation. In the present study, we determined whether local administration of bilirubin attenuates neointima formation following injury of rat carotid arteries. In addition, the ability of bilirubin to regulate the proliferation and migration of human arterial smooth muscle cells (SMCs) was investigated. Local perivascular administration of bilirubin immediately following balloon injury of rat carotid arteries significantly attenuated neointima formation. Bilirubin-mediated inhibition of neointimal thickening was associated with a significant decrease in ERK activity and cyclin D1 and A protein expression, and an increase in p21 and p53 protein expression in injured blood vessels. Treatment of human aortic SMCs with bilirubin inhibited proliferation and migration in a concentration-dependent manner without affecting cell viability. In addition, bilirubin resulted in a concentration-dependent increase in the percentage of cells in the G_0/G_1 phase of the cell cycle and this was paralleled by a decrease in the fraction of cells in the S and G_2M phases of the cell cycle. Finally, bilirubin had no effect on mitochondrial function and ATP content of vascular SMCs. In conclusion, these studies demonstrate that bilirubin inhibits neointima formation after arterial injury and this is associated with alterations in the expression of cell cycle regulatory proteins. Furthermore, bilirubin blocks proliferation and migration of human arterial SMCs and arrests SMCs in the G_0/G_1 phase of the cell cycle. Bilirubin represents an attractive therapeutic agent in treating occlusive vascular disease.

Keywords: bilirubin, stenosis, vascular smooth muscle cells, proliferation, migration

INTRODUCTION

Bilirubin is a heme metabolite that is formed by the sequential action of heme oxygenase (HO) and biliverdin reductase. HO oxidatively degrades heme into equimolar amounts of biliverdin, iron, and carbon monoxide while biliverdin reductase rapidly metabolizes biliverdin to bilirubin. Bilirubin circulates in blood bound to albumin and eventually enters the liver where it is glucuronidated by UDP-glucuronyltransferase 1A1 (UGT1A1) and excreted into the bile ducts for elimination. Although long considered a toxic waste product of heme catabolism, bilirubin has emerged as an important protective molecule in the circulation. Early *in vitro* studies demonstrated that bilirubin is an efficient antioxidant under physiological conditions (Stocker et al., 1987a). Both free and albumin-bound bilirubin scavenges peroxyl radicals and protects low-density lipoprotein against peroxidation (Stocker et al., 1987b; Wu et al., 1996). These findings were subsequently extended to show that bilirubin reacts with other reactive oxygen and nitrogen species including hypochlorous acid, singlet oxygen, hydroxyl radical, nitric oxide, and peroxynitrite (see Stocker, 2004). Indeed, numerous studies have demonstrated a cytoprotective role for bilirubin in response to various oxidative insults (Yamaguchi et al., 1996; Clark et al., 2000; Dore et al., 2009; Wei et al., 2009; Jansen et al., 2010). In addition, bilirubin exerts critical anti-inflammatory actions in the vasculature. It attenuates endothelial expression of leukocyte adhesion molecules and prevents the rolling, adhesion, and infiltration of leukocytes into the vessel wall (Vachharajan et al., 2000; Soares et al., 2004). Given the fundamental role of oxidative stress and inflammation in the pathogenesis of vascular disease, the antioxidant, and anti-inflammatory actions of bilirubin may serve to defend against this disorder. Consistent with this notion, several observational studies have demonstrated an inverse correlation between serum bilirubin levels and coronary artery disease in humans (Schwertner et al., 1994; Hopkins et al., 1996). Similarly, high serum bilirubin is associated with a reduced incidence of atherosclerosis and hypertension (Papadakis et al., 1999; Novotny and Vitek, 2003). Remarkably, the Framingham Offspring Study revealed that men with greater serum bilirubin levels have a lower risk of cardiovascular mortality (Djousse et al., 2001). Furthermore, subjects with mild life-long hyperbilirubinemia arising from Gilbert Syndrome, a genetic disorder where the conjugation and excretion of bilirubin is impaired due to an inactivating polymorphism in the promoter region of the gene coding for UGT1A, results in a significant reduction of risk for cardiovascular death compared

to individuals without the syndrome (Hirschfield and Alexander, 2006; Lin et al., 2006).

Preclinical studies also support a beneficial role for bilirubin in vascular disease. Elevations in endogenous circulating levels of bilirubin attenuate hypertension in mice and rats while the exogenous administration of bilirubin ameliorates ischemia–reperfusion injury in various animal models (Pflueger et al., 2005; Ollinger et al., 2007b; Vera et al., 2009). Parenteral treatment with bilirubin improves endothelial function in atherogenic mice (Kawamura et al., 2005). Interestingly, neointimal lesions that develop following carotid artery balloon injury are dramatically reduced in Gunn rats, a congenital model of hyperbilirubinemia arising from defective UGT1A1 activity (Ollinger et al., 2005). Furthermore, local or systemic administration of the bilirubin precursor, biliverdin, suppresses neointima formation following injury of rat carotid arteries (Nakao et al., 2005; Ollinger et al., 2005) while *ex vivo* application of biliverdin to veins prior to grafting abolishes intimal hyperplasia in a rat arterialized vein-graft model (Nakao et al., 2005). However, it remains to be established whether the exogenous delivery of bilirubin is also able to block intimal thickening following vascular damage. Accordingly, the present study determined whether local administration of bilirubin attenuates neointima formation and the expression of cell cycle regulatory proteins in rat injured carotid arteries. In addition, this study investigated the effect of bilirubin on the proliferation and migration of human arterial smooth muscle cells (SMCs).

MATERIALS AND METHODS

MATERIALS

Sodium dodecyl sulfate (SDS), Tris, dimethyl sulfoxide (DMSO), penicillin, Tes, streptomycin, neomycin, trypan blue, propidium iodide, L-glutamine, trypsin, RNase, ethylenediaminetetraacetic acid (EDTA), bromophenol blue, and glycerol were from Sigma-Aldrich (St. Louis, MO, USA). M199 media, bovine calf serum, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Invitrogen (Carlsbad, CA, USA). Ketamine and xylazine were purchased from Butler Schein Animal Health Corporation (Dublin, OH, USA). Unconjugated bilirubin was from Frontier Scientific (Logan, UT, USA). Antibodies against cyclin D1, cyclin E, cyclin A, p21, p27, p53, and β-actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). A polyclonal antibody against HO-1 was from Assay Designs (Ann Arbor, MI, USA) while antibodies against phospho-ERK1/2 and ERK1/2 were from Cell Signaling (Beverly, MA, USA).

RAT CAROTID ARTERY BALLOON INJURY

Male Sprague Dawley rats (400–500 g) were obtained from Charles River Laboratories, (Wilmington, MA, USA) and maintained on standard rat chow and water *ad libitum* with 12 h light–dark cycles. All experimental procedures conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were approved by the institutional animal use and care committee. Animals were anesthetized with an intravenous injection of ketamine (100 mg/kg) and xylazine (7.5 mg/kg), and a Fogarty 2F embolectomy catheter (Baxter Healthcare Corporation, Deerfield, IL, USA) was introduced into the external carotid branch through an arteriotomy site and advanced to the aortic arch. The balloon was then inflated and withdrawn three times with rotation along the length of the common carotid artery, as we previously described (Tulis et al., 2001a; Granada et al., 2005; Peyton et al., 2009, 2011). The catheter was then removed, the external carotid artery ligated, overlying tissue sutured, and the skin closed with rodent wound clips. In some cases, a local polymer-based delivery system was used to administer bilirubin to the vessel wall immediately after injury and closure of the external carotid arteriotomy. Bilirubin (1 mg) was dissolved in 50 μL DMSO and mixed into 200 μL of a 25% solution of copolymer gel (Pluronic F-127; BASF Corporation, Mount Olive, NJ, USA). The bilirubin-containing gel was applied topically and circumferentially on the exposed adventitia of the carotid artery. A separate control group of animals received a vehicle (DMSO)-containing gel. At various times after arterial injury, animals were sacrificed by an anesthetic overdose followed by pneumothorax and exsanguination, and carotid arteries collected for analysis.

HISTOLOGY

Following sacrifice, the thoracic aorta was clamped and normal saline was perfused trans-cardially followed by 10% buffered formalin in phosphate-buffered saline. The carotid arteries were then excised, paraffin-embedded, and sections (5 μm) stained with Verhoeff–Van Gieson for measurement of vessel dimensions. Microscopic quantification of vessel dimensions was performed using Image-Pro Plus (Media Cybernetics Incorporated, Bethesda, MD, USA) and Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA) linked through a digital camera (QICAM Fast 1394; Hitschfel Instruments Incorporated) to an Olympus model BX41TF light microscope (Olympus America Inc., Center Valley, PA, USA), as previously described (Tulis et al., 2001a; Granada et al., 2005; Peyton et al., 2009, 2011).

WESTERN BLOTTING

Blood vessels were excised, pulverized in liquid nitrogen, and lysed in electrophoresis buffer [125 mM Tris (pH 6.8), 12.5% glycerol, 2% SDS, and trace bromophenol blue] and proteins separated by SDS-PAGE. Following transfer to nitrocellulose membranes, blots were blocked with PBS and non-fat milk (5%) and then incubated with antibodies directed against cyclin D1 (1:500), cyclin E (1:500), cyclin A (1:500), p27 (1:300), p21 (1:500), p53 (1:100), phospho-ERK(1:250), ERK (1;1000), HO-1 (1:1000), or β-actin (1:200). Blots were washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat antibodies and developed with commercial chemiluminescence reagents. Protein expression was quantified by scanning densitometry and normalized with respect to β-actin or ERK1/2.

CELL CULTURE

Human aortic SMCs were obtained commercially (Lonza Inc., Allendale, NJ, USA) and cultured serially in M199 media containing 20% bovine calf serum, 2 mM L-glutamine, 20 mM TES, and 20 mM HEPES, as previously described (Liu et al., 2011b). Culture media were supplemented with 100 units/ml penicillin and streptomycin, and cells propagated in an atmosphere of 95% air – 5% CO_2.

CELL PROLIFERATION

Vascular SMCs were seeded (2×10^4 cells) onto six-well plates and grown overnight. The next day cells were incubated with fresh culture media in the absence or presence of bilirubin. Media with appropriate additions were replenished every 2 days. Cell number determinations were made after 4 days by dissociating cells with trypsin (0.05%):EDTA (0.53 mM) and counting cells in a Beckman Z1 Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

CELL CYCLE PROGRESSION

Cell cycle progression was monitored by flow-activated cell sorting (Peyton et al., 2009). Vascular SMCs were grown in culture media in the absence or presence of bilirubin for 24 h. Cells were then collected, washed in PBS, pelleted by centrifugation ($1000 \times g$ for 5 min), suspended in 70% ethanol, and fixed overnight at 4°C. Fixed cells were then incubated with propidium iodide (50 μg/ml) and RNAse A (100 μg/ml) for 1 h at room temperature. DNA fluorescence was measured in a Becton Dickinson FACScan flow cytometer (Franklin Lakes, NJ, USA) and histograms of DNA content analyzed using Modfit (Verity Software) to determine the fractions of the population in each phase of the cell cycle (Peyton et al., 2009).

CELL MIGRATION

Vascular SMC migration was assessed using a scratch wound assay (Peyton et al., 2011). Confluent cell monolayers were scratched with a sterile 200 μl pipette tip and treated with culture media in the presence or absence of bilirubin. Cells were photographed immediately after injury and 18 h later with a digital camera (QImaging QICAM; Hitschfel Instruments, Inc., St. Louis, MO, USA). The wound area was then measured to determine the extent of cell migration.

CELL VIABILITY

Vascular cell viability was assessed by measuring the uptake of the membrane-impermeable dye trypan blue. Cells were incubated with trypsin (0.25%), collected, diluted (1:4) with trypan blue, and examined by microscopy. Viability was assessed by the fraction of cells that excluded the dye, as previously reported (Wei et al., 2009).

MITOCHONDRIAL ACTIVITY

Mitochondrial function was assessed using the tetrazolium salt MTT, which is efficiently reduced by mitochondrial dehydrogenases to a purple formazan product (Liu et al., 2011a). Cells were incubated with MTT (0.5 mg/ml) at 37°C for 4 h to allow the formation of formazan crystals. Residual MTT was removed and crystals dissolved by incubation with DMSO for 10 min. Absorbance was measured by spectrophotometry at 540 nm wavelength using a plate reader (μQuant spectrophotometer, Bio-Tek Instruments, Winooski, VT, USA). Mitochondrial activity was normalized for cell number and expressed relative to control, untreated cells.

ATP MEASUREMENT

ATP levels were determined using a CellTiter-Glo luciferase/luciferin reaction assay (Promega, Madison, WI, USA). Cells were incubated with the ATP Assay Mix Reagent containing luciferin and luciferase for 10 min at room temperature. Luminescence was measured using a GloMax™Luminometer (Promega, Madison, WI, USA) and ATP levels normalized for cell number and expressed relative to control, untreated cells.

STATISTICS

Results are expressed as mean ± SEM. Statistical analyses were performed with the use of a Student's two-tailed t-test and an analysis of variance with the Bonferroni *post hoc* test when more than two treatment regimens were compared. P values < 0.05 were considered statistically significant.

RESULTS

Balloon injury of rat carotid arteries resulted in significant neointima formation. **Figure 1A** shows representative cross-sections of perfusion-fixed, Verhoeff–Van Gieson-stained tissues obtained from animals 2 weeks after balloon injury. Animals treated with an empty gel exhibited a substantial concentric neointima but a markedly diminished neointima was observed in vessels exposed to a gel containing bilirubin. Morphometric measurements indicate that neointimal area, neointimal thickness, and neointimal/medial wall area ratio were all significantly reduced by bilirubin (**Figure 1B**). Despite a 65% reduction in neointima/medial wall ratio in bilirubin-treated vessels, medial wall area was unaffected by bilirubin. The decrease in neointima formation following the administration of bilirubin was accompanied by pronounced alterations in the expression of cell cycle regulatory proteins (**Figure 2A**). Arterial injury resulted in a significant increase in the levels of cyclin D1, cyclin A, and p21 protein, and a decrease in the expression of p27 protein in the vessel wall. However, bilirubin-treated vessels exhibited diminished expression of cyclin D1 and cyclin A protein following arterial injury whereas p27 protein expression was unaffected. Interestingly, bilirubin exposed vessels demonstrated a dramatic increase in the expression of the transcription factor p53, a moderate yet significant increase in p21 expression, and a significant decrease in ERK activation after vessel injury (**Figures 2A,B**). Arterial injury also caused a pronounced increase in the expression of HO-1 that was unaffected by the administration of bilirubin (**Figure 2C**).

In order to understand the underlying mechanism by which bilirubin inhibits neointima formation, the effect of bilirubin on the function of vascular SMCs was examined. Treatment of human aortic SMCs with bilirubin resulted in a concentration-dependent decrease in cell number beginning at 10 μM (**Figure 3A**). In contrast, bilirubin had no effect on the viability of SMCs, as assessed by trypan blue exclusion (**Figure 3B**). The antiproliferative action of bilirubin was associated with the arrest of SMCs in the G_0/G_1 phase of the cell cycle. Incubation of human aortic SMCs with bilirubin resulted in a concentration-dependent increase in the percentage of cells in the G_0/G_1 phase of the cell cycle and this was paralleled by a decrease in the fraction of cells in the S and G_2M phases of the cell cycle (**Figure 4**). Finally, bilirubin also retarded the migration of SMCs after scratch wounding. Treatment of human aortic SMCs with bilirubin caused a concentration-dependent inhibition of cell migration starting at a concentration of 20 μM (**Figure 5**). In contrast, bilirubin had no effect on mitochondrial function or cellular ATP levels (**Figures 6A,B**).

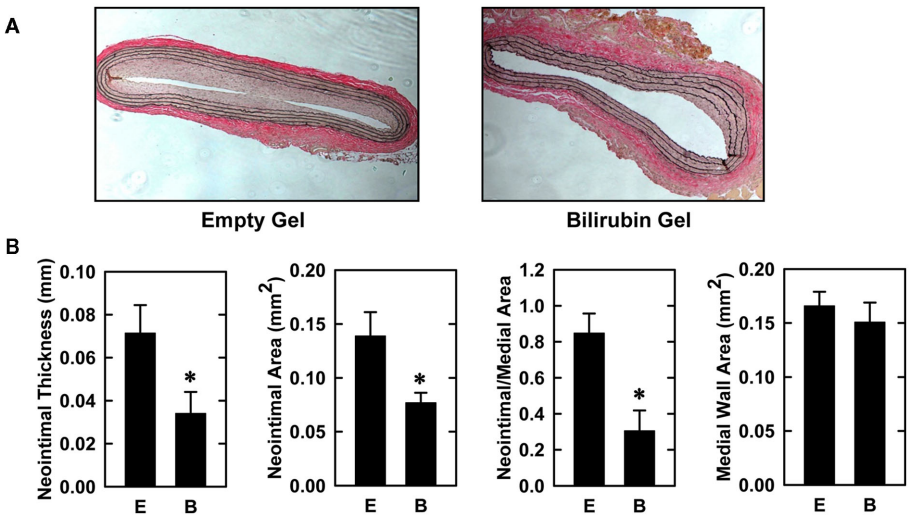

FIGURE 1 | Bilirubin inhibits neointima formation in injured rat carotid arteries. (A) Representative cross-sections of injured carotid arteries treated with an empty gel (E) or a gel containing bilirubin (B) 2 weeks after injury (original magnification, 100×). **(B)** Morphometric analysis of neointima formation 2 weeks after arterial injury. Results are means ± SEM ($n = 7$). *Statistically significant effect of bilirubin.

DISCUSSION

The present study identifies bilirubin as a critical regulator of neointima formation following arterial injury. Topical, local administration of bilirubin inhibits intimal thickening after vascular damage and this is associated with alterations in the expression of several cell cycle regulatory proteins. In addition, bilirubin blocks the proliferation and migration of human arterial SMCs in a concentration-dependent manner that is associated with the arrest of vascular SMCs in the G_0/G_1 phase of the cell cycle, independent of any change in cell viability, mitochondrial function, or intracellular ATP concentration. These findings underscore a potentially important therapeutic role for bilirubin in ameliorating occlusive vascular disease.

The current study is the first to demonstrate that exogenous application of bilirubin attenuates vascular remodeling following arterial injury. Bilirubin was applied topically to the adventitia of blood vessels using a specific local copolymer that has been successfully employed by our laboratory and others (Hu et al., 1999; Granada et al., 2005; Peyton et al., 2009, 2011). This drug delivery approach allows for the sustained release of drugs while circumventing possible non-specific or toxic effects associated with the systemic administration of drugs. This may be especially relevant in the case of bilirubin where high circulating levels of the bile pigment can lead to neurotoxicity (Ostrow et al., 2004). We found that local administration of bilirubin markedly suppresses neointima formation following arterial injury, demonstrating that this bile pigment is capable of blocking the vascular response to injury. The attenuation of lesion formation by bilirubin is associated with diminished expression of the G1 cyclins, cyclin D1 and A, which are critical for entry and progression of cells through S-phase of the cell cycle and DNA synthesis (Sherr, 1994). Interestingly, bilirubin also reduces ERK activity in injured blood vessels. Since the activation of ERK is linked with the induction of cyclin D1 expression following mitogenic stimulation (Weber et al., 1997; Villanueva et al., 2007), the ability of bilirubin to inhibit ERK activity may contribute to the downregulation of cyclin D1 expression observed with this bile pigment after arterial injury. In addition, bilirubin induces the expression of the cyclin-dependent kinase inhibitor p21 and the transcription factor p53, which are established inhibitors of SMC replication and neointimal hyperplasia (Yang et al., 1996; Sanz-Gonzalez et al., 2007). Since p53 has recently been demonstrated to directly induce the expression of HO-1 (Nam and Sabapathy, 2011), which is a key modulator of vascular remodeling (Aizawa et al., 1999; Togane et al., 2000; Duckers et al., 2001; Tulis et al., 2001a), we examined whether bilirubin-mediated increases in p53 expression in damaged blood vessels are associated with elevations in HO-1 expression. Consistent with our previous work (Tulis et al., 2001b), we found that arterial injury results in a marked elevation in HO-1 protein expression. However, this arterial injury-induced rise in HO-1 protein is unaffected by the administration of bilirubin despite a prominent increase in p53 expression, reinforcing the notion that the ability of p53 to stimulate HO-1 gene expression is both stimulus- and cell-specific (Yu et al., 1999; Nam and Sabapathy, 2011). Our *in vivo* findings showing the bilirubin disrupts ERK activity and the expression of cell cycle regulatory proteins following vascular injury are in-line with earlier studies in cultured vascular SMCs and suggest that bilirubin retards injury-induced arterial lesion formation by repressing cell cycle progression and SMC proliferation (Ollinger et al., 2005, 2007c; Stoeckius et al., 2012).

The present study also found that bilirubin is a robust inhibitor of human vascular SMC growth. Bilirubin blocks the proliferation of human aortic SMCs in a concentration-dependent manner. Significantly, bilirubin inhibits vascular SMC growth in the absence of cell death indicating that bilirubin acts via a cytostatic rather than cytotoxic mechanism. Furthermore, analysis of cell cycle distribution reveals that bilirubin inhibits cell cycle progression by

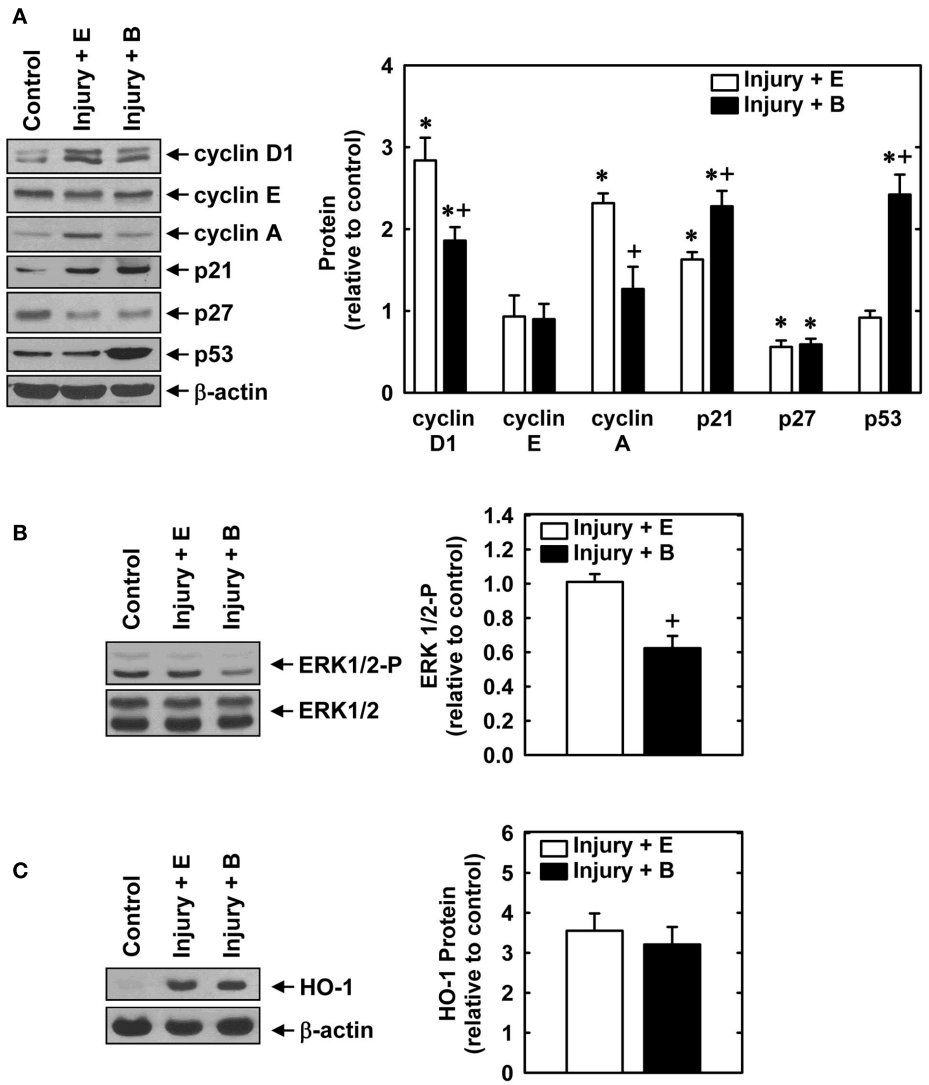

FIGURE 2 | Effect of bilirubin on the expression or phosphorylation of cell cycle regulatory proteins, ERK1/2, or HO-1 following rat carotid artery balloon injury. (A) Expression of cyclin D1, cyclin E, cyclin A, p21, p27, p53, and β-actin protein in control and injured arteries treated with an empty (E) gel or a gel containing bilirubin (B) 2 days after injury. **(B)** Expression of phospho-ERK1/2 and total ERK1/2 in control and injured arteries treated with an empty (E) gel or a gel containing bilirubin (B) 2 days after injury. **(C)** Expression of HO-1 in control and injured arteries treated with an empty (E) gel or a gel containing bilirubin (B) 2 days after injury. Quantification of relative protein levels was achieved by scanning densitometry. Results are means ± SEM ($n = 3$–5). *Statistically significant effect of arterial injury. $^{+}$Statistically significant effect of bilirubin.

specifically arresting human aortic SMCs in the G_0/G_1 phase of the cell cycle. These findings complement previous reports showing that physiologically relevant concentrations of bilirubin attenuates cell cycle progression and DNA synthesis in arterial SMCs derived from different animal species or blood vessels (Ollinger et al., 2005, 2007c; Stoeckius et al., 2012). Growth inhibitory effects of bilirubin have also been reported in human airway smooth muscle and various tumor cell lines demonstrating that the antimitogenic effect of this bile pigment is not unique to vascular SMCs (Taille et al., 2003; Rao et al., 2006; Ollinger et al., 2007a). The antiproliferative action of bilirubin likely involves multiple mechanisms. Consistent with our *in vivo* findings, bilirubin inhibits the activity of ERK, a crucial kinase involved in S-phase entry, in cultured vascular SMCs (Stoeckius et al., 2012). Moreover, inhibition of ERK activation by bilirubin has been implicated in its ability to block airway SMC growth (Taille et al., 2003). However, the transcription factor p53 appears to play an essential role in mediating the antiproliferative action of bilirubin in vascular SMCs. We found that bilirubin is a potent inducer of p53 expression in injured blood vessels. In addition, bilirubin promotes the expression of p53 in numerous cell types and deletion of p53 abrogates the antiproliferative action of bilirubin in murine SMCs (Ollinger et al., 2007a,c). Interestingly, p53 also stimulates apoptotic signaling pathways and may contribute to the apoptotic actions of bilirubin in SMCs grown in serum-free or serum-restricted conditions (Liu et al., 2002; Ollinger et al., 2007c).

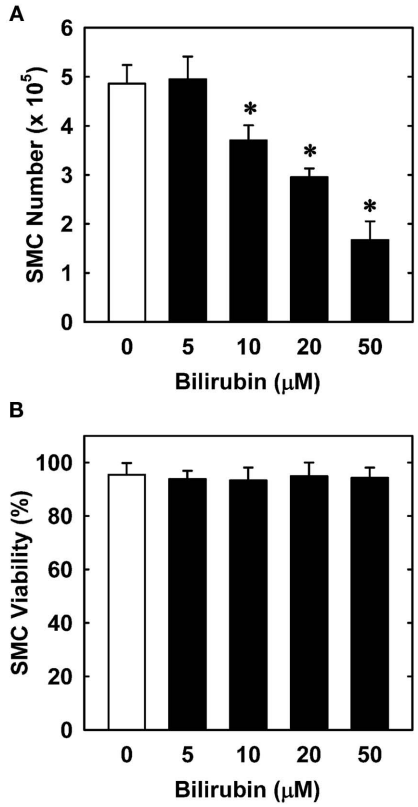

FIGURE 3 | Bilirubin inhibits the proliferation of human vascular SMCs without affecting cell viability. (A) Effect of bilirubin on the proliferation of SMCs. **(B)** Effect of bilirubin on SMC viability. Cells were treated with serum (20%) in the absence or presence of bilirubin (0–50 μM) for 4 days. Results are means ± SEM ($n = 4$–5). *Statistically significant effect of bilirubin.

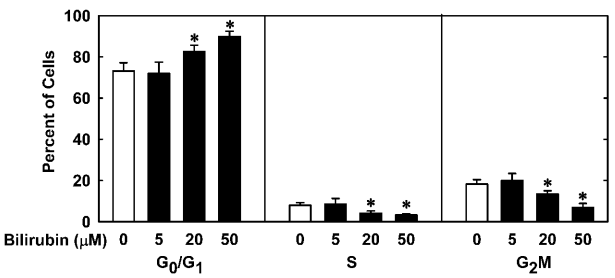

FIGURE 4 | Bilirubin inhibits cell cycle progression of human vascular SMCs. Cells were treated with serum (20%) in the absence or presence of bilirubin (0–50 μM) for 24 h. Results are means ± SEM ($n = 4$). *Statistically significant effect of bilirubin.

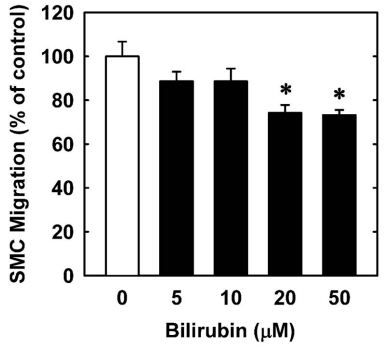

FIGURE 5 | Bilirubin inhibits the migration of human vascular SMCs. Freshly scraped cell monolayers were treated with serum (20%) in the absence or presence of bilirubin (0–50 μM) and cell migration was determined 18 h later. Results are means ± SEM ($n = 3$). *Statistically significant effect of bilirubin.

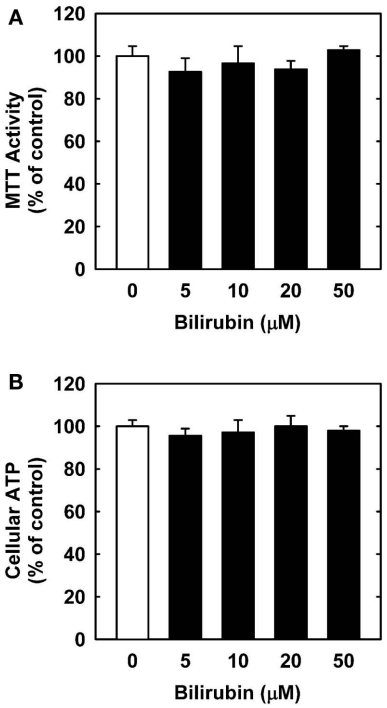

FIGURE 6 | Bilirubin has no effect on mitochondrial function or ATP content in human vascular SMCs. (A) Effect of bilirubin on mitochondrial function. **(B)** Effect of bilirubin on ATP content. Cells were treated with serum (20%) in the absence or presence of bilirubin (0–50 μM) for 24 h. Results are means ± SEM ($n = 3$).

Since vascular SMC proliferation and migration are tightly coupled processes (Fukui et al., 2000), we also investigated whether bilirubin influences vascular SMC migration. Indeed, we found that bilirubin inhibits human aortic SMC migration in a concentration-dependent manner. However, the blockade of SMC migration is not as pronounced as the inhibition of SMC proliferation and is only observed at high bilirubin concentrations. Failure of low concentrations of bilirubin to repress SMC migration is consistent with a recently published study in rat aortic SMCs (Rodriguez et al., 2010). Although the mechanism by which bilirubin attenuates SMC migration is not known it may also involve the induction of p53 since this transcription factor has been demonstrated to block SMC migration (Rodriguez-Compos et al., 2001).

In addition, p21 may also contribute to the antimigratory action of bilirubin since overexpression of p21 in vascular SMCs has been shown to inhibit their migration (Fukui et al., 1997). However, it does not appear that alterations in mitochondrial function or ATP production contribute to the antiproliferative or antimigratory effect of bilirubin.

The ability of bilirubin to inhibit neointima formation by blocking vascular SMC proliferation and migration identifies this bile pigment as an attractive agent in preventing stenosis of blood vessels following surgical interventions to treat atherosclerosis. In particular, perivascular application of bilirubin to veins or arteries prior to or immediately after grafting may promote graft survival following coronary artery bypass surgery. Alternatively, the use of bilirubin-eluting stents may reduce the incidence of restenosis following percutaneous coronary balloon angioplasty. Interestingly, a recent clinical study found that serum bilirubin is an independent predictor of coronary in-stent stenosis (Kuwano et al., 2011). Patients exhibiting elevated levels of bilirubin have a lower rate of stenosis compared to patients with low serum bilirubin, providing additional support for the use of bilirubin-coated stents in patients undergoing coronary stenting. Finally, since clinical studies show that elevations in serum bilirubin protect against coronary and peripheral artery disease and improve clinical outcomes following surgical interventions to treat vascular disease, the development of pharmacological inhibitors that raise circulating levels of bilirubin may provide a useful strategy in preventing occlusive vascular disorders (Schwertner et al., 1994; Hopkins et al., 1996; Perlstein et al., 2008; Kuwano et al., 2011). In this regard, the design of specific pharmacological inhibitors that target the conjugating enzyme UGTTA1 offers a promising approach in raising endogenous bilirubin levels (Vera et al., 2009; Durante, 2010).

In summary, we demonstrated that bilirubin inhibits neointima formation after arterial injury in the rat and the proliferation and migration of human SMCs. The antiproliferative and antimigratory actions of bilirubin are concentration-dependent and associated with the arrest of SMCs in the G_0/G_1 phase of the cell cycle. Thus, local delivery of bilirubin to blood vessels or augmentation of endogenous circulating levels of bilirubin represent promising therapeutic approaches in treating arterial disease.

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Bilirubin, renal hemodynamics, and blood pressure

David E. Stec*, Peter A. Hosick and Joey P. Granger

Department of Physiology and Biophysics, Center for Excellence in Cardiovascular – Renal Research, University of Mississippi Medical Center, Jackson, MS, USA

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Yoseph Caraco, Hadassah University Hospital, Israel
Franz Worek, Bundeswehr Institute of Pharmacology and Toxicology, Germany

****Correspondence:***
David E. Stec, Department of Physiology and Biophysics, Center for Excellence in Cardiovascular – Renal, Research, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA.
e-mail: dstec@umc.edu

Bilirubin is generated from the breakdown of heme by heme oxygenase and the reduction of biliverdin by the enzyme biliverdin reductase. Several large population studies have reported a significant inverse correlation between plasma bilirubin levels and the incidence of cardiovascular disease. Protection from cardiovascular disease is also observed in patients with Gilbert's syndrome which is a disease characterized by mutations in hepatic UGT1A1, the enzyme responsible for the conjugation of bilirubin into the bile. Despite the strong correlation between plasma bilirubin levels and the protection from cardiovascular disease, the mechanism by which increases in plasma bilirubin acts to protect against cardiovascular disease is unknown. Since the chronic antihypertensive actions of bilirubin are likely due to its renal actions, the effects of moderate increases in plasma bilirubin on renal hemodynamics as well as bilirubin's potential effects on renal tubule function will be discussed in this review. Mechanisms of action as well as the potential for antihypertensive therapies targeting moderate increases in plasma bilirubin levels will also be highlighted.

Keywords: biliverdin, heme oxygenase, biliverdin reductase, carbon monoxide, UGT1A1, liver, glomerular filtration rate, renal blood flow

INTRODUCTION

Bilirubin is derived in the plasma from the breakdown of red blood cells in the spleen. In the spleen, heme oxygenase (HO) enzymes catabolize heme released from the breakdown of red blood cells to carbon monoxide (CO) and biliverdin. Biliverdin is then reduced to bilirubin by the enzyme biliverdin reductase (BVR) and released into the blood stream. In the blood, most of the bilirubin is bound to albumin (δ bilirubin) where it travels to the liver and is conjugated by hepatic UDP-glucuronosyltransferase (UGT1A1) enzymes. Most of the conjugated bilirubin then exits the liver through the biliary ducts into the bile where it is eliminated through the digestive system. Gilbert's syndrome is a disease characterized by elevations in the levels of unconjugated bilirubin in the plasma due to mutations in hepatic UGT1A1 which decrease conjugation of bilirubin in the liver. Levels of unconjugated bilirubin in the plasma can also be increased by drugs which compete with bilirubin for conjugation by UGT1A1. Analysis of patients in the Framingham heart study concluded that mutations in UGT1A1 which resulted in moderate increases in plasma bilirubin (twofold) were associated with a decrease risk for the development of cardiovascular disease (Lin et al., 2006). This is in agreement with previous human population studies which found that moderated increases in plasma bilirubin were protective against atherosclerosis, coronary heart disease, metabolic syndrome, diabetic nephropathy, and end stage renal disease (Hopkins et al., 1996; Novotny and Vitek, 2003; Chin et al., 2009a; Han et al., 2010; Wu et al., 2011).

It is well established that the kidney's play a central role the regulation of arterial pressure. Thus, the chronic antihypertensive actions of bilirubin are likely due to its renal actions. As mentioned above, bilirubin is derived from the breakdown of heme by HO enzymes. Our knowledge of the effects of bilirubin in the kidney is mainly derived from studies in which the levels of HO enzymes have been altered. However, the results obtained from these studies are complicated by the fact that CO and bilirubin are generated in equimolar amounts by HO enzymes. This makes it impossible to separate out the contribution of each metabolite to any observed response to alterations in HO activity. Given this fact, we will mainly focus this review on the effects of increases in plasma bilirubin levels on the regulation of renal vascular and tubular function as well as the effects of plasma bilirubin on blood pressure. Studies utilizing alterations of HO enzymes will be discussed in areas in which data from models of hyperbilirubinemia are not available and which point to a potential role for bilirubin.

BILIRUBIN AND THE REGULATION OF RENAL HEMODYNAMICS

Alterations in renal hemodynamics underlie the development and maintenance of hypertension in several forms including angiotensin II (Ang II) dependent hypertension (Granger and Schnackenberg, 2000). Marked elevations in Ang II levels as observed with models of chronic Ang II infusion decrease glomerular filtration rate (GFR) due to effects on the afferent arteriole. The Gunn rat, a rodent model of severe (<20 fold) hyperbilirubinemia due to loss of hepatic UGT1A1, exhibits a significant 50% decrease in the blood pressure response to Ang II infusion. The Gunn rat also does not exhibit a significant decline in GFR in response to chronic, 4 week infusion Ang II infusion as compared to control rats (Pflueger et al., 2005). In a mouse model of moderate hyperbilirubinemia due to blockade of hepatic UGT1A1 with either indinavir, an antiretroviral protease inhibitor, or specific antisense morpholino oligonucleotides, chronic Ang II infusion did not result in a decrease in GFR (**Figure 1A**; Vera and Stec, 2010). Moderate hyperbilirubinemia also preserves renal blood

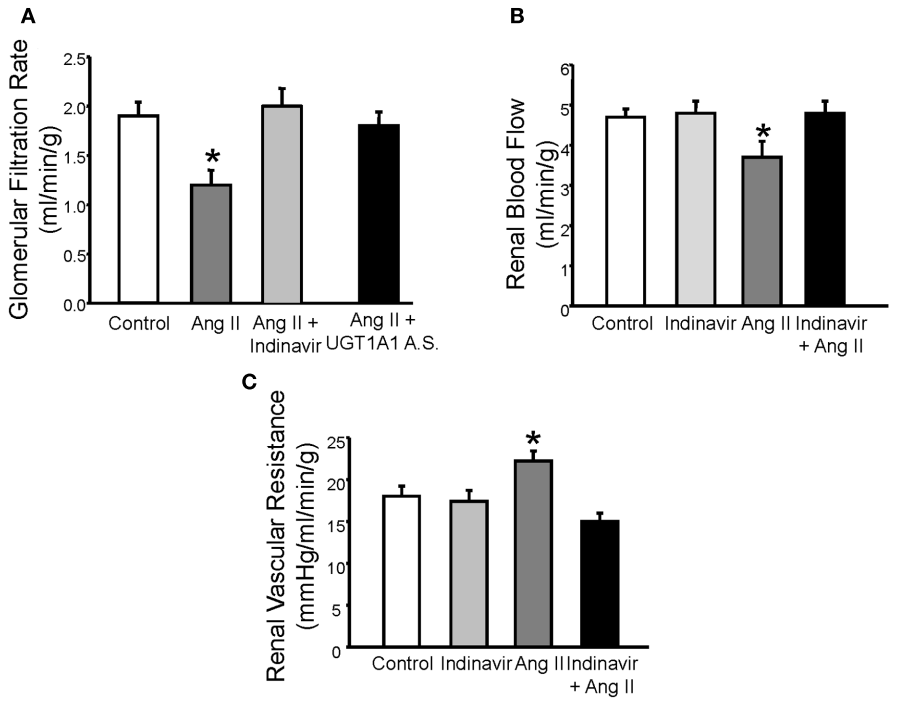

FIGURE 1 | (A) Effects of moderate hyperbilirubinemia with indinavir or UGT1A1 antisense morpholino (UGT1A1 A.S.) on glomerular filtration rate (GFR) in Ang II infused mice. Ang II infusion resulted in a significant decrease in GFR which was normalized by moderate hyperbilirubinemia. **(B)** Effect of moderate hyperbilirubinemia with indinavir on renal blood flow. Ang II infusion resulted in a significant decline in renal blood flow which was normalized by moderate hyperbilirubinemia. **(C)** Effect of moderate hyperbilirubinemia on renal vascular resistance. Renal vascular resistance was significantly increased by Ang II infusion and was normalized by moderate hyperbilirubinemia. * = $P < 0.05$ as compared to control. Figure modified from Vera and Stec (2010).

flow and normalizes renal vascular resistance in Ang II treated mice (**Figures 1B,C**; Vera and Stec, 2010).

Renal afferent arteriole regulation of GFR is accomplished by two major mechanisms; the myogenic response and tubuloglomerular feedback (TGF). The myogenic response is an endogenous response of blood vessels to constrict when stretched by increases in translumenal pressure. The myogenic constriction of the afferent arteriole is believed to help protect the glomerulus from increases in perfusion pressure (Bidani et al., 2009). The role of bilirubin in modifying myogenic vasoconstriction of the afferent arteriole has not been investigated. TGF is the second mechanism by which vascular tone of the afferent arteriole is regulated. TGF response utilizes sodium delivery to macula densa cells of the kidney in order to regulate the tone of the afferent arteriole. The macula densa cells convey a signal to the afferent arteriole to constrict in response to increases in sodium delivery to the macula densa (signal of increase in GFR) and to dilate in response to decreases in sodium delivery to the macula densa (signal of decreased GFR). Recent studies have demonstrated an important role for bilirubin (via conversion from biliverdin) in protecting against excessive TGF mediated constriction of the afferent arteriole (Ren et al., 2008; Wang et al., 2011). The mechanism by which bilirubin can protect against TGF mediated vasoconstriction is not known. It is not clear if moderate increases in plasma bilirubin can have similar effects on TGF mediated vasoconstriction or whether this response is dependent on HO generated bilirubin in the macula densa cells or in the vascular smooth muscle cells of the afferent arteriole.

Current evidence suggests that increases in bilirubin (via conversion from biliverdin) protect against excessive TGF mediated vasoconstriction and can attenuate constriction to high levels of vasoconstrictors such as Ang II; however, the mechanism by which bilirubin can maintain vascular tone is not fully understood. Bilirubin is one of the most potent antioxidants in the body (Stocker et al., 1987; Stocker and Peterhans, 1989; Bulmer et al., 2008). Given that vasoconstrictors such as Ang II can stimulate superoxide anion production in the vasculature, one potential mechanism for the protective role of moderate hyperbilirubinemia in the renal vasculature is quenching of reactive oxygen species (ROS) such as superoxide (Griendling et al., 1994; Rajagopalan et al., 1996; Hanna et al., 2002). This hypothesis is supported by the observation that Ang II mediated superoxide production is significantly attenuated in aortic ring segments from moderately hyperbilirubinemic mice (**Figure 2A**; Vera et al., 2009). Bilirubin can directly scavenge superoxide but also reduces superoxide production via direct inhibition of NAD(P)H oxidases in the vasculature (Lanone et al., 2005). The reduction of superoxide production in the vasculature by bilirubin is associated with the increase in the bioavailability of nitric oxide as reflected in the nitrate/nitrite levels in the plasma of chronically infused Ang II hypertensive

mice made moderately hyperbilirubinemic (**Figures 2B** and **3**; Vera et al., 2009). Nitric oxide (NO) can react with superoxide to produce peroxynitrite which is a potent oxidant (**Figure 3**). It is possible that moderate increases in plasma bilirubin are able to preserve renal blood flow and maintain GFR in Ang II-dependent hypertension through increases in NO; however, the importance of increased NO to this response remains to be specifically tested.

Bilirubin may also preserve renal blood flow and GFR through mechanisms independent of ROS production. For example, bilirubin can attenuate Ang II mediated increase in preproendothelin gene transcription in endothelial cells (unpublished observation). Several studies have demonstrated an important role for endothelin signaling through the endothelin A (ETA) receptor in Ang II-dependent hypertension (d'Uscio et al., 1997; Rajagopalan et al., 1997; Ballew and Fink, 2001). Thus, attenuation of Ang II mediated endothelin signaling could contribute to the preservation of renal blood flow and GFR by moderate hyperbilirubinemia. Bilirubin may also have effects on calcium handling in vascular smooth muscle cells through interactions with calcium channels or the intracellular storing and release of calcium (**Figure 3**). The effects of bilirubin on endothelin production and calcium handling need to be further explored to determine the importance of

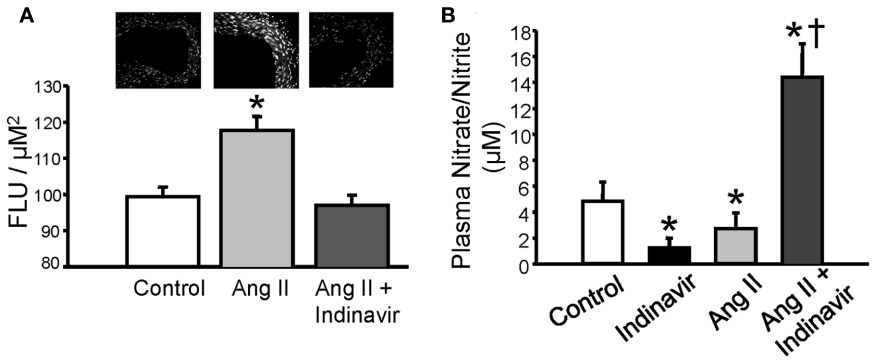

FIGURE 2 | (A) Moderate hyperbilirubinemia decrease Ang II-dependent superoxide production in aortic tissue. Superoxide levels were determined using dihydroethidium (DHE) staining of aortic tissue segments (upper panel). Ang II treatment resulted in a significant increase in aortic superoxide production which was normalized by moderate hyperbilirubinemia. **(B)** Moderate hyperbilirubinemia increases plasma NO levels in Ang II infused mice. * = $P < 0.05$ vs. Control, † = $P < 0.05$ vs. Ang II. Figure modified from Vera et al. (2009).

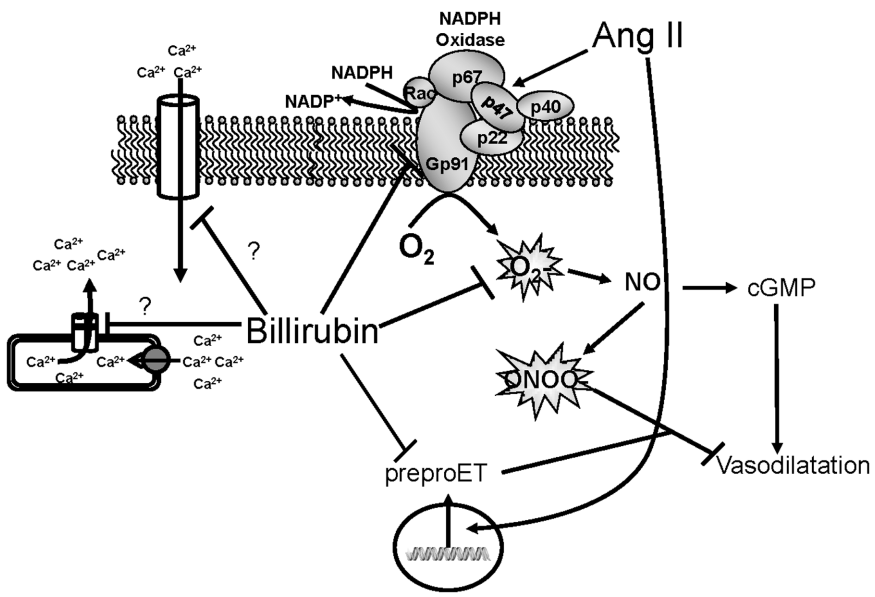

FIGURE 3 | Schematic of vascular actions of bilirubin. Ang II acts on the NADPH oxidase to increase superoxide production. Superoxide can then react with nitric oxide (NO) to form peroxynitrite which inhibits vasodilatation. Ang II can also stimulate the expression of preproendothelin (preproET) which is a vasoconstrictor. Bilirubin can directly scavenge superoxide as well as block NADPH oxidase to increase the bioavailability of NO and promote vasodilatation. Bilirubin can also inhibit Ang II mediated increases in preproET and may also block intracellular calcium through membrane and intracellular calcium channels.

these potential mechanisms which protect the renal vasculature from excessive constriction in Ang II-dependent hypertension.

BILIRUBIN AND THE REGULATION OF RENAL TUBULE FUNCTION

Very little is currently known about the role of bilirubin in the regulation of renal tubule function. Sodium and water homeostasis in models of elevated plasma bilirubin such as the Gunn rat or the moderately hyperbilirubinemic mouse has not been evaluated. The effect that increased renal bilirubin via direct infusion into the kidney has on sodium excretion is not known. Likewise, the effect of blockade of bilirubin production on sodium excretion is also unknown. Several studies in rodents have demonstrated that induction of renal HO increases sodium and water excretion while inhibition of renal HO promotes sodium reabsorption (Rodriguez et al., 2003; Li et al., 2007; Jackson et al., 2011). However, the specific role of bilirubin in this response is not known. Studies in cultured renal thick ascending loop of Henle (TALH) cells have demonstrated that blockade of bilirubin production by targeting BVR results in increased Ang II mediated superoxide production and sodium reabsorption (**Figure 4**; Young et al., 2009). This would suggest a potential role for endogenous cellular bilirubin production in the regulation oxidative stress and sodium reabsorption in the TALH.

Bilirubin may affect tubular function through similar mechanism as proposed in the renal vasculature (**Figure 3**). Several studies have reported an important role for superoxide in stimulating sodium reabsorption in the TALH (Ortiz and Garvin, 2002a; Juncos and Garvin, 2005). Superoxide can also quench the levels of nitric oxide to further increase sodium reabsorption in this nephron segment (Ortiz and Garvin, 2002b). Thus, decreases in bilirubin production via targeting of biliverdin reductase could increase sodium reabsorption in the TALH via increased superoxide production. Whether decreases in BVR activity could result in similar increases in sodium reabsorption in the TALH *in vitro* is not known. The importance of cellular bilirubin generation to the natriuresis exhibited with induction of HO-1 in the kidney is also an area which is unclear. The role of cellular bilirubin generation will require the development of new experimental models in which the activity of BVR is reduced genetically either through chronic knockdown with siRNAs *in vivo* or the creation of specific knockout mice or rats.

BILIRUBIN AND THE REGULATION OF BLOOD PRESSURE

The blood pressure lowering actions of systemic HO-1 induction have been demonstrated in several rodent models of experimental hypertension (Sacerdoti et al., 1989; Sabaawy et al., 2001; Botros et al., 2005; Vera et al., 2007; George et al., 2011). Additional studies have also revealed that alterations in renal heme oxygenase activity alone can have profound effects on the development of hypertension (Li et al., 2007; Vera et al., 2008). While the effects of alterations of HO activity on blood pressure have been established, the specific role for bilirubin generation in this response has remained elusive. As mentioned above, it is impossible to separate the generation of bilirubin from that of CO by HO enzymes so the relative importance of one vs. the other metabolite in the actions of HO on blood pressure remains unresolved. Several large scale human population studies have demonstrated an inverse relationship between plasma bilirubin levels and the incidence of hypertension (Chin et al., 2009b). As stated above, alterations in plasma bilirubin levels are also associated with other cardiovascular risk factors such as atherosclerosis, diabetic nephropathy, and renal disease all of which could contribute to the development of hypertension.

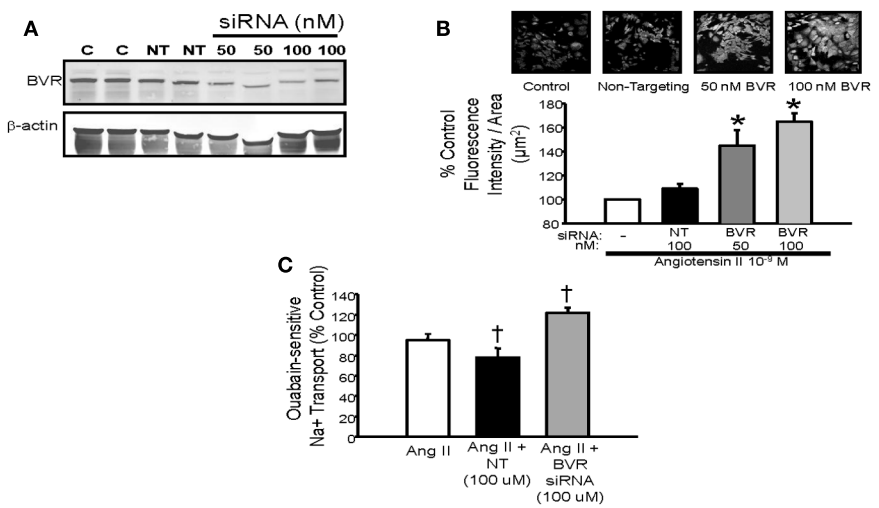

FIGURE 4 | (A) Representative Western blot of protein lysates from mouse thick ascending loop of Henle (TALH) cells treated with biliverdin reductase (BVR) or non-targeting (NT) siRNAs. Treatment with 50 or 100 nM BVR siRNA resulted in significant decrease in BVR protein. **(B)** Top panel – representative images of dihydroethidium (DHE) staining of Ang II treated TALH cells receiving BVR siRNA. **(B)** Bottom panel – treatment with 50 or 100 nM BVR siRNA resulted in significant increase in DHE staining. **(C)** Effect of BVR siRNA treatment on ouabain-sensitive sodium transport in cultured mouse TALH cells. BVR siRNA significantly increase Ang II-mediated sodium transport. $^{*}P < 0.05$ as compared control. $^{\dagger}P < 0.05$ vs. Ang II. Figure modified from Young et al. (2009).

Although it is difficult to determine the specific role of bilirubin in the blood pressure response to alterations in HO activity, several studies have investigated the specific role of plasma bilirubin levels in the development of hypertension using experimental animal models in which confounding factors such as atherosclerosis, diabetes, and renal injury can be carefully controlled. As mentioned above, the Gunn rat is a model of severe hyperbilirubinemia characterized by very high levels of plasma bilirubin resulting from decreased hepatic conjugation due to loss of the UGT1A1 gene (van et al., 1968; van der Wegen et al., 2006). The Gunn rat has been demonstrated to be resistant to the development of both Ang II and deoxycorticosterone acetate (DOCA)-salt dependent hypertension (Pflueger et al., 2005; Nath et al., 2007). The resistance to the development of hypertension in the Gunn rat was associated with preservation of NO dependent vasorelaxation which is altered in both of these forms of hypertension. Both of these forms of hypertension are also characterized by increases in superoxide anion production which is significantly attenuated in the Gunn rat. The results obtained in the Gunn rat clearly demonstrate the difficulty in obtaining a hypertensive response to known pressors; however, the level of bilirubin in the plasma of these rats is significantly higher than those reported to be beneficial in humans (Hopkins et al., 1996; Lin et al., 2006).

In order to determine if physiologic increases in bilirubin (∼twofold) could have similar effects on the development of hypertension, our lab created a mouse model of moderate hyperbilirubinemia achieved through targeting of hepatic UGT1A1 either with a drug which competes with bilirubin for conjugation (indinavir) or antisense oligonucleotides directed against UGT1A1. We were able to find concentrations of these agents which resulted in two to threefold increases in the levels of plasma unconjugated bilirubin (**Figure 5A**) which are in contrast with the greater than 20 fold elevations in unconjugated observed in the Gunn rat (van der Wegen et al., 2006). Using this mouse model, we demonstrated that moderate hyperbilirubinemia achieved with indinavir prevented the development of Ang II mediated hypertension (**Figure 5B**; Vera et al., 2009). Similar effects on the development of Ang II hypertension were also observed in mice which received direct intravenous infusion of bilirubin which increased the levels of unconjugated bilirubin to levels observed in indinavir treated mice (Vera et al., 2009). As mentioned above, moderate hyperbilirubinemia in this mouse model was also associated with increases in renal blood flow and GFR as well as decreases in renal vascular resistance and vascular superoxide production. These results indicate that selective targeting of hepatic UGT1A1 to increase plasma unconjugated bilirubin levels by two to threefold may be a novel antihypertensive approach. In another study, intraperitoneal injection of bilirubin was found to significantly decrease Ang II mediated proteinuria by 60% while having no significant effect on Ang II-dependent hypertension (LeBlanc et al., 2010). The results of this study suggest that alterations in bilirubin may have beneficial effects on the kidney independent of changes in blood pressure. However, since no measurements of plasma or renal bilirubin levels were made in the treated animals; it is difficult to determine whether the bilirubin treatment protocol was sufficient to elevate the levels of unconjugated bilirubin in the plasma enough to lower blood pressure in this model. Nonetheless, this study indicates that bilirubin may have renoprotective effects in the absence of any changes in blood pressure.

FUTURE DIRECTIONS AND PERSPECTIVES

Bilirubin either derived intracellularly by generation through actions of HO or in the plasma regulated by the activity of hepatic UGT1A1 has been demonstrated to play an important role in the regulation of blood pressure as well as renal vascular and tubular function. The challenge in the future is to determine the mechanism by which the levels of bilirubin in both the cell and plasma regulate renal vascular and tubular function in the hopes of creating new targets for therapies. The understanding of cellular bilirubin generation in the regulation of renal function will rely on targeting BVR in the kidney either through siRNA mediated

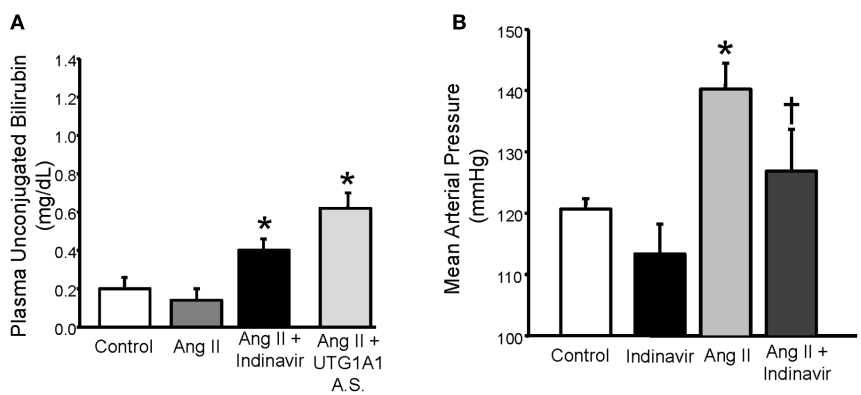

FIGURE 5 | (A) Effect of indinavir or UGT1A1 antisense morpholino oligonucleotides on plasma unconjugated bilirubin levels in Ang II treated mice. **(B)** Effect of moderate hyperbilirubinemia with indinavir treatment on blood pressure in Ang II infused mice. Mice were made moderately hyperbilirubinemic by treatment with Indinavir (500 mg/kg/day, oral gavage) starting 3 days before the implantation of minipumps that delivered Ang II at 1 μg/kg/min and continuing throughout the 12 day infusion. Blood pressure was measured in conscious freely moving mice by fluid filled catheters. Ang II infusion increased blood pressure and this increase was significantly attenuated by moderate hyperbilirubinemia. $^{*}P < 0.05$ as compared control. $^{\dagger}P < 0.05$ vs. Ang II. Figure modified from Vera et al. (2009).

viral approaches or the creation of genetically modified mice or rats. Interestingly, a recent human population study correlated a loss of function mutation in the BVR-A gene with increases in blood pressure suggesting a potential role for this enzyme in the regulation of blood pressure (Lin et al., 2011).

The manipulation of plasma levels of bilirubin is an area which could be further explored as a potential therapeutic approach for the treatment of hypertension. Targeting the conjugation of bilirubin in the liver via UGT1A1 has the potential to be one mechanism to increase plasma bilirubin levels; however, given the important role of UGT1A1 in the elimination of drugs from the body other potential targets in the handling of bilirubin by the liver may need to be developed. For example, modification of proteins responsible for the transport of bilirubin into and out of hepatocytes may be another potential target to increase plasma levels of unconjugated bilirubin for therapeutic purposes. Before targeting of hepatic bilirubin metabolism can be considered for a therapeutic approach to lower blood pressure and improve renal vascular function, the mechanism by which increases in plasma bilirubin levels act to lower blood pressure and improve renal vascular function need to be identified. Determination of these mechanisms may take several years to develop. It is possible that bilirubin may act as more than just an antioxidant to elicit its effects on renal hemodynamics and blood pressure. This possibility also needs to be further tested before targeting of hepatic bilirubin metabolism can move forward as a potential therapeutic option for hypertension. What is clear is that bilirubin has moved beyond a simple component of the plasma responsible for jaundice, to a complex molecule with several beneficial properties that could be exploited for treatment of hypertension and renal disease.

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Physiological antioxidative network of the bilirubin system in aging and age-related diseases

Sung Young Kim and Sang Chul Park*

Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon, South Korea

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Mahin D. Maines, University of Rochester School of Medicine, USA
Shlomo Sasson, The Hebrew University of Jerusalem, Israel

***Correspondence:**
Sang Chul Park, Lee Gil Ya Cancer and Diabetes Institute, Gachon University, 7-45 Songdo dong, Yeonsu gu, Incheon 406-849, South Korea.
e-mail: blueocean@gachon.ac.kr

Oxidative stress is detrimental to life process and is particularly responsible for aging and age-related diseases. Thus, most organisms are well equipped with a spectrum of biological defense mechanisms against oxidative stress. The major efficient antioxidative mechanism is the glutathione system, operating a redox cycling mechanism for glutathione utilization, which consists of glutathione and its peroxidase and reductase. However, this system is mainly effective for hydrophilic oxidants, while lipophilic oxidants require another scavenging system. Since many age-related pathological conditions are related to lipid peroxidation, especially in association with the aging process, the physiological role of the scavenging system for lipophilic oxidants should be considered. In this regard, the biliverdin to bilirubin conversion pathway, via biliverdin reductase (BVR), is suggested to be another major protective mechanism that scavenges lipophilic oxidants because of the lipophilic nature of bilirubin. The efficiency of this bilirubin system might be potentiated by operation of the intertwined bicyclic systems of the suggested redox metabolic cycle of biliverdin and bilirubin and the interactive control cycle of BVR and heme oxygenase. In order to combat oxidative stress, both antioxidative systems against hydrophilic and lipophilic oxidants are required to work cooperatively. In this regard, the roles of the bilirubin system in aging and age-related diseases are reassessed in this review, and their interacting networks are evaluated.

Keywords: bilirubin, biliverdin reductase, heme oxygenase, aging, cellular senescence, reactive oxygen species, antioxidant, lipid peroxidation

INTRODUCTION

Oxidative stress is the result of an imbalance between generation and scavenging of reactive oxygen species (ROS). Old cells have higher levels of ROS than young cells (Hagen et al., 1997; Lee et al., 1999). Senescent states of cells can be readily induced by sub-lethal doses of pro-oxidants (Chen and Ames, 1994; Chen et al., 1998). Thus, any adjustment of the ROS balance is expected to prevent or restore cellular senescence.

Biliverdin reductase (BVR) is an evolutionarily conserved enzyme, converting biliverdin to bilirubin, the potent physiologic antioxidant (Schluchter and Glazer, 1997; Baranano et al., 2002; Sedlak et al., 2009). Bilirubin protects cells against high concentrations of hydrogen peroxide (H_2O_2), for which a redox cycle that amplifies the oxidation of bilirubin into biliverdin, which is then recycled back into bilirubin by BVR, has been proposed (Baranano et al., 2002; Sedlak and Snyder, 2004). Biliverdin is a cleavage product of heme by heme oxygenase (HO). The HOs are heat-shock protein-32 family proteins, comprising a constitutive isoform (HO-2) and an inducible isoform (HO-1). Since BVR can regulate HO-1/HO-2 expression, these intertwined relationships among enzymes of HO and BVR and their metabolites, biliverdin, and bilirubin, may potentiate the efficiency of the cellular physiological defense capacity against oxidative stress. Therefore, it is necessary to assess the physiological role of BVR and the heme degradation pathway in oxidative stress-related phenomena and related diseases. Furthermore, it has recently been reported that knock-down of BVR could induce cellular senescence (Kim et al., 2011a). This relationship of BVR with the aging process, as well as other oxidative stress-associated disorders, led us to consider its roles in age-related diseases as well.

CATALYTIC ROLE OF BILIVERDIN REDUCTASE IN THE BILIVERDIN TO BILIRUBIN PATHWAY

The HO/BVR pathway is the main process for heme degradation and is evolutionarily conserved for control of oxidative stress. Two isozymes of BVR have been characterized in humans: the fetal form, biliverdin reductase B, and the adult form, biliverdin reductase A. Biliverdin reductase A (the major enzyme and hereafter, abbreviated as BVR) is an abundant and ubiquitously expressed enzyme with a high turnover rate. BVR can protect cells against oxidative stress by two different mechanisms: by converting biliverdin to bilirubin and by regulating HO expression (Kravets et al., 2004; Sedlak and Snyder, 2004; Ding et al., 2011). Bilirubin has a strong antioxidant activity, protecting cells against 10- to 1000-fold higher concentrations of H_2O_2 (Sedlak et al., 2009). This high efficiency of bilirubin as a potent antioxidant is proposed to be amplified by the cyclic conversion of bilirubin and biliverdin: the reduction of biliverdin to bilirubin by BVR and the oxidation of bilirubin to biliverdin by lipophilic ROS (Baranano et al., 2002; Sedlak and Snyder, 2004). Despite the nature of recycling bilirubin to biliverdin by peroxyradicals has been questioned recently, the

strong antioxidant effects of biliverdin and bilirubin are evident (McDonagh, 2010).

The major antioxidative defense system *in vivo* is the glutathione redox cycling system, comprised of glutathione and its two redox enzymes, glutathione peroxidase and glutathione reductase. This system is primarily effective for hydrophilic ROS, because of the hydrophilic nature of glutathione. In contrast, the biliverdin and bilirubin pathway is expected to be effective against lipophilic ROS, because of the lipophilic nature of bilirubin. Therefore, this biliverdin to bilirubin pathway system is presumed to be complementary to the glutathione redox cycling system for scavenging both hydrophilic and lipophilic ROS.

The heme degradation pathway provides two enzymatic antioxidants (i.e., HO and BVR) and two hydrophobic antioxidants (i.e., bilirubin and biliverdin) that contribute to the powerful antioxidative mechanism of an organism. The lipophilic nature of bilirubin alone would provide specific protective activity against lipid soluble metabolites, such as stability of phospholipids in multi-lamellar liposomes and rat liver microsomes (Bliuger et al., 1985; Stocker and Ames, 1987; Stocker et al., 1990). Exogenous bilirubin can reduce lipid peroxidation in the heart and kidney and can protect thymus cells against ultraviolet- or sphingosine-mediated apoptosis (Dudnik et al., 2001). Both unconjugated and conjugated bilirubin can protect low density lipoprotein (LDL) against peroxidative attack by scavenging peroxyl radicals (Wu et al., 1996).

META-CATALYTIC ROLES OF BILIVERDIN REDUCTASE

Besides its function as a reductase, BVR has pleiotropic functions in cell signaling, cell metabolism, and gene control. BVR is a dual-specificity (serine/threonine/tyrosine) kinase, involved in various cellular functions (Maines, 2005; Kapitulnik and Maines, 2009). One of the pathways modulated by BVR is the insulin signaling pathway. BVR is a substrate for insulin receptor tyrosine kinase (IRK) activity as well as a kinase for serine phosphorylation of insulin receptor substrate-1 (IRS-1; Lerner-Marmarosh et al., 2005; Wu et al., 2008; Maines, 2010). BVR can also enhance phosphatidyl inositol 3-kinase (PI3K)/Akt activity by binding to SH2 domains, which has been considered a new mechanism of insulin resistance (Lerner-Marmarosh et al., 2005). In addition, BVR plays a role as a carrier protein for nuclear signal transduction, as it has a nuclear localization signal within the carboxy terminal end of its reductase domain. Since BVR has mitogen activated protein kinase (MAPK) docking consensus sequences, it interacts with the MAPK family, in particular, the extracellular signal-regulated kinases 1/2 (ERK1/2), and functions as a nuclear transporter of ERK (Lerner-Marmarosh et al., 2008). BVR is activated and localizes into the nucleus in response to various stress signals, such as bacterial lipopolisaccharide (LPS) and bromobenzene (Maines et al., 2001). In the nucleus, BVR, being a leucine zipper-like DNA binding protein, binds not only to activator protein-1 (AP-1) but also to cyclic adenosine monophosphate (cAMP) response element sites (Kravets et al., 2004; Tudor et al., 2008). Thereby, BVR can bind to the *HO-1* promoter, an AP-1 regulated gene, regulating HO-1 induction in response to oxidative stress (Tudor et al., 2008). BVR is also involved in the inflammatory response via adjusting the levels of PI3K and Akt (Wegiel et al., 2009).

LIPID PEROXIDATION IN AGE-RELATED DISEASES

Since the structure of polyunsaturated fatty acids (PUFAs) is of a bis-allylic nature, lipids are prone to oxidation. Once lipid peroxidation is initiated, a chain reaction is propagated until terminal products are produced. Peroxyl radicals are rearranged via cyclic reactions to endoperoxides (Marnett, 1999). Scission of the oxidized PUFA results in formation of two aldehyde products: phospholipid aldehydes, such as oxidized phosphatidylcholine (OxPC), and α,β-unsaturated aldehyde cleavage fragments, including malondialdehyde (MDA), 4-hydroxynon-enal (HNE), and 4-oxo-2-non-enal (ONE; Kadl et al., 2004; Adibhatla and Hatcher, 2010). Compared to free radicals, the aldehydes, such as MDA, 4-HNE, and other aldehydes, are moderately stable and can diffuse within the cell or be extruded extracellularly to attack distant targets. They show a very high reactivity toward biomolecules, such as proteins, phospholipids and DNA, leading to a variety of intramolecular and intermolecular covalent adducts. At the membrane level, proteins and amino lipids can be covalently modified by lipid peroxidation products, resulting in damages of membrane structure. In addition, these aldehydes can also act as bioactive molecules in physiological and/or pathological events (Catalá, 2009). Therefore, the cumulative damages of these aldehyde products deteriorate cell integrity and function which may eventually result in cellular senescence. Higher levels of lipid peroxidation have been observed in plasma and tissues of aged organisms, including liver, brain, lung, kidney, and muscle (Bourre, 1988; Mizuno, 1990; Pansarasa et al., 1999; Poon et al., 2004; Ward et al., 2005; Gil et al., 2006).

LIPID PEROXIDATION IN CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVD), a family of diseases that include atherosclerosis, coronary heart disease, hypertension, and stroke, are the main causes of death in developed countries. The thickening and stiffening of the arteries in atherosclerosis are due to fatty plaques and mineral deposits, resulting in a shortage of blood supply to the myocardium. Lipid peroxidation markers, such as oxidized low density cholesterol, MDA, 4-HNE, dienoic acid, hydroxyoctadecadienoic acids, and oxidized cholesterol linoleate, are increased in the plaques and blood of atherosclerotic patients (Kuhn et al., 1992; Ahotupa and Asankari, 1999; Valko et al., 2007; Ravandi et al., 2011). A progressive increase of lipid peroxidation in the heart is also observed with aging (Miro et al., 2000). Oxidative stress in cardiac and vascular myocytes has been suggested to be linked with cardiovascular tissue injury (Dhalla et al., 2000). Lipid peroxidation and protein carbonylation lead to disruption of the membrane lipid bilayer and functional deterioration of cellular proteins, eventually resulting in abnormalities of subcellular organelles (Kaneko et al., 1989; Stoyanovsky et al., 1997; Molavi and Mehta, 2004; Valko et al., 2007). In addition, the significant pooling of iron in atherosclerotic lesions implies that the iron-catalyzed formation of free radicals may take place in the development process of atherosclerosis (Yuan and Li, 2003). Therefore, the role of lipophilic antioxidative mechanism for preventing age-related CVD should be considered and evaluated.

LIPID PEROXIDATION IN NEUROLOGICAL DISORDERS

The brain is vulnerable to oxidative damage due to its high lipid content, high oxygen consumption, high concentration of

redox-active metals (e.g., Cu and Fe), and relatively low antioxidant capacity. Therefore, this organ is susceptible to lipid peroxidation (Markesbery and Lovell, 2007). As a consequence, the increased oxidative stress that occurs with aging in the brain may be responsible for the pathogenesis of age-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Niemann–Pick's disease (NPC), and brain traumas (Klein and Ackerman, 2003; Adibhatla and Hatcher, 2007; Zhu et al., 2007). AD, a neurodegenerative disorder of cognitive and memory decline, is represented by a marked accumulation of amyloid-β peptide (Aβ), the main constituent of senile plaques, and deposition of neurofibrillary tangles (Butterfield and Lauderback, 2002; Butterfield et al., 2006). Formation of Aβ is partially induced by oxidative stress, as illustrated by the increased lipid peroxidation, the decreased PUFA content, and the increased MDA and 4-HNE in AD ventricular fluid (Butterfield and Lauderback, 2002; Butterfield et al., 2006; Recuero et al., 2010). The increased lipid peroxidation precedes amyloid plaque formation in an AD animal model (Pratico et al., 2001). Apolipoprotein E (ApoE) is subject to free radical attack and a direct correlation between ApoE peroxidation and AD has been reported (Butterfield and Lauderback, 2002). PD is the second most prevalent age-related neurodegenerative disease after AD. PD is characterized by the selective loss of dopaminergic neurons in the substantia nigra (Lotharius and Brundin, 2002). Oxidative stress-related dopaminergic neuronal damage is the leading theory of PD pathogenesis (Gonzalez-Fraguela et al., 1998; Miller et al., 2009). The important sources of ROS in PD are activation of phospholipases, including cPLA2, and induction of NADPH oxidase in activated microglia (Miller et al., 2009). HD is a rare, inherited neurological disorder, characterized by abnormal body movements and lack of coordination. Although the effect of lipid peroxidation is debatable with respect to the model of HD and type of tissue under consideration, elevated levels of 8-OHdG and HNE in plasma and MDA in striatal and cortical tissues, as well as increased F2-isoprostane, are present in HD (Mariani et al., 2005). In addition, many other neurological diseases are associated with accumulated lipid peroxidation in specific tissues: the spinal cord motor neurons of sporadic ALS patients (Simpson et al., 2004), spinal cord injury, and central nervous system (CNS) injuries, such as stroke, traumatic brain injury (Adibhatla et al., 2006; Adibhatla and Hatcher, 2008), autoimmune diseases, such as MS, all the inflammatory CNS tissues (Muralikrishna Adibhatla and Hatcher, 2006; Qin et al., 2007), and CA1 hippocampal neurons after transient cerebral ischemia (Muralikrishna Adibhatla and Hatcher, 2006). Therefore, in order to develop any strategy to prevent age-related neurological disorders, it is necessary to activate or potentiate the defense mechanism against lipophilic oxidants.

LIPID PEROXIDATION IN OTHER AGE-RELATED DEGENERATIVE DISEASES

Involvement of lipid peroxidation in cardiovascular or neurodegenerative diseases is well established, but the connection of lipid peroxidation with other age-related diseases, such as type II diabetes mellitus and cancer, has not been properly elucidated. Increased oxidative stress has been proposed to be one of the major causes of the hyperglycemia-induced trigger of diabetic complications (Maritim et al., 2003). Hyperglycemia triggers ROS formation from a variety of sources, such as glucose autoxidation, oxidative phosphorylation, NAD(P)H oxidase, lipoxygenase, cytochrome P450 monooxygenases, xanthine oxidase (XO), and nitric oxide synthase (Valko et al., 2007). Among them, XO is one of the major sources of ROS in diabetes mellitus, since treatment of non-insulin dependent diabetic patients with allopurinol, an XO inhibitor, reduces the level of oxidized lipids in plasma and improves blood flow (Butler et al., 2000). Lipoxygenase catalyzes conversion of arachidonic acid into a broad class of signaling molecules, such as leukotrienes, lipoxins, and hydroxyeicosatetraenoic acid. Diabetes is associated with increased lipoxygenase expression, resulting in increased eicosanoid formation (Brash, 1999). β-cells are particularly sensitive to ROS, because they are low in antioxidant enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase, which leads to β-cell dysfunction (Kaneto et al., 1999; Evans et al., 2003). Cancer is a disease that involves a multi-step process of mutations and preferential clonal expansion of highly neoplastic, mutated cells. It is clear that ROS is associated with genomic instability, which predisposes to mutagenesis and carcinogenesis. It has been reported that MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats, and HNE is weakly mutagenic, but appears to be the major toxic product of lipid peroxidation (Basu and Marnett, 1984; Plastaras et al., 2000; Riggins and Marnett, 2001). In addition, MDA correlates with the extent of primary tumor and predicts poor prognosis of oropharyngeal cancer (Salzman et al., 2009). Thereby, in order to prevent these degenerative diseases, the protective mechanism of lipophilic antioxidants should be effectively activated as well.

PREVENTIVE ROLES OF BILIRUBIN SYSTEM AGAINST AGE-RELATED DISEASES

Several lines of evidences have demonstrated the negative relationship of BVR activity and bilirubin concentration with the aging process. An age-dependent decrease in the activity of BVR was detected (Maines, 1990). Together with BVR, HO forms a powerful protective system against various oxidative stresses. But HO-1 induction by various oxidative stressors, such as H_2O_2 and hemin, is markedly impaired in senescent human fibroblasts (Kim et al., 2011a). Serum bilirubin concentration is highest in the 19- to 24-year-old age group, after which it declines, which might be related to the age-related decrease in BVR activity (Rosenthal et al., 1984). Moreover, since oxidized BVR was associated with impairment of its function (Barone et al., 2011a), the age-related increase in ROS generation might influence BVR activity.

With regard to age-related diseases, bilirubin has a significant and beneficial role in preventing oxidative changes in a number of diseases, including atherosclerosis, neurodegenerative disease, diabetes mellitus, and cancer as well as a number of inflammatory and autoimmune diseases. The mild to moderately elevated levels of serum bilirubin are positively related to better outcomes of CVD. For example, the male air force pilot study showed that a 50% decrease in total bilirubin was associated with a 47% increase in the more severe coronary artery diseases (Schwertner et al., 1994). Low serum bilirubin in patients with CVD was

also detected in the Framingham Offspring Study (Djousse et al., 2001, 2003). In addition, positive effects of serum bilirubin were illustrated in the study of Gilbert's syndrome. Gilbert's syndrome is the most common hereditary genetic disorder of impaired glucuronyl transferase activity causing mild to moderate elevations of serum bilirubin and is found in up to 5% of the population. The prevalence of the ischemic heart diseases in Gilbert's syndrome was found to be only 2%, compared with 12% in control individuals (Vitek et al., 2002). It is interesting to note that cigarette smoking, a major risk factor for CVD, was associated with significantly lowered serum bilirubin content (Van Hoydonck et al., 2001). Both unconjugated and conjugated bilirubin can protect human LDL against oxidation by oxy-radicals generated by 2,2′-azo-bis(2-amidinopropane) or Cu (Wu et al., 1996). It seems like BVR/HO-2 link plays an important role in cardiac protection. Recently, it is suggested that BVR would be an upstream stabilizer of HO-2, which prominently expressed in the cardiovascular system, and both entities are intimately linked to cardiomyocyte survival (Ding et al., 2011). There are numerous possible mechanisms by which the HO-1/HO-2 pathway may improve vascular function. It has been reported that HO-2 activation occurs in ischemic hearts in dogs and that inhibition of the HO system reduces vasodilation during ischemia in the presence of NO and COX inhibitors (Nishikawa et al., 2004).

With regard to neurodegenerative disorders, bilirubin is a potent antioxidant against the cellular damage elicited by ROS and contributes to the overall antioxidant network of the brain (Takahashi et al., 2000; Mancuso, 2004). Impairment of the HO/BVR system in the hippocampus of AD, as well as a linkage of BVR with an improvement of cognitive function following atorvastatin treatment, have been reported (Barone et al., 2011b). All these effects contribute to the neuroprotective role of BVR in the brain. In addition, a low concentration of serum bilirubin is related to an increased risk of ALS and mental illnesses, such as winter depression and schizophrenia (Oren et al., 2002; Ilzecka and Stelmasiak, 2003; Iwasaki et al., 2005). HO-2 is selectively enriched in neurons and it is becoming apparent that HO-2 play an important role in cytoprotection in neural tissues. HO-2 expression has been shown to be protective against apoptotic cell death in cortical, hippocampal, and cerebellar granule cultures and an *in vivo* model of ischemic injury (Dore and Snyder, 1999; Dore et al., 1999a, 2000). In the brain, it is suggested that HO-derived CO would function as a neurotransmitter/neuromodulator (Maines et al., 1993; Seki et al., 2000). HO-2 protects against lipid peroxidation mediated cell loss as well as the impaired motor recovery after traumatic brain injury (Chang et al., 2003). Furthermore, a link has been proposed between HO activities and neurodegenerative conditions such as familial AD. Single point mutations in amyloid precursor proteins, binding to HO-1/HO-2, have been associated with a significant reduction in HO activities, resulting in greatly reduced bilirubin levels and increased neurotoxicity (Takahashi et al., 2000). The neuroprotective effects of HO-2 seem to be attributed to the generation of bilirubin (Dore et al., 1999b, 2000).

In diabetes mellitus, a higher level of serum bilirubin is associated with a lower risk of the disease (Fukui et al., 2008, 2011; Han et al., 2010; Dekker et al., 2011). Using the National Health and Nutrition Examination Survey (NHANES) data, an inverse relationship of serum total bilirubin and the incidence of diabetes has been suggested (Cheriyath et al., 2010).

Although controversial, there are some evidences that serum bilirubin may be protective against some forms of cancer. Baseline serum bilirubin concentration was inversely associated with the risk of cancer development (Ko et al., 1994). Cross-sectional analysis demonstrated that an increase in serum bilirubin was associated with a lower rate of colorectal cancer (Zucker et al., 2006). In addition, a possible beneficial role of bilirubin was also described in several diseases, illustrated by the low serum bilirubin levels detected in various oxidative stress-mediated diseases, such as rheumatoid arthritis, systemic lupus nephritis, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (Ohrui et al., 2001; Fischman et al., 2010; Horsfall et al., 2011).

PHYSIOLOGICAL ADJUSTMENT OF THE AGING PROCESS BY BILIRUBIN SYSTEM

The Hayflick limit describes the phenomenon of normal mitotic cells undergoing a finite number of cell divisions before entering replicative senescence, after which they cannot divide further and become unresponsive to mitogenic stimuli (Hayflick, 1965). In addition to replicative senescence, cells can also be induced to become prematurely senescent by exposure to oxidants, DNA damaging agents, histone deacetylase inhibitors, or by overexpression of certain oncogenes, which is referred to as stress-induced premature senescence (Chen et al., 1995; Serrano et al., 1997; Robles and Adami, 1998; Zhu et al., 1998). Regardless of the induction mode, senescent cells generally acquire the enlarged and flattened morphology with high expression of senescence-associated β-galactosidase and lose the ability to proliferate. Senescent cells contain higher levels of oxidative DNA lesions than early passage cells with increased p21, hypo-phosphorylated Rb, reduced E2F activity, and G_1 stage cell cycle arrest (Stein et al., 1990; Noda et al., 1994; Dimri et al., 1995). Over the past few decades, ROS was presumed to be the pivotal determinant factor underlying age-associated decline of physiological functions. ROS levels increase with age in major organs, such as liver, heart, and brain (Gomi et al., 1993; Bejma et al., 2000; Driver et al., 2000; Zhang et al., 2003), and overproduction of pro-oxidants has been reported in many species during aging (Sohal and Weindruch, 1996; Barja, 2002; Zanetti et al., 2010). Oxidative stress has also been implicated in various age-related pathological conditions involving CVD, cancers, neurological disorders, diabetes, and other diseases (Valko et al., 2007). The primary physiological function of BVR is the production of bilirubin, a major natural and potent antioxidant. Moreover, the suggested redox cyclic nature of the bilirubin to biliverdin pathway and induction of HO by BVR would potentiate the physiological antioxidant capacity of this system (Baranano et al., 2002; Sedlak and Snyder, 2004; Sedlak et al., 2009; Ding et al., 2011). Recent studies have revealed that administration of BVR ameliorates the signs of oxidative stress-mediated diseases more efficiently than administration of other antioxidant enzymes, and when cellular BVR activity is suppressed using siRNA, the levels of ROS and cell death markedly increase (Baranano et al., 2002; Sedlak et al., 2009).

When the effects of BVR knock-down on cell viability and cell cycle progression are monitored, human diploid fibroblasts

become growth-arrested for a prolonged period after depletion of BVR, resulting in cellular senescence with decreased levels of cyclin D1, increased levels of p16/INK4a, decreased phosphorylation of Rb, and high expression of senescence-associated β-galactosidase (Kim et al., 2011a). This BVR knock-down-induced cellular senescence could be effectively suppressed by treatment with the ROS scavenger, *N*-acetyl cysteine (NAC). Since simple depletion of BVR can decrease cell viability and induce senescence, BVR might be suggested as the essential component for cellular survival under normal oxidative stress conditions. In addition, overexpression of BVR partially restored young cell-like morphology in senescent fibroblasts, but without resumption of mitogenic potential, implying that BVR overexpression alone was not sufficient to prevent senescence-related cell cycle arrest (Kim et al., 2011a).

Recently, a number of studies have demonstrated that cellular senescence provides an important barrier to genomic instability and tumorigenesis (Campisi and d'Adda di Fagagna, 2007; Schmitt, 2007). Senescent cells also express high levels of p16 (Alcorta et al., 1996), which might be responsible for blocking tumorigenesis. Interestingly, BVR activity is elevated in tumor tissues (Maines et al., 1999), and serum bilirubin levels are inversely related to cancer mortality (McCarty, 2007). Moreover, knock-down of BVR induces cell death in HeLa and A498 cells and sensitizes these cancer cells to oxidative stress (Kim et al., 2011a). Therefore, it can be assumed that down-regulation of BVR might be an important additive strategy for the successful treatment of cancer.

Biliverdin reductase activity is reduced with aging (Maines, 1990). HO-1 induction by BVR, as well as by oxidative stressors, is markedly impaired in senescent human fibroblasts (Kim et al., 2011a). These aging-related decreased responses to oxidative stress might be due to age-dependent differences in the efficiency of oxidative stress-related signal trafficking into the nucleus. We have demonstrated that the nucleocytoplasmic trafficking system was markedly impaired in senescent human diploid fibroblasts (Kim et al., 2010a,b, 2011b). In addition, the nuclear barrier hypothesis of aging has been proposed, in which the aging phenotype might be induced by the inefficiency of nucleocytoplasmic trafficking of a variety of mitogenic or apoptotic signals due to suppression of nuclear pore complex functions (Park, 2011). BVR is a leucine zipper-like DNA binding protein, which serves as a transcription factor for HO-1 (Kravets et al., 2004; Tudor et al., 2008). To perform its activities, such as signal transduction and induction of HO-1 in response to oxidative stress, the nuclear translocation of BVR is prerequisite. In this regard, nuclear trafficking of BVR and subsequent signaling events may be impaired in senescent human diploid fibroblasts, which might help to explain the age-dependent differences in response to oxidative stress.

THE INTERTWINED BICYCLIC NATURE OF THE BILIRUBIN SYSTEM FOR ROS SCAVENGING

Biliverdin reductase in young cells effectively protects the cells from various oxidative stresses through generation of bilirubin, a potent lipophilic ROS scavenger, and by regulating the expression of HO, another potent antioxidant. In this situation, the two cycles of the bilirubin system for ROS scavenging are supposed to be intertwined. The first cycle is the suggested redox cycle of biliverdin to bilirubin by BVR and the cycling back of bilirubin to biliverdin probably by lipophilic oxidants. The second cycle is the induction of HO by BVR and the degradation of heme to biliverdin, which is again metabolized to bilirubin by BVR (**Figure 1**). These two metabolic and transcriptional cycles are intertwined and operate in response to oxidative stress, providing highly efficient ROS scavenging. This intertwined bicyclic bilirubin system, comprised of BVR and HO for heme degradation to biliverdin and bilirubin, could play a significant role in maintaining cellular integrity against oxidative stress. Senescent cells have increased ROS generation and decreased BVR activity due to its oxidative modification, resulting in reduced bilirubin formation. Furthermore, the senescence-mediated impairment of nucleocytoplasmic trafficking prevents the nuclear translocation of BVR, resulting in decreased HO induction (**Figure 1**). Therefore, it is natural to assume that dysfunction of this bicyclic bilirubin system would be responsible for aging and, subsequently, for age-related disease.

THERAPEUTIC STRATEGIES AND FUTURE DIRECTIONS

Although it is claimed that lipid peroxidation and impaired bilirubin system are related to age-related diseases, several important issues still remain to be resolved. The exact role of bilirubin system in the progression of age-related diseases is not fully understood. Further randomized trial data and molecular studies are necessary to evaluate the potential role of bilirubin system in aging and age-related disease progression. Though the causal links between changes in HO/BVR activity, bilirubin/biliverdin level and

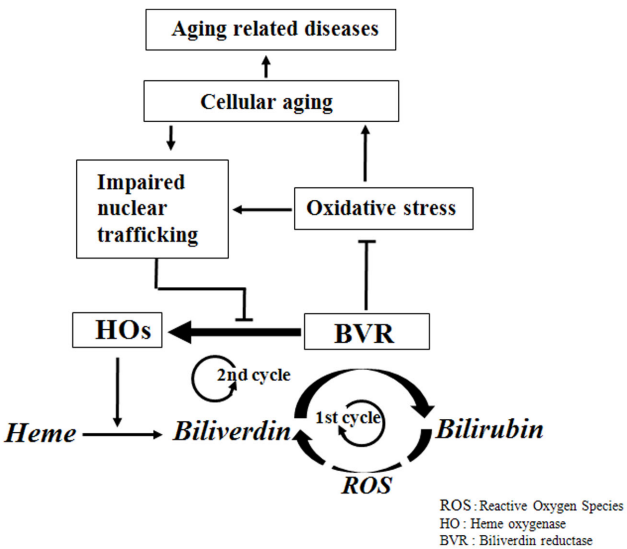

FIGURE 1 | Physiological network of the bicyclic bilirubin system in aging and age-related disease. In normal physiological condition of oxidative stress, BVR catalyzes biliverdin to bilirubin and induces HO, which further provides more biliverdin from heme. And the resulting bilirubin may turn back to biliverdin partially by oxidants. This bicyclic nature of bilirubin system provides the efficient physiological antioxidative capacity. But in harsh conditions of high oxidative stress, BVR is catalytically inactivated and is blocked for nuclear translocation, which inhibits the induction of HO, resulting in inhibition of catalytic turnover of heme to biliverdin and also biliverdin to bilirubin, leading to oxidative damages of the biomolecules and finally to aging and age-related diseases.

upstream/downstream changes in aging process have not been clearly established, the lipid peroxide damages especially at all the cellular membrane fractions would be readily expected to impair cellular functions and its integrity. Therefore, in order to protect the membrane lipid damages from the lipid soluble oxidants, the antioxidants of lipophilic nature rather than hydrophilic nature are expected to play the dominant roles. Therefore, it can be assumed that the bilirubin system might protect the organs effectively from the oxidative stress, especially of lipophilic oxidants, via its potential amplifying antioxidative capacity. If this hypothesis were true, aging and age-related diseases might be prevented by modulating BVR system to a certain degree. In this regard, it can be a novel promising strategy to adjust HO/BVR activity and/or exogenous administration of bilirubin for control of aging process and its related diseases. But it should be reminded that bilirubin possesses both antioxidant and prooxidant properties. Bilirubin shows hormeric dose responses, as illustrated by that it protects red blood cells against oxidative stress at physiologic concentrations, while it is associated with significant cytotoxicity at concentrations of 30 mg/dL or higher (Mireles et al., 1999). The similar results were shown when neuronal cells were exposed to low and high concentrations of bilirubin (Chen et al., 2003). Moreover, since several toxic effects of bilirubin have been demonstrated on erythrocytes, lymphocytes, renal cells, lung, and brain in a dose and time dependent manner (Elias et al., 1987; Alexandra Brito et al., 2006), the specific safe dose response should be considered for its use in regulation of aging process and its related diseases.

CONCLUSION

Since impaired redox homeostasis and defective oxidative stress defense mechanisms are the key contributors to the aging process, the endogenous antioxidative systems have been presumed to play important roles in the control of age-related diseases as well as aging itself. In addition to the glutathione system of hydrophilic nature, when oxidative stress and lipid peroxidation are issued, the another antioxidative mechanism consisting of BVR and the biliverdin to bilirubin pathway should be concerned against lipophilic oxidants. This bilirubin system is comprised of the intertwined bicyclic control mechanisms: the first, the metabolic redox cycle of biliverdin and bilirubin, and the second, the transcriptional control cycle of HO by BVR. The efficiency of this bilirubin system, amplified by the cyclic nature of the control mechanism, enables it to protect cells effectively against oxidative stress. Aging is associated with decreased BVR activity due to oxidative damages, which results in decreased induction of HO, leading to further reduction of bilirubin generation. Prolonged knock-down of BVR in young cells would result in premature senescence. These results provide the strong supports for the concept that a rise in ROS by way of inefficient BVR activity, either by oxidative damage or by impaired nucleocytoplasmic trafficking, could be an intracellular trigger of cellular senescence. Moreover, the inverse relationship of BVR activity and bilirubin status observed in a variety of diseases implies a significant role of the bilirubin system in the pathogenesis of many age-related diseases as well as aging itself. Therefore, it can be suggested that the bilirubin system might provide new opportunities for drug development and therapy for aging and a variety of age-related disorders.

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The role of bilirubin in diabetes, metabolic syndrome, and cardiovascular diseases

Libor Vítek*

Fourth Department of Internal Medicine, and Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
Shlomo Sasson, The Hebrew University of Jerusalem, Israel
Karl Walter Bock, University of Tübingen, Germany

****Correspondence:***
Libor Vítek, Central Research Laboratories, Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University in Prague, Na Bojišti 3, 12808 Prague 2, Czech Republic.
e-mail: vitek@cesnet.cz

Bilirubin belongs to a phylogenetically old superfamily of tetrapyrrolic compounds, which have multiple biological functions. Although for decades bilirubin was believed to be only a waste product of the heme catabolic pathway at best, and a potentially toxic compound at worst; recent data has convincingly demonstrated that mildly elevated serum bilirubin levels are strongly associated with a lower prevalence of oxidative stress-mediated diseases. Indeed, serum bilirubin has been consistently shown to be negatively correlated to cardiovascular diseases (CVD), as well as to CVD-related diseases and risk factors such as arterial hypertension, diabetes mellitus, metabolic syndrome, and obesity. In addition, the clinical data are strongly supported by evidence arising from both *in vitro* and *in vivo* experimental studies. This data not only shows the protective effects of bilirubin *per se*; but additionally, of other products of the heme catabolic pathway such as biliverdin and carbon monoxide, as well as its key enzymes (heme oxygenase and biliverdin reductase); thus, further underlining the biological impacts of this pathway. In this review, detailed information on the experimental and clinical evidence between the heme catabolic pathway and CVD, and those related diseases such as diabetes, metabolic syndrome, and obesity is provided. All of these pathological conditions represent an important threat to human civilization, being the major killers in developed countries, with a steadily increasing prevalence. Thus, it is extremely important to search for novel markers of these diseases, as well as for novel therapeutic modalities to reverse this unfavorable situation. The heme catabolic pathway seems to fulfill the criteria for both diagnostic purposes as well as for potential therapeutical interventions.

Keywords: bilirubin, biliverdin, biliverdin reductase, cardiovascular diseases, diabetes, heme oxygenase, metabolic syndrome, UGT1A1

INTRODUCTION

Bilirubin belongs to the superfamily of tetrapyrrolic compounds, which is one of the most highly conserved groups of molecules in nature. Tetrapyrroles have evolved to have pluripotent functions, including light harvesting in plants; as well as other roles in plants as well as in humans: chronobiology, energy generation, transport, and homeostasis of oxygen by hemoglobins and myoglobin – to mention only a few of them. Although for decades bilirubin was believed to be a potentially toxic metabolite of heme catabolism, in particular for central nervous system, data from the last few decades clearly demonstrates that this tetrapyrrolic compound might also have numerous other beneficial effects for the human body, including the scavenging of overproduced reactive oxygen species, anti-inflammatory actions, or direct effects upon cell signaling.

Bilirubin is the end product of heme catabolism in the systemic circulation. It is formed by the action of heme oxygenase (HMOX), an enzyme that splits cyclic tetrapyrrole heme into biliverdin, carbon monoxide, and ferrous iron. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase (BLVRA). HMOX exists in two isoforms: HMOX1, a highly inducible isoform responsible for reactions of acute phase immediate response against oxidative stress, and a constitutive isoenzyme HMOX2, playing important roles in the brain and testes. The third member of the HMOX family (*HMOX3*) has also been described, but it is generally believed that *HMOX3* is only represented by a pseudogene, with no coding function (Hayashi et al., 2004). Nevertheless, in light of the recent discoveries of the possible regulating functions of pseudogenes (Pink et al., 2011), it is possible that *HMOX3* might also have biological effects by contributing to gene regulation.

Bilirubin, as a non-polar molecule, is solubilized in the vascular bed by binding to albumin. When reaching the liver, it is actively transported by the basolateral OATP transporter, solubilized in the cytoplasm by binding to specific binding proteins, and subsequently conjugated by the action of bilirubin UDP-glucuronosyltransferase (UGT1A1) with two molecules of glucuronic acid. Bisglucuronosyl bilirubin is then actively secreted into bile and proceeds into the intestinal lumen, where it undergoes further metabolic changes. From the metabolic point of view, there are several crucial enzymatic steps (in particular those catalyzed by HMOX1, BLVRA, and UGT1A1 enzymes) which play an

important role in bilirubin homeostasis with subsequent impacts on the risk of metabolic diseases, including cardiovascular diseases (CVD), diabetes, metabolic syndrome, arterial hypertension, and obesity.

Serum bilirubin concentrations are affected by many factors including cigarette smoking, gender, fasting, intake of numerous drugs and/or plant products, altitude, race, and age (for review see Vítek and Schwertner, 2007b). All these factors are likely to influence biological impact of bilirubin on human body.

BIOLOGICAL PROPERTIES OF BILIRUBIN

Bilirubin has been recognized as a substance with potent antioxidant properties. The first report on the antioxidant effects of bilirubin was published as early as 1954 (Bernard et al., 1954), however, it took several more decades until the antioxidant properties of bilirubin attracted major scientific attention (Stocker et al., 1987). Indeed, bilirubin has been shown to be more effective at protecting lipids from oxidation than the water-soluble antioxidants such as glutathione, which primarily protect proteins from oxidation (Sedlak et al., 2009). However, bilirubin has also been demonstrated to be almost 30 times more potent toward the prevention of LDL oxidation compared to a vitamin E analog, Trolox, which represents a lipid-soluble antioxidant substance (Wu et al., 1994). Even more importantly, serum bilirubin has been demonstrated to be a major contributor to the total antioxidant capacity in blood plasma (Frei et al., 1988). Additionally, bilirubin has been proven to have anti-inflammatory properties (for review see Vítek and Schwertner, 2007b). Bilirubin inhibited tumor necrosis factor α-induced up-regulation of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule (ICAM-1) *in vitro* (Mazzone et al., 2009). The negative association between serum bilirubin levels and soluble forms of CD40 ligand and P-selectin which have also been demonstrated in *in vivo* study on subjects with Gilbert syndrome (Tapan et al., 2009). Bilirubin has also been shown to significantly interfere with the complement system, with all of the possible protective consequences (reviewed in Basiglio et al., 2010). Consistent with this data, several studies have demonstrated a negative relationship between serum bilirubin and C-reactive protein levels (Vítek et al., 2007a; Hwang et al., 2011; Yoshino et al., 2011). In addition, the modulatory effects of bilirubin on T regulatory cell differentiation were recently reported (Rocuts et al., 2010), further underlining the protective role of bilirubin in the pathogenesis of chronic inflammatory as well as in autoimmune conditions.

NEGATIVE ASSOCIATION BETWEEN BILIRUBIN AND CARDIOVASCULAR DISEASES

The first report of a negative relationship between serum bilirubin levels and coronary artery disease was published as early as 1994 (Schwertner et al., 1994). Since then, numerous studies have been published, which have consistently demonstrated that subjects with lower bilirubin levels are at increased risk of both coronary and peripheral atherosclerotic disease (for reviews see recent papers; Vítek and Schwertner, 2007b; Schwertner and Vítek, 2008; Vítek and Ostrow, 2009; Lin et al., 2010). Serum bilirubin concentrations were also found to be negatively related to coronary artery calcification (Tanaka et al., 2009), and also to ischemic stroke (Kimm et al., 2009); all consistent with our own data which demonstrated a marked postponement in the progression of the intimo-medial thickness of carotid arteries in hyperbilirubinemic subjects with Gilbert syndrome, when compared to normobilirubinemic individuals (Vítek et al., 2006). In addition, our meta-analysis of studies focused on the association between CVD and bilirubin, involving almost 15,000 men, showed that each 1.0 μmol/L increase in serum bilirubin was associated with a 6.5% decrease in CVD risk (Novotný and Vítek, 2003). The same association was also reported in a recent Taiwanese prospective study on patients with cardiac X syndrome followed for 5 years, in which patients with the lowest serum bilirubin levels had a higher incidence of non-fatal myocardial infarction, ischemic stroke, rehospitalization for unstable angina, and coronary revascularization procedures (Huang et al., 2010).

Logically, the opposite relationship is also true, i.e., subjects with moderately elevated levels of serum bilirubin, such as those with Gilbert syndrome, are at decreased risk of CVD (Vítek et al., 2002).

Although the association of serum bilirubin levels and CVD has been known for almost two decades, the relationship between bilirubin and other players of the heme catabolic pathway, as well as pathologic conditions such as metabolic syndrome and/or diabetes has only been investigated in the last few years.

HEME OXYGENASE AND DIABETES – EXPERIMENTAL EVIDENCE

Diabetes mellitus, the hallmark of which consists of elevated plasma glucose, is consistently associated with increased oxidative stress, as well as enhanced formations of advanced glycation end products (AGEs; Yamagishi et al., 2011). Overproduction of oxidizing molecules results in the progressive loss of pancreatic β-cells, depleting insulin levels. However, also numerous other mechanisms contributing to the pathogenesis of diabetes play an important pathogenic role, including reduction of the adiponectin levels (Soares et al., 2005), interference with insulin signaling, endothelial dysfunction, or direct damages of the endothelial wall (Unoki and Yamagishi, 2008; Potenza et al., 2009; Giacco and Brownlee, 2010). Thus, it is not surprising that HMOX1, as a key antioxidant enzyme, has consistently been shown to protect from the development of diabetes (for review see Ndisang, 2010). The mechanisms by which HMOX1 mediated these effects are pluripotent, and apart from the antioxidant action, HMOX1 also directly affects glucose metabolism, which might be due to the presence of a binding site for a glucocorticoid-responsive element in the *HMOX1* gene promoter (Lavrovsky et al., 1996). In fact, HMOX1 was shown to stimulate insulin production in experimental animal models (Ndisang, 2010), at least partially, *via* the release of carbon monoxide (Henningsson et al., 1999), which is an immediate product of HMOX. Indeed, HMOX1 induction by hemin has been demonstrated to improve insulin signaling and glucose metabolism, and to have lowered insulin resistance in various animal models, including those of streptozotocin-induced diabetes (Ndisang and Jadhav, 2009a), insulin-resistant type 2 diabetes (Ndisang and Jadhav, 2009b), primary hyperaldosteronism (Ndisang and Jadhav, 2010a), as well as essential hypertension (Ndisang et al., 2010b). Apart from an improvement of insulin

sensitivity, HMOX1 induction was also reported to reduce visceral and subcutaneous obesity in diabetic and obese mice (Li et al., 2008; Nicolai et al., 2009; Burgess et al., 2010) through mechanisms involving the attenuation of the inflammatory processes, as well as modulation of PPARγ signaling (Ndisang, 2010). Furthermore, chronic HMOX1 induction was shown to increase metabolic turnover, heat production, and physical activity in an experimental model of obesity (Csongradi et al., 2012), suggesting the complexity of the beneficial effects of HMOX1 induction upon these metabolic disorders.

HEME OXYGENASE AND DIABETES – CLINICAL EVIDENCE

Experimental data on the role of HMOX in the pathogenesis of diabetes, metabolic syndrome, and obesity are also supported by the clinical evidence. Plasma concentrations of HMOX1 have been demonstrated to strongly correlate with type 2 diabetes mellitus (Bao et al., 2010b). In fact, subjects in the highest quartile of HMOX1 plasma levels had more than an 8X higher risk for development of type 2 diabetes, compared to the lowest quartile (Bao et al., 2010b). In this study, the HMOX1 plasma level might reflect increased oxidative stress preceding the expression of diabetes. In addition, it is known that high glucose exposure leads to HMOX1 induction (Jonas et al., 2003; Won et al., 2006), which also might account for the observed phenomenon. On the other hand, the same authors recently published a meta-analysis of six studies focused on the role of *HMOX1* promoter gene variants and the risk of diabetes, covering almost 2,000 diabetic patients and 3,500 controls. These results revealed that subjects carrying longer (GT)n repeats in the *HMOX1* gene promoter (associated, nevertheless, with decreased HMOX1 activity), had an increased risk of type 2 diabetes (Bao et al., 2010a). In addition to the effect on diabetes risk, diabetic subjects with the L/L genotype had an almost threefold increase in CVD risk, even after controlling for conventional risk factors in one of the studies involved in the reported meta-analysis (Chen et al., 2008). Further detailed clinical studies are needed to elucidate all of the mechanisms related to the roles of HMOX in the risks of development of diabetes.

BILIVERDIN REDUCTASE AND DIABETES – EXPERIMENTAL EVIDENCE AND POSSIBLE CLINICAL IMPLICATIONS

Biliverdin reductase, similarly as HMOX, is an evolutionarily conserved enzyme, responsible for the hydrogenation of biliverdin on bilirubin. Besides that, BLVRA has multiple other functions affecting cell signaling, modulating immune system response, and also exerting substantial metabolic effects (Maines, 2005; Kapitulnik and Maines, 2009; Wegiel et al., 2011). BLVRA also serves as a unique serine/threonine/tyrosine kinase, significantly modulating (among other molecules) the phosphorylation of the insulin receptor substrate-1, as well as other insulin/IGF-1 signaling pathway targets (Kapitulnik and Maines, 2009). As a result, BLVRA diminishes insulin signaling, and acts as a negative regulator of glucose uptake (Lerner-Marmarosh et al., 2005). BLVRA also binds to the p85 regulatory subunit of PI3K/Akt (Pachori et al., 2007), a mechanism which might also lead to improved insulin sensitivity (Terauchi et al., 1999). Based upon this data, small BLVRA-based peptides which activate the insulin receptor signaling axis have been suggested as possible therapeutics to combat insulin resistance in diabetic patients (Lerner-Marmarosh et al., 2008).

Not only BLVRA itself, but also the products of its basic enzymatic activity, biliverdin (and consequently bilirubin), might significantly contribute to protection from diabetic complications. Oral administration of biliverdin to *db/db* mice partially prevented a worsening of hyperglycemia and glucose intolerance (Ikeda et al., 2011), as well as diabetic nephropathy in these animals, principally *via* inhibition of oxidative stress-induced damage, mediated by the interference with the NADPH oxidase pathway (Fujii et al., 2010). Biliverdin-treated animals had less albuminuria, and complete protection against the progression of mesangial expansion, accompanied by normalization of transforming growth factor-β1 and the expression of fibronectin (Fujii et al., 2010).

There are no clinical data on the role of BLVRA/biliverdin in diabetes, metabolic syndrome, or obesity. Only recently, a paper describing the effect of a BLVRA gene variant (rs699512) on the risk of essential hypertension has just been reported (Lin et al., 2011). Detailed studies are certainly needed to fully elucidate the clinical relevance of the experimental findings discussed.

BILIRUBIN AND DIABETES – EXPERIMENTAL EVIDENCE

It is not surprising that bilirubin, a subsequent reduction product of biliverdin, also has salutary effects in terms of the prevention of diabetes mellitus and its complications. In addition, bilirubin was also reported to provide protection against metabolic syndrome, and to be negatively associated with overweight and obesity.

In fact, in a manner similar to biliverdin substantially improving renal pathology in *db/db* mice, specifically *via* inhibition of NADPH oxidase (as discussed above), a practically identical effect was observed in hyperbilirubinemic Gunn rats exposed to streptozocin (Fujii et al., 2010). Compared to their diabetic normobilirubinemic heterozygous siblings, diabetic Gunn rats developed less albuminuria, exhibited practically normal urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), as well as 8-epi-prostaglandin F2α levels; both markers of systemic oxidative stress. Additionally, expression of NADPH oxidase was markedly attenuated in the kidneys of diabetic Gunn rats, who also exhibited complete inhibition of mesangial expansion when compared to normobilirubinemic diabetic controls (Fujii et al., 2010). Consistent with this data, Korean researchers reported substantial resistance of hyperbilirubinemic Gunn rats to developing diabetes after intraperitoneal exposure to streptozocin, compared to their normobilirubinemic littermates (Fu et al., 2010). Markers of diabetes, such as fasting blood glucose and HbA1c levels were much less pronounced in Gunn rats; on the other hand, insulin secretion by the pancreatic islets was preserved in hyperbilirubinemic animals. In addition, concentrations as low as 1.7 μmol/L bilirubin prevented streptozocin-induced apoptosis in rat insulinoma cell culture; significantly supporting the *in vivo* data (Fu et al., 2010).

BILIRUBIN, DIABETES, METABOLIC SYNDROME, AND OBESITY – CLINICAL EVIDENCE

Negative associations between serum bilirubin concentrations and abnormal glucose tolerance tests were reported as early as 1996 (Ko et al., 1996). Since then, numerous clinical studies evaluating the possible associations between serum/plasma bilirubin levels

and the risk of diabetes (and its complications) have been published. Inoguchi et al. (2007) first indicated that diabetic patients who have concomitant Gilbert syndrome have a lower prevalence rate of vascular complications, compared to normobilirubinemic diabetics. Although not specifically mentioned in their study, the prevalence of Gilbert syndrome among more than 5,000 consecutive diabetics was at least three times lower than would be expected in the general population. This data is in line with our observation of low prevalence of diabetes in subjects with $(TA)_7$ promoter variation in *UGT1A1* responsible for systemic bilirubin homeostasis (Jirásková et al., 2011), and with low levels of AGEs in Gilbert syndrome subjects, compared to the normobilirubinemic population (Kalousová et al., 2005). Furthermore, low serum bilirubin levels were identified as an important predictor of CVD in Type 2 diabetes patients receiving haemodialysis (Fukui et al., 2011). Just recently, another group of Japanese investigators reported in their cross-sectional study on more than 3,000 participants, that compared to subjects within the lowest bilirubin quartile, those with the highest bilirubin levels had a four times lower prevalence of diabetic retinopathy (Yasuda et al., 2011). In a large Korean cross-sectional study on almost 94,000 subjects, high serum bilirubin was also found to be associated with the reduced risk of diabetes mellitus and diabetic nephropathy (Han et al., 2010). Higher serum bilirubin levels were also shown to protect from diabetes mellitus in the US National Health and Nutrition Examination Survey (NHANES) on almost 16,000 subjects. In this study, subjects with a bilirubin level above 10 μmol/L had 20% less risk of developing diabetes, compared to those with bilirubin levels below this cut-off value, even after multiple adjustments (Cheriyath et al., 2010).

Similarly, additional studies have supported the protective effects of bilirubin on both clinical and laboratory outcomes of diabetic subjects. Serum bilirubin concentrations were demonstrated to be negatively associated with albuminuria in patients with type 2 diabetes (Fukui et al., 2008), HbA1c levels (being an independent risk factor of CVD) in Japanese populations (Oda, 2010; Oda and Kawai, 2010; Ohnaka et al., 2010); additionally, with both insulin resistance and prevalence rate of the metabolic syndrome among children and adolescents (Lin et al., 2009a), as well as in adult populations (Kwon et al., 2011; Wu et al., 2011). Consistent with this data, low prevalence of metabolic syndrome in subjects with phenotypic Gilbert syndrome was described in a recent large Korean study on more than 12,000 participants (Choi et al., 2011).

In line with these negative relationships between serum bilirubin levels and metabolic syndrome, a negative association between bilirubin levels and abdominal obesity *per se* has been shown in several recent studies (Bhuiyan et al., 2008; Lin et al., 2009a; Choi et al., 2011; Kwon et al., 2011; Wu et al., 2011). Since weight reduction is known to reduce several cardiovascular risk factors, it is important to note that each one percent decrease in weight loss was associated with a linear increase in serum bilirubin concentration (Andersson et al., 2009). The close relationship between serum bilirubin levels and the *UGT1A1* gene promoter variants, responsible for manifestation of Gilbert syndrome in Asians, and the risk of non-alcoholic fatty liver disease (a condition commonly associated with obesity and metabolic syndrome) was recently demonstrated in Taiwanese children (Lin et al., 2009b); further underlining the importance of the heme catabolic pathway in the pathogenesis of obesity, metabolic syndrome, diabetes mellitus and its complications.

PHARMACOLOGIC AND/OR NUTRACEUTIC INTERVENTIONS

Interventions which induce HMOX1 and/or BLVRA, or those that inhibit UGT1A1 activities might be effective in increasing serum and tissue bilirubin concentrations, as well as in modulating signaling pathways involved in energy homeostasis.

Heme oxygenase 1, as a highly inducible enzyme, which can be upregulated by many common drugs routinely used in clinical medicine (Bach, 2005), including non-steroidal anti-inflammatory drugs, hypolipidemics (Muchová et al., 2007; Wu et al., 2012), PPARα agonists, certain immunosuppressive drugs, to mention just a few. It is likely that at least part of their therapeutic action might be mediated *via* induction of HMOX1. The therapeutic potential of HMOX1 induction is also supported by recent experimental data demonstrating that chronic HMOX1 induction lowers body weight, corrects hyperglycemia and hyperinsulinemia, as well as increases oxygen consumption, heat production, and activity in obese mice (Csongradi et al., 2012).

Based on recent data, it also becomes evident that BLVRA might have great therapeutic implications (Maines, 2010). In fact, small residue peptides designed according to the primary structure of BLVRA were shown to block important pathways implicated in the pathogenesis of diabetes, including the inhibition of the MAPK (Lerner-Marmarosh et al., 2008) and TNF-α/PKC ζ/NF-κB pathways (Lerner-Marmarosh et al., 2007), as well as the inhibition of activation and membrane translocation of PKC-βII (Maines et al., 2007). There are also pharmaceuticals capable of a partial inhibition of UGT1A1, which in turn result in a mild elevation of systemic bilirubin levels. Among the substances having UGT1A1 inhibiting activity is a uricosuric drug probenecid (McCarty, 2007), and also the antiviral drug atazanavir (Dekker et al., 2011). In fact, atazanavir has been demonstrated to improve endothelial function in patients with type 2 diabetes mellitus, most likely due to its bilirubin-increasing activities (Dekker et al., 2011).

However, the heme catabolic pathway might also be potentiated through non-pharmacologic means, and its salutary effects can, for instance, be mimicked by tetrapyrollic compounds widely occurring in plants and algae. There are numerous natural HMOX1 inducers originating from plants, including polyphenols, but also compounds such as curcumin, or silymarin (Bonifaz et al., 2009). Indeed, curcumin was reported to increase insulin secretion from rat-isolated pancreatic islets *via* HMOX1 induction (Abdel Aziz et al., 2010), as well as to improve clinical and laboratory markers of experimental diabetes (Gutierres et al., 2011; Soetikno et al., 2011); the same effect was also demonstrated for silymarin-treated diabetic rats (Vessal et al., 2010). Although the action of both compounds is certainly multifactorial, HMOX1 induction seems to play an important role in their anti-diabetic effects.

It is also tempting to speculate that oral supplementation with nutraceuticals containing plant tetrapyrolles, such as phycocyanobilins (Lee et al., 2008; Moura et al., 2011), or their increased consumption in the form of natural foods (Tonstad et al., 2009) might be used as a novel approach of the chemoprevention of obesity, metabolic syndrome, and diabetes.

CONCLUDING REMARKS

In this review, the experimental as well as clinical studies on the associations between CVD, diabetes mellitus, metabolic syndrome, and obesity with the heme catabolic pathway have been discussed. These studies unanimously show that low serum bilirubin concentrations are associated with an increased risk of these pathologic conditions; whereas, mildly elevated serum bilirubin levels provide protection. Moreover, it is clear that apart from the direct beneficial effects of bile pigments, the enzymes HMOX1 and BLVRA also play an important role in the development of these metabolic diseases. Based on recent data, it seems that there is a therapeutical potential to modulate HMOX1, BLVRA, and UGT1A1 activities, and/or mildly increase bilirubin levels (or its analogs) in the systemic circulation to suppress pathways leading to the development of diabetes and its complications. Obviously, detailed studies to explore this potential further appear warranted, and will definitely provide much needed evidence that the whole heme catabolic pathway is also likely beneficial for this group of metabolic diseases.

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Endothelial cells derived from the blood-brain barrier and islets of Langerhans differ in their response to the effects of bilirubin on oxidative stress under hyperglycemic conditions

Jaime Kapitulnik*, Clara Benaim and Shlomo Sasson

Department of Pharmacology, Institute of Drug Research, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel

Edited by:
Hege Christensen, University of Oslo, Norway

Reviewed by:
Nils Tore Vethe, Oslo University Hospital, Norway
Stanislav Yanev, Department of Drug Toxicology, Bulgaria

****Correspondence:***
Jaime Kapitulnik, Department of Pharmacology, Institute of Drug Research, School of Pharmacy, The Hebrew University of Jerusalem, P.O.B. 12065, Ein Kerem, Jerusalem 91120, Israel.
e-mail: jaimek@savion.huji.ac.il

Unconjugated bilirubin (UCB) is a neurotoxic degradation product of heme. Its toxic effects include induction of apoptosis, and ultimately neuronal cell death. However, at low concentrations, UCB is a potent antioxidant that may protect cells and tissues against oxidative stress by neutralizing toxic metabolites such as reactive oxygen species (ROS). High glucose levels (hyperglycemia) generate reactive metabolites. Endothelial cell dysfunction, an early vascular complication in diabetes, has been associated with hyperglycemia-induced oxidative stress. Both glucose and UCB are substrates for transport proteins in microvascular endothelial cells of the blood-brain barrier (BBB). In the current study we show that UCB (1–40 μM) induces apoptosis and reduces survival of bEnd3 cells, a mouse brain endothelial cell line which serves as an *in vitro* model of the BBB. These deleterious effects of UCB were enhanced in the presence of high glucose (25 mM) levels. Interestingly, the bEnd3 cells exhibited an increased sensitivity to the apoptotic effects of UCB when compared to the MS1 microcapillary endothelial cell line. MS1 cells originate from murine pancreatic islets of Langerhans, and are devoid of the barrier characteristics of BBB-derived endothelial cells. ROS production was increased in both bEnd3 and MS1 cells exposed to high glucose, as compared with cells exposed to normal (5.5 mM) glucose levels. While UCB (0.1–40 μM) did not alter ROS production in cells exposed to normal glucose, relatively low ("physiological") UCB concentrations (0.1–5 μM) attenuated ROS generation in both cell lines exposed to high glucose levels. Most strikingly, higher UCB concentrations (20–40 μM) increased ROS generation in bEnd3 cells exposed to high glucose, but not in similarly treated MS1 cells. These results may be of critical importance for understanding the vulnerability of the BBB endothelium upon exposure to increasing UCB levels under hyperglycemic conditions.

Keywords: bilirubin, glucose, blood-brain barrier, jaundice, diabetes, apoptosis, oxidative stress, reactive oxygen species

INTRODUCTION

Bilirubin is a linear tetrapyrrole that is formed during the process of heme degradation. Heme is released from a series of hemeproteins, including hemoglobin and cytochromes P450, and metabolized by heme oxygenase to form carbon monoxide, biliverdin, and free iron. Biliverdin is subsequently transformed to unconjugated bilirubin (UCB) by biliverdin reductase. UCB binds to plasma albumin, which transports it to the liver, where it is conjugated to hydrophilic acceptors. The major conjugates are bilirubin glucuronides formed by UDP-glucuronosyltransferase 1A1 (UGT1A1). These polar derivatives are thereafter excreted in the bile (**Figure 1**).

Unconjugated bilirubin is a molecule which behaves as a "double-edged sword" (Kapitulnik, 2004); it exerts both cytotoxic and cytoprotective effects which are dose- and target-dependent. When the blood levels of UCB are excessively elevated and surpass the capacity of albumin for high-affinity binding of UCB, the unbound (free) fraction of the pigment increases. Free UCB can easily enter cells by passive diffusion and cause toxicity. The most vulnerable target is the central nervous system (CNS). UCB binds to discrete brain areas, such as the basal ganglia (kernicterus), and produces a wide array of neurological deficits collectively known as bilirubin encephalopathy.

The cytotoxic effects of UCB include programmed cell death (apoptosis) of a variety of cell types such as neurons (Grojean et al., 2000; Rodrigues et al., 2002), aortic smooth muscle cells (Liu et al., 2002), brain microvascular endothelial cells (Akin et al., 2002), and hepatoma cells (Seubert et al., 2002).

The pioneering studies conducted in the laboratory of Bruce Ames (Stocker et al., 1987a,b) introduced the concept that UCB, which was until then regarded as a toxic waste product of heme catabolism, possesses a beneficial role at low ("physiological") plasma concentrations (<1 mg/dl, <17 μM) by acting as a potent antioxidant that scavenges reactive oxygen species (ROS). Almost two decades ago it was suggested that moderately elevated plasma UCB levels may reduce the risk of coronary artery

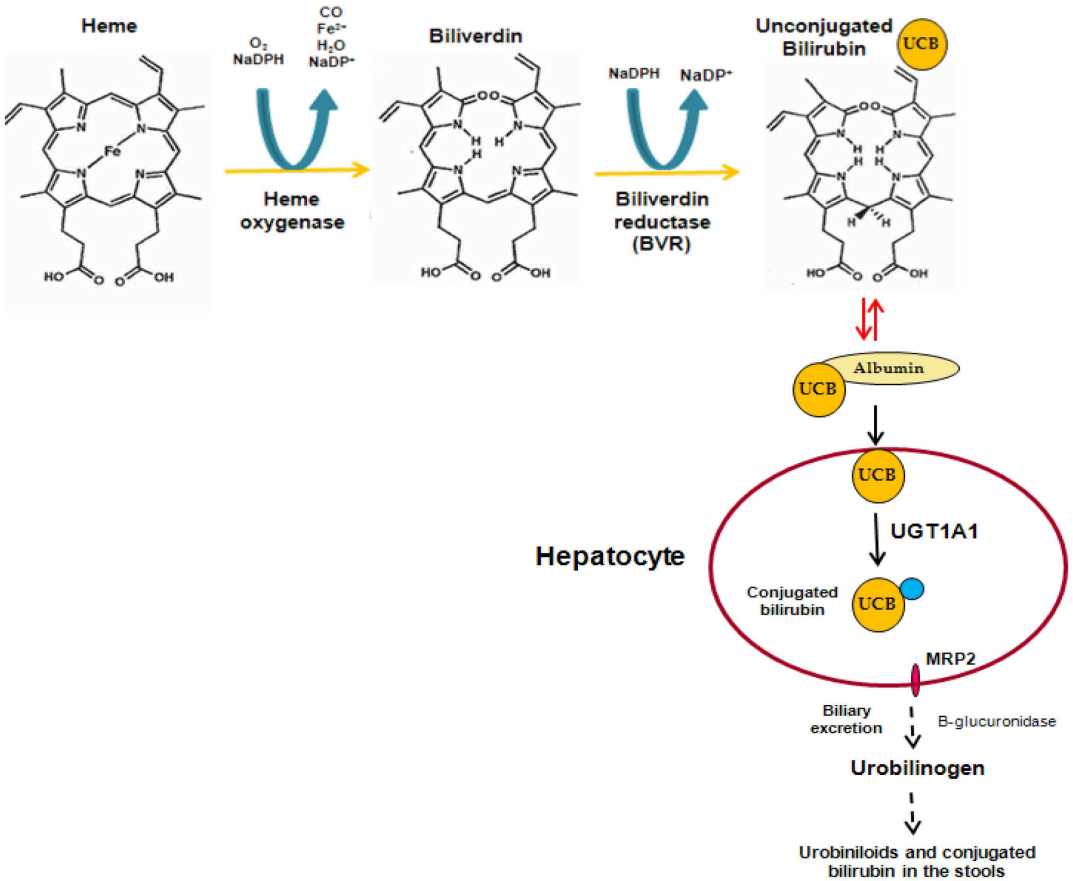

FIGURE 1 | Formation and elimination of unconjugated bilirubin.

disease (Schwertner et al., 1994). Indeed, individuals with Gilbert syndrome, a mild form of hyperbilirubinemia, who display moderately increased plasma UCB concentrations (~40 μM) due to a decreased UGT1A1 activity, show a lower risk of cardiovascular and related diseases such as diabetes, as compared with normobilirubinemic subjects (reviewed by Lin et al., 2010; Vitek, 2012).

High glucose (hyperglycemia) augments production of ROS, which play a major role in the etiology of diabetes complications, such as endothelial dysfunction (Guzik et al., 2002) and astrocyte activation in the CNS (Wang et al., 2012). Of interest are the findings that ROS generated by hyperglycemia cause apoptosis of vascular endothelial cells (Hulsmans et al., 2012) and are responsible for the endothelial cell sloughing associated with hyperglycemia (Rodella et al., 2006).

Glucose is an essential nutrient for brain cells and crosses the blood-brain barrier (BBB) via the influx glucose transporter-1 (GLUT1). The entry of the neurotoxic UCB into the brain is prevented by the efflux transporter P-glycoprotein (P-gp). Thus, it was of great interest to examine the interplay between UCB and glucose in BBB-derived endothelial cells. In the current study we examined the effects of low ("physiological") and moderately elevated UCB concentrations (0.1–40 μM), alone or in combination with high glucose, on apoptosis and cellular ROS levels in bEnd3 cells, which are a model for studying BBB characteristics. The responses of these cells to UCB and glucose were compared with those of MS1 cells, microvascular endothelial cells derived from pancreatic islets of Langerhans. The latter cells do not exhibit barrier characteristics and may serve as a model for studying free glucose passage into pancreatic islets.

MATERIALS AND METHODS

CHEMICALS

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), HEPES, SDS 10% (wt/vol) solution, L-glutamine solution (2 mM), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) solution, sodium pyruvate, and trypsin were purchased from Biological Industries (Beth-Haemek, Israel). Micro BCA™protein assay kit, bovine serum albumin (BSA; fraction V), DMSO, PBS, NaOH, and staurosporine solution were purchased from Sigma-Aldrich (Rehovot, Israel). UCB was purchased from MP Biomedicals (Santa Ana, CA, USA), hydrogen peroxide 30% (wt/vol) from J. T. Baker (Phillipsburg, NJ, USA), 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) from Molecular Probes (Eugene, OR, USA), and D(+) glucose anhydrous from Riedel-de Haën (Seelze, Germany).

CELL CULTURE

The bEnd3 microvascular endothelial cell line is derived from mouse brain. These cells display structural and functional barrier properties owing to their high expression of tight junction proteins and high transendothelial resistance, thus resembling the BBB (Brown et al., 2007; Li et al., 2010). The MS1 microvascular endothelial cell line is derived from mouse pancreatic islets. These cells are highly fenestrated and do not show barrier characteristics (Konstantinova and Lammert, 2004). The bEnd3 cells were a gift from Dr. P. Lazarovici (School of Pharmacy, Hebrew University of Jerusalem), and MS1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

Both cell types were grown in plastic flasks in DMEM culture medium supplemented with 1% (vol/vol) antibiotics, 1% (vol/vol) L-glutamine, 10% (vol/vol) FBS, and 5.5 mM glucose (37°C, 5% CO_2). After reaching confluency, cells were plated to 12 well-plates in 2 ml of fresh culture medium per well. The culture medium was replaced 24 h after seeding with fresh medium containing 5.5 or 25 mM glucose. After an additional 24 h-incubation period, the culture medium was replaced with fresh medium containing increasing concentrations of UCB. A fresh stock solution of UCB (40 mM, in 0.1 M NaOH) was prepared for each experiment. Serial dilutions of the UCB stock solution were made in fresh culture medium containing 20 mM HEPES, to keep a stable pH of 7.5–7.6, and 5.5 or 25 mM glucose. Cells were further incubated for an additional 24 h-period. Addition of UCB and further handling of cell cultures was done in dim light.

CELL NUMBER DETERMINATION

Cells were detached from the plate with 0.25 ml of a solution of trypsin (1:2000). Volume was adjusted to 0.5 ml with culture medium and cells were counted in a hemacytometer.

APOPTOSIS

Apoptosis was assayed using the FAM-FLICA™Poly Caspases Assay Kit (ImmunoChemistry Technologies, LLC, Bloomington, MN, USA). This assay detects the presence of caspase activity in apoptotic cells by quantifying the intensity of fluorescence in cells exposed to a membrane-permeant, Fluorescent Labeled Inhibitor of Caspases (FLICA) probe. The fluorescent tag is carboxyfluorescein. This probe binds covalently with the active caspases and is retained only in apoptotic cells.

After incubation with increasing concentrations of UCB, the cells were analyzed according to the manufacturer's instructions. Briefly, cells were trypsinized, followed by centrifugation and resuspension in culture medium. Triplicate samples of 2.5×10^6 cells from each treatment were transferred into plastic tubes and incubated in the dark with the carboxyfluorescein-FLICA solution for 1 h (37°C, 5% CO_2). Cell suspensions were thereafter washed twice with wash buffer and resuspended in PBS, keeping a final uniform cell density (2.5×10^6 cells/sample). One hundred microliters of each cell suspension was placed into each of three wells of a black microtiter plate for measurement of fluorescence intensity (excitation: 490 nm; emission: 520 nm). Thus, triplicate readings were obtained for each triplicate caspase sample. A positive control was prepared by incubating naive (untreated) cells with 1 μM staurosporine for 3 h. Staurosporine induces apoptosis by caspase activation (Thuret et al., 2003).

Caspase activity was expressed as relative fluorescence units (RFU), calculated by dividing the individual fluorescence values for the different treatments by that obtained for cells exposed to 5.5 mM glucose in the absence of UCB (the latter were assigned a value of 1). Experiments were repeated at least three times.

REACTIVE OXYGEN SPECIES

Reactive oxygen species production was examined using the cell-permeant probe 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). This dye enters the cell by passive diffusion, undergoes hydrolysis to generate chloromethyl-dichlorodihydrofluorescein (CM-H_2DCF), which remains trapped intracellularly. Upon oxidation of CM-H_2DCF by ROS, the fluorescent product chloromethyl-dichlorofluorescein (CM-DCF) is formed (Kehrer and Paraidathathu, 1992). After incubation with UCB, cells were rinsed three times with PBS and incubated with CM-H_2DCFDA for 30 min (37°C, 5% CO_2). The cells were thereafter washed three times with PBS and solubilized with 600 μl SDS 0.1% (wt/vol). One hundred microliters of each sample was placed into each of three wells of black microtiter plates, and fluorescence intensity was measured (excitation: 490 nm; emission: 520 nm). A positive control was prepared by incubating naive (untreated) cells with 1 mM H_2O_2 for 2 h.

Reactive oxygen species production was normalized to protein content. Twenty microliters aliquots of each sample were placed into each of three wells of 96-multiwell plates. Volume was adjusted to 150 μl with SDS 0.1%, and 150 μl of Micro BCA working reagent was added. The plates were covered and incubated for 2 h (37°C, 5% CO_2), and the colored product was quantitated by measuring absorbance at 562 nm.

Reactive oxygen species production is expressed as RFU, calculated by dividing the individual fluorescence values for the different treatments (normalized to protein content) by that obtained for cells exposed to 5.5 mM glucose in the absence of UCB (the latter were assigned a value of 1). Experiments were repeated at least three times.

STATISTICAL ANALYSIS

The experiments were carried out in replicates of $n \geq 3$ and results are presented as Mean ± Standard Deviation. Statistical differences were evaluated using the Student's two-tailed t test. A "p" value of ≤ 0.05 indicates statistical significance.

RESULTS

APOPTOTIC EFFECTS OF BILIRUBIN AND/OR GLUCOSE

Confluent cultures of bEnd3 and MS1 cells were incubated for 48 h with culture medium containing normal (5.5 mM) or high (25 mM) glucose, in the absence or presence of increasing concentrations of UCB added during the last 24 h of incubation. UCB (1–40 μM) caused a concentration-dependent increase of caspase activity in both cell types (**Figure 2**). This effect was more pronounced in bEnd3 than in MS1 cells. The 40 μM concentration of UCB caused a 7.6-fold increase in caspase activity in the normal glucose-containing cultures (**Figure 2A**), as compared with a 2.5-fold increase in MS1 cells under similar conditions (**Figure 2B**).

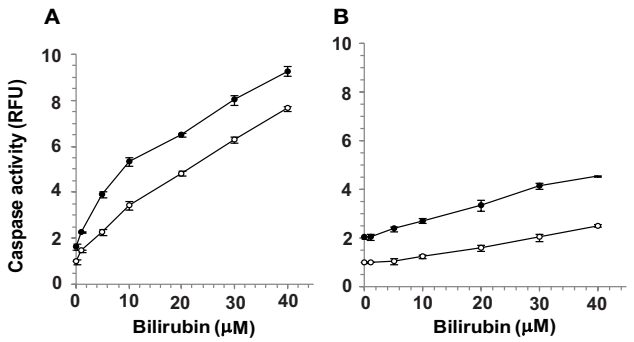

FIGURE 2 | Apoptotic effects of UCB and glucose in microvascular endothelial cells. Confluent cultures of bEnd3 **(A)** or MS1 **(B)** cells were incubated for 48 h with culture medium containing normal (5.5 mM; -O-) or high (25 mM; -●-) glucose, in the absence or presence of increasing concentrations of UCB added during the last 24 h of incubation. Caspase activity was determined using the carboxyfluorescein-FLICA probe (see Materials and Methods) and was expressed as relative fluorescence units (RFU), calculated by dividing the individual fluorescence values for the different treatments by that obtained for cells exposed to 5.5 mM glucose in the absence of UCB (the latter were assigned a value of 1). Experiments were repeated at least three times. The results of a representative experiment are shown as Mean ± Standard Deviation. Values for the bEnd cells at all UCB concentrations differ from the values obtained in the absence of UCB (statistically significant at $p < 0.05$). Values for the MS1 cells at UCB concentrations of 10–40 μM differ from the values obtained in the absence of UCB (statistically significant at $p < 0.05$).

Noteworthy, in contrast with the marked apoptotic effect of UCB in bEnd3, but not in MS1 cells, exposure of both cell types to 1 μM staurosporine caused a 6.2-fold increase in caspase activity. Elevation of the glucose concentration to 25 mM in the absence of UCB, increased caspase activity nearly twofold in both cell types, and further increased the effect of 40 μM UCB to 9.3- and 4.5-fold for bEnd3 and MS1 cells, respectively (**Figures 2A,B**). It should be noted that the relative degree of apoptosis in high glucose-containing bEnd3 cultures was more pronounced for the lower (1–10 μM) than for the higher (20–40 μM) UCB concentrations (**Figure 2A**). UCB also reduced the number of viable bEnd3 cells, and high glucose increased this effect (**Table 1**). As described above for the caspase activity determinations, the MS1 cells were also less sensitive to the cytotoxic effects of UCB. The fraction of non-viable/apoptotic cells was less than 1% for all treatments of both cell lines (data not shown).

EFFECTS OF UCB ON THE GENERATION OF ROS IN ENDOTHELIAL CELLS CULTURED IN NORMAL AND HIGH GLUCOSE-CONTAINING MEDIA

Given the differential sensitivity of bEnd3 vs. MS1 cells toward the apoptotic effect of UCB, and the involvement of ROS in apoptosis of endothelial cells, it was of a great interest to determine whether these two cell types also differ in their capacity to produce ROS in response to either UCB or high glucose, or to their combination.

Exposure of both bEnd3 and MS1 cells to increasing concentrations of UCB in normal glucose-containing medium had no significant effect on ROS production (**Figures 3A,B**, respectively). In the bEnd3 cells, elevation of the glucose concentration to 25 mM approximately doubled ROS production in the absence of UCB.

Table 1 | Viability of microvascular endothelial cells exposed to UCB in normal or high glucose-containing culture media.

UCB (μM)	bEnd3 cells		MS1 cells	
	5.5 mM glucose	25 mM glucose	5.5 mM glucose	25 mM glucose
0	100	100	100	100
10	94.5 ± 1.0	91.6 ± 1.7	94.8 ± 1.6	95.1 ± 3.5
40	85.0 ± 0.7	77.5 ± 2.1	90.3 ± 1.0	84.5 ± 0.5

Cells were counted at the end of incubations with UCB in normal (5.5 mM) or high (25 mM) glucose-containing media. The fraction of non-viable/apoptotic cells was less than 1% for all treatments of both cell lines (data not shown). Results are expressed as percentages of viable cells, and are presented as Mean ± Standard Deviation of triplicate samples. Numbers of cells in the absence of UCB are taken as 100% values: bEnd3 cells in normal glucose – 1.47 × 10^5 cells/ml; in high glucose – 1.78 × 10^5 cells/ml. MS1 cells in normal glucose – 1.55 × 10^5 cells/ml; in high glucose – 1.23 × 10^5 cells/ml.

The combined exposure of bEnd3 cells to UCB (0.1–40 μM) and high glucose had a biphasic effect on ROS production (**Figure 3A**). UCB (0.1–5 μM) caused an initial decrease in ROS production: similar ROS values were obtained at 25 and 5.5 mM glucose in the presence of 2 μM UCB. Higher UCB concentrations (20–40 μM) markedly elevated ROS production in the presence of 25 mM glucose.

Exposure of MS1 cells to high glucose caused a minor elevation in ROS production (about 40%) in the absence of UCB (**Figure 3B**). Again, low (0.1–5 μM) concentrations of UCB abolished this increase, as shown for the bEnd3 cells. However, in contrast to the latter cells, increasing further the UCB concentration (up to 40 μM) did not increase ROS production in MS1 cells above the value obtained in the absence of UCB. In a control experiment, 1 mM H_2O_2 caused 1.5- and 1.6-fold increases in ROS production in bEnd3 and MS1 cells, respectively.

DISCUSSION

The CNS is extremely sensitive to UCB, particularly in the immediate postnatal period. UCB-induced neurotoxicity is affected by the interplay between neurons and glial cells (Silva et al., 2011). Relatively low UCB levels may cause apoptotic cell death, particularly in immature neurons (Grojean et al., 2000; Rodrigues et al., 2002).

We show here that endothelial cells derived from the brain microvasculature exhibit a marked and dose-dependent apoptotic response to UCB, as compared with a weaker response of microvascular endothelial cells obtained from pancreatic islets of Langerhans. Apoptosis in the former cells occurs already at UCB concentrations which are considered "physiological" in humans, namely 1–10 μM, and is accompanied by a reduction in the number of viable cells remaining in culture after a 24 h-exposure to UCB. To our knowledge, this is the first report on a greater sensitivity to UCB of brain-derived microvascular endothelial cells, when compared with their counterparts in other microvessels.

The brain-derived bEnd3 cells are useful for studying BBB function *in vitro* since they display structural and functional barrier properties owing to their high expression of tight junction

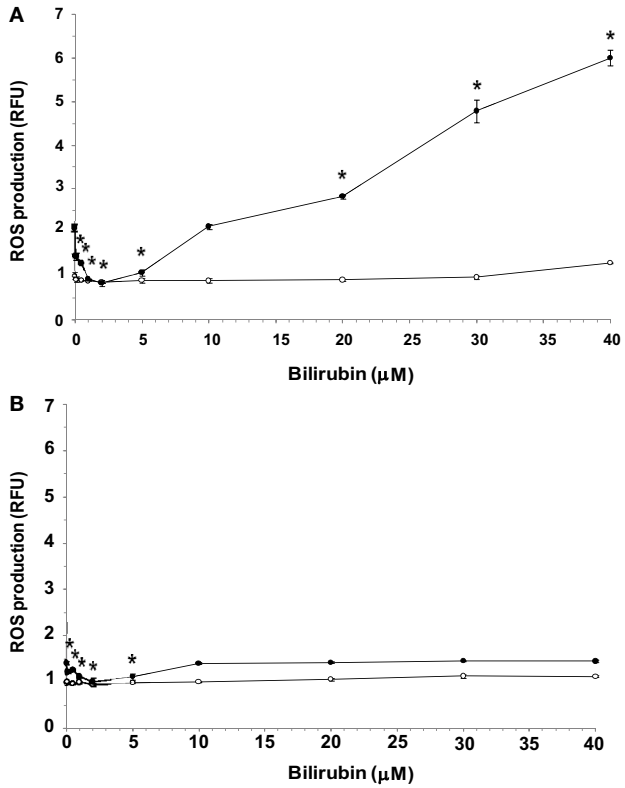

FIGURE 3 | Effects of UCB on the generation of ROS in microvascular endothelial cells cultured in normal or high glucose-containing media. Confluent cultures of bEnd3 **(A)** or MS1 **(B)** cells were incubated for 48 h with culture medium containing normal (5.5 mM; -O-) or high (25 mM; -●-) glucose, in the absence or presence of increasing concentrations of UCB added during the last 24 h of incubation. ROS production was assessed using the cell-permeant probe CM-H_2DCFDA, as described in Materials and Methods. ROS production was normalized by determining protein content and is expressed as relative fluorescence units (RFU), calculated by dividing the individual fluorescence values for the different treatments by that obtained for cells exposed to 5.5 mM glucose in the absence of UCB (the latter were assigned a value of 1). Experiments were repeated at least three times. The results of a representative experiment are shown as Mean ± Standard Deviation. *Statistically significant difference from 0 μM UCB at $p < 0.05$.

proteins and high transendothelial resistance (Brown et al., 2007; Li et al., 2010). Glucose enters these cells via the GLUT1 transporter. In contrast, the MS1 cells are highly fenestrated and lack barrier characteristics (Konstantinova and Lammert, 2004). Glucose passage through pancreatic islet microvascular endothelium is free and elicits the release of insulin from β-cells in a glucose concentration-dependent manner.

Increasing the glucose concentration of the culture medium from 5.5 to 25 mM in cultures of both endothelial cell types approximately doubled the caspase activity in the absence of UCB. The combination of a high glucose level and increasing UCB concentrations further increased the apoptotic response of the cells, suggesting that UCB and hyperglycemia induce apoptosis in these cells by independent mechanisms. In developing rat brain neurons, UCB facilitates glutamate-mediated apoptosis through the activation of *N*-methyl-D-aspartate receptors (Grojean et al., 2001) or by directly disrupting the integrity of the mitochondrial membrane, thus inducing mitochondrial depolarization and Bax translocation (Rodrigues et al., 2002). On the other hand, high glucose-mediated apoptosis of vascular endothelial cells results from increased ROS production (van den Oever et al., 2010; Hulsmans et al., 2012).

Under normal glucose conditions (5.5 mM glucose) there was no significant effect of UCB (0.1–40 μM) on ROS production in either cell type, suggesting that ROS are not responsible for UCB-mediated apoptosis in microvascular endothelial cells. In contrast, in the absence of UCB, high glucose levels induced a 2.1-fold increase in ROS production in the brain-derived cells and a 1.4-fold increase in the islet-derived cells.

Most strikingly, the two cell types studied showed different patterns of ROS production in response to the combined exposure to UCB and high glucose (**Figures 3A,B**). Such an exposure of brain-derived bEnd3 cells affected ROS production in a biphasic manner. Low ("physiological") concentrations of UCB (0.1–2 μM) attenuated high glucose-induced ROS production, while higher UCB concentrations markedly elevated ROS production under these conditions (**Figure 3A**). A similar initial decrease in ROS production was observed in the MS1 islet-derived cells exposed to UCB and high glucose. In both cell types the levels of ROS produced at 10 μM UCB resembled the ROS levels measured in the absence of UCB. However, contrary to the bEnd3 cells, further increases in UCB concentration did not cause further changes in ROS production in the MS1 cells (**Figure 3B**).

Thus, in the presence of high glucose, which by itself is an ROS generator, low UCB concentrations exhibit a protective effect by counteracting the excessive production of ROS in microvascular endothelial cells. The bEnd3 cells responded to higher UCB concentrations by exacerbating the high glucose-mediated oxidative stress, whereas the MS1 cells tolerated well this condition and did not increase further ROS production. These disparate responses suggest that hyperglycemia might compromise the function of the BBB during hyperbilirubinemia, while islets of Langerhans are more resistant to hyperbilirubinemia, and thus may preserve their insulin secreting capacity in response to hyperglycemia. Interestingly, hyperbilirubinemic Gunn rats do not develop diabetes in response to streptozotocin exposure, as compared to normobilirubinemic animals (Fu et al., 2010). Moreover, insulin secretion by the pancreatic islets was preserved in these hyperbilirubinemic animals.

Our data suggest that UCB *per se* does not affect ROS production in microvascular endothelial cells under normal glucose conditions. However, although ROS production under high glucose conditions was diminished by low UCB concentrations in both microvascular cell types, UCB-mediated apoptosis of these cells was not affected by this decrease in ROS production. These findings support a general concept that as much as ROS play a key role in inducing apoptosis, there are other pro-apoptotic factors that act independently of the redox state of cells.

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Evaluating the beneficial and detrimental effects of bile pigments in early and later life

Phyllis A. Dennery [1,2] *

[1] Division of Neonatology, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA, USA
[2] Division of Neonatology, Department of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA

Edited by:
Jaime Kapitulnik, The Hebrew University of Jerusalem, Israel

Reviewed by:
David K. Stevenson, Stanford University School of Medicine, USA
Michael Kaplan, Shaare Zedek Medical Center, Israel

***Correspondence:**
Phyllis A. Dennery, Division of Neonatology, Department of Pediatrics, University of Pennsylvania, 34th and Civic Center Boulevard, Philadelphia, PA 19104-4318, USA.
e-mail: dennery@email.chop.edu

The heme degradation pathway has been conserved throughout phylogeny and allows for the removal of a pro-oxidant and the generation of unique molecules including bile pigments with important cellular functions. The impact of bile pigments on health and disease are reviewed, as is the special circumstance of neonatal hyperbilirubinemia. In addition, the importance of promoter polymorphisms in the UDP-glucuronosyl transferase gene (*UGTA1*), which is key to the elimination of excess bilirubin and to the prevention of its toxicity, are discussed. Overall, the duality of bile pigments as either cytoprotective or toxic molecules is highlighted.

Keywords: neonatal jaundice, kernicterus, UDP-glucuronyltransferase, antioxidant, polymorphisms

INTRODUCTION

The generation of bile pigments occurs through a unique pathway for the degradation of heme, limited by the enzyme hemeoxygenase (HO). This enzymatic reaction requires molecular oxygen (O_2) and NADPH as a reducing equivalent and results in the formation of biliverdin and the release of carbon monoxide (CO) in an equimolar ratio. In addition, reduction of heme iron from Fe^{2+}–Fe^{3+} occurs with the transfer of electrons from oxygen with NADPH as a source of reducing equivalents. Also, oxidative cleavage of the α-methene carbon bridge in the heme molecule forms biliverdin, releases carbon monoxide and iron (**Figure 1**). In most circumstances, heme does not accumulate freely but rather, it is bound to hemo proteins that are essential for cellular metabolism. An intricate enzymatic cascade regulates the production of heme (Ryter and Tyrrell, 2000). In pathological conditions, free heme is released from hemoglobin and can deposit in tissues because it is lipophilic and can lead to the formation of oxygen radicals therefore, the enzymatic reaction of HO is essential to preventing this since HO-1 is highly inducible by the substrate heme or by oxidative stress (Chang et al., 2005). The constitutive isoenzyme HO-2 can also catalyze the degradation of heme and is found in high abundance in the brain and testes (Maines et al., 1986; Maines, 1988). In the last steps of the reaction, biliverdin reductase (BVR), a microsomal enzyme, converts biliverdin into bilirubin in a non-rate-limiting fashion (**Figure 1**). Bilirubin, unlike biliverdin is not water soluble, but rather lipophilic, and can penetrate cellular membranes. To be made water soluble and therefore excretable in the gastrointestinal tract, it must be conjugated. The latter is regulated by UDP-glucuronosyl transferase 1A1 (UGT1A1), an enzyme that adds two glucuronide residues to bilirubin to render it water soluble (**Figure 1**).

The byproducts of HO-mediated heme degradation can have both positive and negative effects on cellular function. These will be outlined in this review.

BILIRUBIN

EFFECTS OF BILIRUBIN IN THE NEONATE

Newborns have elevated numbers of red blood cells with a shortened life-span. When these cells lyse, heme is released from hemoglobin. In addition, due to a reduced ability to conjugate bilirubin formed during the degradation of heme, this pigment can accumulate in the serum in the first days of life leading to a transient hyperbilirubinemia, which typically resolves within the first weeks of life. In fact, the average full-term newborn infant has a peak serum bilirubin concentration of 5–6 mg/dL (86–103 μmol/L). This level is referred to as physiologic jaundice. However, in some circumstances, such as increased accumulation of heme (i.e., birth trauma, bruising, hemolysis), serum bilirubin levels can increase beyond the physiologic range. If serum bilirubin values are between 7 and 17 mg/dL (104–291 μmol/L), this is then referred to as exaggerated physiologic jaundice. However, this must be judged according to the infant's age in hours on the bilirubin nomogram, as serum bilirubin levels change rapidly during the first week of life. Serum bilirubin concentrations higher than 17 mg/dL in full-term infants are considered pathologic and can be associated with adverse sequelae. Fortunately, most infants will not be affected until their bilirubin levels are significantly higher than 17 mg/dL because the toxicity of bilirubin is dictated by many factors including age (the younger, the more vulnerable), maturity (prematures are more vulnerable), and associated illnesses (hemolysis, sepsis, acidosis worsen bilirubin toxicity) amongst other factors. The most severe manifestation of bilirubin toxicity is kernicterus, a rare but devastating condition with acute

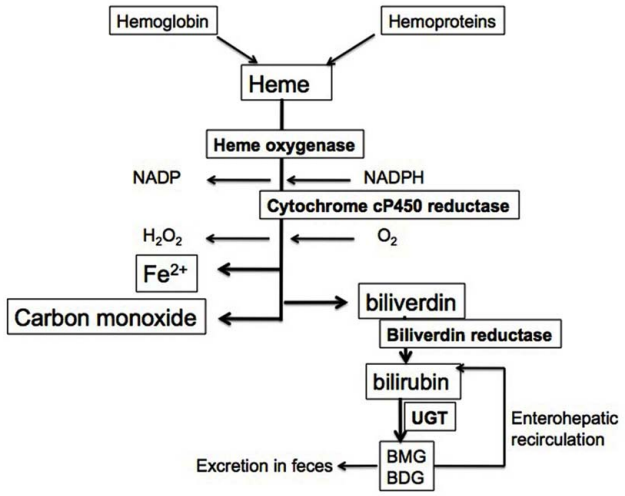

FIGURE 1 | Heme degradation pathway. Heme from hemoglobin and cellular hemo proteins is metabolized in a rate-limiting step by HO. The reaction utilizes molecular oxygen and NADPH as a reducing equivalent. This leads to the release of iron, water, and CO as well as biliverdin. The latter is converted in a non-rate-limiting fashion to bilirubin by BVR. Since bilirubin is not water soluble, it is conjugated to monoglucuronide and diglucuronide forms, which can then be excreted. These glucuronide residues can be removed by intestinal bacteria and allow unconjugated bilirubin to re-enter the circulation.

neurological abnormalities including seizures, opisthotonus, and hypertonia and long lasting sequelae including sensorineural deafness, athetoid cerebral palsy, and delayed motor skills (Dennery et al., 2001).

Prior to the late 1980s, physicians had a very aggressive approach to the management of hyperbilirubinemia in neonates, which included institution of phototherapy at low levels of bilirubin and exchange transfusion if the level exceeded 20 mg/dL in all cases. This approach changed radically since kernicterus was such a rare condition and, with the advent of Rhogam prophylaxis for Rh-negative pregnant mothers, kernicterus was thought by some to be nearly eradicated in developed nations (Watchko and Oski, 1983). In addition, reports of the beneficial, antioxidant effects of bilirubin (Stocker et al., 1987, 1990; McDonagh, 1990) made this aggressive approach seem even more unwarranted. Alas, with this change in attitude, pediatricians saw a resurgence of bilirubin neurotoxicity, and were faced with litigation for the negligent practice of not having taken simple measures to prevent the devastating consequences of hyperbilirubinemia (Maisels and Newman, 2007; Bhutani et al., 1999). In the present day, we have adopted a more thoughtful approach to this preventable problem by using strict guidelines for universal screening and by instituting therapy guided by age-based serum bilirubin thresholds (Bhutani et al., 1999; Maisels and Newman, 2007).

It is not clear why bilirubin accumulates in the first days of life and what is its value. Physiologic hyperbilirubinemia is a phenomenon only seen in mammals. Many have speculated a teleological benefit to this reaction (McDonagh, 1990) and laboratory investigations do confirm that bilirubin has significant antioxidant properties both *in vitro* (Stocker et al., 1987; Mireles et al., 1999; Granato et al., 2003) and *in vivo* (Dennery et al., 1995; Mayer, 2000). Perhaps this may be useful in the transition from the relatively hypoxic environment of the wound to ambient air. This is not yet clear. Also, the exact dose at which bilirubin is toxic to cells vs. beneficial is also not yet known.

In infants with Rh hemolytic disease, peak serum bilirubin concentrations above 20 mg/dL predict poor outcome however, many infants without an obvious hemolytic etiology for their jaundice are normal at serum bilirubin concentrations of 25 mg/dL or higher. Looking at this more closely, 8% of infants with Rh-associated hemolysis and serum bilirubin concentrations of 19–24 mg/dL had kernicterus whereas this condition was observed in 73% of infants with concentrations of 30–40 mg/dL demonstrating the dose effect of bilirubin, at least in hemolytic children. Bilirubin enters the brain and targets the basal ganglia (Johnston and Hoon, 2000) and the auditory nerve preferentially (Shapiro and Nakamura, 2001). This occurs if bilirubin is not bound to albumin or is unconjugated or if there has been damage to the blood–brain barrier. As an example, a newborn infant with a serum albumin concentration of 3 g/dL can bind 25 mg/dL of bilirubin. In current clinical practice, the bilirubin albumin ratio is taken into consideration to determine levels at which physicians should proceed to more aggressive management of hyperbilirubinemia (i.e., exchange transfusion). If the serum albumin concentration is low, the risk of kernicterus increases because free bilirubin enters tissues and causes its toxic effects (Ahlfors et al., 2009). Conditions that alter the blood–brain barrier, such as infection, acidosis, hyperoxia, sepsis, prematurity, and hyperosmolarity, may also affect the entry of bilirubin into the brain (Dennery et al., 2001).

MECHANISMS OF TOXICITY OF BILIRUBIN

Bilirubin has high affinity for membrane phospholipids thereby entering cells and inhibiting mitochondrial enzymes (Chuniaud et al., 1996; Rodrigues et al., 2002a), interfering with DNA synthesis, inducing DNA-strand breaks (Rosenstein et al., 1983), and inhibiting protein synthesis and phosphorylation. In the brain, bilirubin inhibits the uptake of tyrosine, reduces synaptic transmission, and inhibits *N*-methyl-D-aspartate-receptor ion channels. Overall, bilirubin can interfere with neuro excitatory signals and impair nerve conduction particularly in the auditory nerve (Bratlid, 1990). Unconjugated bilirubin directly interacts with mitochondria influencing membrane lipid and protein properties, redox status, and cytochrome c content (Rodrigues et al., 2002b). It can work in concert with amyloid β peptide to activate apoptosis in neural cells (Rodrigues et al., 2000). Interestingly, younger animals are less susceptible to bilirubin-related mitochondrial injury (Rodrigues et al., 2002c) and the toxicity of bilirubin is not restricted to neonates. In patients with Crigler–Najjar I syndrome and absent activity of the UGTA1, therefore a complete lack of ability to conjugate bilirubin, the risk of kernicterus is quite high especially in the face of intercurrent illnesses and these patients require life-long phototherapy and/or liver transplantation (Strauss et al., 2006).

In the nervous system, the susceptibility of bilirubin varies with cell type (Ngai et al., 2000). In brain endothelial cells, bilirubin resulted in apoptosis in a time-dependent manner (Akin et al.,

2002). Unconjugated bilirubin induced protein oxidation and lipid peroxidation and reduced antioxidant defenses in neuronal cells in culture (Brito et al., 2008). Although toxic to cultured neuroblastoma cells, exposure to unconjugated bilirubin induced genes involved in the endoplasmic reticulum stress response in surviving cells thereby enhancing cellular homeostasis (Calligaris et al., 2009). In astrocytes, unconjugated bilirubin up-regulated the multidrug resistance-associated protein-1 and increased its trafficking to the plasma membrane, thus reducing its cytotoxicity by preventing its intracellular accumulation (Gennuso et al., 2004).

The toxic effects of bilirubin are not limited to the brain. Unconjugated bilirubin can mediate apoptosis in cultured hepatocytes by increasing oxidative stress and enhancing caspase-9 activity (Oakes and Bend, 2005). In erythrocytes, high bilirubin concentrations can induce hemolysis and lead to membrane disruption, which could theoretically worsen hemolytic anemia (Brites et al., 1997). We observed that in erythrocytes derived from cord blood, concentrations of bilirubin equal to or exceeding 30 mg/dL were associated with increased protein oxidation, decreased erythrocyte glucose-6 phosphate dehydrogenase and adenosine triphosphatase activity as well as altered cell membrane integrity (Mireles et al., 1999). There was a correlation between the release of unconjugated bilirubin and hepatotoxicity after TNF-a administration, in mice and this was resolved with HO inhibitors (Van Molle and Libert, 2003). Another important cytotoxicity of bilirubin involves its effects on complement activation, a key element of immune defense. Unconjugated bilirubin interferes with the interaction between C1q and immunoglobulins, which results in decreased complement activation via the classical pathway (Basiglio et al., 2010).

ANTIOXIDANT BENEFITS OF BILIRUBIN

Despite the fact that bilirubin may be toxic at higher concentrations, there is still significant evidence that it is a potent antioxidant at micromolar concentrations *in vitro* and *in vivo*. In fact, bilirubin is the most abundant cellular antioxidant. *In vitro*, bilirubin is a chain-breaking molecule that can scavenge the hydroxyl radical better than a-tocopherol, a well-known antioxidant against lipid peroxidation (Stocker et al., 1987; Mireles et al., 1999). Although incubation with bilirubin and albumin at concentrations greater than 30 mg/dL was associated with dose-dependent injury in erythrocytes derived from cord blood, protection against lipid peroxidation was seen at lower concentrations (Mireles et al., 1999), indicating the duality of bilirubin as a cytotoxic and cytoprotective molecule. Similarly, hemeoxygenase is both detrimental and beneficial based on levels of activity. Nevertheless, in that study, no change in bilirubin levels could be detected to explain the toxicity of hemeoxygenase (Suttner and Dennery, 1999).

Similarly, *in vivo*, there are several examples of the beneficial effects of hyperbilirubinemia. In 50 patients older than 40 years with Gilbert syndrome, a relatively benign condition leading to mild to moderate unconjugated hyperbilirubinemia because of impaired glucuronidation, occurrence of ischemic heart disease was compared to that of a large cohort of patients without the disease. Ischemic heart disease occurred in only 2% of the Gilbert patients compared to 12.1% of the controls and, interestingly, hyperbilirubinemia rather than elevation of HDL cholesterol levels seemed to be more important in protection from ischemic heart disease (Vitek et al., 2002). In a case report, resolution of corticosteroid- and cyclophosphamide-resistant pulmonary fibrosis occurred with onset of hyperbilirubinemia due to biliary obstruction in a patient who developed elevated conjugated bilirubin levels (Ohrui et al., 2001), suggesting that higher serum bilirubin levels could reverse pulmonary fibrosis. The mechanisms by which this could occur are not yet explored. In organ transplantation, bilirubin can be protective against graft rejection (Ollinger et al., 2007). Injection of bilirubin in mouse organ recipients prolonged islet allograft survival and induced tolerance induction and graft acceptance via a regulatory T cell-dependent mechanism involving CD4(+) and CD25(+) cells. In fact, bilirubin enhanced *de novo* generation of regulatory T cells in the recipients thereby preventing rejection (Rocuts et al., 2010). Another novel mechanisms by which bilirubin may be protective is by the regulation of rapid eye movement sleep and by mediating some of the antidepressant effects of ambient light (Oren, 1997). Whether the antioxidant effects mediate these benefits is not yet clear.

Overall, the beneficial effects of bilirubin have been demonstrated in various models but beyond a certain threshold, bilirubin is clearly toxic.

EPIDEMIOLOGIC EVIDENCE OF BILIRUBIN AS A CYTOPROTECTIVE MOLECULE

To further understand whether bilirubin is cytoprotective in humans, epidemiologic studies can provide a clue. Regulation of bilirubin conjugation is key in the accumulation of bilirubin and its potential benefits or toxicity, therefore, studies comparing patients with differences in the ability to conjugate bilirubin may provide clues. The promoter of the *UGT1A1* gene has regions of TA repeats, which regulate its transcriptional efficiency. In Caucasian populations, an additional TA repeat (TA_7 vs. TA_6) is necessary but not sufficient to cause Gilbert syndrome (Bartlett and Gourley, 2011). Strong associations between polymorphisms in the *UGT1A1* gene and human disease have been shown. In particular, there have been associations with altered bilirubin conjugation and the occurrence of various cancers. The common *UGT1A1**28 allele results in elevated plasma bilirubin levels and is strongly associated with Gilbert syndrome in Caucasians. Low serum bilirubin levels observed in a Caucasian cohort with predicted high activity of *UGT1A1* were associated with an increased risk of esophageal cancer. Interestingly, the *UGT1A8* and *UGT2B4* genotypes, associated with decreased UGT enzyme activity and increased unconjugated bilirubin levels, were also significantly associated with increased risk of esophageal cancer (Dura et al., 2012). In another study, the *UGT1A* gene cluster on chromosome 2q37.1 was identified in a cohort of patients with bladder cancer suggesting that enhanced UGT1A may protect from bladder cancer by increasing the removal of carcinogens from bladder epithelium (Tang et al., 2012). In a meta-analysis of 21 case-control studies cancer risk was associated with intermediate, and low activity of *UGT1A7* genotypes, found predominantly in Asians (Lu et al., 2011). In contrast to the other studies, the TA repeat polymorphism of *UGT1A1* gene did not alter prostate cancer risk susceptibility in Caucasian men (Karatzas et al., 2010).

Overall, these studies suggest that the concentration of bilirubin in the serum determines whether it is beneficial or detrimental.

Not only does the *UGT1* gene play a role in cancer, it appears to have important effects in other diseases. For example, the homozygous state associated with higher serum bilirubin levels appeared to be protective against Crohn's disease (de Vries et al., 2012).

Serum bilirubin, independent of variation in *UGT* promoter activity, is also associated with diseases in large populations, in particular in cardiovascular disease. In a Swedish cohort, plasma bilirubin was lower in 231 cases of ischemic stroke than in 462 matched controls but the difference reached significance only in women (Ekblom et al., 2010). In males with coronary artery disease, there was inverse association between serum total bilirubin and coronary artery calcification score. Additionally, bilirubin was associated with reduced c-reactive protein levels, which could explain the lower calcification scores (Zhang et al., 2012). In another study, bilirubin levels were also inversely associated with the presence of coronary heart disease. Interestingly, bilirubin levels were significantly raised after treatment with 80 mg simvastatin independent of changes in liver enzymes (Nolting et al., 2011).

Despite the beneficial effects of bilirubin, the biggest challenge remains determining a specific threshold at which bilirubin is toxic vs. beneficial. It seems paradoxical that early events in bilirubin toxicity may involve increased oxidative stress and changes in redox status (Tell and Gustincich, 2009) yet conversely, bilirubin alleviates oxidative stress.

ANTIOXIDANT PROPERTIES OF BILIVERDIN

Although biliverdin does not accumulate in mammals, since it is rapidly converted to bilirubin through the action of biliverdin reductase (BVR), it may have important signaling effects. In macrophages, biliverdin activates endothelial nitric oxide, resulting in NO-dependent S-nitrosylation of BVR. The mechanisms by which biliverdin mediates this effect was via the repression of Toll-like receptor-4 (Wegiel et al., 2011). In another study, rats injected intraperitoneally with biliverdin before undergoing lung transplantation had less evidence of inflammation, oxidative injury, and apoptosis suggesting that biliverdin has anti-inflammatory and anti-apoptotic effects (Wang et al., 2010). Despite these data, the most plausible effect of biliverdin is to serve as a signaling molecule that may regulate BVR (Lerner-Marmarosh et al., 2008). The properties of this enzyme have been reviewed at length in a previous issue of this journal (Gibbs et al., 2012).

OTHER POSSIBLE MEDIATORS OF HO-RELATED CYTOPROTECTIVE EFFECTS

Although the byproducts of the HO-1 reaction are important cytoprotective molecules, a likely factor that influences the beneficial effects of the HO reaction is the degradation of a potent oxidant, heme. In one example, induction of HO-1 prevented photodynamic therapy-induced tumor necrosis, but neither bilirubin, biliverdin nor CO was responsible for this cytoprotection. In fact, the iron chelator desferrioxamine enhanced the cytotoxic effects of photodynamic therapy suggesting that heme was key important to enhancing the tumor killing effects of this therapy (Nowis et al., 2006).

SUMMARY

Overall, the byproducts of the HO reaction are important cytoprotective molecules that have clinically significant effects in various diseases. Nevertheless, in most cases, these molecules can also be cytotoxic under specific circumstances and/or at high concentrations. Potential therapeutic interventions will need to balance the potential benefits with the risk of toxicity to be most effective.

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Regulation of bilirubin clearance by ligand-activated transcription factors of the endo- and xenobiotic metabolism system

Karl Walter Bock*

Department of Toxicology, Institute of Pharmacology and Toxicology, University of Tübingen, Tübingen, Germany
**Correspondence: bock@uni-tuebingen.de*

Bilirubin, the product of heme catabolism, represents an important endobiotic of the endo-and xenobiotic metabolism system including drug-metabolizing enzymes, drug transporters, and drug-inducible ligand-activated transcription factors (LATFs). Bilirubin is of clinical concern since severe neonatal jaundice may lead to "kernicterus" and neurotoxicity (Kapitulnik, 2004). Hyperbilirubinemia in the newborn is particularly a problem in preterm infants owing to more profound hemolysis, and immaturity of liver function. However, bilirubin is also an interesting antioxidant which may attenuate atherosclerosis (Lin et al., 2008). Hence, bilirubin synthesis and its catabolism has to be strictly controlled. The key to bilirubin's elimination is glucuronidation by UDP-glucuronosyltransferase UGT1A1. However, alternative pathways of bilirubin catabolism have also been observed. For example, the dioxin TCDD, a "classical" ligand of the Ah receptor (AhR) stimulates bilirubin elimination in congenitally jaundiced, UGT1A1-defective Gunn rats (Kapitulnik and Ostrow, 1977). Furthermore, bilirubin itself activates the AhR postnatally, leading to increased transcription of CYP1A1 and CYP1A2 in Gunn rats (Kapitulnik and Gonzalez, 1993). Bilirubin and its extrahepatically formed glucuronides are taken up from blood into hepatocytes by OATP1B1 and OATP1B3 (Nies et al., 2008). Following conjugation bilirubin glucuronides are biliary secreted by ABCC2.

UGT1A1 and all the above transporters are transcriptionally regulated by the constitutive androstane receptor CAR (Huang et al., 2003). In CAR-defective mice phenobarbital-mediated induction of these proteins is absent. When human CAR is transfected into CAR-null mice phenobarbital and bilirubin itself activate CAR, the latter finding suggesting homeostatic control between enzyme substrate and activation of its major transcription factor. Bilirubin is a ligand of the AhR, a multifunctional transcription factor which induces a different set of genes than CAR in the drug metabolism system (Nguyen and Bradfield, 2008). It was discovered by the induction of aryl hydrocarbon metabolism and as mediator of dioxin toxicity. However, the AhR is also involved in development, cell proliferation, differentiation, and immune biology. The importance of the AhR in UGT1A1 induction has been demonstrated in human hepatocyte cultures treated with 3-methylcholanthrene (Ritter et al., 1999). Notably, phenobarbital and bilirubin are not ligands of CAR but activate CAR via phosphorylation/dephosphorylation pathways (Timsit and Negishi, 2007). Binding sites for CAR and the AhR have been identified in a 290-bp cluster of the human UGT1A1, termed gtPBREM (phenobarbital responsive enhancer module; **Figure 1**; Sugatani et al., 2005). The gtPBREM cluster contains binding sites for a number of ligand-activated transcription factors including pregnane X receptor (PXR), glucocorticoid receptors GR1 and GR2, CAR, antioxidant-responsive Nrf2 (Yueh and Tukey, 2007), AhR, and fibrate-inducible peroxisome proliferator-activated receptor PPARα (Seneko-Effenberger et al., 2007). It is important to note that the CAR binding site (gtNR1) plays a central role in UGT1A1 induction by both CAR and PXR, which is supported by site-directed mutagenesis and electrophoretic mobility shift assays (Sugatani et al., 2005). The cluster appears to be evolutionary conserved since the same arrangement of LATF-binding sites was found in the baboon (Caspersen et al., 2007). Allelic variants involved in bilirubin clearance have also been detected in the gtPBREM cluster. For example, the $(TA)_7$ promoter polymorphism UGT1A1*28, responsible for Gilbert's syndrome, is often in linkage disequilibrium with the T3279G polymorphism in the gtPBREM cluster (Sugatani et al., 2008).

PERINATAL UGT1A1 INDUCTION

It is tempting to speculate that the gtPBREM cluster of UGT1A1 is related to perinatal UGT1A1 induction and bilirubin homeostasis in the adult (Bock and Köhle, 2010; Bock, 2011). In perinatal stressful conditions glucocorticoids may be involved in UGT1A1 induction by CAR and PXR (Pascussi et al., 2008). In addition to CAR (Huang et al., 2003), activated PXR has also been demonstrated to induce UGT1A1 and enhance bilirubin clearance (Xie et al., 2003). With regard to AhR-mediated induction evidence was obtained that both AhR and Nrf2 are required for UGT induction (Yeager et al., 2009). Interestingly, CAR was found to be low in neonatal livers. Hence, the deficit of CAR activity may contribute to neonatal jaundice (Huang et al., 2003).

BILIRUBIN'S ANTIOXIDANT FUNCTION

Ligand-activated transcription factors which bind in the distal gtPBREM cluster are operating in concert with other transcription factors such as HNF1 and HNF4 and with multiple factors of the proximal promoter. This is demonstrated by the $(TA)_7$ and T3279G polymorphisms which synergistically reduce CAR- and AhR-mediated UGT1A1 induction not only in transfected cells (Sugatani et al., 2008) but also in human liver samples (Li et al., 2009). Decreased UGT1A1 expression leads to increased serum bilirubin which may contribute to bilirubin's antioxidant function (Stocker et al., 1987a). Albumin-bound bilirubin or conjugated bilirubin have also been shown to exhibit antioxidant activity (Stocker et al., 1987b; Wu et al., 1996; Kapitulnik and Maines, 2009). Evidence was obtained that heme oxygenase/biliverdin reductase activity and biliverdin–bilirubin redox cycles are

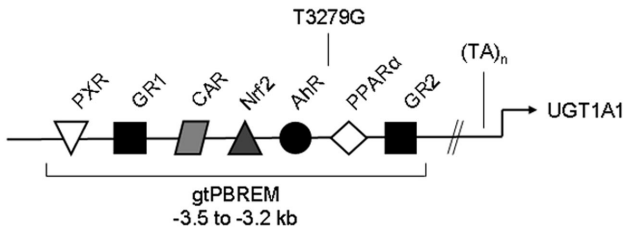

FIGURE 1 | Schematic illustration of allelic variants and the position of response elements binding ligand-activated transcription factors in the enhancer/promoter region of human UGT1A1. Positions are taken from Sugatani et al. (2005), and Bock and Köhle (2010). For discussion see text.

essential for bilirubin's antioxidant and local atheroprotective functions (Baranano et al., 2002). The antioxidant action of bilirubin for lipids can be complementary to glutathione (Sedlak et al., 2009). Bilirubin may be particularly effective in reducing lipid peroxides as it easily enters the lipid environment (Zucker et al., 1999). However, bilirubin catabolism by hepatic UGT1A1 activity is also decisive for bilirubin's antioxidant function, as evidenced by many epidemiologic studies with UGT1A1*28 homozygotes (Lin et al., 2008; Bock and Köhle, 2010, for references). Notably, UGT1A1-regulating LATFs may be therapeutic targets (Kapitulnik and Maines, 2009; Navarro et al., 2009). In conclusion, bilirubin has both neurotoxic and antioxidant functions which are keeping clinicians and basic scientists busy.

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