Products of heme oxygenase and their potential therapeutic applications

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Heme proteins play a critical role in many physiological processes including oxygen transport, mitochondrial respiration, and signal transduction (87). The majority of heme is present in hemoglobin (Hb), whereas other sources of heme proteins include myoglobin, mitochondrial and microsomal cytochromes, and various catalytic enzymes such as nitric oxide synthases, catalase, and respiratory burst oxidase (35). Free heme exerts cytotoxic effects through formation of oxygen free radicals and lipid peroxidation (7, 8, 33, 37, 73, 108). The kidney is particularly sensitive to free heme molecules, and heme-induced injury appears to be an important component of rhabdomyolysis (72), nephrotoxin (3, 97, 119), and ischemia-reperfusion (56, 96)-induced acute renal failure (ARF) in animal models.

Heme is degraded by the enzyme heme oxygenase (HO) to produce equimolar quantities of carbon monoxide (CO), iron, and biliverdin (BV). The latter is reduced to bilirubin (BR) by the enzyme biliverdin reductase. Experimental evidence suggests that induction of the HO system is an important endogenous mechanism for cytoprotection and that the downstream products of heme degradation, CO, BR, and BV, may mediate these powerful beneficial effects. These molecules, which were once considered to be toxic metabolic waste products, have recently been shown to have dose-dependent vasodilatory, antioxidant, and anti-inflammatory properties that are particularly desirable for tissue protection during organ transplantation. In fact, recent work has demonstrated that administration of exogenous CO, BR, or BV may offer a simple, inexpensive method to substitute for the cytoprotective effects of HO-1 in a variety of clinically applicable models. This review will attempt to summarize the relevant biochemical and cytoprotective properties of CO, BR, and BV, and will discuss emerging studies involving the therapeutic applications of these molecules in the kidney and other organ systems.

bilirubin; biliverdin; carbon monoxide; ischemia-reperfusion injury

Indispensable to survival and, in particular, to protection from oxidant stress (3, 41, 88). The HO-1 −/− mice exhibited a decreased birth rate, growth retardation, microcytic hypochromic anemia, tissue iron deposition, hepatosplenomegaly, lymphadenopathy, leukocytosis, and glomerulonephritis (88). Interestingly, not long after these findings were reported in mice, Yachie et al. (115) documented the first human case of HO-1 deficiency in a 6-yr-old boy. The child suffered from similar symptoms as the (−/−) mice, including stunted growth, anemia, iron deposition, and leukocytosis. Other findings included persistent proteinuria and hematuria, coagulation defects, hyperlipidemia, and hypobilirubinemia. Microscopic examination of renal biopsy samples revealed mesangial cell proliferation, lymphocyte infiltration, and detachment of the glomerular capillary endothelium (115).

Further investigations employing HO-1 knockout mice have indicated the importance of this gene in mediating ARF. Shiraishi et al. (97) administered cisplatin, a potent nephrotoxin, to HO-1 −/− mice and found significantly greater renal injury and more severe renal failure compared with wild-type mice. Conversely, overexpression of HO-1 caused a substantial reduction of cisplatin-induced renal injury (97). In a similar experiment, Nath et al. (74) noted severe, irreversible renal failure and 100% mortality in HO-1 −/− mice after heme protein-induced renal injury, whereas HO-1 +/+ mice displayed mild, reversible renal injury and 0% mortality.

Although the cytoprotective effects of HO-1 induction have been confirmed in a number of experimental models, the mechanism of action has not been completely elucidated. Degradation of the prooxidant heme molecule by HO-1 is likely to aid in tissue protection; however, recent evidence suggests that it is the downstream products of HO-1 activity (BV, BR, and CO) that mediate many of the anti-inflammatory,
antiapoptotic, antioxidant, and immune-modulatory effects associated with induction of HO-1. Iron is also liberated during the breakdown of heme. Whereas it has been shown that the induction of ferritin is enhanced in conjunction with HO-1 upregulation (6, 72), iron is an extremely prooxidative molecule. A number of studies have demonstrated prooxidative effects following HO-1 induction, primarily through the accumulation of iron and endothelial cell damage (16, 45, 48, 91, 92). For this reason, the administration of CO and BV/BR, rather than the induction of HO-1, may prove to be a safer and more practical means of conferring cytoprotection in a clinical setting. Administration of micromolar doses of BV, BR, and CO have now been shown to substitute for the effects of HO-1, providing dose-dependent cytoprotection (1, 5, 23, 52, 75, 82).

This review will focus on the downstream products of HO-1, including pertinent aspects of biochemistry, the mechanisms of cytoprotection in the kidney and in other organ systems, and current challenges in the therapeutic application of these molecules.

CO

CO was first discovered in the late 18th century and, until recently, was regarded solely as a toxic air pollutant. It is a colorless, odorless gas that is liberated by natural sources, or through incomplete combustion of organic material including wood, coal, and natural gas. The toxic effects of CO lie in its strong affinity to Hb, which is nearly 245 times that of oxygen (90). CO displaces oxygen from Hb, shifting the oxygen dissociation curve to the left, resulting in tissue hypoxia (28, 90). Symptoms of CO toxicity begin to occur at carboxyhemoglobin (CO-Hb) levels of 20% and include dizziness, shortness of breath, and headache. CO-Hb levels of 50 – 80% may result in death (90). The dangers of CO exposure have led the US Environmental Protection Agency to publish recommendations for allowable exposure levels [9 parts/million (ppm) averaged over 8 h or 35 ppm in 1 h] and to recommend the routine installation of CO detectors in homes and businesses.

In healthy nonsmoking adults, basal levels of CO-Hb range between 1 and 3% (90), and exhalation of CO varies from 0 – 6 ppm, depending on the amount of environmental exposure and normal endogenous production. (In smokers, CO-Hb can reach 10–15% and exhaled CO 7–70 ppm). HO cleavage of the α-meso carbon bridge of b-type heme accounts for >85% of endogenous CO, with the remainder originating from other metabolic pathways (64, 90). Elevated levels of exhaled CO are associated with chronic disease states such as asthma, bronchitis, and diabetes (85, 116). Intuitively, it seems that this increase in CO would be deleterious, yet experimental evidence now suggests that this “toxic” gas may exert cytoprotective effects in response to cellular stress. Several investigators have supplied exogenous CO to rats at levels far exceeding the EPA’s recommended exposure levels for humans (e.g., 250 ppm for 25 h) and have noticed significant improvements in transplanted organ survival (75). Thus despite the irrefutable
tivity that occurs following prolonged exposure to high concentrations of CO, it is apparent that that a physiological dose range exists wherein CO exerts vasodilatory, antiapoptotic, and anti-inflammatory effects.

Initial investigations into the beneficial physiological effects of CO revealed that this molecule exerts vasodilatory effects through cGMP-dependent smooth muscle relaxation. Similar to the well-established vasodilator nitric oxide (NO), CO binds to the heme moiety of soluble guanylyl cyclase (sGC), causing activation of cGMP and resultant vascular relaxation (19, 27, 39, 46, 51, 89, 106, 120). Whereas the affinity of CO for sGC is equivalent to NO, the potency of NO-stimulated cGMP production is 30–100 times greater than that for CO (81, 90). Both nitric oxide synthase (NOS) and HO-1 are coinduced in times of stress, and the majority of evidence suggests that HO may serve both to regulate and to continue the effects of NOS following the initial stress response. NO has been demonstrated to induce HO-1 and subsequent production of CO (21), whereas HO-1 and CO appear to serve as a feedback mechanism, inhibiting NOS activity (60, 113, 123). Because NO is both a potent vasorelaxant and a potential free radical (through the formation of peroxynitrite radicals), these results imply that HO-1 regulation of NOS functions to limit NO free radical production, while maintaining the vasodilatory properties of the molecule by means of CO-stimulated cGMP production. Additional cGMP-mediated effects of CO include neurotransmission (98, 109), inhibition of platelet aggregation (13) and vascular smooth muscle proliferation (62, 63), protection of pancreatic β cells from apoptosis (29), and bronchodilation (14, 26).

CO-mediated cytoprotection has also been described through interaction with MAPK signaling pathways. There are three subfamilies of MAPK pathways that modulate gene transcription: ERK, JNK, and p38. Several aspects of the protective effects of CO appear to be mediated through the p38 MAPK pathway, a system that is involved in the physiological response to stress signals (47). For example, exogenously supplied CO (300 ppm) was shown to decrease portal venous resistance via stimulation of the p38 MAPK pathway and preserve hepatic function in the isolated, perfused rat liver following 24 h of cold ischemia (5). The significant anti-inflammatory (83) and antiapoptotic (121) effects of CO following lung injury also appear to be mediated through p38 MAPK-dependent mechanisms.

A number of studies have analyzed the specific effects of CO on the inflammatory response. Endothelial injury induced by sepsis or organ transplantation leads to a cascade of events, including leukocyte margination and extravasation, increased expression of endothelial cell adhesion proteins (ICAM-1), secretion of inflammatory cytokines (IL-1β, IL2, IL-6, and TNF-α), and smooth muscle proliferation. In solid organ transplantation, the end result of these processes is vessel lumen occlusion and graft failure. CO has been demonstrated to modulate the inflammatory pathway in a variety of experimental models, reducing the production of inflammatory cytokines and preventing smooth muscle proliferation, while increasing the production of anti-inflammatory cytokines (IL-10) through interaction with the MAPK pathways. For example, CO has been shown to depress T cell proliferation and IL-2 production in vitro via inhibition of the ERK pathway (84) and to inhibit TNF-, IL-1β, and macrophage inflammatory protein-1β production while increasing IL-10 in vivo and in vitro (80). Additionally, CO has been shown to decrease IL-6 production in vivo through the JNK pathway in response to sepsis (65).

In addition to its role in response to tissue injury, CO also appears to have a regulatory role in vascular tone. CO is endogenously produced by HO-2 in the interlobar arteries and inhibits vascular sensitivity to phenylephrine via a mechanism involving the tetraethylammonium-sensitive 105-pS K channel (38). Similarly, ANG II-induced CO expression has been demonstrated in the rat renal vasculature, where it counteracted the pressor effects of ANG II (50). The ability of CO to offset such vasoconstrictive substances illustrates that HO and its metabolites exist as an endogenous regulatory system, offering cytoprotection in times of stress.

The beneficial vasodilatory, anti-inflammatory, and immunomodulatory effects of CO suggest that this molecule may have potential therapeutic applications. Recently, several investigators have presented evidence of cytoprotection in the lungs, kidneys, small intestines, liver, and pancreatic islet cells of rats following CO inhalation or induction (68–70, 75, 79, 100, 111, 123). Song et al. (100) performed orthotopic lung transplantsations and exposed the recipient animals to 500 ppm CO following surgery, demonstrating a marked reduction of apoptosis, suppression of proinflammatory genes, and preservation of tissue architecture in the CO-treated vs. control rats. Neto et al. (75) demonstrated similar protective effects in a syngeneic rat kidney transplant model. Recipient rats were exposed to CO (250 ppm) for 1 h before and 24 h following orthotopic kidney transplantation. Results indicated a marked decrease in inflammatory mediators, improved renal cortical blood flow, preservation of glomerular and tubular architecture, and increased survival in CO-treated rats vs. control (75). Exogenous CO administration has also been shown to produce an analogous reduction of proinflammatory molecules and improved survival in rats undergoing orthotopic small intestinal transplantation (68, 69) and protect against intestinal inflammation in a rat model of necrotizing enterocolitis (123). Most recently, CO induction, following oral administration of methylene chloride, was shown to protect against chronic rejection of rat renal allografts when administered to the donor 4 h before organ harvesting (58).

It is evident that CO is not merely an injurious byproduct of heme catabolism but that it serves a clear physiological role in cellular defense. While exogenous supplementation of the molecule effectively ameliorates inflammatory and ischemic injury in rats, the logistics (i.e., dose, timing of delivery, and safety) of supplying this potentially toxic gas to human patients have not been determined and could complicate the clinical application of this technique. Recently, novel water-soluble CO-releasing molecules are being investigated, which may facilitate therapeutic delivery of CO (25, 66). Unfortunately, CO therapy is most effective when administered before the onset of renal injury in rats (107), and clinical applications may be limited to preconditioning of organs before elective procedures that involve known tissue injury.

BR

In adults, ~250–350 mg of BR are produced daily, primarily through the breakdown of Hb. HO opens the heme (Fe-protoporphyrin IX) molecule ring, liberating iron and CO and...
forming the linear tetrapyrrole molecule BV, which is then reduced to BR. The BR molecule consists of two rigid planar dipyrrole units joined by a methylene bridge at carbon 10 and can exist as three isomers, i.e., IXα, IXβ, and XIIIβ, with IXα being the natural structure formed from heme catabolism (Fig. 2) (77). BR also exists as three different pH-dependent ionic species, and the proportions of each species are determined by the pKₐ values of the −COOH groups on the carboxymethyl sidechains: 8.1 and 8.4 (31). At physiological pH (7.4), ~83% of BR is present as the protonated diacid (H₂B), whereas 16% is monoanion (HB⁻) and <1% dianion (B²⁻) (Fig. 3) (77). The solubility of BR (H₂B) at neutral pH is low, ~70 nM, due to internal hydrogen bonding of the polar groups. However, if pH rises above the pKₐ values for the COOH, ionization of these groups leads to greater proportions of HB⁻ and B²⁻, and the solubility of BR increases dramatically, reaching 1 mM at pH 9 and 60 mM above 9.5 (77).

The solubility of unconjugated BR at neutral pH is improved by the high degree of protein binding that occurs between BR (IXα) and albumin in the plasma, whereas intracellular proteins, such as glutathione-S-transferase (GST), are bound to BR in the cytosol. Albumin has one high-affinity binding site and one or more lower-affinity sites for BR (77). In adults, the normal plasma concentration of unconjugated BR is between 5 and 15 μM, and >99% is bound to albumin. The association of BR with albumin serves not only to improve solubility at physiological pH but also to sequester the potentially toxic molecule because unbound, nonionized BR is capable of crossing cell membranes and can interfere with mitochondrial respiration when concentrations are >50 μM (67). Toxicity can develop when albumin binding becomes saturated and BR concentrations are >200–300 μM (78). The potential for BR toxicity is also increased at pH <7.4, as BR dissociates readily from albumin and is able to bind to cell and mitochondrial membranes, leading to cell lysis or disruption of mitochondrial function (12, 67).

Unconjugated, albumin-bound BR is transported to the liver, where it dissociates from albumin and spontaneously diffuses through phospholipid bilayers (122) into hepatocytes. Within the hepatocyte, BR is bound to cytosolic proteins and glucuronic acid is attached to one or both of the propionic side chains of BR by the microsomal enzyme BR uridine-diphosphosphate glucuronosyltransferase (UDPGT), forming water-soluble BR monoglucuronide and diglucuronide, which are then excreted into the bile and eliminated from the body.

Like CO, unconjugated BR has long been considered solely as a toxic waste product of heme metabolism. Indeed, hyperbilirubinemia is responsible for diseases such as neonatal jaundice and kernicterus, and BR is capable of contributing to other forms of cytotoxicity (40). The question of why BV, a nontoxic and water-soluble compound, is reduced to the potentially toxic and insoluble BR molecule has long been unanswered. However, in the past three decades, beneficial properties of BR have been identified that begin to elucidate the physiological role of BR.

The primary mechanism for BR-mediated cytoprotection in various types of stress appears to be due to the powerful antioxidant activity of this molecule. In 1987, a landmark study by Stocker et al. (101) introduced the idea that BR served as one of the most important endogenous antioxidants in the serum. In fact, at physiological oxygen concentration (2%), micromolar amounts of BR were able to scavenge peroxyl radicals more effectively than α-tocopherol, which had previously been considered the most powerful serum antioxidant. Subsequent studies have demonstrated that superinduction of HO leads to BR-mediated reductions in oxidative stress following renal ischemia (57) and provides cytoprotection in cardiomyocytes (24) and neurons (20) subjected to oxidative stress. New evidence indicates that BR may also serve as an important mediator of nitrosative injury (44) through a similar...
mechanism. BR and BV were shown to scavenge peroxynitrite, an extremely potent and stable oxidant formed by the interaction of NO and superoxide anion (44, 61), and NO has been revealed as an inducer of HO-1 expression. These results are particularly noteworthy, because NO production is often increased in the kidney during ischemic injury or oxidative stress. Although NO is a well-recognized signaling molecule with numerous beneficial properties (19, 27, 46, 51, 120), excessive production of the molecule can contribute to oxidative damage through the formation of peroxynitrite (120).

Along with potent antioxidant properties, BR also exerts anti-inflammatory effects. In 1999, a study by Hayashi et al. (32) explored the relationship between HO-1 and endothelial cell-leukocyte interactions in vivo. The investigators induced HO-1 expression in mesenteric tissues by intraperitoneal injection of hemin and subsequent oxidative stress by either hydrogen peroxide infusion or ischemia-reperfusion injury (IRI). Leukocyte adhesion and rolling were inhibited in the HO-1-induced rats compared with control rats. Inhibition of HO-1 expression using zine protoporphyrin-IX reversed these findings. However, further supplementation of BR or BV, but not CO, again prevented leukocyte adhesion (32).

The effects of BR appear to be particularly valuable in preventing cardiovascular disease. Mildly increased serum BR levels have been shown to decrease risk for the development of coronary artery disease (CAD) and atherosclerosis in humans (59). A report by Schwertner et al. (93) indicated that a 50% decrease in total BR was associated with a 47% increase in the chance of having severe CAD. A similar study compared the protective effects of BR with that of HDL cholesterol (36). Furthermore, the prevalence of ischemic heart disease in individuals affected by Gilbert syndrome, a deficiency of UDPGT resulting in sustained unconjugated hyperbilirubinemia, is 2% compared with 12% in the general population (110). A similar UDPGT deficiency in the Gunn rat confers resistance to the pressor effects of ANG II, presumably through scavenging of reactive oxygen species (ROS) by BR (86). High serum BR levels have also been associated with decreased cancer mortality (102), resolution of asthma symptoms (76), and a decreased incidence of retinopathy of prematurity (34).

The aforementioned properties of BR suggest that the molecule may be a vital factor in mediating ARF due to toxic or ischemic injury, which are characterized by varying degrees of cell injury, leukocyte infiltration, and the generation of inflammatory mediators and ROS. Based on the documented antioxidant and anti-inflammatory properties of BR, several investigators have pursued the direct use of exogenous BR therapy to minimize the effects of IRI associated with organ transplantation. One such study compared the protective effects of heme-induced HO-1 vs. administration of micromolar amounts of BR in a rat liver transplantation model (42). Results indicated that flushing the liver with BR was equally as effective at defending against oxidative stress as HO-1 induction. These results suggest that supplementation of BR may provide a simple means of organ protection during graft harvest, which is inevitably associated with a period of ischemia and oxidative injury. Recently, work in our laboratory demonstrated that micromolar doses of exogenous BR offered similar protective effects in the isolated, perfused rat kidney during IRI (1). Rat kidneys flushed with 10 μM BR demonstrated significant improvements in urine output, glomerular filtration rate, tubular function, and mitochondrial integrity after 20 min of warm ischemia. A study by Leung et al. (49) described the marked reduction of glycerol-induced ARF following ligation of the common bile duct. This group also demonstrated that micromolar concentrations of BR were protective against heme-induced renal cell injury in vitro (49). Exogenous BR supplementation has also been shown to ameliorate oxidative injury in the spinal cord in a rat model of multiple sclerosis (53) and to preserve mucosal integrity in a rat model of intestinal ischemia (15).

The most effective dose and timing of administration of BR for prevention of IRI are unclear and appear to vary widely among different organs and experimental models. One method for establishing a proposed dose of exogenous BR is to approximate the physiological levels of BR produced after induction of HO-1. Previous studies have reported that in the glycerol model of ARF, heme content increases in the kidney to ~10 μM within 3 h (73), a time point that corresponds to maximal induction of HO-1. Because BV is produced in an equimolar ratio to the amount of heme degraded by HO-1, 10 μM BR could potentially be generated in this model. The exogenous BR concentrations used in the previously mentioned studies (1, 17, 42, 49) ranged from 0.05 to 10 μM and fell within the reference range for serum BR concentrations in the rat (0 – 0.64 mg/dl) (95). In the rat kidney, 10 μM BR provided a maximal protective effect in vitro (1), although the in vivo dose response has not been determined. Similar to CO, it appears that supplying BR before an ischemic insult results in superior protection (17). The requirement for pretreatment with BR may limit the clinical applicability of this protective agent to elective situations in which renal injury is anticipated. Even with this caveat, use of BR may have wide therapeutic application in organ transplantation or for use before administration of nephrotoxic drugs and radiographic contrast agents.

**BV**

Unlike BR, BV is a soluble and nontoxic compound; however, it is quickly reduced to BR by the enzyme biliverdin reductase (BVRA). The conversion of BV to BR is a powerful redox cycle that results in augmentation of the BR molecule. Baranano et al. (9) demonstrated that 10 nM BR was able to protect cells against 10,000-fold higher concentrations of hydrogen peroxide. This was possible because BR is oxidized to BV, which is recycled back to BR by BVRA, resulting in a 10,000-fold amplification of BR available to sequester ROS (9).

Recently, several investigators have chosen to supply exogenous BV as a means of harnessing the antioxidative properties of BR. Yamashita et al. (117) found that pretreatment with BV (50 μmol/kg) resulted in an increased survival of heart allografts. Similarly, Nakao et al. (71) described significantly decreased inflammatory mediators, leukocyte infiltration, and organ injury in BV-treated (50 mg/kg) transplanted small bowel. Moreover, Fondevila et al. (23) revealed a dramatic decrease in apoptosis, inducible NOS expression, leukocyte infiltration, and proinflammatory cytokine expression with a profound increase in hepatic function, antiapoptotic genes, and animal survival when BV (10 and 50 μmol/l) was added to the perfusate of an ex vivo orthotopic liver transplantation model. Whereas administration of BV alone was ineffective in rat syngeneic kidney and heart transplant models, coadministra-
tion of BV and CO provided significant protection of organ function and improved survival (70). BV may provide a safer and potentially more effective means of BR delivery; further studies are needed to determine the most appropriate dose and timing of BV delivery.

IRON
Along with the generation of CO and BV, iron is also liberated from the degradation of heme. Free iron is an extremely prooxidative molecule, primarily through its role in the Fenton reaction (30). Ferritin is a ubiquitously existing intracellular protein that is able to effectively sequester intracellular iron and, hence, limit its prooxidative capacity. Whereas no cytoprotective properties of free iron have been described, the induction of HO-1 has been linked to the upregulation of ferritin (6, 72). Some suggest that the induction of ferritin is equally, if not more, advantageous than the induction of HO-1 and that the antioxidative property of ferritin is superior to that of BR (6). Recently, Berberat et al. (10) found that overexpression of heavy chain ferritin was associated with the inhibition of endothelial cell and hepatocyte apoptosis following IRI in vivo and ex vivo. This finding may offer another potentially therapeutic method of diminishing oxidative damage in organ transplantation.

CONCLUSION
Induction of HO-1 by cellular stress leads to the production of molecules with antioxidant, antiapoptotic, and immunomodulatory properties. Although it is apparent that administration of exogenous CO, BR, or BV alone can lead to potent cytoprotective effects, experimental evidence suggests that the coordinated response of all elements may be necessary for maximal cellular defense (70). Further research involving HO and its downstream products will certainly provide important insights and relevant information. As our understanding of the cellular and molecular events surrounding IR, ARF, and transplant rejection continues to expand, so should our capability of manipulating the involved pathways for the preservation of organ function and, ultimately, patient survival.

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REFERENCES
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