Carbon Monoxide and Bilirubin

Potential Therapies for Pulmonary/Vascular Injury and Disease

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Heme oxygenase (HO)-1, an inducible, low-molecular-weight stress protein, confers cellular and tissue protection in multiple models of injury and disease, including oxidative or inflammatory lung injury, ischemia/reperfusion (I/R) injuries, and vascular injury/disease. The tissue protection provided by HO-1 potentially relates to the endogenous production of the end products of its enzymatic activity: namely, biliverdin (BV)/bilirubin (BR), carbon monoxide (CO), and iron. Of these, CO and BV/BR show promise as possible therapeutic agents when applied exogenously in models of lung or vascular injury. CO activates intracellular signaling pathways that involve soluble guanylate cyclase and/or p38 mitogen-activated protein kinase. Although toxic at elevated concentrations, low concentrations of CO can confer antiinflammatory, antiapoptotic, antiproliferative, and vasodilatory effects. BV and BR are natural antioxidants that can provide protection against oxidative stress in cell culture and in plasma. Application of BV or BR protects against I/R injury in several organ models. Recent evidence has also demonstrated antiinflammatory and antiproliferative properties of these pigments. To date, evidence has accumulated for salutary effects of CO, BV, and/or BR in lung/vascular injury models, as well as in models of transplant-associated I/R injury. Thus, the exogenous application of HO end products may provide an alternative to pharmacologic or gene therapy approaches to harness the therapeutic potential of HO-1.

Keywords: bilirubin; carbon monoxide; heme oxygenase-1; inflammation; ischemia/reperfusion

In the context of lung/vascular injury and pathophysiology, inducible stress protein responses have emerged as important components of innate tissue defenses against oxidative stress, inflammation, and other injurious insults (1–3). Preconditioning strategies, involving the manipulation of stress protein gene expression for therapeutic gain, have been developed for the lung and cardiovascular systems, as well as for other organs, as a means to limit tissue injury associated with ischemia/reperfusion (I/R), whether arising from vascular disease, surgical procedures, or organ transplantation (4). In such an approach, organ preconditioning occurs in response to pretreatment (of the organ) with an inducing agent or condition sufficient to invoke a transcriptional response and subsequent accumulation of stress protein product, which, in turn, confers tissue protection. Experimentally, the manipulation of stress protein gene expression remains

CLINICAL RELEVANCE

This review discusses the use of heme metabolites as therapeutic agents for lung/vascular disease, with potential clinical applications.

a feasible therapeutic approach. Among the major stress protein families, the heat shock proteins (Hsps), which respond transcriptionally to hyperthermia and confer tissue thermotolerance, can also confer protection against myocardial I/R injury when overexpressed or preinduced with hyperthermia (5, 6). The low-molecular-weight stress protein, heme oxygenase (HO)-1, a distinct stress protein not directly related to the Hsps, also confers tissue protection in multiple injury models, including oxidative and inflammatory lung injury, vascular injury, and multiorgan and/or transplant-associated I/R injury (1). The importance of HO-1 in tissue homeostasis is underscored by studies using HO-1 knockout mice (*ho-1*^{-/-}), which show enhanced oxidative stress sensitivity, as well as aberrations in tissue iron content (7, 8). Similarly, the only documented case of HO-1 deficiency in humans presented with extensive endothelial cell damage, anemia, iron deposition, hyperbilirubinemia, and other disorders (9). The transcriptional upregulation of HO-1 responds to a variety of chemical and physical conditions, including noxious agents (i.e., heavy metals, arsenite, nitric oxide, and oxidants), ultraviolet-A radiation, as well as natural antioxidants (1). This broad induction profile renders HO-1 a viable candidate for pharmacologic manipulation, as relatively nontoxic inducing agents could be exploited or developed. Thus the pharmacologic induction of HO-1 and its artificial overexpression by gene therapy approaches have been proposed as therapeutic strategies. HO-1 represents a unique stress protein in that it exerts a metabolic (enzymatic) activity. HO catalyzes the oxidative conversion of heme, to biliverdin (BV)-IX α , releasing carbon monoxide (CO) and ferrous iron as reaction products (10) (Figure 1). HO activity represents the rate-limiting step in the heme metabolic pathway leading to the production of bilirubin (BR)-IX α , which arises from the subsequent reduction of BV-IX α by reduced nicotinamide adenine dinucleotide phosphate (NADPH):BV reductase (BVR) (10). The protective function of HO-1 has been attributed to several mechanisms. HO-1 may provide an antioxidative effect by removing heme, which, when accumulating in excess of its utilization for hemoprotein synthesis, may exert pro-oxidant effects in unbound form. Furthermore, all three HO reaction products have been extensively discussed as potentially contributing to HO-mediated cytoprotection. CO, as discussed subsequently here, confers cyto- and tissue protection by modulating intracellular signaling pathways (reviewed in Refs. 1, 11, 12), culminating in antioxidative, antiinflammatory, antiproliferative, and vasodilatory effects (Figure 2). Iron and BV/BR potentially modulate intracellular redox homeostasis (reviewed in Refs. 1, 13). BV and BR act as potent *in vitro* and serum antioxidants, whereas iron is generally regarded as a pro-oxidant. The iron released

(Received in original and final form September 5, 2006)

This work was supported by an American Heart Association award 0335035N (S.W.R.), a Veterans Administration Career Development Award (D.M.), and National Institutes of Health grants R01-HL60234, R01-AI42365, R01-HL55330, R01-HL-079904 (A.M.K.C.).

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Am J Respir Cell Mol Biol Vol 36, pp 175–182, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2006-0333TR on September 15, 2006

Internet address: www.atsjournals.org

Figure 1. Therapeutic application of heme metabolites mimics endogenous cytoprotective pathways. CO, BV, and ferrous iron (Fe-II) are generated from the oxidative degradation of heme in an endogenous enzymatic reaction catalyzed by HO-1. BV is converted to BR by the action of NADPH:BV reductase. The application of CO gas or CO-releasing molecules can mimic many of the cytoprotective effects of endogenous HO-1 activation. These include general cytoprotective effects involving antiapoptotic, antiinflammatory, and antiproliferative mechanisms. Vascular effects of CO include modulation of vessel tone, the inhibition of platelet aggregation, and the inhibition of vascular smooth muscle proliferation, leading to prevention of tissue remodeling. Similarly, exogenous application of bile pigments BV or BR, which are natural antioxidants, can confer tissue protection. Several of the protective effects of exogenous CO are mimicked by application of BV/BR, including antiinflammation and antiproliferation.

from heme, however, may promote the synthesis of the iron sequestering molecule, ferritin, which is a potent cytoprotectant (14). Furthermore, HO-1 potentially couples with an iron pump mechanism for exportation of released heme iron (15). Because iron itself is predicted to have little therapeutic potential in lung/vascular systems due to inherent toxicity, a discussion of the role of HO-derived iron in cellular protection is beyond the scope of the current review (*see reviews in Refs. 1, 13*). Neverthe-

less, the possibility that the remaining HO-derived end products directly participate in tissue protection opens unique avenues for therapeutic intervention. Thus, the pharmacologic or exogenous application of BV, BR, and/or CO may represent novel strategies to precondition or protect organs, with demonstrable success in several models of lung/vascular injury and multiorgan I/R injury. This review places a particular emphasis on pulmonary injury and disease models. Specifically, models of I/R injury, oxidative

Figure 2. Signaling pathways activated by CO and BR. CO exerts pleiotropic cellular effects by modulating intracellular signaling pathways. sGC represents one possible target of CO action. Activation of sGC leads to enhanced synthesis of cGMP and subsequent activation of G-kinase. This pathway has been implicated in vascular and neural effects of CO. CO treatment also modulates major MAPK pathways, including upregulation of p38 MAPK and downregulation of c-Jun N-terminal protein kinase (JNK) or extracellular signal-regulated kinase (ERK) 1/2 pathways. p38 MAPK has been implicated in antiinflammatory, antiapoptotic, and antiproliferative effects of CO by sGC-dependent and sGC-independent pathways, depending on the model system.

Recently, the tumor suppressor protein, caveolin-1, and the heat shock factor-1 (*hsf1*) have been shown to act as intermediates in the antiproliferative and antiinflammatory effects of CO, respectively. Additional potential targets of CO include Ca²⁺-activated K⁺ channels (K_{Ca}) implicated in vasodilation. The pathways by which BV or BR elicit cytoprotective actions are less well studied. Recent evidence implicates p38 MAPK in the antiproliferative effect of BR.

and/or inflammatory injury, ventilator-induced lung injury (VILI), fibrosis, asthma, and lung transplantation will be discussed. Other recent articles have described the application of heme metabolites in cardiovascular and renal systems (1, 16). The molecular biology and regulation of HO-1, as well as the characterization of its related constitutive isozymes, HO-2 and HO-3, have been extensively reviewed elsewhere (1).

CARBON MONOXIDE

CO is a low-molecular-weight gas, well known for its noxious properties. In nature, CO originates primarily from the oxidation or combustion of organic matter, such as from the burning of wood, coal, natural gas, and tobacco (17). Significant quantities of CO are produced endogenously in humans during the course of ordinary metabolism (18). Biological CO in cells and tissues originates as the product of the oxidation of the α -methene bridge carbon of heme by HO enzymes (10). Thus, the endogenous production of CO arises primarily from the degradation of hemoglobin, the major circulating hemoprotein (18). By the same mechanism, the degradation of other cellular or tissue hemoproteins, such as myoglobin, cytochrome p-450, and mitochondrial cytochromes, contributes to endogenous CO production, according to their relative abundance and turnover rate (19). CO competes with O₂ for the heme binding sites of hemoglobin, with an affinity $\sim$ 245 times that of O₂ (20). In the absence of significant ambient CO, the majority of blood carboxyhemoglobin arises from endogenous production, corresponding to blood CO levels of 0.4–1.0% (17). Less than 15% of endogenous CO production potentially arises from nonheme sources, including lipid oxidation and xenobiotic metabolism (21). CO is relatively inert and participates in few known biochemical interactions. CO can bind to iron chelates, (i.e., hemes) and, thereby, acts as a potential ligand for several known hemoproteins in addition to hemoglobin (i.e., myoglobin, cytochrome p-450, inducible nitric oxide synthase, etc.) (1).

The proposition of using CO gas as an inhalation therapeutic appears at first counterintuitive, due to the well characterized toxic properties of this gas. The binding of CO to hemoglobin interferes with O₂ transport and delivery to tissues. Thus, at high concentrations, CO acts as a potent asphyxiant that causes tissue hypoxia associated with a number of clinical symptoms, including dizziness, loss of consciousness, and death with prolonged or excessive exposure (17). Symptoms of CO poisoning in humans begin to appear at a carboxyhemoglobin saturation of 20%, whereas loss of consciousness (coma) leading to death occurs in the range of 50–80% carboxyhemoglobin saturation (17). High inhaled concentrations of CO in rodent models cause tissue injury and apoptosis, particularly in the brain (22).

Vasodilatory Properties

CO, like its cognate gas, nitric oxide (NO), has long been recognized as a vasodilator. Similar to the response to NO, this action is mediated by activation of soluble guanylate cyclase (sGC). CO binds directly to the heme moiety of the enzyme, resulting in a conformational change and subsequent increase in the production of guanosine 3',5'-monophosphate (cGMP). *In vitro* studies demonstrate that CO is a much weaker activator of sGC than NO (< 300 -fold), raising questions of its physiologic relevance relative to NO (23). Nevertheless, application of exogenous CO can dilate large animal aortas, as well as relax precontracted isolated aortas in an endothelium- and NO-independent fashion (23, 24). These vasorelaxant properties may possibly contribute to CO-mediated protection observed in I/R injury models by improving circulation (25). It should be noted that, in certain vascular beds, CO has also been shown to exert vaso-

constrictive properties, potentially by antagonizing endothelium-dependent NO production (26).

Antiapoptotic Properties

CO can confer protection against apoptosis in several cell culture models, including endothelial cells. Exogenous CO has been shown to inhibit tumor necrosis factor (TNF)- α -initiated apoptosis in mouse fibroblasts (27) and endothelial cells (28). A similar *in vitro* antiapoptotic effect was observed with HO-1 overexpression (27). In the endothelial cell model, the inhibitory effect of CO on TNF- α -induced apoptosis could be abolished with the selective chemical inhibitor, SB203580, or a p38 mitogen-activated protein kinase (MAPK) dominant negative mutant, implying a critical role for the p38 MAPK pathway (28). In mouse lung endothelial cells and fibroblasts, protection against TNF- α /actinomycin-D-induced cell death likewise involved p38 MAPK activation, in this instance leading to upregulation of heat shock factor-1 and Hsp-70 expression (29). In the specific case of Fas ligand-induced apoptosis, CO promoted, rather than inhibited, apoptosis in Jurkat T cells. This was associated with the downstream activation of caspases, and the inhibition of antiapoptotic Bcl family members (i.e., Bcl-X_L) (30). In this cell type, the proapoptotic effect of CO was associated with downregulation of extracellular signal-regulated kinase 1/2 activation (30).

Antiinflammatory Properties

CO has been shown, in a number of models, to exert a potent antiinflammatory effect at low concentration. In murine models of endotoxemia, CO preconditioning reduced the production of serum TNF- α , IL-1 β , IL-6, and prolonged survival after LPS challenge (31, 32). Antiinflammatory effects of CO, with respect to modulation of pro- or anti-inflammatory cytokines were diminished in heat shock factor knockout mice, indicating a potential role for the heat shock response in CO-dependent cytoprotection (29). *In vitro*, CO prevented the LPS-induced production of proinflammatory cytokines, such as TNF- α , IL-1 β , and macrophage inflammatory protein-1 β in cultured macrophages. This protection mimicked that which could be achieved by the artificial overexpression of HO-1 in this model, suggesting a link between CO and the cytoprotective effect of HO-1 induction. CO treatment also promoted the increased production of the antiinflammatory cytokine, IL-10, during LPS challenge. Recent studies also suggest an antiinflammatory mechanism involving increased reactive oxygen species production and expression of peroxisome proliferator-activated receptor- γ (33).

Antiproliferative Properties

At low concentration, CO exerts potent antiproliferative effects on cells, including endothelial cells and vascular smooth muscle, and thus has potential application in preventing tissue remodeling. CO inhibits smooth muscle proliferation in mechanisms dependent on the activation of sGC and p38 MAPK, resulting in the upregulation of p21^{Waf1/Cip1} (25, 34–36). The antiproliferative effects of CO were associated with protection in several *in vivo* models of vascular injury, transplantation, and fibrosis (25, 36, 37).

CO IN OXIDATIVE/INFLAMMATORY LUNG INJURY

The first evidence of tissue-protective properties of low-dose CO emerged in rodent models of oxidative (hyperoxic) lung injury. When rats were exposed to hyperoxia ($> 95\%$ O₂), they developed inflammatory lung injury characterized by neutrophil influx in the airways, pulmonary edema, pleural effusion, and increased lung cell apoptotic index. The presence of CO at a concentration of 250 ppm in the hyperoxic environment

prevented these manifestations of lung injury. The presence of CO also prolonged the survival time of rats subjected to lethal hyperoxia (38). This same tissue protection afforded by CO was also demonstrated in a mouse model (39). Genetic studies in the mice revealed that the antiinflammatory effect of CO was associated with the upregulation of p38 β MAPK and required the presence of the MAPK kinase (MKK) 3/p38 β MAPK pathway (39). Comparable tissue protection against hyperoxia-mediated injury was also observed with the adenoviral-directed expression of HO-1 in the rat lung (40).

PROTECTIVE EFFECTS OF CO IN I/R INJURY OF THE LUNG AND OTHER ORGANS

A beneficial effect of CO inhalation has been demonstrated in a rodent model of I/R-induced lung injury. Homozygous *ho-1*-null mice (*ho-1*^{-/-}) are highly susceptible to lung I/R injury. CO inhalation compensated for the HO-1 deficiency in *ho-1*^{-/-} mice, and improved survival after lung I/R (41). The protection provided by CO involved the stimulation of fibrinolysis, by the cGMP-dependent inhibition of plasminogen activator inhibitor-1, a macrophage-derived activator of smooth muscle cell (SMC) proliferation (41). CO pretreatment of mechanically ventilated rats subjected to lung I/R injury also conferred tissue protection associated with reduced lung cell apoptosis (42). *In vitro* experiments using pulmonary endothelial cells demonstrated that exogenously applied CO at low concentrations inhibited anoxia-reoxygenation-induced apoptosis, associated with the CO-dependent activation of the p38 α MAPK isoform with parallel suppression of extracellular signal-regulated kinase and c-Jun N-terminal protein kinase activation (42). Chemical inhibition of p38 MAPK activity, or the use of the MKK3^{-/-} mice, abolished the antiapoptotic effects of CO during lung I/R by preventing the modulation of caspase-3 activity (43). In addition to activation of p38 α MAPK and its upstream MKK3, the antiapoptotic effect of CO involved inhibition of Fas/FasL expression and other apoptosis-related factors, including caspase-3, -8, and -9, mitochondrial cytochrome-*c* release, Bcl-2 proteins, and poly (ADP-ribose) polymerase cleavage (42, 43). Further studies also demonstrated that antiapoptotic functions of CO after anoxia-reoxygenation *in vitro* depended on activation of the phosphatidylinositol 3-kinase/Akt and the p38 MAPK-dependent signal transducer and activator of transcription-3 pathway (44).

In addition to the lung, antiinflammatory and antiapoptotic effects of CO have been demonstrated during I/R injury in several other tissues, including heart, kidney, liver, and small bowel (45–47). Inhalation of CO (1,000 ppm) reduced the infarct area after occlusion of a cardiac artery. The effect was mediated by the increased production of cGMP and activation of p38 MAPK, leading to activation of the Akt-endothelial NO synthase (NOS) pathway (48). Similarly, the pharmacologic application of CO by CO-releasing molecules reduced the infarction size after I/R in the heart (49, 50).

PROTECTIVE ACTION OF CO IN LUNG/VASCULAR TRANSPLANTATION

Expression of HO-1 in rodent allografts (kidney, heart, and liver) and xenografts (heart) correlates with long-term graft survival in several transplantation models (51–54). In xenotransplantation models, HO-1 gene expression correlates with xenograft survival (51, 55). Increased HO-1 expression has been detected in alveolar macrophages from lung tissue in lung transplant recipients with acute or chronic graft failure when compared with stable recipients (56). The level of HO-1 expression correlated with the acute rejection grade scores in lung fibroblasts taken from

biopsies from a lung transplant recipient (57). CO treatment conferred protection in a rat model of lung transplantation. During orthotopic left lung transplantation in rats, the transplanted lungs developed severe intralveolar hemorrhage and intravascular coagulation. In the presence of continuous CO exposure (500 ppm), histologic analysis showed remarkable graft preservation, with reduction in hemorrhage, fibrosis, and thrombosis after transplantation. Furthermore, CO reduced lung cell apoptosis and downregulated lung and proinflammatory cytokine and growth factor production (IL-6, macrophage inflammatory protein-1 α , macrophage migration inhibitory factor, platelet-derived growth factor), which were induced during transplantation (57). In a similar rat model, CO treatment conferred protection during cardiovascular transplantation. When transplant recipients of aortic grafts were maintained in a CO environment (250 ppm) with preconditioning, these animals displayed significantly less intimal hyperplasia, and reduced accumulation of leukocytes, macrophages, and T cells in the graft (25). Bubbling of CO through the organ buffer prevented cold I/R injury during subsequent intestinal transplantation, implicating the *ex vivo* application of CO as an alternative therapeutic strategy (58).

The antiapoptotic effect of CO during transplantation was further demonstrated to involve the downregulation of Bax, and the upregulation of Bcl-2, in a cGMP-dependent pathway (59, 60). The induction of Hsp-70 potentially contributed to the antiapoptotic effect of CO after liver transplantation (61). The antiapoptotic and antiinflammatory effects of CO have been proposed to be of major influence to protect transplanted organs from dysfunction and failure (62), though improvement of blood circulation by CO within the reperfused transplanted organ cannot be discounted (25, 45, 63).

CARBON MONOXIDE IN OTHER LUNG DISEASE MODELS

Salutary effects of low-dose exogenous CO exposure have been observed in several models of lung injury and disease other than I/R.

VILI

Recent studies demonstrate an antiinflammatory effect of inhaled CO in rats subjected to experimental VILI (64). Rats ventilated with an injurious ventilator setting, (26 ml/kg tidal volume without positive end-expiratory pressure) in the presence of intraperitoneal LPS exhibited increased expression of HO-1, suggesting that HO-1 may play a role in the defense against VILI. CO mixed in the ventilator air at low concentration (250 ppm) significantly reduced the inflammatory cell count in bronchoalveolar lavage fluid (BALF). In the absence of significant cardiovascular changes, CO dose-dependently decreased TNF- α and increased IL-10 in the BALF. Lung tissue extracts displayed increased activation of p38 MAPK after ventilation with CO. Chemical inhibition of p38 MAPK *in vivo* attenuated IL-10 production in VILI. These experiments suggested that mechanical ventilation in the presence of CO may protect against VILI (64).

Asthma

Given the known antiinflammatory properties of CO, several studies have examined the potential for CO in modulating airway inflammation and reactivity in preclinical asthma models. Mice develop an airway hyperresponsiveness, similar to that seen in human asthma, when challenged with aerosolized ovalbumin after initial sensitization. CO treatment of ovalbumin-challenged animals has been shown to reduce inflammatory cell counts, especially of eosinophils and macrophages, in the BALF at 24 h

after challenge. Exogenous CO administration also significantly reduced IL-5 production and eicosanoid mediator levels (INF- γ , leukotriene B₄, and prostaglandin E₂) (65). Furthermore, CO was recently shown to attenuate airway hyperreactivity in mouse models. An acute dose of CO (0.5–1.0%; 10 min) reduced methacholine-induced airway resistance in ovalbumin-challenged C57 mice and in airway-hyperresponsive A/J mice. Repeated administrations of low-dose CO (250–500 ppm) over 5 d in both naive and inflamed A/J mice significantly reduced airway resistance (66).

Bleomycin-Induced Pulmonary Fibrosis

CO can provide protection against bleomycin-induced fibrotic lung injury in mice (37). Mice treated intratracheally with bleomycin and then exposed to CO displayed reduced lung hydroxyproline, collagen, and fibronectin levels relative to air-treated bleomycin-injured controls. The protective effect of CO in this model was associated with an antiproliferative effect of CO on fibroblast proliferation, dependent on p21^{Waf1/Cip1} induction and suppression of cyclin A/D expression (37). In a similar model, adenoviral-mediated gene transfer of HO-1 in the lung protected against bleomycin-induced pulmonary fibrosis. The protection was associated with decreased epithelial cell apoptosis and increased IFN- γ production (67).

BV/BR

The bile pigments, BV and BR, originate primarily from the turnover of hemoglobin heme (68). BV and BR have long been regarded as toxic metabolites of heme degradation. BV formed in cells and tissues is rapidly converted to BR by BVR (10). BR is circulated through plasma in complex with serum proteins, and conjugated in the liver by phase-II UDP glucuronosyltransferases to form mono- and diglucuronides. Ultimately, BR is excreted by the biliary-fecal route. The excessive accumulation of BR can cause neurologic complications in newborn infants. In cases of hyperbilirubinemia, phototherapy with ultraviolet radiation is often applied to remove preformed BR (reviewed in Refs. 1, 13). In 1989 and thereafter, Stocker and colleagues demonstrated that BV and BR could act as potent chemical antioxidants in multiple *in vitro* models. In experiments conducted with phospholipid micelles, the inclusion of BR inhibited chemically induced lipid peroxidation by acting as a chain-breaking antioxidant, whereas BV acted as a peroxyl radical trap (69). These observations led to the proposal that endogenous bile pigment generation as the result of elevated HO activity could confer antioxidative protection to cells and tissues. This hypothesis was supported by observations of cytoprotection afforded by exogenous BR in various cell culture models of oxidative and/or nitrosative stress. For example, direct addition of BR to tissue culture media protected endothelial cells and various other cell types against oxidant-induced cytotoxicity *in vitro* (70–73). Cellular depletion of BR by siRNA directed against BVR increased the intracellular levels of reactive oxygen species and promoted apoptotic cell death in HeLa cells and primary neuronal cultures (70).

BV/BR in Vascular Disease and Therapy

In several *in vivo* models, elevations of serum BR have been correlated with vascular protection and resistance to oxidative stress (74). In a rat model of hyperbilirubinemia, jaundiced Gunn rats displayed reduced plasma biomarkers of oxidative stress after exposure to hyperoxia relative to normal controls. These experiments led to the suggestion that hyperbilirubinemia may represent an antioxidative state (74). Recent clinical studies have indicated correlations between circulating BR levels and risk of

vascular disease. For example, serum BR levels were indicated as an independent, inverse risk factor for coronary artery disease and peripheral vascular disease (75, 76). In a large-scale prospective study of men, subjects in the midrange of serum BR concentration were at the lowest incidence of ischemic heart disease relative to those subjects displaying values in the lowest or highest fifth of serum BR distribution (77). In healthy subjects, serum BR levels were inversely correlated with two indicators for atherosclerosis (78).

Therapeutic application of BR has been shown to preserve myocardial function during cardiac I/R injury (72). In an isolated, perfused heart model, heme preconditioning conferred protection against myocardial infarction after I/R injury, associated with increased HO-1 expression and BR formation. Administration of exogenous BR at nanomolar concentrations in this model also improved cardiac performance and reduced infarct size and mitochondrial dysfunction after I/R injury (72). BR or BV provided tissue protection in a number of models of organ I/R injury associated with transplantation, including hepatic (79), renal (80), and cardiac (81) I/R injury. In an isolated perfused kidney model, inclusion of BR (10 μ M) in the perfusate protected against warm I/R-induced tissue injury and preserved renal function (80). BV provided tissue protection in an *ex vivo* model of cold hepatic I/R injury. Furthermore, inclusion of BV in the perfusate increased the survivability of rats undergoing orthotopic liver transplantation by preserving liver function (79). This protection afforded by BV occurred in association with the decreased expression of proinflammatory markers, including neutrophil influx, proinflammatory cytokine expression, and inducible NOS (iNOS) activation (79). BV treatment also improved the survivability of rat cardiac allografts by reducing leukocyte infiltration and inhibiting T-cell proliferation (81). In independent experiments, little effect of BV or CO was observed individually, whereas the coadministration of BV and CO provided a synergistic tissue protection against transplant-associated cold I/R injury of heart and kidney grafts (47). In addition to possible antioxidative effects, BR, like CO, has been shown to inhibit the proliferation of SMCs (82, 83). Exogenous BV administration inhibited neointimal hyperplasia associated with vascular balloon injury in rats (82, 83), whereas hyperbilirubinemic animals also displayed increased resistance to vascular injury (83). The antiproliferative effect of BV/BR was demonstrated in vascular SMC culture, whereby exogenous BV/BR arrested cells in the G1 phase associated with inhibition of p38 MAPK and retinoblastoma protein phosphorylation *in vitro* (83). Several limitations of the pharmacotherapeutic applications of bile pigments must be considered. Although BV is soluble in aqueous media, the abundance of BVR in many cells and tissues implies that BV, provided exogenously, is rapidly converted to BR. On the other hand, BR is lipophilic and soluble only in dimethylsulfoxide or other organic media, thereby posing challenges for therapeutic delivery.

BV/BR as Lung Therapy

To date, despite the protective potential demonstrated in other organ systems, relatively few studies have examined the protective potential of BV or BR in the lung. For example, the protective potential of BV or BR has not yet been tested in the lung with respect to I/R injury models, as shown for kidney, liver, and intestinal I/R. Recent studies, however, have demonstrated beneficial effects of BV or BR in inflammatory lung injury. Administration of BV to rats protected against systemic inflammation and lung injury, and extended survival after exposure to a lethal dose of LPS. The protection afforded by BV was associated with a reduction of proinflammatory cytokines in the serum (i.e., IL-6) and upregulation of serum IL-10 levels, as

well as reduction of various lung injury markers, such as lung permeability. This protection against LPS-induced injury also extended to cultured lung endothelial cells and macrophages (84). In a murine model of asthma, the application of BR inhibited vascular cell adhesion molecule-1-associated airway inflammation and lung leukocyte influx, as well as inhibited leukocyte migration *in vitro* (85). Furthermore, rats that were rendered hyperbilirubinemic by infusion of BR were relatively resistant to bleomycin-induced lung injury (fibrosis) and associated mortality compared with normobilirubinemic animals. The BR-treated rats displayed reduced lung injury markers in response to bleomycin, including lowered lung hydroxyproline content, and reduced polymorphonuclear lymphocyte and leukocyte counts, as well as reduced levels of transforming growth factor- β in the BAL (86).

INTERRELATIONSHIPS BETWEEN STRESS-RESPONSIVE SYSTEMS

Recent evidence suggests that cytoprotection elicited by exogenous pharmacologic agents, such as CO, potentially involves multiple factors, which may cross-regulate each other. Although CO is the end product of the stress protein, HO-1, it may regulate the expression of a number of downstream factors, also potentially contributing to cellular defense. Low-dose CO was shown to upregulate the expression of Hsp-70 in mouse lung endothelial cells, which itself can confer cross-resistance to a number of agents in addition to thermal stress. In mouse lung endothelial cells, this expression of Hsp-70 contributed to the apparent cytoprotection conferred by CO against proapoptotic stimuli (29). HO-1 expression and/or CO also potentially upregulate the expression of the antioxidant enzyme, manganese superoxide dismutase (87). In a model of acute liver failure, low-dose CO conferred organ protection associated with the transcriptional upregulation of iNOS. The authors proposed a complementary adaptive response, whereby the upregulation of iNOS by CO treatment promoted the subsequent NO-dependent induction of HO-1. The protection afforded by CO was diminished in *inos*-deleted hepatocytes, but could be compensated for, in this case, by HO-1 expression. On the other hand, *ho-1* was essential for the cytoprotective potential of CO in this model (88). In contrast, the cytoprotection afforded by CO during kidney and intestinal transplant-associated I/R injury was associated with the inhibition, rather than the upregulation, of iNOS expression (45). In addition to apparent transcriptional modulation of *inos* and *ho-1* after CO exposure, it should be noted that CO also potentially inhibits the corresponding enzymatic activities, especially at elevated concentrations (10, 26). More recent studies have implicated that the antiproliferative effects of CO potentially involve caveolin-1, a candidate tumor suppressor protein that resides in lipid rafts, and which regulates a number of downstream signaling pathways (36).

CONCLUSIONS AND FUTURE DIRECTIONS

Elevated expression of HO-1 as an inducible transcriptional response to stress can occur in the context of a variety of disease states, including lung and vascular diseases. As in other models, this response is typically associated with tissue adaptation and activation of survival pathways. The precise underlying mechanisms for HO-1-directed tissue protection remain incompletely understood, but have been shown to involve downstream effects of the reaction products on intracellular signaling pathways. Aside from the tentative role of heme metabolites in endogenous cellular defense, which is a separate topic of investigation, it has become evident in recent years that the pharmacologic applica-

tion of these heme metabolites to cells and animals can confer tissue protection in stress/injury models. The protective properties of CO have been demonstrated in lung and other organ I/R injury models, lung and vascular transplantation, endotoxemia, and, recently, in specialized lung disease models, such as VILI and bleomycin-induced pulmonary fibrosis. The recently developed prodrugs (i.e., transition metal carbonyls), which release CO (CO-releasing molecules), may provide an effective alternative strategy to inhalation gas therapy (89). The success of CO as a therapeutic strategy in preclinical animal models lends itself to future translational studies in human diseases. Like all new therapies in human diseases, the use of CO for clinical applications must be tested for safety and appropriate dosing regimens in carefully controlled and executed clinical trials. In this regard, a recent human study, representing a first step in this direction, failed to demonstrate antiinflammatory effects of CO inhalation, although the study encompassed only a limited dose and kinetic regimen (90). Furthermore, a recent study demonstrated cardiac dysfunction in rats after treatment with CO at 250 ppm, a dose that was associated with tissue protection in oxidative lung injury models (91). Clearly, rigorous pharmacokinetics and dosing studies in clinical trials are needed before routine use as a molecular medicine.

Interestingly, the bile pigments BV and BR, applied independently or even in combination with CO, can also confer tissue protection in several of the models established for CO. The beneficial effects of bile pigments *in vivo* are originally attributed to antioxidative effects, as exemplified by oxidative stress resistance in hyperbilirubinemic animals. Currently, BV and BR remain viable experimental therapeutic agents for the treatment of several inflammatory disease states, including transplant-associated I/R injury and vascular injury (reviewed in Ref. 1). Taken together, the selection of studies presented here suggest that heme metabolites (CO, BV, BR) could be exploited as natural protective agents in the context of lung disease, or, more broadly, in any disease state involving components of apoptosis, inflammation, oxidative stress, and/or tissue remodeling.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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