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Targeting Heme Oxygenase-1/Carbon Monoxide for Therapeutic Modulation of Inflammation

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Abstract

The heme oxygenase-1 (HO-1) enzyme system remains an attractive therapeutic target for the treatment of inflammatory conditions. HO-1, a cellular stress protein, serves a vital metabolic function as the rate-limiting step in the degradation of heme to generate carbon monoxide (CO), iron, and biliverdin-IXα (BV) which is converted to bilirubin-IXα (BR). HO-1 may function as a pleiotropic regulator of inflammatory signaling programs, through the generation of its biologically active end-products, namely CO and BV/BR. CO, when applied exogenously, can affect apoptotic, proliferative, and inflammatory cellular programs. Specifically, CO can modulate the production of pro- or anti-inflammatory cytokines and mediators. HO-1/CO may also have immunomodulatory effects with respect to regulating the functions of antigen-presenting cells, dendritic cells, and regulatory T-cells. Therapeutic strategies to modulate HO-1 in disease include the application of natural inducing compounds, as well as gene therapy approaches for the targeted genetic overexpression or knockdown of HO-1. Several compounds have been used therapeutically to inhibit HO activity, including competitive inhibitors of the metalloporphyrin series, or non-competitive isoform-selective derivatives of imidazole-dioxolanes. The end-products of HO activity, BV/BR and CO may be used therapeutically as pharmacological treatments. CO may be applied by inhalation, or through the use of CO releasing molecules (CORMs). This review will discuss HO-1 as a therapeutic target in diseases involving inflammation, including lung and vascular injury, sepsis, ischemia/reperfusion injury and transplant rejection.

INTRODUCTION

The heme oxygenase (HO) enzyme system continues to intrigue researchers across the spectrum of biological sciences, from those engaged in the study of basic metabolism and enzymology, to those investigating the pathogenesis of human disease with the ultimate goal of developing molecular medicine.[1] HO provides an essential enzymatic activity by catalyzing the rate-limiting step in the oxidative catabolism of heme, in a reaction that generates carbon monoxide (CO), ferrous iron, and biliverdin-IXα (BV); the latter which is

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converted to bilirubin-IXα (BR) (Figure 1).[2–3] Heme, the natural substrate and enzyme cofactor for HO, serves as a key mediator of many vital biological processes including oxygen transport and delivery to tissues, peroxide metabolism, cell signaling, xenobiotic detoxification, and mitochondrial bioenergetics. Thus, HO enzymes may fulfill a crucial metabolic function by regulating heme bioavailability and turnover in cells and tissues.[4] In addition to this well-characterized metabolic function, heme oxygenase-1 (HO-1), the inducible form of HO, has gained recognition as a ubiquitous 32-kDa stress protein whose expression is highly upregulated in mammalian cells or tissues during cellular stress.[5–6]

In mammals, the gene(s) that encode HO-1 (HMOX1 in humans, *Hmox1* in rodents), are highly transcriptionally-regulated by injurious stimuli. In additional to the natural substrate heme, and oxidizing cellular stress, such as generated by ultraviolet-A radiation, hydrogen peroxide (H_2O_2), and redox-cycling compounds, HO-1 responds to induction by a multiplicity of chemical and physical agents, including heat shock (in rodents), fluctuations in oxygen tension, nitric oxide, thiol-reactive substances, heavy metals, cytokines, and natural phytochemicals (*see* Table 1 for summary).[5–22] Elevated HO-1 expression in tissue is commonly associated with increased inflammation or oxidative stress, as exemplified by models of acute lung injury (ALI) and ischemia/reperfusion (I/R) injury.[22–24]

The central importance of HO-1 in human physiology and tissue homeostasis is accentuated by studies of naturally occurring genetic deficiency of HO-1 in humans. A patient with human HO-1 deficiency presented with severe hemolytic anemia, endothelial degradation, reduced serum bilirubin, renal and hepatic iron accumulation, and a pro-inflammatory phenotype.[25] Similarly, HO-1 gene-deleted mice (*Hmox1*$^{-/-}$) displayed increased inflammation accompanied by tissue iron accumulation, whereas cells isolated from these animals displayed increased susceptibility to oxidative stress.[26–27] Several studies, which have used *Hmox1*$^{-/-}$ mice or HO-1 transgenic mice, have demonstrated the tissue protective properties of HO-1 in mouse models of cardiovascular, pulmonary, cardiac, or skin injury and disease (*see* Table 2 for summary).[28–37] Despite these observations, deleterious consequences of HO-1 or HO-2 overexpression have been reported *in vitro* and *in vivo* associated with toxic levels of iron accumulation.[38–43]

The mechanisms by which HO-1 expression is associated with context-specific cytoprotection remain incompletely clear, but may reside in the combined effects of the removal of heme (a pro-oxidant iron-chelate) with the enzymatic generation of biologically-active end-products from heme catabolism.[43] This hypothesis has provided the basis for the development of new fields focused on the pharmacological delivery of HO-1 reaction products. In this regard, application of CO has demonstrated tissue protective effects in models of acute inflammation and organ injury.[28, 44] These studies, using inhaled CO gas, include endotoxemia,[45–47] hyperoxia-induced ALI,[48–49] ventilator-induced lung injury (VILI),[50–52] sepsis and pneumonia,[53–55] I/R injury[56–57], vascular injury and disease,[58–60] and organ transplantation[58,61–83] (*see* Table 3 for representative summary). The protective effects observed in these models were attributed to the effects of CO on apoptosis, cell proliferation, inflammation and immunomodulation.[28,44] Similarly, the pharmacological applications of BV or BR, enzymatic products of heme metabolism, have shown protective effects in models of organ injury and transplantation.[63,67,81,84–86]

In addition to pleiotropic cellular effects of HO-1, including reported effects on the regulation of programmed cell death and proliferation programs,[87] current research points to profound anti-inflammatory and immunomodulatory properties of HO-1 and its reaction products (*e.g.,* CO)[88] (Figure 2). This review will focus on the crucial impact of HO-1/CO in inflammation and the underlying mechanisms, in human diseases. Emphasis will be placed on the modulation of HO-1 expression and activity as a potential therapeutic strategy in human diseases that implicate inflammation as a key mediator of pathogenesis. Such strategies may include natural inducing compounds and gene therapy approaches to elevate HO-1 expression, the pharmacological delivery of reaction products such as CO or BV/BR, as well as gene silencing approaches and chemical inhibitors to reduce HO expression and activity in a context-specific fashion. (Figure 3). [1,28,44,89]

HEME OXYGENASE ACTIVITY AND ISOZYMES

HO activity [heme, hydrogen-donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC: 1:14:99:3] catalyzes the rate-limiting step in the oxidative catabolism of heme-*b*. The HO reaction cleaves heme at the α-methene bridge carbon which is liberated as carbon monoxide (CO), with stoichiometric generation of the open-chain tetrapyrrole biliverdin-IXα (BV), and ferrous iron (Fe II).[2–3, 90–91] The reaction proceeds through three sequential oxidation steps each requiring one mole of molecular oxygen (O_2).[92–94] Each oxidation cycle requires reducing equivalents from NADPH: cytochrome p450 reductase (CPR; E.C. 1.6.2.4).[95–97] In the first oxidation step, which converts heme to α-meso-hydroxyheme, one electron from NADPH is utilized for the reduction of the ferric heme iron to its ferrous form, which accepts the binding of O_2. A second electron is utilized for the activation of O_2 to form the hydroxylating species, Fe-OOH.[93–94, 98–100] Additional electron transfer steps are required for the conversion of α-meso-hydroxyheme to verdoheme, which releases CO, and the subsequent conversion of verdoheme the Fe (III)-biliverdin complex[90, 94, 101–103] Upon univalent reduction, the Fe (III)-biliverdin complex dissociates to release BV and free Fe (II).[99] The completion of enzymatic heme degradation involves the divalent reduction of BV by NAD(P)H: biliverdin reductase (BVR; E.C. 1.3.1.24), which produces the lipid soluble pigment bilirubin-IXα (BR). [2–3, 104] HO activity occurs highest in the spleen, testes, and brain, with moderately high levels reported in the liver, kidney and lung.[4] HO activity is represented by two major molecular species, HO-1 and HO-2.[105–106] HO-1 expression is highest in the spleen, the site of erythrocyte turnover. In most tissues HO-1 is undetectable under basal conditions, but highly inducible under conditions of stress or inflammation.[107] The constitutive isoform HO-2 is expressed highly in many tissues including testes, spleen, liver, kidney, cardiovascular and nervous systems.[4,107] HO-1 and HO-2 represent the products of distinct genes and differ in primary amino acid sequence and biochemical/biophysical properties.[108–109] HO-1 and HO-2 share a highly conserved heme catalytic domain as well as hydrophobic regions at the carboxyl-terminus.[110–111] HO-2 contains additional non-catalytic heme binding sites termed heme regulatory domains, with unclear functional significance.[112]

HO-1 and CPR localize to the smooth ER, though recent evidence suggests an association of these proteins with other intracellular membranes, including the inner mitochondrial membrane,[113–114] and plasma membrane caveolae.[115] Immunological evidence supports the

existence of intramolecular interactions between HO-1, CPR, BVR, and between HO-1, BVR, and caveolin-1.[115–117] Recent studies suggest that the plasma membrane scaffolding caveolin-1 can act as a natural inhibitor of HO activity.[115–116] Additional studies suggest the occurrence of immunoreactive forms of HO-1 in the nucleus which lack catalytic activity. A putative role for nuclear forms of HO-1 in transcriptional regulation has been proposed.[118]

REGULATION OF HEME OXYGENASE GENE EXPRESSION

Transcriptional Regulation

The mechanisms by which HO-1 is regulated at a transcriptional level have been elucidated from functional analyses of the 5′ regulatory regions of the corresponding genes in rodents and humans. Studies of the mouse *Hmox1* gene promoter have revealed two major regulatory enhancer regions which are located at −4 kb and −10 kb upstream of the transcriptional start site. These enhancer elements are essential for the transcriptional regulation of the *Hmox1* gene in response to many inducing chemicals including bacterial LPS, and heavy metals such as $CdCl_2$.[119–121] The mouse proximal and distal enhancer regions contain tandem repeats of the stress-responsive element/antioxidant response element (StRE/ARE) motif.[121]

The nuclear factor erythroid 2 (NFE2)-related factor-2 (NRF2) recognizes and binds to the StRE/ARE motifs, and represents the major transcriptional regulator of the *Hmox1* gene.[122] Nrf2 is anchored in the cytoplasm by the Kelch-like ECH-associated protein (Keap1), which inhibits its transcriptional activity. Keap1 facilitates the targeted ubiquitination of Nrf2 by the Cullin 3-based E3 ubiquitin ligase complex, which marks Nrf2 for proteasomal degradation.[123–125] Under basal conditions, Keap1 forms a complex with Nrf2 and prevents its nuclear translocation. Due to the reversible oxidation state of critical cysteine residues, Keap1 has been proposed to act as a redox sensor.[126] The selective autophagy cargo adaptor protein p62 interacts with Keap1 at the Nrf2 binding site, thus promoting the displacement of Keap-1 from Nrf2 to enhance Nrf2 transcriptional activity.[127] Keap1 has also been found to undergo constitutive degradation through the lysosome-dependent autophagy pathway.[128] When cells are exposed to inducing stimuli, such as heme, electrophiles and oxidants, Nrf2 dissociates from Keap1 and then translocates to the nucleus where its transcription factor activity requires the formation of stable heterodimers with small Maf proteins (*i.e.,* MafF, MafG).[122–124] The heme binding protein BTB and CNC homologue 1 (Bach1) has been identified as a negative transcriptional regulator of *Hmox1.* Bach1 forms a complex with small Maf proteins and competes against Nrf2 for binding at the StRE. The heme complex with Bach1 impairs its DNA binding activity and promotes its nuclear export.[129–130] Although LPS acts as a positive inducer of HO-1 in many cell types, including murine monocytes and macrophages, the paradoxical inhibition of HO-1 by LPS in primary human monocytes and macrophages was attributed to activation of Bach1.[131–132]

In addition to the proximal and distal enhancer regions, the *Hmox1* gene contains other functional *cis*-acting elements that contribute to transcriptional regulation, including the heat shock and hypoxia-responsive elements identified in rodents.[20–21] The human HMOX1 gene also contains regions analogous to the upstream enhancer regions described in rodents, but differs from the rodent genes in sequence-specific variation in the regulatory elements at −4

kb, and the presence of an early growth response-1 protein (Egr-1) binding site at −9.5 kb.[133–134] The multiplicity of functional as well as putative regulatory sequences occurring in the genes encoding HO-1 in rodents or humans suggests that the regulation of these genes involves complex transcription factor interactions that may vary in an inducer-specific fashion.[135–137]

HMOX1 Promoter Polymorphisms

Microsatellite $(GT)_n$ dinucleotide length polymorphisms have been identified in the regulatory regions of the human HMOX1 gene (located on chromosome 22q13.1) which may result in the impaired transcriptional regulation and decreased expression of HO-1 in individuals that carry the long (L) allele [(GT)n ≥30] of this polymorphism. A number of studies have described positive correlation between the incidence of HMOX1 promoter polymorphisms with disease severity, mortality, or risk in various human diseases.[138–139] These studies include associations of HMOX1 promoter polymorphisms with diseases such as atherosclerosis and cardiovascular disease,[140] chronic kidney disease,[141] coronary artery disease,[141] necrotizing acute pancreatitis,[142] and preeclampsia.[143] Additional studies reported associations of the long allele of (GT)n with chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome, vascular restenosis, rheumatoid arthritis and outcome of renal transplantation.[144–150] In contrast, short [(GT)n ≥20] HMOX1 promoter polymorphisms were associated with susceptibility to neonatal hyperbilirubinemia.[151] Microsatellite polymorphisms can affect alternative splicing in the HMOX1 5′ untranslated region, thus providing an additional regulatory mechanism for HMOX1 translation.[152] Additional SNPs in HMOX1 have been identified, including rs2071746 T/A which has been associated with increased risk of Parkinson's disease, and with protection from cardiovascular disease.[153–154]

In contrast, various negative studies have reported no associations between HMOX1 polymorphisms in the outcome of cardiac or renal transplantation,[155–156] coronary artery disease,[157] and alcoholic liver disease.[158] Tanaka *et al.* recently reported no association between five SNPs (*i.e.,* rs3761439, rs2071746 and $(GT)_n$) and HMOX1 gene expression or lung functional decline in COPD patients.[159] In conclusion, association studies, when positive, have suggested that a genetically-dependent downregulation of HO-1 expression may arise in subpopulations, possibly linked to increased susceptibility to oxidative stress, inflammation and associated diseases. This hypothesis remains inconclusive and obscured by negative studies. Further research will be needed to validate these observations.

Regulation of HO-1 by MicroRNA

MicroRNAs (miR) are small non-coding RNAs that function in post-transcriptional regulation of gene expression. Emerging studies suggest the critical involvement of miRs in the regulation of HO-1 gene expression either directly by decreasing mRNA stability and/or translation, or indirectly by modulating the expression of upstream regulatory factors (*e.g.,* Nrf2/Keap1, Bach1, *etc.*).[160] A number of miRs may regulate the expression of Nrf2, which may result in the altered regulation of HO-1.[160–163] Several miRs identified in direct HO-1 translational regulation include miR-24[164–166] miR-200c,[162] miR-204/miR-211,[167] miR-155,[168] miR-378,[169] miR-377,[170] and miR-217.[170] Additional indirect mechanisms for

miR-dependent regulation of HO-1 have been proposed. For example, several miRs (*e.g.,* miR-155) can potentially activate HO-1 expression through the downregulation of the transcriptional repressor Bach1.[171–173] Additionally, miR-101 promoted HO-1 expression by targeting the Cullin 3: E3 ubiquitin ligase, resulting in Nrf2 stabilization,[174] whereas miR-200a promoted Nrf2 activation by targeting its cytoplasmic anchor Keap-1.[175]

HO-1 can also regulate miR networks, implicating downstream miR regulation in the functional effects of HO-1 in differentiation, angiogenesis, proliferation, inflammation and tumorigenesis.[176] For example, expression of HO-1 in myoblasts downregulated specific miRs (*e.g.,* miR-1, miR-133a/b, and miR-206) associated with the inhibition of myoblast differentiation.[177] Expression of HO-1 in tumor cells was associated with downregulation of miR-378.[169] These observations point to an emerging complexity of HO-1 regulation and function that may involve a network of miR-dependent regulatory events.

HEME OXYGENASE/CARBON MONOXIDE AS MODULATORS OF INFLAMMATION AND THE IMMUNE RESPONSE

Acute Inflammation Models

HO-1 has been identified as a major modulator of the acute inflammatory response, as demonstrated in *in vitro* and *in vivo* models of inflammation and acute lung injury.[45, 178–179]. Early studies by Willis *et al.* suggested the involvement of HO-1 in the resolution of acute inflammation *in vivo*.[178] In a model of carageenin-induced inflammation of the pleural cavity associated with neutrophil influx, the expression of HO-1 in pleural macrophages was highest at the time of resolution. Inhibition of HO activity by treatment with the HO inhibitor Sn-protoporphyrin-IX (SnPPIX) increased inflammatory exudates in this model whereas hemin-dependent upregulation of HO-1 reduced inflammation.[178] The enhanced gene expression of HO-1 by intratracheal adenoviral-mediated gene transfer protected against LPS-induced lung injury in mice involving the increased production IL-10.[179] Anti-inflammatory effects of low-dose inhaled CO (*i.e.,* 250 ppm) were also demonstrated *in vivo* using a mouse model of endotoxemia.[45] CO preconditioning reduced the production of serum TNF-α, IL-1β, IL-6, whereas increased the production of the anti-inflammatory cytokine IL-10; reduced organ injury and prolonged survival following LPS challenge.[45, 180] The anti-inflammatory potential of CO with respect to modulation of pro- or anti-inflammatory cytokines production was reduced in heat shock factor-1 knockout (*hsf1*$^{-/-}$) mice, implicating a role of the heat shock response *in vivo*.[181] Similar anti-inflammatory effects were observed in mice treated with inhalation CO prior to hyperoxia exposure, a model of ALI.[48–49] The anti-inflammatory protection against LPS-induced organ injury conferred by CO was also observed in association with inhibition of inducible nitric oxide synthase (iNOS) expression and activity in the lung, and enhancement of iNOS expression and activity in the liver.[182] Pharmacological application of BV, an enzymatic by-product of HO activity, also decreased pro-inflammatory cytokine production, upregulated IL-10 levels, and reduced lung injury markers in LPS-treated rats.[183]

In cultured RAW 264.7 macrophages challenged with LPS, HO-1 gene transfer or application of low concentrations of CO (*e.g.,* 250 ppm) inhibited the production of pro-

inflammatory cytokines, including TNF-α, IL-1β, and macrophage inflammatory protein-1β (MIP-1β), and simultaneously increased the expression of the anti-inflammatory cytokine IL-10.[45] The effect of CO on the downregulation of pro-inflammatory cytokines production was associated with the modulation of mitogen activated protein kinase (MAPK) activities, including p38 MAPK and c-Jun NH_2-terminal kinase (JNK).[45, 180] Lee *et al.* demonstrated that HO-1 can be upregulated by IL-10, and proposed that the anti-inflammatory effect of this cytokine depended on HO-1 activation.[184] The anti-inflammatory effect of CO in macrophages involved the downregulation of reactive oxygen species (ROS)-dependent recruitment of TLR4 to plasma membrane lipid rafts.[185] Pretreatment of RAW 264.7 macrophages with heme, or transfection of HO-1 significantly inhibited the production and nuclear translocation of the pro-inflammatory factor high-mobility group box-1 (HMGB1), and impaired the release of pro-inflammatory cytokines in response to LPS challenge. These effects were mimicked by CO donor compounds and reversed by CO scavengers.[54] Additional studies have associated the anti-inflammatory effect of CO with activation of the heat shock factor-1 (HSF-1), and the upregulation of the immunoresponsive gene-1 protein in macrophages.[181,186]

Anti-inflammatory effects of CO have also been demonstrated in pre-clinical models employing higher mammals. In swine, CO reduced the development of disseminated intravascular coagulation and inhibited serum levels of the pro-inflammatory cytokine IL-1β in response to LPS, whereas upregulated IL-10 levels.[46] Similar, though less pronounced anti-inflammatory effects were observed in a non-human primate model of *Cynomolgous macaques* subjected to LPS challenge.[47] CO exposure following LPS inhalation decreased TNF-α release in broncoalveolar lavage (BAL) fluid, but had no apparent effect on IL-6 and IL-8 release, in addition to reducing pulmonary neutrophilia at the highest concentration (500 ppm). The therapeutic efficacy of CO in this model required relatively high doses that resulted in elevated carboxyhemoglobin (CO-Hb) levels (>30%). This study was the first to examine the therapeutic index and dose-response relationships of CO therapy in nonhuman primates in an acute inflammation model.[47]

The potential for protective effects of HO-1/CO have been evaluated in a clinically-relevant model of sterile inflammation induced by mechanical ventilation (MV) which causes ventilator-induced lung injury (VILI) in rats and mice.[50–52] Rats ventilated with an injurious (high tidal volume) ventilator setting in combination with LPS injection, exhibited lung injury. The inclusion of CO (250 ppm) during MV reduced the inflammatory cell infiltration, decreased TNF-α and increased IL-10 in BAL fluid.[50] CO application conferred tissue protection in a mouse model of VILI, at moderate tidal volume ventilation.[51–52] CO reduced MV-induced cytokine and chemokine production and prevented lung injury during ventilation, involving the reduction of BAL protein and cell count, lung neutrophil recruitment, and pulmonary edema.[51–52] These effects of CO were associated with the activation of caveolin-1[51] the activation of PPARγ, and the inhibition of the pro-inflammatory factor Egr-1.[52] These studies, taken together, suggest that CO may protect the lung in animal models of VILI. More studies are required to determine the underlying mechanisms of the therapeutic effectiveness of CO in rodent VILI models, and whether these observations may be translatable to humans.

Regulation of the Inflammasome by HO-1/CO

Inflammasomes are cytosolic protein complexes that promote the cleavage of caspase-1, which leads to the maturation and secretion of pro-inflammatory cytokines including interleukin-1β (IL-1β) and IL-18. Among the known inflammasomes, the NOD-, leucine rich region- and pyrin domain-containing-3 (NLRP3)-dependent inflammasome is crucially involved in the pathogenesis of various acute or chronic inflammatory diseases.[187–188]

Current research suggests a potential regulatory role for HO-1/CO in the inflammasome system. Recent studies demonstrate that heme pre-conditioning to induce HO-1 can reduce IL-1β maturation and downregulate inflammasome activation in a model of sepsis-associated lung injury.[189] Furthermore, induction of HO-1 by heme conditioning was associated with protection from acute liver injury induced by D-galactosamine and LPS, and with downregulation of the associated NLRP3 inflammasome-dependent activation of caspase-1.[190]

Our recent studies demonstrate that application of CO gas can inhibit caspase-1 activation and secretion of IL-1β and IL-18 in bone marrow-derived macrophages in response to LPS and ATP treatment, a NLRP3 inflammasome activation model.[191] CO also inhibited IL-18 secretion in response to LPS and nigericin, an alternate NLRP3 inflammasome activator. LPS and ATP stimulation induced the formation of complexes between NLRP3 and ASC, or NLRP3 and caspase-1. CO treatment inhibited these molecular interactions induced by LPS and ATP. In addition, CO inhibited mitochondrial depolarization induced by LPS and ATP in macrophages.[191] These results suggest that CO negatively regulates NLRP3 inflammasome activation in part by preventing mitochondrial dysfunction.[191] Application of the CO donor compound CORM-2 downregulated caspase-1 activation and IL-1β maturation and secretion in the context of ER-stress induced inflammation.[192] In contrast to these observations, another report shows that CO can promote ATP secretion from bacteria which in turn can upregulate NLRP3 inflammasome activation by stimulating the purinergic receptor (P2X7R) in bacteria-treated macrophages.[193] The reasons for these contrasting findings remain unclear at present, but may be related to differences between live bacterial sepsis and inflammation induced by cell free LPS. Although further studies will be needed to clarify the precise mechanisms, HO-1 and CO show potential as novel regulators of the NLRP3 inflammasome and secretion of IL-1β and IL-18.

HO-1/CO in Sepsis and Infectious Disease

A protective anti-inflammatory function of HO-1 has been demonstrated in the context of of microbial sepsis.[54,194–196] Using the cecal ligation and puncture (CLP) method to induce polymicrobial sepsis, HO-1-deficient mice (*Hmox1*$^{-/-}$) displayed higher mortality rates compared with wild type mice. The *Hmox1*$^{-/-}$mice also had increased levels of free circulating heme and reduced levels of the heme sequestering protein hemopexin, rendering them more susceptible sepsis-induced mortality.[195] Conversely, targeted over-expression of HO-1 to smooth muscle cells and myofibroblasts, and bowel protected against sepsis-induced mortality associated with *Enterococcus faecalis* infection, and enhanced bacterial clearance by increasing phagocytosis and the endogenous antimicrobial response.[194] These studies suggest that endogenous heme is a pro-pathogenic mediator which is associated with

propagation of tissue injury and inflammation in sepsis. The pro-inflammatory effects of heme have been demonstrated in cultured cells, and involve activation of TLR4-mediated signaling pathways leading to increased production of ROS in macrophages and activation of endothelial cells.[197–198] In contrast, pharmacological application of heme has been used as a preconditioning agent, which can confer protection by virtue of its ability to induce HO-1 in tissues in advance of injury, resulting in cytoprotection. For example, hemin pre-conditioning of healthy animals, which induces HO-1, can protect mice from subsequent lethal endotoxemia and sepsis induced by LPS or CLP, respectively.[54]

Several studies have demonstrated that circulating levels of HMGB1, a protein that can regulate chemotaxis and accumulation of pro-inflammatory cytokines, contributes to LPS-induced mortality in *Hmox1*$^{-/-}$ mice.[54,196] Furthermore, pharmacological preconditioning with heme, to induce HO-1, significantly reduced plasma levels of HMGB1 in mice challenged with LPS or CLP, which was associated with the reduction of serum TNF-α, and IL-1β levels.[54,196] Transfection of HO-1 or induction of HO-1-derived CO resulted in a significant reduction in the translocation and release of HMGB1 in CLP-induced sepsis *in vivo*. In conclusion, HO-1-derived CO attenuated HMGB1 release during sepsis, which may represent a therapeutic intervention against sepsis.[54]

Emerging studies suggest that CO can enhance bacterial clearance, though the mechanisms remain incompletely understood.[193–194,199] The pharmacological application of CO releasing pro-drugs enhanced bacterial phagocytosis *in vivo* and rescued *Hmox1*$^{-/-}$ mice from sepsis-induced mortality.[194] Furthermore, CO releasing pro-drugs improved intestinal bacterial clearance in an *S. typhimurium infection* model.[199] In an *E. coli* infection model, endogenous HO-derived CO was associated with enhanced macrophage phagocytosis and this was shown to require NLRP3 and caspase-1 dependent immune responses.[193] In accord with these studies, we have recently shown that CO inhalation (250 ppm) either as pre-treatment or post treatment improved mouse survival in the CLP model. The salutary effects of CO in this model were related to the induction of autophagy and phagocytosis, the reduction of inflammation, and enhanced bacterial clearance from organs and blood. The pro-survival effects of CO in CLP were dependent on the autophagy program, as they were reduced in mice heterozygous for genetic deletion in the autophagy regulator molecule Beclin 1.[53]

The therapeutic potential of HO-1/CO has also been investigated in infectious disease models. The enhanced gene expression of HO-1 in rat lungs by intratracheal adenoviral mediated gene transfer limited murine acute lung injury following influenza virus infection.[200] Current studies have evaluated the therapeutic potential of CO using an *S. pneumoniae* model of pneumonia in baboons.[55] Plasma obtained from *S. pneumoniae* infected baboons displayed significantly reduced levels of lipid mediator/specialized pro-resolving mediators (SPM), including eicosapentaenoic acid-derived E-series resolvins (RvE) and lipoxins, suggesting that pneumonia can deregulate pro-resolution programs in baboons. In these animals, CO inhalation increased the levels of plasma RvE and lipoxins relative to control levels. These results suggested that altered SPM profiles during pneumonia can be partially restored with inhaled low-dose CO.[55]

HO-1/CO in Vascular Injury and Disease

Protective roles for HO-1/CO in vascular injury and disease have been reported.[29, 58] In injured pig arteries, HO-1 gene transfer increased vasodilation and inhibited vascular smooth muscle proliferation.[29] *Hmox1*$^{-/-}$ mice displayed increased intimal hyperplasia after aortic injury relative to corresponding wild type mice.[29] HO-1 expression *in vitro* reduced vascular smooth muscle cell (SMC) proliferation through upregulation of p21^{Cip1}.[29] Inhaled CO prevented arteriosclerotic lesions associated with aorta transplantation in rodents, and reduced intimal hyperplasia associated with vascular injury. Exposure to a low level of CO (250 ppm) for 1 hour before injury inhibited intimal hyperplasia associated with balloon injury.[58] The protective effect of CO was associated with inhibition of graft leukocyte infiltration/activation as well as with inhibition of SMC proliferation through upregulation of p21^{Cip1}.[58]

HO-1 gene transfer was also shown to reduce hypoxia/reoxygenation-induced stasis in mice with sickle cell disease (SCD). The increase of HO-1 expression in this model promoted anti-inflammatory effects associated with the activation (phosphorylation) of p38 MAPK and Akt, decreased the nuclear accumulation of p65 NF-κB in liver tissue, as well as reduced serum levels of soluble vascular cell adhesion molecule-1 (sVCAM-1).[201] Pharmacological application of heme to increase HO-1 expression in the liver, inhibited vascular stasis and leukocyte adhesion, and inhibited NF-κB activation. CO inhalation also reduced stasis and conferred similar anti-inflammatory effects in this model.[59,60] Additionally, CO inhalation reduced hepatic necrosis, reduced myeloid and lymphoid markers in the bone marrow, and reduced blood cell counts in SCD mice.[60]

HO-1/CO in Organ Ischemia/Reperfusion Injury

Tissue protective effects of HO-1 or CO have been demonstrated in rodent models of organ I/R injury. For example, overexpression of HO-1 protected against myocardial injury in a mouse cardiac I/R injury model.[202] Mice genetically deficient in HO-1 (*Hmox-1*$^{-/-}$) were sensitized to the lethal effects of lung I/R injury. Inhaled CO compensated for the HO-1 deficiency in *hmox-1*$^{-/-}$ mice, and improved survival during pulmonary I/R.[30] The protection provided by CO involved activation of fibrinolysis in a guanylate cyclase-dependent mechanism, involving downstream inhibition of plasminogen activator inhibitor-1.[30] CO inhibited fibrin deposition and improve circulation in ischemic lungs, by inhibiting the pro-inflammatory transcription factor Egr-1.[203] Additional studies in the pulmonary I/R model demonstrate protective effects of CO involving downregulation of apoptosis pathways.[56,204]

In organ transplantation, I/R injury subsequent to transplantation may represent a major causative factor in graft failure. Transgenic systemic overexpression of HO-1 was shown to improve cardiac graft tolerance and reduce inflammation associated with I/R injury in a cardiac transplantation model.[205] CO has been intensively studied as an anti-inflammatory therapeutic in experimental organ transplantation. CO has a demonstrated potential for reducing transplant associated I/R injury and also reducing the probability of graft rejection when applied at low concentration, when added to organ preservation fluid or when applied to donors and/or recipients in gaseous form at low concentration. The application of CO can

confer protection during transplantation of multiple organs (see Table 3). In a model of orthotopic lung transplantation in rats, exogenous application of CO (500 ppm), significantly protected the graft, and reduced hemorrhage, fibrosis, and thrombosis after transplantation.[61] Furthermore, CO inhibited lung cell apoptosis and downregulated lung and systemic pro-inflammatory cytokine production.[61] In a vascular transplantation model, when transplant recipients of aortic grafts were exposed to CO (250 ppm), these animals displayed reduced intimal hyperplasia, and reduced leukocyte, macrophage, and T cell infiltration in the graft.[58] The modulation of responses, and the inhibition of apoptosis and inflammation associated with I/R injury, likely represent the main mechanisms by which CO promotes the survival and function of transplanted organs, though effects on circulation and cell proliferation may also contribute.[58]

Metabolic Disease and Inflammation

HO-1 and CO have been implicated in the modulation of metabolic disease, including high fat diet (HFD)-induced obesity, insulin resistance, hyperglycemia, and diabetes. These topics has been reviewed elsewhere.[206–207] Chemical induction of HO-1 using hemin protected against obesity-induced adipose inflammation through M2 macrophage phenotype switching in mice fed a high fed diet (HFD).[208] In models of metabolic disease, inhalation of CO gas reduced hepatic steatosis in mice subjected to 30% fructose or methionine-deficient and choline-deficient-diets.[209] CO exposure (administered as CORM-2) was shown to confer cardioprotection and restore mitochondrial function in a HFD-induced model of metabolic syndrome.[210] Furthermore, the application of CORM-A1, a CO releasing molecule, prevented HFD-induced hypoglycemia, insulin resistance and weight gain in mice.[211] The application of BR, an HO end-product, also improved insulin sensitivity in HFD-induced obese mice.[212]

In contrast to these observations, the adipose-specific transgenic expression of HO-1 in mice failed to modulate insulin resistance in HFD mice.[213] Despite reported anti-inflammatory effects associated with HO-1 in models of endotoxemia, ALI, and I/R injury, recent research has indicated a potential pro-inflammatory effect of HO-1 in the context of inflammation associated with metabolic disease.[214] Hepatocyte and macrophage conditional HO-1 deletion in mice conferred protection against diet-induced insulin resistance and inflammation, dramatically reducing secondary disease such as steatosis (fatty liver) and liver toxicity.[214] Heterozygous *Hmox1* knockout mice were also shown to be protected against HFD-induced insulin resistance by reducing macrophage migration.[215] These observations, suggestive of pro-pathogenic roles for HO-1, emphasize that the functions of HO-1 in chronic inflammation are not yet fully understood.

Immunomodulation by HO-1/CO

HO-1 can exert significant immunomodulatory potential, though the mechanisms remain incompletely understood. HO-1 expression may promote macrophage class switching from activated M1 macrophages to alternatively activated anti-inflammatory M2 macrophages.[216] M2 macrophage subtypes have been identified which display high levels of HO-1 expression.[216] HO-1 has also been shown to regulate interferon-β (IFN-β) production in myeloid cells.[217] Myeloid cell-specific immunomodulatory functions of HO-1 were

observed in myeloid-specific HO-1 knockout mice. Mice with myeloid cell-specific *Hmox1* deletion exhibited a defect of the IFN-β pathway. These mice were characterized by aberrant immune responses in experimentally-induced infections, were resistant to *Listeria monocytogenes* infection, but displayed an aggravated autoimmune response and accelerated pathology in a model of experimental autoimmune encephalomyelitis.[217]

A number of studies have previously implicated HO-1 in T-cell mediated immunosuppression. Upregulation of HO-1 and associated CO production was proposed to influence the function of $CD4^+CD25^+$ regulatory T cells (Treg) in immunosuppression and peripheral tolerance.[218] HO-1 dependent tolerance to cardiac transplantation depended on $CD4^+CD25^+$ Treg functions.[219] HO-1 induction with hemin was also associated with the suppression of allergic airway inflammation through upregulation of $CD4^+CD25^+$ Tregs.[220–222] The transcription factor Foxp3 which functions in development and function of $CD4^+CD25^+$ Tregs was shown to induce HO-1, which in turn was proposed to mediate Foxp3-dependent immunosuppressive functions.[224] More recent studies have challenged these conclusions and present evidence that the $CD4^+CD25^+$ Treg function does not directly depend on HO-1 expression in these cells.[223]

Emerging evidence suggests that the impact of HO-1 in immunomodulation may be related to the effects of its expression on activated T cells, dendritic cells (DCs) and antigen-presenting cells (APCs). The absence of HO-1 in APCs abolished the suppressive activity of Treg cells on effector T cells.[224] Stimulation of HO-1 induction *ex vivo* restored $GARP^+CD4^+CD25^+$ Tregs populations in isolated T lymphocyte samples from patients with acute coronary syndrome, and promoted the expression of effector molecules (*i.e.,* LAP and GARP) on activated T cells.[225] HO-1 may be important for the functioning of DCs in antigen-presentation and adaptive immune responses. Targeted up-regulation of HO-1 can influence the maturation and cell-specific functions of DCs in humans and mice.[226–227] Induction of HO-1 or treatment with exogenous CO inhibited LPS-induced maturation of DCs and protected against *in vivo* and *in vitro* antigen-specific inflammation.[228] Current work also shows that HO-1 induction or application of CO blocks antigen presentation by DCs at the step of endosome-lysosome fusion.[230] In a Type-1 diabetes model, *ex vivo* treatment of DCs with gaseous CO was shown to augment dendritic-cell based therapy. Application of CO-conditioned DCs was shown to effectively impair the accumulation and pathogenic activity of autoreactive $CD8^+$ T cells in the pancreas.[230] These examples illustrate the complex and incompletely understood role for HO-1 in adaptive immune response.

THERAPEUTIC STATEGIES INVOLVING HO-1 MODULATION

Heme oxygenase inhibitor compounds—HO activity can be inhibited in cells or tissues by several natural and synthetic compounds. The application of HO activity inhibitors was initially proposed as a clinical therapy for neonatal jaundice, where HO promotes the excess formation of BR which may lead to neurological injury in neonates.[231]

HO activity inhibitors may also potentially have therapeutic value in diseases such as neurological disorders where excess HO activity has been implicated in the pathology.[232]

First generation competitive inhibitors of HO are represented by synthetic metalloporphyrins (Figure 4). These include heavy metal (*i.e.,* Zn^{2+}, Sn^{2+}, and Cr^{2+}) chelates of protoporphyrin (PP), mesoporphyrin (MP), or deuteroporphyrin (DP).[231, 233] Of these SnMP was described as the most potent HO inhibitor.[234] Modified derivatives for improved oral absorption have been described, exemplified by Zinc deuteroporphyrin bis-glycol (ZnDP-BG).[234–235] Despite the potency of these compounds, their principle drawback is their lack of isoform selectivity (*e.g.,* HO-1 *vs.* HO-2).[236] Furthermore, pleiotropic off-target effects of metalloporphyrins have been reported in some experimental settings, such as inhibition of iNOS or guanylate cyclase, phototoxicity, or ability to induce *Hmox1* gene transcription.[235–236] Several clinical trials were performed which demonstrated reduction of total BR after SnMP application in neonates. Phototoxicity resulting in erythemia, and increased toxicity with combination phototherapy were the principle reported concerns.[237]

The imidazole-dioxolanes represent a second generation of HO activity inhibitors, the first of which is represented by azalanstat (QC-1) (Figure 4).[238–244] Additional derivatives of this molecule (*e.g.,* QC-13, QC-15 and others) have been shown to inhibit HO activity, with QC-15 reported as highly selective for HO-1.[238–240, 243] To date, limited *in vivo* experiments have been performed using these novel inhibitors. In mice, the intra-renal medullary interstitial infusion or peritoneal injection of QC-13 resulted in the inhibition of HO activity in the kidney.[245] Renal delivery of QC-13 aggravated angiotensin-II induced hypertension in this model as effectively as the metalloporphyrin SnMP.[246] Recent reports also describe therapeutic effects of azole-based HO-1 inhibitors (e.g., QB-28) in mouse models of neurodegenerative disease.[232]

Natural and Synthetic HO-1-inducing Compounds—A subclass of HO inducing agents includes electrophilic antioxidant compounds, many of which are plant-derived polyphenols found in the human diet. These compounds activate the Nrf2 system resulting in the enhanced expression of several Nrf2 target genes coding for detoxification-associated proteins (e.g., HO-1, glutathione S-transferase A2 and NADPH: quinone oxidoreductase).[247–248] Among the natural compounds that can induce HO-1 in model systems include the plant-derived compounds curcumin,[12–14] caffeic acid phenethyl ester,[13–14] resveratrol,[11] quercetin,[15–17] epigallocatechin gallate,[18–19] carnosol,[248] sulforaphane[248] and others. The effectiveness of these dietary compounds at inducing HO-1 using *in vitro* model systems has led to a hypothesis that these or related compounds may be used as pharmaceuticals for human conditions such as cardiovascular disease.[249] A recent study has evaluated the effectiveness of high dose oral curcumin administration in humans.[250] This study concluded that oral curcumin was ineffective at inducing HO-1 in human peripheral blood mononuclear cells after administration, due to poor absorption.[250] Further studies are needed to identify safe and effective oral compounds that can induce HO-1 in humans.

In addition to dietary compounds, several pharmaceutical compounds have been identified that can activate Nrf2/Keap1 axis, including dimethyl fumarate (DMF) and related compounds.[248] Fumarates have been used in the treatment of psoriasis, while DMF has shown therapeutic effects in Phase III clinical trials for multiple sclerosis. An oral formulation of DMF (*e.g.,* BG-12, Tecfidera) has been approved for clinical use in the

treatment of multiple sclerosis in the USA, Canada, Europe and Australia.[251–252] The mechanism of action of DMF, or its downstream metabolite monomethyl fumarate, involves the direct modification of a critical cysteine thiol group of Keap1 (Cys151) resulting in Nrf2 stabilization.[253–254] DMF can act as a potent inducer of HO-1 in microglia cells and can confer anti-inflammatory effects in this cell type.[248] Application of DMF can confer neuroprotection, reduce macrophage inflammation, and increase IL-10 production in mouse models of chronic experimental autoimmune encephalomyelitis.[253, 255] DMF also reduced intimal hyperplasia in a model of vascular injury, in an Nrf2-dependent mechanism involving upregulation of $p21^{Cip1}$.[256] The effects of Nrf2-inducing compounds such as DMF are not necessarily specific for HO-1, as Nrf2 regulates multiple effector enzymes. Further research may lead to the development of additional Nrf2-activating compounds for safe and effective activation of the antioxidant response in the context of human disease.

HO-1 Gene therapy

Gene therapy approaches to regulate HO-1 in tissues were initially proposed based on preclinical data associating HO-1 with cytoprotection.[257] Adenovirus and retroviral-based vectors have been evaluated for HO-1 gene transfer *in vivo*. For example, HO-1 expression by adenovirus-mediated gene transfer inhibits lung cell injury in response to hyperoxia *in vivo* after intratracheal administration.[258] Microinjection of a human HO-1 transgene into rabbit eyes resulted in the site-specific expression of human HO-1 protein.[259] Delivery of human HO-1 using a replication-deficient retroviral vector to spontaneously hypertensive rats reversed the hypertensive phenotype.[260] Adenoviral-dependent expression of HO-1 also protected against myocardial injury in a mouse cardiac I/R injury model.[202] Gene transfer of HO-1 using the Sleeping Beauty transposase vector was shown to effectively increase hepatic HO-1 expression in SCD mice, and conferred anti-inflammatory effects in this model.[201]

Recent studies have explored the possibility of targeted downregulation of HO-1 using the retroviral expression of miRNAs. Lung endothelial specific lentiviral expression of miR was used successfully to knockdown HO-1 in the lung, and this resulted in susceptibility to oxidant stress, enhanced pulmonary apoptosis and reduced autophagy.[261] These examples support the feasibility of modulating HO-1 expression in a tissue-targeted fashion. Further progress in HO-1 gene therapy depends on the continued development of cell or organ-specific vectors for gene delivery.[257] Furthermore, the safety and efficacy of retroviral vectors for human application also remains an important concern.[257]

THERAPEUTIC STATEGIES INVOLVING APPLICATION OF HO-1 END PRODUCTS

Inhalation CO: Mechanism of action

In addition to genetic or pharmacological strategies aimed at altering HO-1 expression or activity, the direct application of the HO activity end-products (*e.g.,* CO) remains a promising strategy to harness the therapeutic potential of HO-1. Much initial research has focused on the direct application of CO gas by inhalation (*in vivo*) or ambient application (*in vitro*). Such experiments have typically utilized relatively low concentrations of CO (e.g.,

250 ppm in air) for cell culture, with up to 500 ppm used *in vivo*.[45–49] CO is an odorless and colorless low molecular weight gas. The principle consideration of inhaled CO, albeit at supraphysiological concentrations, is the inherent toxicity associated with this gas, which is a common contaminant of indoor and outdoor air. At high ambient concentrations, CO can act as a chemical asphyxiant. CO has a high affinity for hemoglobin (approximately 250 times that of oxygen), which results in the formation of CO-Hb in the circulation. Partial occupation of CO at the O_2 binding sites of hemoglobin inhibits the release of O_2 from the remaining heme groups. Accumulation of CO-Hb results in hypoxemia, and impaired oxygen delivery to tissues, which can result in morbidity or death.[262] CO also has a high affinity for other heme iron chelates, and thus may target other biological hemoproteins, including guanylate cyclase, cytochrome *c* oxidase and cytochrome p-450.[262] The impairment of mitochondrial respiration by CO may contribute to cellular toxicity, though hypoxemia remains the principle toxic mechanism of action *in vivo*.

At low concentrations, below the threshold of toxicity, CO may exert physiological effects (e.g., vasoregulation)[263–265] and cellular effects, the latter which include modulation of cellular signaling pathways that regulate apoptosis, proliferation, and inflammation, as demonstrated in various cell culture and animal models.[28,44,266] Similar to NO, CO serves as an agonist of soluble guanylate cyclase (sGC), *via* heme liganding, which results in the formation of 3′,5′-cyclic guanosine monophosphate (cGMP).[267–268] The sGC/cGMP axis has been implicated in vasoregulation, antiproliferative effects, and neurotransmitter effects of CO.[263–265, 269–271] The agonist effect of CO on sGC, however is much less potent than NO.[268] In addition to cGMP formation, CO may also activate potassium channels, such as the large conductance voltage- and Ca^{2+} activated K^+ channel (i.e., BK_{Ca}), and other ion channels such as the voltage gated L-type Ca^{2+} channels, which may contribute to vasodilatory and other signaling effects.[272–274]

CO can modulate the activation of the mitogen activated protein kinases (MAPK), including p38 MAPK, JNK and ERK1/2 activites, though the proximal target(s) remain unclear.[45,49,56,180,204,275] MAPKs represent crucial mediators of inflammatory and stress responses. The anti-inflammatory effect of CO as demonstrated in LPS-stimulated macrophages, required activation of p38 MAPK pathway,[45] and may also may require the downregulation of Toll-like receptor-4 (TLR4) trafficking and activation,[276–277] and inhibition of NADPH oxidase-dependent signaling.[276] Additional signal transduction pathway molecules have been implicated in CO dependent anti-inflamamtory effects, including heat shock factor-1 (HSF-1),[181] the PPARγ/Egr-1 pathway[278] and caveolin-1, which inhibits TLR4 signaling.[277]

CO has been shown to regulate apoptotic signaling pathways in cultured cells.[56,204,275] When applied at low concentration, CO inhibited cell death caused by pro-apoptotic agents (*e.g.,* TNFα) in endothelial cells, which required the p38 MAPK pathway, and modulation of NF-κB signaling.[275,279] Additional targets for CO-dependent regulation of cellular apoptosis in various stress models include the STAT3 and PI3K/Akt pathways,[280] inhibition of NADPH: oxidase dependent ROS formation,[281] and modulation of caspase-8.[204, 281–282] CO can exert anti-proliferative effects *in vitro*, with respect to the proliferation of vascular SMC.[269–270] In SMC, both sGC/cGMP and p38 MAPK signaling pathways have been

implicated in the anti-proliferative effects of CO these cells.[58,269–270] Signaling mechanisms involved in CO-dependent regulation of cell proliferation and/or migration, include the regulation of the lipid-raft associated protein caveolin-1,[283] and the modulation of NADPH oxidase.[284–285]

Additional hemoprotein targets of CO that may be relevant to modulation of cellular signaling, include modulation inducible NOS (iNOS),[182, 286] inhibition of cytochrome p-450 degradation,[287] and inhibition of mitochondrial cytochrome *c* oxidase associated with increased mitochondrial ROS formation.[288–290] The latter pathway has been linked to autophagy signaling in epithelial cells,[290] and to activation of hypoxia-inducible factor (HIF-1) mediated gene regulation.[289] Finally, several studies have shown that CO can activate the Nrf2 system,[291–292] and thus act as a feedback regulator of HO-1 expression. The activation of the Nrf2 pathway was recently implicated in CO-dependent protection against neural ischemia.[293] Endogenous CO, generated from HO-1, can activate Nrf2 nuclear translocation through upregulation of PI3K/Akt and downregulation of glycogen synthase kinase-3β (GSK-3β). This pathway has been implicated in the cardiac upregulation of the nuclear regulatory factor-1 (NRF-1), a cardinal regulator of mitochondrial biogenesis.[292] Taken together, these intriguing modulatory effects of CO on the signal transduction pathways that culminate in the regulation of inflammation, apoptosis, cell proliferation, and vascular function all may contribute to the proposed therapeutic effects of this gas.

Pharmacological CO

As an alternative to the inhalation of CO gas for therapeutic delivery, chemical CO-donor compounds termed carbon monoxide releasing compounds (CORMs) have been developed as experimental therapeutics over the last decade.[44,294–295] An advantage of CORMs is that they deliver CO to tissues with reduced CO-Hb accumulation as compared with inhalation CO.[41,294] CORMs can deliver small amounts of CO in a controlled manner and may represent an experimental therapy for sepsis and inflammatory disorders.[44,294]

The CORMs that have been developed for experimental applications include the original prototype compound Mn_2CO_{10} (CORM-1) and the ruthenium-based compounds tricarbonyldichlororuthenium-(II)-dimer (CORM-2) and tricarbonylchoro(glycinato)-ruthenium (II) (CORM-3).[44, 294–297] CORM-1 and CORM-2 have a hydrophobic backbone, whereas CORM-3 is water-soluble and rapidly releases CO in physiological fluids. A water-soluble boron-containing CORM (CORM-A1) has been developed, which slowly releases CO in a pH and temperature-dependent fashion.[298] New generation CORMs based on iron carbonyl structures have been described. The hydrophilic iron carbonyl in this line displayed improved cytotoxicity and slower CO release than the hydrophobic analogues.[299] Novel compounds also include light-activated CORMs such as CORM-S1, a compound based on iron and cysteamine.[300] New photoactivatable CORMs (Photo-CORMs) based on manganese tricarbonyl structure have recently been introduced. Among these, Mn(CO)3(tpa-κ(3)N)](+) is stable in the dark, releases CO in solution upon photoactivation at 365 nm, and has demonstrated anti-bacterial activity.[301–303] Recent innovation in this area also includes

the development of nanoparticle delivery systems for CORMs,[303–304] as well as bifunctional hybrid molecules with both Nrf2-activating and CORM functions.[305]

CORMs can exert vasomodulatory effects with CORM-3 shown to produce a rapid vasodilatory response.[297] Similar to inhalation CO, protective effects have been demonstrated in preclinical injury and disease models with pharmacological application of CORMs. Among the therapeutic effects of CORMs include the inhibition of pro-inflammatory cytokine production in LPS-stimulated macrophages,[306] and inhibition of the inflammatory response in LPS-stimulated endothelial cells.[307] *In vivo,* CORMs have been shown to attenuate systemic inflammation and pro-adhesive vascular cell properties, as well as prolong survival, and reduce liver injury during sepsis and inflammatory injury.[308–310] The protective effects of CORMs in sepsis were related to stimulation of mitochondrial biogenesis.[311] Furthermore, the beneficial effects of CORMs in sepsis, like gaseous CO, may involve stimulation of bacterial clearance.[194,199,312] CO, when generated by application of CORMs, can also exert direct bactericidal activity.[313–317] CORM3 exerted antibacterial activity against *P. aeruginosa* and reduced bacterial counts in a model of live bacterial infection.[314–315] CORM3 was also shown to have antibacterial activity against *E. coli*,[313,318] and *S. typhimurium* after bacterial uptake of the drug.[316] Both CORM2 and CORM3 exerted antibacterial activity against antibiotic-resistant strains of *H. pylori*.[319] Recent advances using PhotoCORMs demonstrate photoactivatable toxicity of these compounds toward *E. coli* bacteria.[301] CORMs may also find application in reducing gastrointestinal inflammation in various models including post-operative ileus and necrotizing enterocolitis.[312] Finally, applications for CORMs in transplantation models have also been described. For example, in a cardiac transplantation model, inclusion of CORM-3 in the preservation fluid was shown to improve cardiac function following transplantation.[320]

These examples of salutary effects of CORMs are suggestive of possible therapeutic applications of CORMs in human disease, though more research regarding safety and efficacy of these compounds for human application is needed.

BV/BR therapy

BV and BR are known to have potent chemical antioxidant properties, which have been well documented using *in vitro* model systems,[321–323] as well as cytoprotective effects in cultured cells.[324–327] Elevated serum BR conferred oxidative stress resistance in hyperbilirubinemic animals.[328] In human subjects, circulating BR has been correlated to reduced risk of cardiovascular disease.[329–332] Exogenous applications of BV or BR, can confer anti-inflammatory and protective effects in models of organ injury and transplantation.[63,67,81,84–86] For example, exogenous application of BR preserved myocardial function during cardiac I/R injury.[326] BR or BV conferred tissue protection from I/R injury in various organ transplantation models, including liver,[84] kidney,[85] and heart.[86] Perfusion with BV increased the survival in rats undergoing orthotopic liver transplantation by preserving liver function,[84] by decreasing neutrophil influx, reducing pro-inflammatory cytokine expression, and reducing iNOS activation.[84] In an isolated perfused kidney model, perfusion with BR protected against warm I/R-induced tissue injury and preserved renal function.[85] BV improved the survivability of rat cardiac and pancreatic allografts, by

reducing leukocyte infiltration and inhibiting T-cell proliferation.[81,86] Application of both BV and CO provided a synergistic tissue protection against transplant-associated cold I/R injury of heart and kidney grafts.[67] Hyperbilirubinemic rats were resistant to bleomycin-induced lung injury (fibrosis) and associated mortality, and displayed reduced lung hydroxyproline content, and reduced polymorphonuclear lymphocyte and leukocyte counts, as well as reduced levels of TGF-β in the BAL.[333] Administration of BV to rats protected against systemic inflammation and lung injury, and extended survival after exposure to a lethal dose of LPS, associated with a reduction of pro-inflammatory cytokines in the serum (i.e., IL-6) and upregulation of serum IL-10 levels, as well as reduced lung permeability.[183] Recent studies have proposed a novel pathway for anti-inflammatory effects of BV in macrophages, which depend on eNOS upregulation, and eNOS/NO-dependent downregulation of TLR4 expression.[334] Exogenous BV administration inhibited neointimal hyperplasia associated with vascular balloon injury in rats,[335–336] while hyperbillirubinemic animals also displayed increased resistance to vascular injury.[336] An antiproliferative effect of BV/BR was demonstrated in vascular SMC culture, whereby exogenous BV/BR arrested cells in G1 phase associated with inhibition of p38 MAPK and retinoblastoma protein phosphorylation *in vitro*.[336] Taken together, these studies suggest that BV/BR may have therapeutic applications in disease.

Heme and Iron modulation

Free circulating hemoglobin can represent a toxic insult to the vascular endothelium. Heme, the prosthetic group of hemoglobin and other hemoproteins, in free form is regarded as a potentially toxic molecule based on its nature as an iron chelate, which can catalyze free radical generating reactions, leading to LDH oxidation and NO depletion, causing injury to endothelium, and promoting inflammatory pathways.[43, 337–338] Thus, the removal of cell-free hemoglobin or free heme with scavenging proteins such as haptoglobin or hemopexin, respectively, has been proposed as a therapeutic strategy in inflammatory diseases.[339–340] HO-1 has a well recognized role in iron metabolism by redistributing heme iron. *Hmox*$^{-/-}$ mice are known to display aberrant tissue iron deposition.[26] HO-1-derived iron drives the synthesis of ferritin, which in turn sequesters the liberated iron in an inert state, and this may represent a cytoprotective pathway that limits the potential of iron to catalyze harmful reactions.[341–343] Iron chelators, although not specific for HO-derived iron, may be of therapeutic value in iron-dependent pathologies.[344–345] In diseases where HO-1 derived iron has been directly implicated in the pathology (e.g., Alzheimer's disease), such a neurodegenerative diseases, inhibition of HO activity may provide therapeutic benefit.[232,346]

IMPLICATIONS FOR CO THERAPY IN HUMAN DISEASE

The widespread and general success of CO as an anti-inflammatory agent in preclinical rodent models, as well as additional progress in primate and porcine models of inflammatory disease have fueled the continued aspiration that CO will eventually provide useful clinical applications as a gaseous molecular medicine for human disease. Ongoing Phase I/II clinical trials may soon yield additional information. In a randomized, double-blinded, placebo-controlled, two-way cross-over trial, experimental endotoxemia was induced in healthy volunteers by injection of 2 ng/kg LPS. The potential anti-inflammatory effects of CO

inhalation were investigated under these conditions by inhalation of 500 ppm CO (which increased CO-Hb to 7%) versus synthetic air as a placebo for 1 h. Under these conditions, CO inhalation had no effect on the inflammatory response as measured by systemic cytokine production. In this study, no adverse effects of CO inhalation were observed.[347] Another human clinical trial demonstrated the feasibility of administering inhaled CO to humans with chronic obstructive pulmonary disease (COPD).[348] In this study, ex-smoking patients with stable COPD were subjected to CO inhalation (100–125 ppm for 2 hours/day for 4 days), which increased CO-Hb levels to 4.5%. Inhalation of CO by patients with stable COPD displayed a trend in reduction of sputum eosinophils and improvement of methacholine responsiveness, without adverse effects.[348] In summary, the protective phenotype of CO in with respect to reducing inflammation in mice has been partially recapitulated in higher animals. Further experiments are required to confirm the safety and efficacy of CO inhalation as a treatment for inflammatory lung diseases in humans. Ongoing clinical trials in sepsis, acute lung injury, and fibrosis may yield additional information in the foreseeable future.

CONCLUDING REMARKS

HO-1 continues to fascinate the research community as a molecule with a multiplicity of implications in the pathogenesis and therapeutics of human disease. Although many pre-clinical research studies have pointed to anti-inflammatory effects of HO-1 in tissue injury, recent studies now also propose a pro-pathogenic effect of HO-1 in the propagation of chronic inflammation.[214] Thus, the function of HO-1 in inflammation remains complex and incompletely understood. The putative role of HO-1 in the modulation of the adaptive immune response represents an area of active investigation.

The modulation of HO-1 by genetic overexpression or counter-regulation remains a candidate for gene therapy applications, yet this approach is limited by the overall constraints of human gene therapy approaches, as well as potential for overdosing and off-target effects. Similarly, the application of synthetic chemical inhibitors of HO activity may find therapeutic application, though further development of isoform-selective and specific inhibitors is ongoing. The modulation of HO-1 by dietary antioxidants and related compounds has long been proposed as a therapeutic avenue based on model studies and has met with limited success in humans using the model compound curcumin, though the pharmaceutical DMF shows promise.

Finally, the pharmacological application of HO activity end-products including systemic administration of BV/BR or inhalation of CO gas has now been tested in multiple pre-clinical studies. It should be noted that the exogenous application of these substances as pharmaceuticals, even at low concentrations, may result in non-physiological effects that may not necessarily have the identical effect as the endogenous production of these substances in their natural contexts.[349]

As an alternative to inhaled CO, pharmacological application of CO using CORMs or PhotoCORMs may provide a promising therapeutic strategy.[44] Other novel CO-based

therapies may include the use of CO-saturated hemoglobins as well as the combination of CO preconditioning with cell based therapies.[230, 350]

The success of inhalation CO in preclinical animal studies using rodents and higher mammals has led to continued efforts toward the clinical application of inhaled CO. To date, human studies recapitulating the therapeutic effects of CO observed in animals are ongoing. Several studies have been completed which lend support for the safety and feasibility of low-dose CO application in humans. Further progress in clinical CO awaits the completion and analysis of several ongoing clinical trials in organ transplantation, pulmonary fibrosis, and ARDS.

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Abbreviations

ALI	Acute lung injury
APCs	Antigen presenting cells
ARE	Antioxidant responsive element
Bach 1	BTB and CNC homologue 1
BAL	Bronchoalveolar lavage
BR	bilirubin-IXα
BV	biliverdin-IXα
BVR	Biliverdin reductase, CO, carbon monoxide
CO-Hb	Carboxyhemoglobin
COPD	Chronic obstructive pulmonary disease
CORM	CO releasing molecule
CPR	Cytochrome p-450 reductase, DCs, Dendritic cells
DMF	Dimethylfumarate
EGR-1	Early growth response-1
ERK1/2	Extracellular regulated protein kinase 1/2
FoxP3	Forkhead Box P3
HO	Heme oxygenase
HFD	High fat diet
HO-1	Heme oxygenase-1

HO-2	Heme oxygenase-2
HMGB1	high-mobility group box-1
HSF-1	Heat shock factor-1
IFN-β	Interferon-β
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I/R	Ischemia/reperfusion
JNK	c-Jun NH_2-terminal kinase
Keap1	Kelch-like ECH-associated protein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
miR	MicroRNA
MV	Mechanical ventilation
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NLRP3	NOD-, leucine rich region- and pyrin domain-containing-3
Nrf2	Nuclear factor erythroid 2-related factor-2
p38 MAPK	p38 Mitogen activated protein kinase
PI3K/Akt	Phosphatidylinositol-3-kinase/Akt
PhotoCORMs	Photoactivatable carbon monoxide releasing molecules
ROS	Reactive oxygen species
SCD	Sickle cell disease
SMC	smooth muscle cells (SMC)
SNP	small nucleotide polymorphism
SnPPIX	Tin Protoporphyrin-IX
StRE	Stress-responsive element
SPM	Specialized pro-resolving mediators
TLR4	Toll-like receptor-4
Treg	Regulatory T lymphocyte
VILI	Ventilator-induced lung injury

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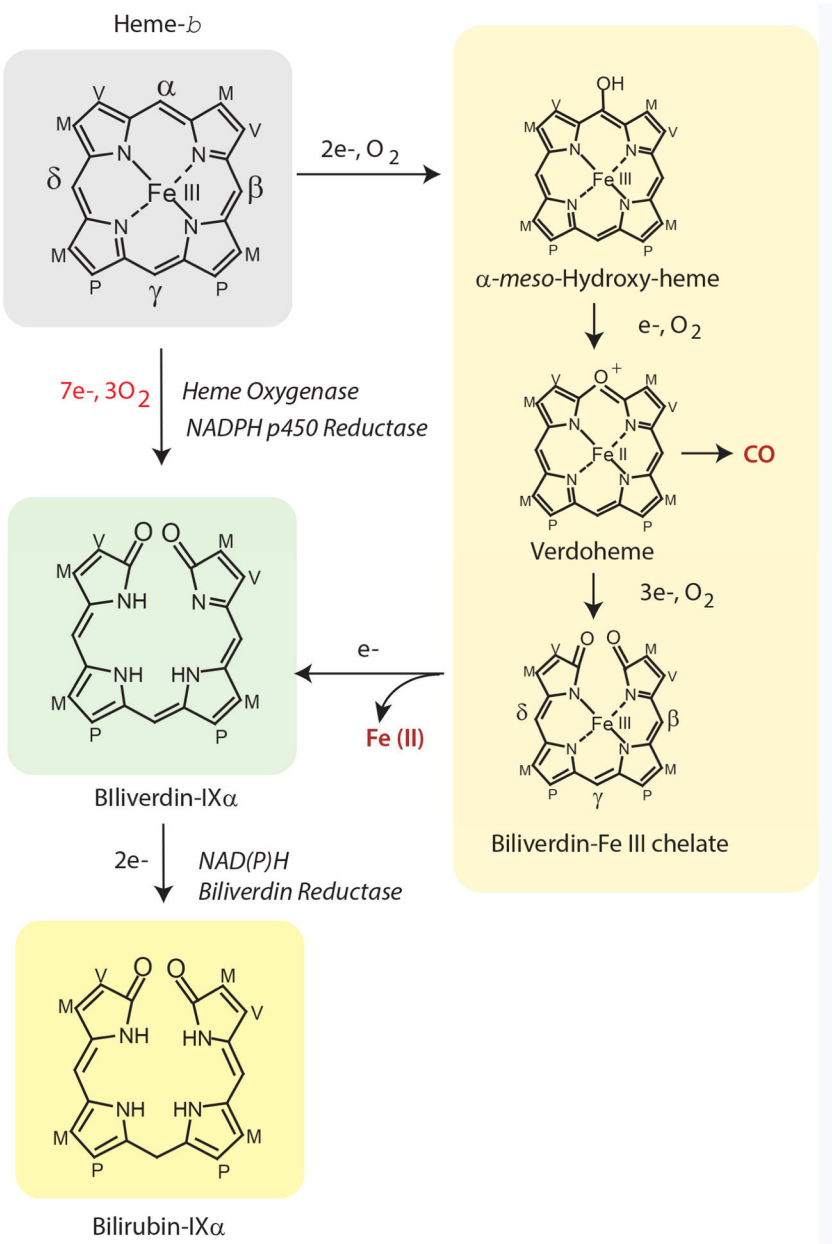

Figure 1.

The heme oxygenase (HO) reaction cleaves heme at the α-methene bridge carbon and generates carbon monoxide (CO), biliverdin-IXα. and ferrous iron (Fe II). The reaction proceeds through three sequential oxidation steps each requiring one mole of molecular oxygen (O_2), and a total of seven electrons from NADPH: cytochrome p450 reductase. Three reaction intermediates have been proposed: α-meso-hydroxyheme, verdoheme, and the Fe (III)-biliverdin complex. Upon univalent reduction, the Fe (III)-biliverdin complex dissociates to form biliverdin-IXα and free Fe (II). The completion of enzymatic heme degradation involves the divalent reduction of biliverdin-IXα by NAD(P)H: biliverdin

reductase (BVR; E.C. 1.3.1.24), which produces the lipid soluble pigment bilirubin-IXα. Heme side chains are designated: M=Methyl, V=Vinyl, P=Propionate.

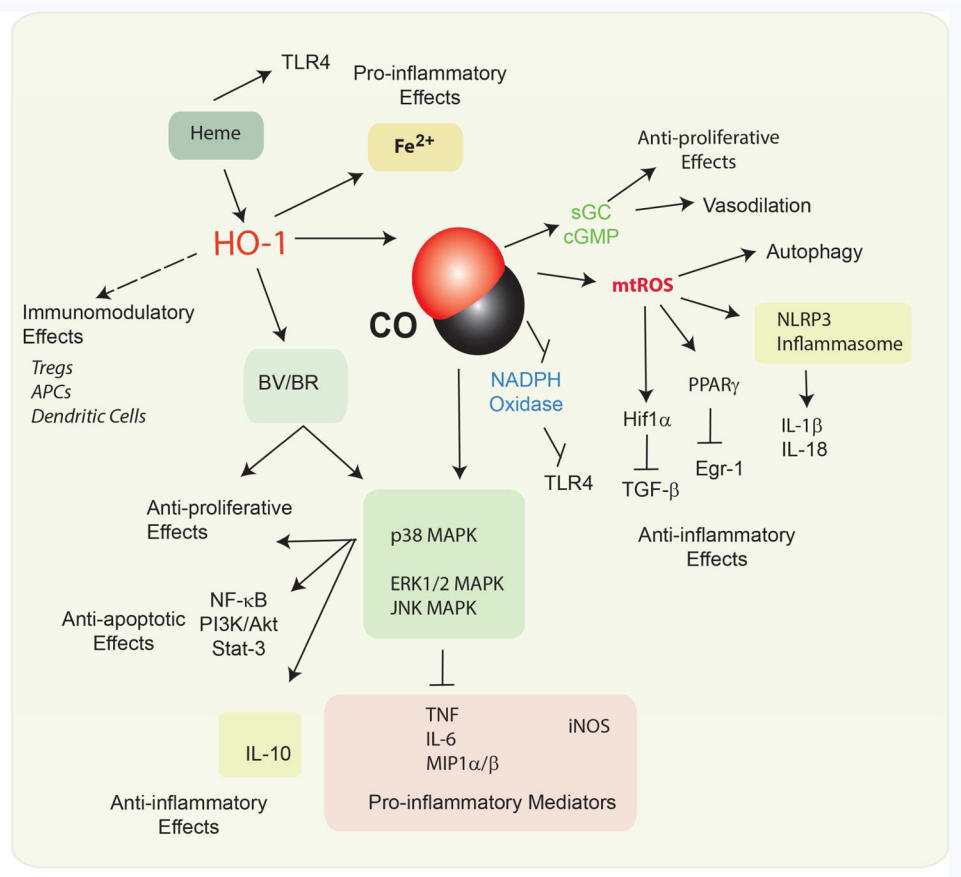

Figure 2.

Pivotal Functions of HO-1 in inflammation. HO-1 may have immunomodulatory effects with respect to regulating the functions of antigen presenting cells, dendritic cells, and regulatory T-cells. Heme may exert pro-inflammatory effects. HO-1 end products generated from heme degradation may modulate inflammation. Iron release from HO activity may be pro-inflammatory in the case of excess activation, and has been associated with neurodegenerative diseases. CO whether endogenously produced or applied as a pharmacological treatment, has been shown to modulate apoptotic, proliferative, and inflammatory cellular programs. In particular, CO can downregulate the production of pro-inflammatory cytokines (*e.g.,* IL-1β, IL-6, TNFα, Mip1α/β, and upregulate the anti-inflammatory cytokines (IL-10). These effects were attributed to alterations of MAPK activities including p38 MAPK. CO can stimulate mitochondrial ROS production, which can promote the autophagy program, activate HIF-1α, and downregulate pro-inflammatory transcription factor Egr1. Recent evidence also suggests that CO can modulate the activation of the NLRP3 inflammasome, which regulates the production of IL-1β, and IL-18. BR, a product of heme degaradtion, also may exert anti-inflammatory and anti-proliferative effects.

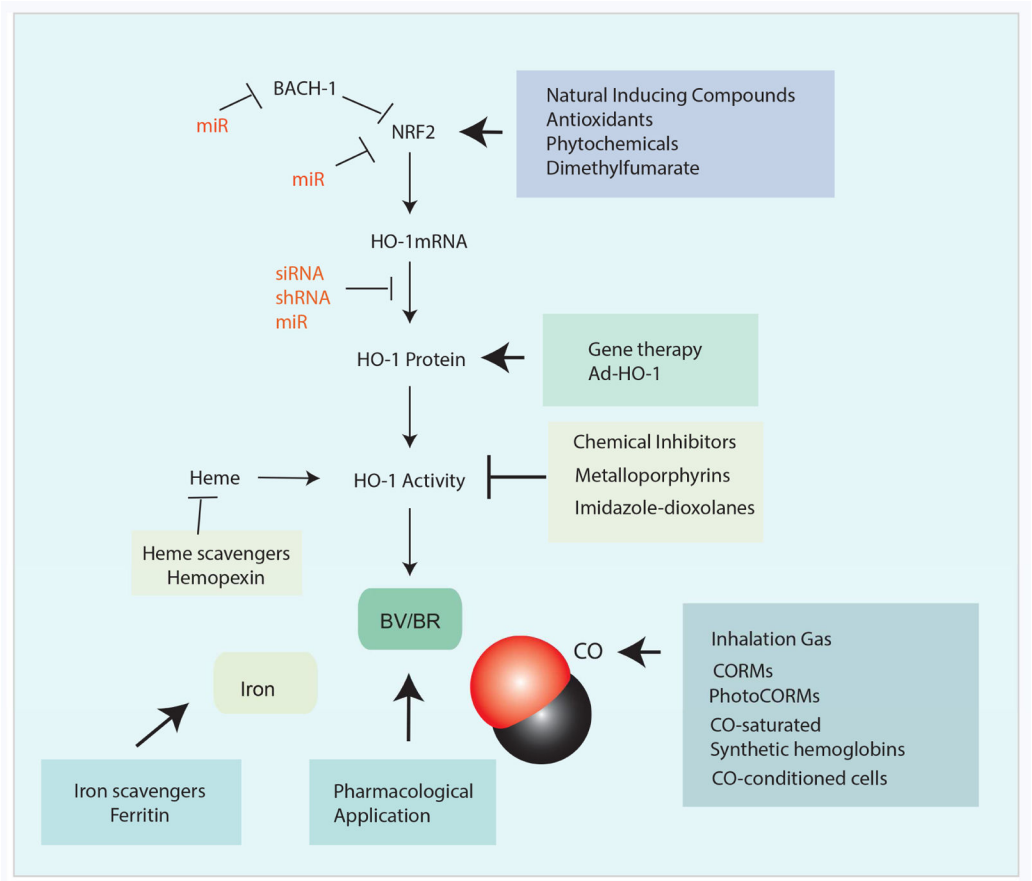

Figure 3.

Therapeutic modulation of the HO-1/CO system. HO-1 can be upregulated by natural antioxidants which act upon the NRF2/Keap1 system to upregulate the transcription of the Hmox1 gene. The application of antioxidant compounds may represent a possible strategy for therapeutic manipulation of HO-1. Gene therapy approaches using retroviral vectors may be used to upregulate HO-1. HO-1 is subject to natural up or downregulation through mIRs that target Hmox1 or other genes involved in its regulation (Bach1, Nrf2). The targeted expression of specific mIRs may also be combined with gene therapy approaches. Several compounds have been used therapeutically to inhibit HO activity. These include competitive inhibitors of the metaloporphyrin series, or non-competitive isoform selective derivatives of imidazole-dioxolanes. The end-products of HO activity, namely BV or BR, and CO may be used therapeutically as pharmological reagents. CO may be applied by inhalation, or through the use of CO releasing molecules (CORMs and PhotoCORMs). Additional therapeutic strategies involving CO include CO-saturated hemoglobins, or the use of CO preconditioned cells for cell based therapy. Furthermore, the removal of hemoglobin/heme or iron by scavenger compounds or chelation, respectively, have been proposed as intervention strategies.

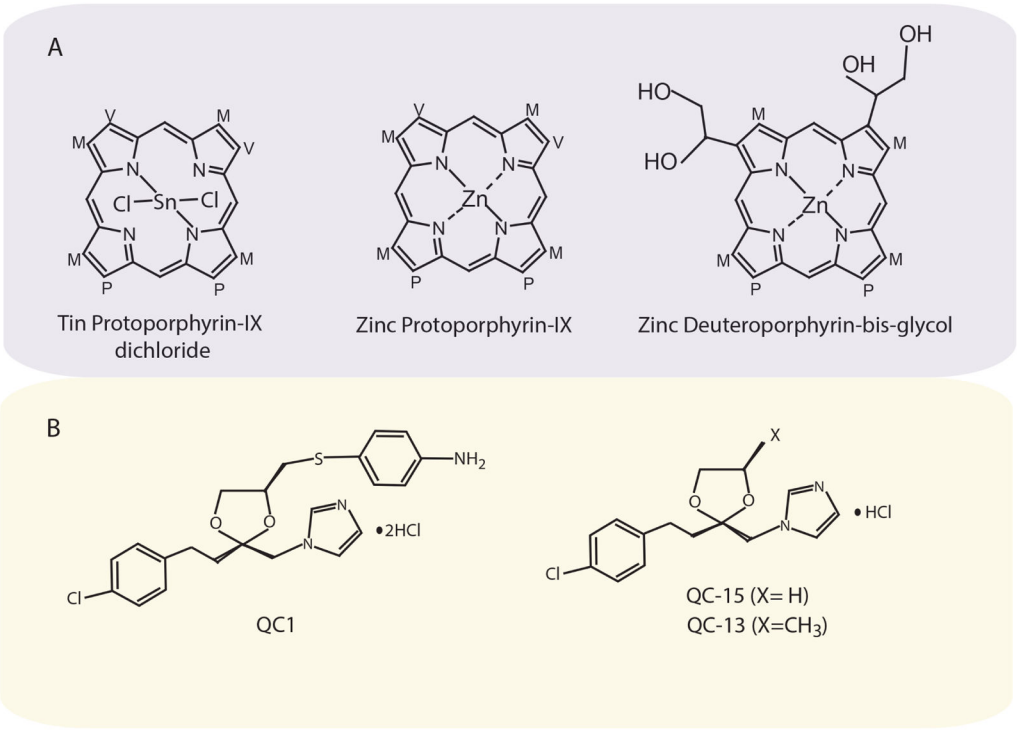

Figure 4.

Structures of HO inhibitor compounds. (*Top*) Representative metalloporphyrin compounds that act as competitive inhibitors of HO activity. Tin protoporphyrin IX (SnPPIX), Zinc Protoporphyrin (ZnPPIX), and Zinc Deuteroporphyrin Bis Glycol (ZnDPBG). (*Bottom*) Representative imidazole-dioxolanes that act as non-competitive inhibitors of HO activity. **QC-1:** (azalanstat) (2*S*,4*S*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-[((4-aminophenyl)thio)methyl]-1,3-dioxolane. **QC-13:** (2*R*,4*R*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-methyl-1,3-dioxolane hydrochloride **QC-15:** (2*R*,4*R*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride.[238–244]

Table 1

Representative Chemical and Physical Inducers of HO-1 in cell culture models.

Inducer Class	Chemical/Agent	Cell/Tissue Type	Refs
Metalloporphyrins	Heme Cobalt-Protoporphyrin	Mouse hepatoma cells	5
Oxidants	H_2O_2 UVA (320–380 nm) radiation Menadione	Human skin fibroblasts	6–7
Heavy Metals	$CdCl_2$	Mouse hepatoma cells	5
Thiol-Reactive Substances	Diethylmaleate	Chinese hamster ovary cells	8
	Sodium arsenite	Human skin fibroblasts	6
	Nitric oxide	Vascular smooth muscle cells	9
Pro-inflammatory Agents	LPS	RAW 264.7 Cells	10
Phytochemicals/Natural Antioxidants	Resveratol	PC-12 Cells	11
	Curcumin	Endothelial cells, Renal epithelial cells, Astrocytes	12–14
	Quercetin	Rat aortic smooth muscle cells, Human hepatocytes	15–17
	Epigallocatchin gallate	Human aortic endothelial cells, B-lymphoblasts	18–19
Physical Stress	Heat	Rat glioma, mouse melanoma	20
Oxygen tension	Hypoxia	Vascular smooth muscle cells	21
	Hyperoxia	Epithelial cells, fibroblasts, macrophages, and smooth muscle cells.	22

Table 2

Preclinical Studies Demonstrating the Importance of HO-1 in Disease

Strain	Model	Phenotype	Refs
Hmox1−/−	Vascular Injury	Increased intimal hyperplasia	29
	Pulmonary Ischemia/Reperfusion	Repression of fibrinolysis	30
	Hypoxia	Increased myocardial infarction, Right ventricular dilation	31
Hmox1TG (*Cardiac*)	Ischemia/Reperfusion	Protection from I/R injury	32
		Reduction of cardiomyocyte apoptosis	33
Hmox1TG (*Cardiac, Inducible*)	Cardiotoxicity (Cre-recombinase)	Protection from cardiotoxicity	34
Hmox1TG (Pulmonary)	Hypoxia-induced pulmonary hypertension	Reduced symptoms of hypertension	35
		Anti-inflammatory protection	36
Hmox1TG (Keratinocyte)	Skin lesions	Improved wound healing and vascularization	37

Table 3

Representative studies demonstrating tissue protective effects of CO in pre-clinical models of inflammatory disease

Model	Target Organ	Species	Phenotype	Ref
Endotoxemia	Systemic	Mice	Increased survival, Reduced pro-inflammatory cytokine production, increased IL-10	45
		Pigs	Reduced coagulation. Reduced pro-inflammatory cytokine production, increased IL-10	46
		Monkeys (*Macaques*)	Reduced TNF, Reduced Neutropenia	47
Hyperoxic Injury	Lung	Mice	Increased survival, Reduced pro-inflammatory cytokine production	48–49
Mechanical Ventilation	Lung	Rats, Mice	Reduction of BAL protein and cell count, lung neutrophil recruitment, and edema	50–52
Sepsis	Lung, Liver	Mice	Increased survival, increased bacterial clearance from organs and blood, induction of autophagy	53–54
		Baboons	Normalization of pro-resolving mediator profiles	55
Ischemia/Reperfusion	Lung Liver	Mice	Inhibition of apoptosis Reduced inflammation	56–57
Vascular Injury	Vascular Tissue	Mice, Rats	Reduced intimal hyperplasia Reduced inflammation	58
Sickle Cell Disease	Vascular Tissue	Mice	Reduced vascular stasis Reduced lung NF-κB activation	59–60
Organ Transplantation	Lung	Rat	Inhibition of apoptosis and inflammation, reduced macrophage infiltration	61–63
		Swine	Graft tolerance, Inhibition of inflammation	64
	Vascular	Rat	Reduced intimal hyperplasia Reduced inflammation	58, 65
	Heart	Mouse-to-Rat, Rat	Inhibition of platelet aggregation, thrombosis, apoptosis, infarction	66–70
	Kidney	Rat	Improved renal function, survival	67; 71–74
		Swine	Improved graft function, reduced I/R injury	75–76
	Liver	Rat	Reduced I/R injury	77–78
	Intestine	Rat	Reduced I/R injury	79–80
	Pancreas	Rats, Mice	Improved graft survival, Islet function	81–83