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Heme Oxygenase-1, a Critical Arbitrator of Cell Death Pathways in Lung Injury and Disease

Danielle Morse[1], **Ling Lin**[2], **Augustine M. K. Choi**[1], and **Stefan W. Ryter**[1]

[1]Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

[2]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh, PA 15213.

Abstract

Increases in cell death by programmed (*ie.*, apoptosis, autophagy) or non-programmed mechanisms (*ie.*, necrosis) occur during tissue injury, and may contribute to the etiology of several pulmonary or vascular disease states. The low molecular weight stress protein heme oxygenase-1 (HO-1) confers cytoprotection against cell death in various models of lung and vascular injury by inhibiting apoptosis, inflammation, and cell proliferation. HO-1 serves a vital metabolic function as the rate-limiting step in the heme degradation pathway and in the maintenance of iron homeostasis. The transcriptional induction of HO-1 occurs in response to multiple forms of chemical and physical cellular stress. The cytoprotective functions of HO-1 may be attributed to heme turnover, as well as to beneficial properties of its enzymatic reaction products: biliverdin-IXα, iron, and carbon monoxide (CO). Recent studies have demonstrated that HO-1 or CO inhibits stress-induced extrinsic and intrinsic apoptotic pathways *in vitro*. A variety of signaling molecules have been implicated in the cytoprotection conferred by HO-1/CO, including autophagic proteins, p38 mitogen activated protein kinase, signal transducer and activator of transcription proteins, nuclear factor-κB, phosphatydylinositol-3-kinase/Akt, and others. Enhanced HO-1 expression or the pharmacological application of HO end-products affords protection in preclinical models of tissue injury, including experimental and transplant-associated ischemia/reperfusion injury, promising potential future therapeutic applications.

Must not all things at the last be swallowed up in death?

-Plato

Introduction

Heme oxygenase-1 (HO-1) provides an inducible defense mechanism that can be activated ubiquitously in cells and tissues in response to noxious stimuli, conferring cellular protection against injury inflicted by such stimuli [1–3]. HO-1 serves a vital metabolic function as the rate-limiting step in the oxidative catabolism of heme-*b*, to generate equimolar carbon

Address correspondence to: Stefan W. Ryter, Ph.D., Department of Medicine, Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115. Tel: 617 732 7599, Fax: 617 732 7421, Sryter@partners.org.

monoxide (CO), biliverdin-IXα (BV), and ferrous iron; the BV generated is subsequently converted to bilirubin-IXα (BR) by NADPH biliverdin reductase (BVR)[4]. The essential role of HO-1 in stress adaptation has been demonstrated by the phenotype of *ho-1*$^{-/-}$ (*null*) mice and in a unique case of human HO-1 deficiency. The *ho-1*$^{-/-}$ mice exhibit altered tissue iron distribution, increased susceptibility to pulmonary ischemia/reperfusion (I/R) injury, but paradoxical resistance to hyperoxic lung injury [5–8]. The HO-1 deficient child exhibited extensive endothelial cell damage, anemia, hypobilirubinemia, aberrant tissue iron deposition, and increased inflammatory indices [9]. Furthermore, vascular endothelial cells derived from *ho-1*$^{-/-}$ mice or the HO-1 deficient child exhibited enhanced susceptibility to oxidative stress *in vitro* [6,9].

HO-1 expression can confer cytoprotection in many lung and vascular injury and disease models [1–3,8]. The cytoprotective effects of HO-1 are related to end-product formation (Figure 1) [1,10–11], though non-catalytic functions of the protein have also been proposed [12]. The pharmacological application of CO and BV/BR can mimic the HO-1 dependent cytoprotection in many injury models [1,10–11]. Tissue protection generally involves inhibition of apoptosis and related cell death pathways, although inhibition of inflammation and/or cell proliferation may also be involved, depending on the specific injury model [1]. In recent years, intensive investigation has focused on potential therapeutic tools to manipulate apoptosis in order to alter the outcome of pulmonary or vascular diseases. Such approaches have involved the use of agonists or inhibitors of specific signal transduction components, (*eg.*, mitogen activated protein kinases, MAPK), or antioxidants, thus far with limited clinical efficacy [13–14]. In this regard, the targeted manipulation of HO-1 or of its end-products remains a promising experimental and translational strategy. This review will focus on the role of HO-1/CO in modulating cell death mechanisms in tissue injury and pathology models. The specific roles of HO-1 in inflammation and carcinogenesis have been reviewed elsewhere [15–16]. Recent works examining the therapeutic potential of HO-1/CO in models of oxidative stress, I/R injury, cigarette smoke exposure, and other forms of acute and chronic lung or vascular injuries will be described.

Mechanisms of Cell Death

Just like the multi-cellular organisms they constitute, cells must die. The method of cell death may be traumatic, resulting from acute, accidental, or non-physiological injury (*ie.*, necrosis), or may arise as the consequence of genetic programs, (*ie.*, apoptosis or Type I programmed cell death). Apoptosis provides essential homeostatic functions in regulating growth and development of organs, and in tissue responses to injurious stimuli, such as exposure to xenobiotics or adverse environmental conditions [17]. Disruption of apoptosis may lead to tumorigenesis or autoimmune disease, whereas excessive apoptosis may cause organ failure [18–20]. The role of apoptosis in the pathogenesis of specific diseases remains controversial, but has been implicated in cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD), oxidative lung injury, pulmonary I/R injury, transplant-associated I/R injury, and others [20–23].

Apoptosis is distinguished from necrosis on the basis of morphological and biochemical features [17,24]. Apoptosis requires the activation of proteases (*eg.*, caspases) and nucleases within an intact plasma membrane, resulting in organelle decomposition [25]. On the other hand, necrosis is characterized by membrane damage and leakage of cytosol into the extracellular space, which may promote local inflammation and damage to surrounding tissues [24]. The cardinal biochemical features of apoptosis include DNA fragmentation, mitochondrial dysfunction, and increased expression or activation of pro-apoptotic (*eg.*, Bax, Bid) relative to anti-apoptotic (*eg.*, Bcl-2, Bcl-X_L) proteins of the Bcl-2 family [25–27].

Cells can initiate apoptosis by two distinct pathways, a receptor-dependent “extrinsic” pathway and an “intrinsic” pathway involving mitochondrial dysfunction [28–30] (Figure 2). In response to stimuli, (*ie.*, nutrient deprivation, DNA-damaging agents), proapoptotic Bcl-2 family proteins such as Bax initiate the intrinsic pathway by permeabilizing the outer mitochondrial membrane, thereby releasing cytochrome *c* (Cyt-*c*) from the inter-membrane space [31–32]. Cytosolic Cyt-*c* forms a complex with apoptotic protease-activating factor-1, and dATP which activates caspase-9, and its downstream caspase-3, resulting in the activation of the apoptosis [3].

In contrast, the extrinsic pathway initiates when a death ligand, (*ie.*, FasL) interacts with its cell surface receptor (*i.e.*, Fas), forming a death-inducing signal complex (DISC)[28,33]. Activation of Fas triggers the rapid recruitment of FADD (Fas-associated death domain protein) and caspase-8, resulting in the activation of caspase-8. Active caspase-8 cleaves Bid into truncated Bid (tBid), which translocates to the mitochondrial membrane, assists in Bax activation, and triggers Cyt-*c* release [33]. Thus, the extrinsic and intrinsic pathways converge at the mitochondria, and share similar features downstream of Bax activation and Cyt-*c* release.

Recent studies indicate that autophagy, a regulated pathway for internal organelle or protein degradation influences the outcome of cell death pathways [17,34]. During autophagy, cytoplasmic double membrane-bound vesicles (*ie.*, autophagosomes) sequester damaged organelles for delivery to the lysosome where they are degraded and recycled [35–37]. The Bcl-2 interacting protein Beclin 1 and the microtubule-associated protein-1 light chain-3 (LC3), represent major regulators of autophagosome formation in human cells [39–40]. Autophagy plays a central role in cellular adaptation to environmental stress, such as oxidative stress, serum starvation, endoplasmic reticulum stress, hypoxia, and infection [34–38]. Activation of autophagy is regarded as a survival mechanism during nutrient deprivation, whereas excessive autophagy may promote cell death under certain conditions [37,41]. Recent studies suggest that autophagic proteins may cross-regulate the initiation of the apoptotic program in response to stress [42–44]. Though increased autophagosome formation is frequently observed in dying cells, the casual relationship between autophagy and cell death remains a matter of current controversy [45]. The role of HO-1 in regulating specific pathway elements of autophagy and apoptosis will be discussed in subsequent sections.

Heme Oxygenase-1: biochemical properties and subcellular localization

HO-1, the inducible HO isozyme, is the major inducible low molecular weight (32–34 kDa) stress protein of mammalian cells and tissues [46]. Heme oxygenase has a major constitutively expressed isozyme, heme oxygenase-2 (HO-2) [47]. HO-1 and HO-2 represent the products of two distinct genes [48]. Although HO-1 and HO-2 both catalyze heme-*b* to biliverdin, the two enzymes differ in primary structure and biochemical/biophysical properties [48–49]. A highly conserved sequence of 24 amino acid residues corresponding to the heme catalytic domain has been identified in common to both HO-1 and HO-2 [50]. Furthermore, both HO-1 and HO-2 hare similar hydrophobic regions at the carboxyl-terminus that anchor the proteins in cellular membranes [51–53]. A gene potentially encoding a third HO isozyme, *ho-3* was cloned in the rat, though further analysis has suggested that the rat *ho-3* genes represent processed pseudogenes, and to date, no natural protein has been found [54–55].

The expression of HO-1 occurs at very low levels in most tissues under physiological conditions, with the exception of the spleen, the site of erythrocyte hemoglobin turnover, where high levels of HO-1 are constitutively present. In contrast, HO-2 is expressed under

basal conditions in most tissues, including testes, spleen, liver, kidney, brain, and vasculature (*Reviewed* in [1]).

HO-1 is an integral membrane component of the smooth endoplasmic reticulum (ER), evidenced by its abundance in microsomal membrane preparations. Recent studies have identified possible subcellular compartmentalization of HO-1 beyond ER membranes [56–61]. For example, a nuclear localization of HO-1 has been described [56]. These studies have suggested that HO-1 undergoes a proteolytic truncation of the carboxyl terminal hydrophobic region to facilitate nuclear entry. Despite apparent lack of heme catalytic activity of the truncated nuclear HO-1, recent work suggests that HO-1 may have a non-catalytic function in regulating nuclear transcription factor activities [12,56]. Increased nuclear localization of HO-1 has been associated with several clinical disease conditions, as highlighted by recent studies in prostate cancer [57], suggesting a possible role for nuclear HO-1 accumulation in malignancy.

Furthermore, HO-1 and functional HO activity can be detected in detergent-resistant fractions (lipid rafts) of plasma membrane. Sucrose density gradient fractionation revealed the presence of HO-1 in low-density caveolin-1 containing fractions (caveolae). Within this compartment, caveolin-1 was shown to bind to HO-1 and negatively regulate HO activity [58]. Caveolin-1 regulates a number of signaling-related molecules in caveolae, (*ie.*, endothelial nitric oxide synthase)[59]. The regulation of HO activity in this compartment may represent a mechanism for the controlled production of CO for signaling purposes [58]. However, the functional significance of HO-1 in plasma membrane lipid rafts remains unclear at present.

HO-1 also localizes in part to the mitochondria as shown in pulmonary epithelial cells [60] and in rat hepatocytes [61]. In epithelial cells, the expression of HO-1 protein and activity in the mitochondria increases in response to toxic stimuli, including cigarette smoke, bacterial lipopolysaccharide, and hemin [60]. While the mitochondria play central roles in the regulation of apoptosis, it remains unclear whether mitochondria-localized HO-1 modulates the apoptotic program by acting on specific components of the mitochondria. In conclusion, the specificity and function of the novel compartmentalization (*ie.*, nucleus, mitochondria, lipid raft) of HO-1 remains unclear, but further study may reveal additional mechanisms relevant to the anti-apoptotic or cytoprotective potential of HO-1.

Regulation of HO-1 transcription

The transcriptional upregulation of the *ho-1* gene, and subsequent *de novo* synthesis of the corresponding protein occurs in response to elevated levels of its natural substrate heme, and to a multiplicity of endogenous factors including NO, cytokines, heavy metals, hormones, and growth factors (*Reviewed* in [1]). Many agents that induce HO-1 are associated with oxidative stress in that they (I) directly or indirectly promote the intracellular generation of reactive oxygen species (ROS), (II) fall into a class of electrophilic antioxidant compounds which includes plant-derived polyphenolic substances [62–64], or (III) complex intracellular reduced glutathione and other thiols [65–66]. HO-1 expression responds to pro-inflammatory stress, associated with the production of ROS from activated inflammatory cells [67]. Furthermore, HO-1 expression responds to changes in ambient O_2 tension, such as hyperoxia (increased pO_2) or hypoxia (decreased pO_2), both which can modulate mitochondrial ROS production. The regulation of HO-1 by hypoxia can vary in a cell type and species-specific manner, with transcriptional repression rather than induction reported in human cell types [68].

Because expression of HO-1 is regulated by a wide array of compounds in a cell-type and inducer-specific fashion, defining unifying transcriptional regulatory mechanisms has been

challenging [69]. Two enhancer regions located at approximately −4kb and −10kb relative to the *ho-1* transcriptional start site have been identified in the mouse gene [70–71]. The dominant sequence element of the enhancers is the stress-responsive element (StRE), which is structurally and functionally similar to Maf response element (MARE) and the antioxidant response element (ARE)[69,72]. Several transcriptional regulators bind these sequences, including nuclear factor erythroid 2-related factor-2 (Nrf2) and Bach1. These are both members of the Cap'n'collar/basic-leucine zipper family capable of heterodimerizing with small Maf proteins and regulating MARE-driven genes. Nrf2 contains a transcription activation domain and positively regulates HO-1 transcription, whereas Bach1 competes with Nrf2 and represses transcription [73–76]. Mice lacking the gene for Bach1 have dramatic increases in HO-1 expression in the heart, lung and liver, indicating a role for Bach1 in tonic suppression of HO-1 transcription. Conversely, inhibition of Nrf2 gene function results in impairment of transcriptional responses to known inducers of HO-1 [73–74,77–79]. Thus, HO-1 expression depends upon changes in the balance of activity of Nrf2 and Bach1. Heme can relieve Bach1 of DNA binding activity through complex formation, promote the nuclear export of Bach1 and inhibit the proteasomal degradation of Nrf2, thus enhancing HO-1 expression [73–74,79–82]. The Kelch-like ECH-associated protein (Keap1) inhibits HO-1 expression by sequestering Nrf2 in the cytoplasm. Keap1 facilitates the targeting of Nrf2 by Cullin 3-based E3 ubiquitin ligase complex, which marks Nrf2 for proteasomal degradation [83–86]. Under basal conditions, Keap1 binds to Nrf2 and sequesters it in the cytoplasm, which results in a lower accumulation of Nrf2 in nucleus and reduced transcription of the HO-1 gene [85,87–88]. When cells are exposed to electrophiles or oxidants, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to target genes [89–90]. In Keap1 null mice, Nrf2 constitutively increases in the nucleus and inappropriately stimulates its target genes, leading to growth retardation and early death. However, the phenotypic abnormalities can be reversed by reduction of Nrf2 abundance in the Keap1-null mutant background [91]. Nrf-2 knockout mice were more susceptible to tobacco smoke-induced lung apoptosis and emphysema relative to wild type mice, illustrating a critical role for Nrf2 in the regulation of adaptive responses to stress [92].

While the Nrf2 system has been characterized extensively in the mouse as the major stress inducible operator of the *ho-1* gene, many additional *cis*-acting promoter elements have been identified in the context of the mouse, rat, and human *ho-1* upstream regulatory regions which may also contribute to *ho-1* regulation in a cell type an inducer-specific fashion. Examples of elements that respond in an inducer-specific or selective fashion include those responsive to hyperthermia [93] or hypoxia [94] which represent the targets of distinct DNA-binding proteins such as heat shock factors (HSF) and hypoxia-inducible factor-1 (HIF-1), respectively.

While the human *ho-1* promoter contains regions analogous to the upstream enhancer regions described in the mouse, further studies have revealed additional complexities specific to the human *ho-1* promoter. The human *ho-1* gene contains an additional 10 bp sequence element, termed the cadmium responsive element (CdRE), occurring within the –4kB enhancer region but extrinsic to the StRE sequence homologies, which mediates $CdCl_2$ induction of the gene [95]. Furthermore, the human −4kb enhancer region contains a binding site preferentially targeted by cyclic AMP responsive element binding factor (CREB) [96]. A region at −9.5 kb corresponds to a binding site for the early growth response-1 (Egr-1) factor, which mediates the response to metalloporphyrin induction [97]. A number of proximal promoter elements have also been identified as potentially important in human *ho-1* gene regulation [98–100]. These include E-box motifs potentially recognized by the upsteam stimulatory factor (USF), and/or basic helix loop helix (bHLH) proteins [101–102], ETS binding sites (EBS) [103], as well as binding sites for nuclear factor-kappa B (NF-κB) [104], STAT-3 [105], activator protein-2 (AP-2) [104], and HSF-1[99]. An

upstream region contains potential binding sites for hepatocyte nuclear factors (HNE1 and HNE4), AP-1, STAT-x, c-Rel, and GATA-X [106–107]. These studies reveal the complex nature of *ho-1* gene regulation, which potentially involves the coordinated interaction of multiple transcription factors. The functional significance of all of these factors however, remains incompletely understood [10,65,98].

A microsatellite $(GT)_n$ dinucleotide length polymorphism has been described in the promoter region of the human *ho-1* gene, which can result in the impaired transcriptional regulation and decreased expression of HO-1 in individuals that carry the long (L) allele [*ie*., (GT)n ≥30] of this polymorphism [108]. Multiple human genetic epidemiology studies have examined the association between this length polymorphism in the human *ho-1* gene, and COPD-related traits. The "Long" allele of the GT repeat (≥25, ≥30, ≥32, or ≥33 repeats, depending on study) has been associated with COPD [109], emphysema [108], COPD severity [110], lung function decline in COPD [111] and lung function decline in the general population [112–114]. For example, in a retrospective study of French smokers the L allele [(GT)n ≥ 33] was associated with decreased lung function parameters relative to non-carriers. The greatest decline in lung function was observed in heavy smokers that carried the L allele [112]. However, the association with lung function decline was not replicated in COPD subjects in the National Heart, Lung, and Blood Institute Lung Health Study (115), while other studies have demonstrated associations with different alleles [*ie*., (GT)n ≥30] of the GT repeat [116]. Thus additional studies are required before a consensus can be realized. Interestingly, lymphoblastoid cells cultured from homozygous carriers of the L allele displayed increased susceptibility to oxidant (H_2O_2)-mediated apoptosis *in vitro* [117]. These studies suggest that a genetically-dependent downregulation of HO-1 expression may arise in subpopulations, possibly linked to increased susceptibility to oxidative stress-related disease.

Heme Oxygenase-1/CO Confers Protection Against Cell Death in Pulmonary/Vascular Cell Injury

Cytokine mediated apoptosis

The antiapoptotic effects of CO were originally described *in vitro*, and several potential mechanisms were implicated. Exogenous CO was shown to inhibit tumor necrosis factor-α (TNFα)-initiated apoptosis in mouse fibroblasts [118] and endothelial cells [119]. In fibroblasts, an anti-apoptotic effect was also observed with HO-1 overexpression [118]. In endothelial cells, the inhibitory effect of CO on TNFα-induced apoptosis could be abolished with the selective p38α/β MAPK inhibitor, SB203580, or a p38 MAPK dominant negative mutant, implying a critical role for the p38 MAPK pathway [119]. Furthermore, a role for NF-κB and the downstream activation of anti-apoptotic genes was also described [120]. In mouse lung endothelial cells, protection against TNFα/actinomycin-D induced cell death involved p38β MAPK activation, leading to upregulation of heat shock factor-1 and expression of the 70-kDa heat shock protein [116]. The cytoprotective effects of CO were diminished in heat shock factor-1 knockout ($hsf1^{-/-}$) mice, indicating a role of the heat shock response in CO-mediated cytoprotection [121].

In cultured vascular smooth muscle cells, CO inhibited cytokine (TNFα, IL1-β, INF γ)-induced apoptosis, dependent on activation of soluble guanylate cyclase (sGC), whereas p38 MAPK was not implicated in this cell type [122–123]. Despite numerous reports of antiapoptotic activity of HO-1/CO in cultured cells, evidence for pro-apoptotic effects for HO-1/CO have also been described. For example, Thom *et al*. reported induction of apoptosis in cultured endothelial cells with CO application [124]. Liu *et al*. reported that adenoviral-directed expression of HO-1 in smooth muscle cells promoted apoptosis in a

dose-dependent fashion [125]. Though the reasons for these contrasting observations are unclear, they are consistent with the notion that HO-1/CO can modulate apoptosis in vascular cells with the outcome dependent on specific experimental conditions, cell type, or nature of inducing stimuli.

Hyperoxia-induced lung cell death

Hyperoxia, or high O_2 tension, induces the expression of HO-1 in several cell types including endothelial cells and fibroblasts [126–127]. HO-1 overexpression or CO treatment protected A549 alveolar epithelial cells against cell killing by exposure to hyperoxia [128,129]. Treatment with the p38 MAPK inhibitor or transient transfection with dominant negative mutants of p38β or mitogen activated protein kinase kinase-3 (MKK3) abolished the cytoprotective effect of CO against hyperoxia [129]. Recent studies have also implicated the signal transducer and activator of transcription protein-3 (STAT3), as a mediator of CO-dependent antiapoptotic protection in hyperoxic lung cell injury [130].

CO (250 ppm) specifically inhibited the initiation and propagation of extrinsic apoptosis pathways in mouse lung endothelial cells subjected to hyperoxia [131]. CO co-treatment inhibited the hyperoxia-induced DISC formation as well as downstream caspase-8 and Bid activation. Antiapoptotic cytoprotection culminated with the inhibition of caspase-9/3 activation and protection against cell death. CO also inhibited hyperoxia-induced ROS production, as well as the activation of the DISC, and its plasma membrane assimilation. Furthermore the protective effects of CO in this model depended on the downregulation of the ERK1/2 MAPK pathway, which was shown to regulate NADPH oxidase-dependent ROS production in this cell type during hyperoxia [131].

Hypoxia/reoxygenation induced lung cell death

Hypoxia/reoxygenation (H/R) or anoxia/reoxygenation (A/R) are commonly used as *in vitro* models of the oxygen flux that occurs during I/R injury, though these models do not recapitulate the systemic and hemodynamic stress components of I/R injury *in vivo*. H/R and A/R protocols cause apoptosis in cultured endothelial cells. *In vitro* experiments using pulmonary endothelial cells demonstrated that exogenously applied CO at low concentrations inhibited A/R-induced apoptosis. The antiapoptotic effect of CO involved inhibition of Fas/FasL expression, and other apoptosis-related factors including caspases (-3,-8,-9), mitochondrial Cyt-*c* release, Bcl-2 proteins, and poly (ADP-ribose) polymerase (PARP) cleavage [132]. CO activated the p38α MAPK isoform and its upstream kinase (MKK3), whereas it inhibited ERK and JNK activation [132–133]. The antiapoptotic effects of CO in A/R exposed endothelial cells were inhibited in cells from *mkk3*$^{-/-}$ mice or by treatment with a p38α/β MAPK-specific inhibitor [132]. Further studies revealed that CO activated the phosphatydylinositol-3 kinase (PI3K)/Akt pathway, which mediated the activation of the p38 MAPK pathway and activated the STAT3 pathway. Increased phosphorylation and DNA binding activity of STAT3 in response to CO was accompanied by suppression of STAT-1 activation. STAT3 mediated both the inhibition of Fas expression and the inhibition of caspase-3 activation in response to CO treatment; whereas a dominant negative mutant of STAT3 increased endothelial cell apoptosis in response to A/R and antagonized the effects of CO [134].

HO-1 in cytoprotection against cigarette smoke-induced cell death

Cigarette smoke (CS) contains over 4,500 distinct chemical species. Since CS contains ROS, NO and other free radicals, electrophilic substances and heavy metals, and furthermore can trigger the intracellular production of ROS and deplete natural antioxidants, CS is generally regarded as an oxidative cellular stress [135]. While animals in inhalation studies are typically exposed to mainstream or side stream CS, cell culture studies typically utilize

aqueous cigarette smoke extract (CSE) [136]. Though some reports have suggested that CSE causes primarily necrotic cell death in bronchial epithelial cells [137], apoptotic phenotypes have also been observed depending on cell model and experimental conditions [60,138]. Lower dose and shorter kinetics of CSE exposure generally favor apoptosis [60].

We found that CSE activated the extrinsic apoptotic pathway in human epithelial cells (Beas-2B) and primary human lung fibroblasts. CSE induced assimilation of the Fas-dependent DISC at the cell surface plasma membrane, as the result of mobilization of pre-formed complexes in the *Golgi* apparatus. CSE induced DISC formation resulted in activation of caspase-8, and subsequent activation of downstream caspases. The primary involvement of an extrinsic pathway in CSE-induced cell death is supported by reduced cell death in $Fas^{-/-}$ fibroblasts exposed to CSE [139].

The induction of HO-1 by CS or CSE was observed in a number of pulmonary and non-pulmonary cell types, including fibroblasts, alveolar macrophages, and epithelial cells [60,140–142]. In our recent studies, human bronchial epithelial cells (Beas-2B) subjected to CSE responded with a time- and dose-dependent upregulation of HO-1 protein and enzymatic activity. HO-1 protected against CSE-induced cell death and preserved cellular ATP levels which were depleted by the CSE treatment. This protection afforded by HO-1 at high concentrations of CSE, as well as preservation of cellular ATP levels suggest a general anti-necrotic effect of HO-1 against CSE-exposure [60]. Anti-necrotic protection of HO-1 was also described in HO-1 expressing epithelial cells subjected to direct NO exposure, in this case attributed to decreased membrane lipid peroxidation [143].

At lower, apoptosis inducing, concentrations of CSE, expression of HO-1 was also shown to inhibit the activation of the extrinsic pathway in Beas-2B cells by inhibiting the formation of the DISC and subsequent activation of downstream capsases -8,-9,-3 [144]. Interestingly, HO-1 expression also inhibited the expression of autophagic regulator proteins, LC3B and Beclin 1. Further experimentation revealed that these autophagic proteins were critical mediators of the initiation of extrinsic apoptosis in these cells in response to CSE exposure. Infection with siRNA specific for either LC3B or Beclin 1 inhibited CSE-induced activation of DISC formation and caspase-8 activation in Beas-2B cells. These experiments suggest that the complex interplay of signaling molecules affected by HO-1 include not only regulators of apoptosis pathways, but also of autophagic pathways. In the case of CSE-induced cell death, enhanced autophagy correlated with increased cell death, therefore the homeostatic function of HO-1 is consistent with the downregulation of both pathways. These experiments suggest that inducible stress responses such as HO-1 act as a first-line defense against oxidative cell injury, which can reduce the progression of the cell toward stress-inducible autophagy as well as apoptosis [144]. The direct mechanisms by which HO-1 downregulates Beclin 1 or LC3B expression in epithelial cells, and the overall significance of HO-1 in the regulation of autophagy in general remain unclear.

Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a vital organelle involved in the protein secretory pathway and lipid biosynthesis. Various stress conditions, including alterations in intracellular calcium homeostasis, inhibition of protein glycosylation, and/or accumulation of misfolded proteins, can induce an ER stress response, characterized by increased synthesis of ER stress proteins. Excessive ER stress and ER dysfunction can promote apoptotic cell death, through the activation of caspases (*ie.*, caspase-12, -9, -8) [145–146]. Recent studies identify HO-1 as a critical component of the ER stress response in smooth muscle cells (SMC) [146–147]. HO-1 expression responded to transcriptional induction by canonical ER stress-inducing chemicals (*ie.*, thapsigargin, brefeldin-A, homocysteine, proteosome inhibitors) in SMC [147]. Exogenous application of CO inhibited apoptosis

induced by ER stress-inducing agents in SMC, which was associated with the downregulated expression of the propapoptotic protein GADD153 [147]. In human endothelial cells an antiapoptotic mechanism of HO-1/CO against ER stress was demonstrated involving the activation of p38 MAPK and downregulation of the proapoptotic C/EBP homologous protein (CHOP) [148]. Pharmacological induction of HO-1 or application of the CO-releasing molecule (RuCO) downregulated CHOP and protected against thapsigargin-induced apoptosis. CO also activated protein kinase R–like endoplasmic reticulum kinase (PERK), resulting in Nrf-2 activation and nuclear translocation. Activation of Nrf-2 in turn led to an induction of HO-1 which contributed to the cytoprotective response [148]. These results, demonstrate that HO-1/CO confers antiapoptotic cytoprotection against apoptotic signals originating from ER compartment stress.

Role of biliverdin/bilirubin in cytoprotection

The bile pigments, biliverdin (BV) and bilirubin (BR) generated from the action of HO and BVR activities respectively, have also been implicated in both toxicity and cytoprotection, depending on the dose and model system. The overproduction of BR in neonates is neurotoxic, thus strategies to remove BR (phototherapy) or inhibit its formation (competitive inhibitors of HO activity) are implemented clinically. BV and BR have been demonstrated to have antioxidant properties in *in vitro* model systems which include the inhibition of LDL oxidation, protein damage and peroxidation of phospholipid micelles. Albumin bound BR acts as a co-antioxidant with α-tocopherol. The relative significance of BR as a circulating antioxidant *in vivo* remains unclear due to the existence of multiple circulating antioxidants [149]. BR has been shown to confer cytoprotection against oxidative or nitrosative stress conditions in various cultured cells, including endothelial, vascular smooth muscle, HeLa cells, cardiomyocytes, and neural cells (*Reviewed* in [150]). Addition of BR to cultured HeLa cells has been shown to protect against H_2O_2 dependent cytotoxicity [151]. Moreover, various *in vivo* studies investigating disease models such as I/R, transplantation, pulmonary fibrosis, or organ injury have demonstrated a reduction of cell death when BR or BV were administered or were present at elevated levels (*Reviewed* in [15]). BV adjuvant therapy has been shown to protect rat liver grafts from I/R injury. In this model, BV suppressed activation of apoptosis (Cyt-*c* release) and inhibited JNK activation [152]. In addition to possible antioxidative effects, BR, has been shown to inhibit the proliferation of vascular smooth muscle cells [153–154]. Exogenous BV administration inhibited neointimal hyperplasia associated with vascular balloon injury in rats [153–154], and hyperbilirubinemic animals also displayed increased resistance to vascular injury [153].

Recent studies have suggested that BVR, in addition to its metabolic capacity in the conversion of BV to BR, may have additional functions in the activation of cellular signaling pathways (*Reviewed* in [155]). Experiments using siRNA approaches support a role for BVR in cytoprotection. For example, siRNA directed knockdown of BVR increased total cellular ROS production in HeLa cells [151]. The siRNA directed knockdown of BVR increased apoptosis in response to sodium arsenite treatment, as evidenced by increased Cyt-*c* release and PARP cleavage, and other biochemical markers [156]. siRNA directed knockdown of BVR augmented cardiomyocyte apoptosis in response to H/R stress *in vitro* [157]. The antiapoptotic protection of BVR was related to not only its metabolic activity, but also to activation of the PI3K/Akt pathway, through an intramolecular interaction with PI3K [157].

Role of iron in cytotoxicity and protection

Despite the essential requirement for iron in various cellular processes, such as heme synthesis, DNA replication, mitochondrial function, and oxygen sensing, iron potentially

poses a toxic insult to cells by catalyzing free radical generating reactions. Iron loading sensitizes endothelial cells to oxidant-mediated cell killing [158]. On the other hand, complete deprivation of iron leads to apoptosis, as shown by proapoptotic effects of metal chelator treatments, suggesting that at least a minimal amount of iron is needed to serve for vital cellular processes [159]. Nevertheless, the role of intracellular iron in the regulation of apoptosis remains incompletely understood. Though HO activity is generally associated with cellular protection, HO activity releases ferrous iron from heme, with potential deleterious effects. Evidence from *in vitro* models, describes a potential toxic threshold for HO-1 overexpression, related to iron accumulation [160–161]. For example, hyperoxia sensitivity was observed at high levels of HO-1 overexpression, whereas hyperoxia-resistance at moderate levels of HO-1 expression [160–161]. Similarily, HO-2 overexpressing clones displayed short-term hypersensitivity to UVA radiation in the presence of heme loading, relative to wild type cells [162]. The apparent toxicity of HO-1 or HO-2 in these models was likely related to gene overdosing and/or substrate load conditions resulting in a transient rise in intracellular iron under pro-oxidant conditions.

The antioxidative protection afforded by HO activity cannot be explained alone by the conversion of heme in exchange for increased intracellular iron, which is potentially toxic. This antioxidant protection of HO with respect to iron metabolism is possible by further coupling with proteins that either promote the sequestration or export of the liberated iron. HO-1 has been associated with iron efflux pathways [163], and also promotes the synthesis of ferritin, which eventually neutralizes potentially reactive iron [164]. Iron release from HO activity leads to post-translational release of translational repression of ferritin, therefore ferritin synthesis is increased [164]. The HO-1 dependent increase in ferritin was linked to cytoprotection in several models of oxidative stress, including UVA radiation and photosensitizer-mediated oxidative stress [164–165]. Ferritin overexpression also reduced apoptosis in a model of I/R injury after liver transplantation [166]. Thus ferritin appears to confer cytoprotection against oxidative challenge, and potentially plays an intermediate role in HO-mediated antiapoptotic protection. Furthermore, an ATP-dependent iron pump has been shown to colocalize with HO-1 in the microsomal membrane. This pump also might facilitate the exocytosis of HO-derived iron [167]. A possible antiapototic mechanism of intracellular HO-derived iron has recently been proposed, whereby HO-derived iron activates NF-κB which in turn mediates an antiapoptotic pathway [168]. These studies, taken together, suggest that HO-derived iron contributes to phenotypic effects of HO activation, and cannot be discounted in therapeutic applications of HO-1, though these processes remain incompletely understood.

Cytoprotective actions of HO-1 in preclinical models of lung injury

Pulmonary I/R

Pulmonary I/R caused by temporal clamping of the pulmonary artery induced the biochemical features of apoptosis in rodent lungs, including increased expression of Fas, FasL, activation of caspases (3-,8-,9-), modulation of Bcl2-related proteins, PARP cleavage, and Cyt-*c* release [132]. The protective effect of CO pretreatment on mice subjected to lung I/R injury *in vivo* was associated with the inhibition of apoptosis markers, including caspase-3 activation, and depended on activation of p38α MAPK [133]. Homozygous *ho-1* null mice (*ho-1*$^{-/-}$) were highly susceptible to lung I/R injury. CO inhalation compensated for the HO-1 deficiency in *ho-1*$^{-/-}$ mice, and improved survival following lung I/R [7]. 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 proliferation [7]. CO also inhibited fibrin deposition and improved circulation in ischemic lungs [169]. These protective effects were recently associated with downregulated expression of early growth response-1 (Egr-1), a multifunctional transcription factor, with

the concomitant downregulation of Egr-1 target genes, many which contribute to inflammatory or pro-thrombotic processes. The downregulation of Egr-1 depended on the enhancement of cGMP signaling by CO treatment, leading to the inhibition of the ERK1/2 MAPK pathway [169]. Recent *in vivo* studies indicate that *ho-1* knockdown using siRNA dramatically increased apoptosis during pulmonary I/R [170].

Transplantation

One of the best studied models of I/R injury is organ transplantation. Generally, these models involve cold preservation of organs followed by warm reperfusion of the grafts. The transplanted organs are therefore at risk of injury during both preservation and reperfusion phases. The involvement of HO-1 in chronic graft dysfunction due to immunologic rejection has also been well studied [23], but this subject is beyond the scope of this current review. During the ischemic stage, ATP depletion can lead to altered intracellular electrolyte balance, hypoxanthine accumulation and activation of cytotoxic enzymes, resulting in cell damage or death. Upon reperfusion and reintroduction of oxygen, ROS are generated, potentially extending cellular injury, and promoting cell death by both necrosis and apoptosis [171]. HO-1 is consistently upregulated in models of transplantation [123], and there is ample evidence that HO-1 confers protection in this setting. The bulk of experimental evidence has been derived from rodent I/R transplantation models, where HO-1 induction pharmacologically or by gene transfer has been shown to provide cytoprotection in transplanted heart [172], liver [173], kidney [174], small bowel [175], pancreatic islets [176], and muscular flap [177]. In addition, a recent report correlating donor HO-1 polymorphisms with post-transplant renal graft function suggests that HO-1 function may contribute to clinical disease outcome [178].

The mechanism by which HO-1 provides protection in transplant-associated models of I/R injury appears to be multifactorial. Hemoglobin, the major source of extracellular heme protein, is known to contribute to I/R injury [179]. Heme contributes to oxidative injury, which may trigger programmed cell death, and thus increased catabolism of heme by HO-1 may attenuate injury. Scavenging of ROS by BV and/or BR may protect cells from oxidative injury. Increased ferritin synthesis may promote iron redistribution and neutralization in the setting of transplantation [172]. CO may also contribute to protection by regulating vascular tone and platelet aggregation [180–182], and more importantly, through direct inhibition of apoptosis.

In addition to manipulation of HO-1, the direct application of CO has also been shown to confer protection in I/R transplantation models, including vascular transplantation [182], cardiac [181], small intestinal [183], kidney, [184], liver [185], and lung transplantation [186]. During orthotopic left lung transplantation in rats, the transplanted lungs developed severe intra-alveolar hemorrhage and intravascular coagulation. In the presence of continuous CO exposure (500 ppm), histologic analysis showed markedly improved 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-1α, macrophage migration inhibitory factor, platelet-derived growth factor), which were induced during transplantation [186]. CO treatment also 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 [182]. 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 [187]. In recent studies, inclusion of the water soluble carbon monoxide releasing molecule (CORM)-3 in the preservation fluid improved cardiac

functional parameters following cardiac transplantation [188]. Several studies have recently examined the mechanisms underlying the antiapoptotic effects of CO in transplantation models. The antiapoptotic effect of CO during transplantation of pancreatic islets or intestinal grafts involved activation of a cGMP-dependent pathway [189–192]. The induction of Hsp-70 potentially contributed to the antiapoptotic effect of CO after liver transplantation [185]. The inhibition of apoptosis and inflammation are currently believed to represent the major mechanisms by which CO protects transplanted organs from dysfunction and failure [192], though improvement of blood circulation by CO within the reperfused transplanted organ cannot be discounted [182,192–193].

The pharmacological application of BR or BV also has been documented to confer tissue protection in I/R transplantation models, including liver [194], kidney [195], and cardiac [188] transplantation. 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 liver transplantation by preserving liver function [194]. This protection afforded by BV occurred in association with the decreased expression of pro-inflammatory markers, including neutrophil influx, proinflammatory cytokine expression, and inducible NOS (iNOS) [194]. In the isolated perfused kidney model, inclusion of BR in the perfusate protected against warm I/R–induced tissue injury and preserved renal function [195]. BV treatment also improved the survivability of rat cardiac allografts by reducing leukocyte infiltration and inhibiting T-cell proliferation [196]. The combined application of BV and CO provided a synergistic tissue protection against transplant-associated cold I/R injury of heart and kidney grafts, under conditions where little protection was observed with either agent alone [197]. In conclusion, protection by HO-1 and its end products BV/BR and CO in I/R injury [167–168,172–174,176,186], as well as other models of oxidative injury [198] has in many cases been correlated in part, with the inhibition of apoptosis, in a complex interplay of protective mechanisms that also may include anti-inflammatory [199] and antiproliferative [182] effects.

Conclusions

There now exists ample evidence that the HO-1 system, as well as the pharmacological application of HO pathway end products, provides robust protection against cellular stress. While considerable progress has been made in defining the therapeutic potential of HO-1 *in vitro* and in preclinical models of organ injury and disease, the clinical benefit of therapies involving HO-1 have yet to been demonstrated in humans. Of the preclinical disease models exploring medical applications for HO-1, transplantation is perhaps the best developed. Transplantation is in many ways the ideal clinical setting for exploiting the antiapoptotic function of HO-1, since the time of injury is predictable and well defined, and the opportunity exists to apply the therapy not only to the patient but to the donor organ as well. Nevertheless, the use of HO-1 as a treatment strategy has yet to be adopted. Concern for toxicity from gene therapy or from pharmacologic use of BV or CO has delayed progress in the clinical arena, although enthusiasm for further research in this area remains strong. The development of non-toxic inducers of HO-1 or Nrf2 activation remains a viable strategy [89]. Further progress in gene therapy approaches that allow tissue or cell type specific gene delivery may also facilitate progress in this area. As an alternative to inhalation of CO, pharmacological application of CO with the transition-metal carbonyl CO-releasing molecules, (CORMs) may provide an additional therapeutic avenue [200]. Whether direct application of CO by either method will provide a safe modality for the treatment of human disease requires further research directed at understanding the pharmacokinetics and toxicology of CO application in humans. Further progress awaits the completion of several ongoing clinical trials in transplantation and sepsis.

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List of Abbreviations

A/R	anoxia/reoxygenation
BR	Bilirubin
BV	Biliverdin
BVR	biliverdin reductase
cGMP	
CO	carbon monoxide
CORM	carbon monoxide releasing molecule
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
CSE	cigarette smoke extract
Cyt-c	cytochrome-c
DISC	death inducing signaling complex
HO-1	heme oxygenase-1
HO-2	heme oxygenase-2
H/R	hypoxia/reoxygenation
IL	interleukin
I/R	ischemia/reperfusion
JNK	c-Jun NH_2-terminal kinase
Keap1	Kelch-like ECH-associated protein
LC3	microtubule-associated protein-1 light chain-3
MAPK	mitogen activated protein kinase
MKK3	mitogen activated protein kinase kinase-3
NF-κB	nuclear factor kappa-B
Nrf2	NF-E2 related factor-2
PARP	poly (ADP-ribose) polymerase
PI3K/Akt	phosphatydylinositol-3 kinase/Akt
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
siRNA	small interfering ribonucleic acid
STAT3	signal transducer and activator of transcription-3
TNFα	tumor necrosis factor-alpha.

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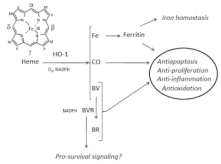

Figure 1. Relationship between the HO-1 system and cytoprotective functions

Heme oxygenase activity catalyzes the rate-limiting step in the oxidative catabolism of heme-*b*, to generate equimolar quantities of carbon monoxide (CO), biliverdin-IXα (BV), and ferrous iron. The reaction requires three moles of molecular oxygen (O_2) and reducing equivalents from NADPH: ferrihemoprotein (cytochrome p-450) reductase. The biliverdin-IXα generated is rapidly converted to bilirubin-IXα (BR) by NADPH biliverdin reductase (BVR). The iron generated in the HO reaction induces a compensatory elevation in the synthesis of ferritin, which sequesters the iron. The HO system provides cellular and tissue protection through the concerted action of these end-products, through anti-inflammatory, antiapoptotic, anti-proliferative, or anti-oxidative effects. Non-catalytic functions of BVR in the regulation of pro-survival cell signaling pathways have also been proposed.

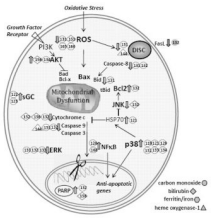

Figure 2. Inhibition of apoptosis by HO-1 and its enzymatic products

Cells may undergo apoptosis by two major pathways, an extrinsic receptor-dependent pathway and an intrinsic (mitochondrial) apoptosis pathway, in response to various acute injurious stimuli. In the intrinsic pathway, environmental stress may cause direct mitochondrial damage, or promote signaling pathways dependent on ROS generation, resulting in the activation of Bax. Bax translocates to the mitochondrial outer membrane, where it oligomerizes or forms complexes with other proapoptotic Bcl-2 related proteins such as Bid or Bad. Bax oligomers form pores in the outer mitochondrial membrane, which facilitate the release of pro-apoptotic molecules such as cytochrome-c. Cytochrome c forms a complex with Apaf-1 and caspase-9, leading to caspase-9 activation. In extrinsic apoptosis, a death inducing ligand such as Fas ligand (FasL), initiates the death program upon interacting with its corresponding receptor (*ie.*, Fas).These interactions lead to the recruitment and activation of caspase-8 in a death-inducing signal complex (DISC). Caspase-8 activation can lead to caspase-3 activation, or to the activation of Bid. Bid assists in the activation and mitochondrial translocation of Bax. Bid activation also results in the release of cytochrome c from the mitochondria. This diagram indicates those points in the apoptotic pathway at which HO-1 and its enzymatic products have been shown to inhibit cell death. Each number corresponds with an individual reference, and the shape of each symbol indicates which effector molecule has been implicated: HO-1, CO, BV/BR or ferritin/iron.