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Heme oxygenase/carbon monoxide signaling pathways: Regulation and functional significance

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Abstract

Carbon monoxide (CO), a gaseous second messenger, arises in biological systems during the oxidative catabolism of heme by the heme oxygenase (HO) enzymes. HO exists as constitutive (HO-2, HO-3) and inducible isoforms (HO-1), the latter which responds to regulation by multiple stress-stimuli. HO-1 confers protection *in vitro* and *in vivo* against oxidative cellular stress. Although the redox active compounds that are generated from HO activity (i.e. iron, biliverdin-IXα, and bilirubin-IXα) potentially modulate oxidative stress resistance, increasing evidence points to cytoprotective roles for CO. Though not reactive, CO regulates vascular processes such as vessel tone, smooth muscle proliferation, and platelet aggregation, and possibly functions as a neurotransmitter. The latter effects of CO depend on the activation of guanylate cyclase activity by direct binding to the heme moiety of the enzyme, stimulating the production of cyclic 3′:5′-guanosine monophosphate. CO potentially interacts with other intracellular hemoprotein targets, though little is known about the functional significance of such interactions. Recent progress indicates that CO exerts novel anti-inflammatory and anti-apoptotic effects dependent on the modulation of the p38 mitogen activated protein kinase (MAPK)-signaling pathway. By virtue of these effects, CO confers protection in oxidative lung injury models, and likely plays a role in HO-1 mediated tissue protection. (Mol Cell Biochem **234/235**: 249–263, 2002)

Key words: antioxidant, carbon monoxide, heme oxygenase, hypoxia, iron, oxidative stress, stress response

Introduction

Carbon monoxide (CO) arises in biological systems principally during heme degradation as the oxidation product of the α-methene bridge of heme, a process catalyzed by the heme oxygenase (HO) enzymes [EC 1:14.99.3, heme, hydrogen donor: oxygen oxidoreductase, (α-methene hydroxylating, decyclizing)] [1, 2]. The inducible form of HO, heme oxygenase-1 (HO-1), confers protection against oxidative stress conditions *in vitro* and *in vivo*, through anti-oxidative, anti-apoptotic and anti-inflammatory actions [3–10]. Although the underlying mechanisms in HO-dependent cytoprotection remain incompletely understood, recent evidence has strongly implicated contributory role(s) for endogenous CO generated from HO activity [6, 11]. Previously regarded as metabolic waste, CO affects vascular function by influencing the regulation of vessel tone, platelet aggregation, and smooth muscle proliferation [12–16]. Studies of HO-1 localization in the brain have implicated HO-derived CO as a neurotransmitter [17]. These potential effects of CO involve its complexation to the heme moiety of soluble guanylate cyclase (sGC), stimulating the production of guanosine 3′,5′-cyclic monophosphate (cGMP), a second messenger molecule [14–18]. As a consequence of heme binding, intracellular CO potentially influences the activity of other cellular hemoproteins such as cytochrome p-450, nitric oxide synthase (NOS), NADPH oxidase, and cytochrome-c oxidase, which are involved in vital processes including drug detoxification, inflammation, respiration, and possibly oxygen sensing [19–22].

Recent studies have discovered a potent anti-inflammatory effect of CO: the inhibition of pro-inflammatory cytokine production following inducing stimuli, dependent on

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the modulation of mitogen activated protein kinase (MAPK)-signaling cascades [6, 11]. The effects of CO on MAPK apparently occur independently of sGC activation and cGMP production; however the direct physical target of the CO, in this case, remains unknown.

In addition to CO, redox-active heme metabolites may also participate in cellular defense mechanisms (Fig. 1) [23–24]. HO exerts anti-oxidative functions by converting heme, whose intercellular accumulation may elevate intracellular pro-oxidant status [25], into the bile pigments, biliverdin-IXα, and bilirubin-IXα, which have potent antioxidant properties [26]. The reactive iron released from heme by HO activity may follow detoxification pathways involving either sequestration or extracellular efflux [27–29]. By inactivating iron regulatory protein (IRP) activity, iron stimulates the synthesis of the iron sequestration protein ferritin [30–31], promoting a secondary cellular desensitization to oxidative stress [10, 32].

This review will (I) describe the regulation of HO-1 as an inducible source of endogenous CO, (II) describe evidence that HO-1 acts as a mediator of cellular and tissue protection against oxidative stress, and (III) emphasize recent studies that introduce novel anti-apoptotic and anti-inflammatory properties of HO-derived CO in oxidative lung injury models.

Heme oxygenase isozymes: Properties and significance

Heme oxygenase activity generates equimolar CO, ferrous iron (Fe^{2+}), and biliverdin-IXα per mole of heme-b oxidized, in a reaction requiring NADPH: cytochrome p-450 reductase [EC 1.6.2.4] as electron donor [1–2, 33–34]. The reduction of biliverdin-IXα to bilirubin-IXα by NAD(P)H: biliverdin reductase [EC 1:3:1:24] completes heme degradation [1–2, 35]. In addition to HO-1, the inducible form, the HO system consists of two constitutively expressed isozymes (HO-2, and HO-3) which represent the products of distinct genes [36–39]. While the *ho-1* gene responds to induction by a broad spectrum of chemical and physical agents, the *ho-2* and *ho-3* genes do not respond to xenobiotic induction [37]. Thus, HO-1 protein occurs at undetectable levels in most tissues and cell types until a stress condition arises, whereas HO-2 may exist at detectable levels in most tissues in the absence of stress. HO-2 occurs abundantly in the central nervous system and vasculature [37, 40], and responds to regulation by adrenal glucocorticoids in the brain [41–42]. HO-1 and HO-2 differ in primary structure and molecular weight (32 and 36 kD respectively, for the rat isozymes), and in their K_m values (0.24, 0.67 μM, respectively) and reaction rates toward heme [37–38]. HO-2 contains two high affinity heme-binding sites termed heme regulatory domains (HRD) that are distinct from the catalytic heme-binding site. Accessory heme molecules bound to HO-2 HRD possibly act as a reservoir for small gas molecules, including NO and CO [43–44]. The significance of HO-3, a homolog of HO-2, remains unclear as it demonstrates poor heme catalytic activity [39]. HO enzymes perform a vital physiological function in the turnover of hemoglobin-heme during the metabolism of senescent erythrocytes in reticuloendothelial tissues, especially the spleen, liver and kidney [45]. HO regulates the intracellular concentration of heme, from the turnover of intracellular hemoproteins and cytochromes, and thus governs the redistribution of heme iron in tissues [45–46].

Regulation of *ho-1* by chemical and physical stress

In 1989 Keyse and Tyrrell, using hybrid-selection cloning techniques, identified the major 32-kDa mammalian stress-protein inducible by hydrogen peroxide, ultraviolet-A (UVA, 320–380 nm) radiation, and sodium *m*-arsenite ($NaAsO_2$), as identical to the rate limiting enzyme in heme degradation, HO-1 [25, 47]. In addition to oxidants, the induction of the *ho-1* gene also follows cellular exposure to agents such as heme [48], pro-inflammatory cytokines [49–53], bacterial endotoxins [49, 51, 54–58], growth factors [59–60], nitric oxide [61–66] and tumor promoters [67–70]. These agents share the ability to directly or indirectly generate intracellular reactive oxygen species (ROS) and/or modulate intracellular redox equilibrium. HO-1 elevation appears as a general indicator of oxidative stress in cells and tissues [25, 71].

Regulation of HO-1 by oxidative stress exemplified by UVA radiation and H_2O_2: The role of intracellular glutathione and iron status

UVA radiation imposes an oxidative stress in cultured cells by exciting intracellular chromophores to produce ROS [72]. Exposure to either UVA radiation or the oxidant H_2O_2 increased the transcriptional rate of the *ho-1* gene, and the steady-state levels of HO-1 mRNA or protein in human skin fibroblasts [25, 47, 73]. The response to UVA-treatment involved singlet molecular oxygen ($^1O_2$), since it could be enhanced in deuterium oxide (D_2O), which prolongs $^1O_2$ lifetime relative to aqueous media, or inhibited by semi-specific $^1O_2$ reactive agents [74]. The expression of HO-1 mRNA and protein also increase following cellular exposure to photosensitizers that produce $^1O_2$ and other ROS upon light-activation [74–76].

The induction of HO-1 mRNA by ROS generating systems may be enhanced by the depletion of intracellular reduced glutathione GSH, using the drug D,L-buthionine-(*S,R)*-sulfoximine (BSO) which inhibits γ-glutamyl-cysteinyl-synthetase (γ-GCS), the rate limiting step in GSH biosynthesis [77–78].

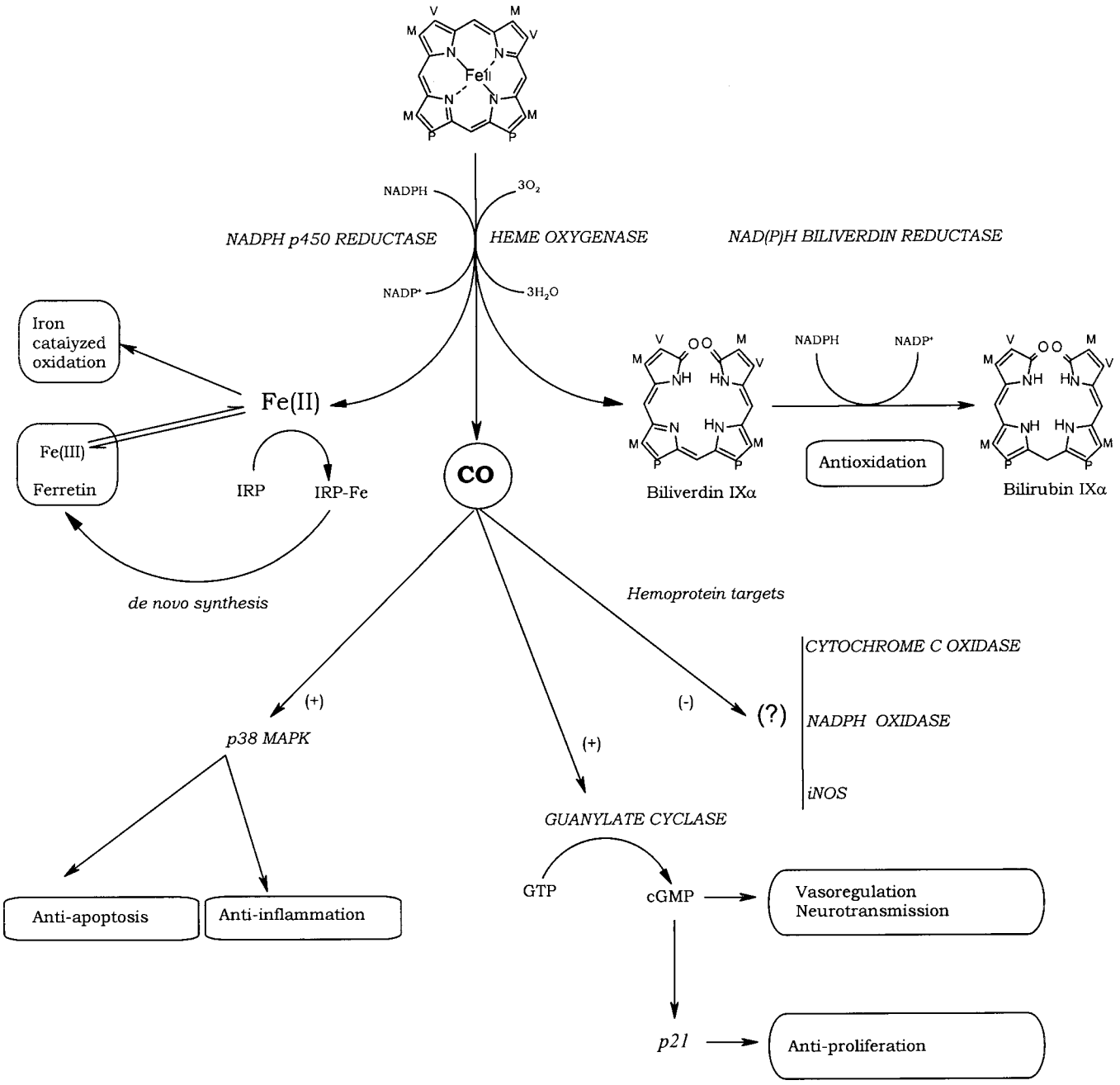

Fig. 1. Functional consequences of HO activity. Heme oxygenase degrades heme to biliverdin-IXα, carbon monoxide, and iron. Biliverdin-IXα is converted to bilirubin-IXα by NAD(P)H biliverdin reductase. Both bile pigments have potent *in vitro* antioxidant activity. Redox-active iron released from HO activity may promote oxidative damage. However, by inactivating iron regulatory protein (IRP) activity, iron stimulates the synthesis of ferritin, an iron-sequestration protein and possible cytoprotectant. CO derived from the HO reaction has possible significance in the regulation of vascular and neural functions. The stimulation of cGMP-dependent signal transduction pathways may account for the vasodilatory and anti-proliferative effects. CO has potent anti-inflammatory effects, which depend on downregulation of pro-inflammatory cytokine production mediated by modulation of p38 MAPK. The abbreviations used in this figure include: cGMP = guanosine 3′,5′-cyclic monophosphate; CO = carbon monoxide; Fe(II) = ferrous iron; Fe (III) = ferric iron; GTP = guanosine triphosphate; IRP = iron regulatory protein; NOS = nitric oxide synthase; p38 MAPK = p38 mitogen activated protein kinase.

BSO treatment sensitizes human fibroblasts to the cytotoxic effects of UVA and ultraviolet B (UVB, 290–320 nm) radiation, and H_2O_2 treatment [79–80]. BSO treatment alone had moderate to little effect on HO-1 mRNA or protein accumulation in human skin fibroblasts or rodent cell lines, respectively [77, 81–82]. However, BSO treatment in combination with H_2O_2 or UVA, amplified the induction response and lowered the UVA fluence necessary to induce HO-1 mRNA levels in human skin fibroblasts [77]. Supplementation with the GSH precursor *N*-acetyl-L-cysteine (NAC) inhibited HO-1 induction in many systems [53, 57–59, 61–62, 83–87]. The induction of HO-1 by oxidants could be inhibited by iron chelators such as desferrioxamine (DFO) or *o*-phenanthroline, suggesting either a pro-oxidative or regulatory role for intracellular chelatable iron [88]. Iron chelators also attenuate HO-1 induction under hyperoxia [89], or hypoxia [86, 90]. Iron may aggravate ROS production and sensitize cells under oxidative stress conditions by acting as a catalyst in membrane lipid peroxidation and Fenton-type reactions [91–92]. Iron may also have more direct roles in the transcriptional and post-transcriptional regulation of gene expression. For example, iron chelation upregulates the DNA binding activity of the hypoxia-inducible factor (HIF1) [93], whereas iron complexation controls the activity of the iron regulatory protein-1, a translational regulator of proteins involved in iron metabolism [31, 94].

Induction of HO-1 by thiol reactive substances: Role of GSH complexation

HO-1 activation responds to numerous thiol (–SH)-reactive compounds that form complexes with intracellular reduced glutathione (GSH), including sodium *m*-arsenite ($NaAsO_2$), diethylmaleate (DEM), and heavy metal salts [25, 81, 95]. $NaAsO_2$ reacts with free –SH groups of GSH, and protein, exerting lethal effects by inactivating –SH dependent enzyme functions. *In vivo*, $NaAsO_2$ by injection increases rat hepatic and renal HO activity [96]. $NaAsO_2$ and related arsenicals increase HO-1 (32-34-kDa) protein synthesis and mRNA steady state levels as a general response in many cell types [69, 71, 82, 87, 97–101].

Other thiol reactive substances that induce HO-1 include chemicals which conjugate GSH in glutathione *S*-transferase (GST) catalyzed reactions (i.e. diethylmaleate, DEM) to form mixed disulfides (GSSR), many which undergo prior biotransformation to electrophilic intermediates by cytochrome p450/p448 enzymes (i.e. halogenated hydrocarbons) [37]. The complexation and subsequent depletion of GSH by DEM to a degree exceeding 80% induced HO-1 in various cell types [81–82, 102–103]. Sulphydril oxidants such as diamide, which promote the formation of GSSG are typically ineffective at inducing HO-1 in cell culture [81–82]. While GSSG may be regenerated to GSH by NADPH:glutathione reductase, GSSR species may not undergo enzymatic reduction, but are detoxified as *N*-acetyl-cysteine (mercapturic acid) derivatives. The –SH reactive substance *N*-ethylmaleimide has little effect on HO-1 induction, due to its preferential reactivity for protein –SH groups rather than GSH [103].

Metal salts (i.e. $CdCl_2$, $CoCl_2$, $NiCl_2$, $SiCl_2$, $HgCl_2$ etc.) potently activate HO-1 *in vivo* [37, 95, 104], as well as in many cell types [25, 48, 68, 81–82, 97, 99–101, 105]. Heavy metals form complexes with thiol groups including cysteine and GSH. When injected into rats, heavy metals depress hepatic GSH levels, which in turn rebound to elevated levels in a compensatory response. Metal-dependent induction of hepatic HO activity may be inhibited by the prior complexation of the metals with thiol compounds, and potentiated by GSH depletion [95]. Transgenic mice lacking the metallothionein –I and –II genes, which code for low molecular weight thiol-rich proteins involved in metal detoxification, display more pronounced hepatic and renal HO-1 mRNA and protein expression following $CdCl_2$ injection, than wild-type mice [107]. The induction of HO-1 expression by metals is regulated at the transcriptional level, demonstrated *in vitro* and *in vivo* using nuclear run-on analysis [48, 106, 108–109]. Certain metals (i.e. Fe^{2+}, Co^{2+}, Cu^{2+}) undergo ferrochelatase-dependent incorporation into protoporphyrin IX (PPIX) to form metalloporphyrins [104, 109]. Non-heme synthetic metalloporphyrins (i.e. SnPPIX, ZnPPIX) paradoxically inhibit HO enzyme activity but stimulate HO-1 transcription [104, 106, 110–111].

Regulation of HO-1 expression by nitric oxide

The free radical gas nitric oxide (NO) mediates a number of physiological functions, including vasoregulation, neurotransmission, and inflammation. NO serves as a cytotoxic effector species of the macrophage respiratory burst. At high concentrations, NO may exert a 'nitrosative' cellular stress, reacting with thiols (including GSH) to form *S*-nitrosothiols, and with O_2^-, to form the pro-oxidant peroxynitrite ($ONOO^-$) [112]. Exogenous NO gas administered to human embryonic lung fibroblasts potently induced HO-1 protein and mRNA levels in a concentration and time-dependent manner [66]. NO donor compounds such as sodium nitroprusside (SNP), *S*-nitroso-N-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), and spermine NONOate (SNN) dose and time dependently increased HO expression in various cell culture systems [61–65, 113–114]. The activation of HO-1 by NO donors or NO gas is independent of cGMP production, since cGMP analogues had no effect and involves transcriptional regulation of the *ho-1* gene [62–63, 65–66]. In human fibroblasts, however, NO donors or NO gas stabilized HO-1 mRNA in a NO concentration-dependent fashion [66, 115]. Furthermore, NO donation by SNAP increased detectible non-heme iron levels

in PAEC and stimulated the synthesis of ferritin in a HO-activity dependent manner [64]. The NO metabolite peroxynitrite ($ONOO^-$) induced HO-1 in endothelial cells, which could be inhibited by the antioxidants NAC or uric acid [116].

Regulation of HO-1 by pro-inflammatory states

HO-1 elevation may occur as a consequence of inflammation, infection, sepsis and other pathophysiological conditions associated with increased ROS production and may play a protective role in these contexts [51, 58, 117–118]. HO-1 elevation appears as a component of the hepatic acute phase response in humans, and rodents. The lipopolysaccharide (LPS) component of bacterial endotoxin induces HO activity in rat peritoneal macrophages, and in hepatic parenchyma and sinusoidal cells following intraperitoneal injection [56]. In mice, injection of LPS, or the pro-inflammatory cytokines interleukin-1 (IL-1), tumor necrosis factor-α, (TNFα), and interleukin-6 (IL-6) induced hepatic HO-1 mRNA, with the response to IL-1 verified as a transcriptional regulation [51]. The induction of hepatic HO-1 mRNA levels by LPS could be enhanced by GSH depletion and diminished by NAC, suggesting an influence of cellular redox status in the induction mechanism [58]. Likewise, HO-1 expression responded *in vitro* to cellular stimulation with LPS [54], or pro-inflammatory cytokines (IL-1, IL-6, TNFα) [51–53, 119]. In HUVEC, the TNFα mediated induction of HO-1 required protein kinase-C and phospholipase A2, and responded to inhibition by NAC, and intracellular calcium chelation [53]. Interestingly, HO-1 induction (in HUVEC) also responded to treatment with the thrombopoietic cytokine interleukin-11 [119]. Growth factors that mimic cytokine responses with respect to HO-1 induction include transforming growth factor-β (TGF-β), which induced HO-1 protein in human retinal pigment epithelial cells [60], and platelet derived growth factor (PDGF), which stimulated HO-1 mRNA in VSMC [59]. Like the cytokine-mediated responses, the growth factor responses occurred in association with increased intracellular ROS production, and responded to inhibition by NAC treatment.

Regulation of HO-1 by oxygen tension

HO-1 expression responds to fluctuations in the 'normal' or acclimated oxygen (O_2) tension of the system [120–122, 86]. Hypoxia, or lowered pO_2, may occur in the cardiovascular system as a consequence of restricted oxygen intake, ischemia, or disease states such as atherosclerosis. Acute hypoxia dilates the systemic vasculature, whereas chronic hypoxia may constrict the pulmonary vasculature, leading to pulmonary hypertension [123–124].

The exposure of mammalian cells to hypoxia *in vitro* triggers cell type-specific alterations in protein expression patterns [125–128]. Following the original observation by Murphy *et al*. that described HO-1 as the major hypoxia-inducible protein in CHO cells [122], the response has also been demonstrated in vascular systems. For example, acute hypoxia induced HO-1 mRNA accumulation in rat organs, including lung, liver, heart, and aorta [121]. Chronic hypoxia induced HO-1 mRNA in both ventricles of the rat heart [129].

In bovine aortic endothelial cells (BAEC), hypoxia treatment induces HO-1 protein levels and HO enzymatic activity, which persisted during subsequent reoxygenation [86]. This response could be abolished by inclusion of iron chelators or NAC in the hypoxic phase, and conversely increased by prior iron loading [86]. Inhibitors of iNOS or NO scavengers, inhibited the induction of HO activity by hypoxia, while treatment with *S*-nitrosoglutathione augmented the response [130]. These reports, taken together, suggest a critical role for iron and intracellular redox equilibrium in the hypoxic activation of HO-1 gene expression.

Hypoxia induced *ho-1* transcription and HO-1 mRNA accumulation in rat aortic vascular smooth muscle cells (VSMC) [14, 121], and pulmonary artery endothelial cells (PAEC) [131]. In PAEC the response occurred in association with increased AP-1 DNA binding activity, whereas in VSMC, involved activation of HIF-1 DNA binding activity [121,131]. In contrast to wild-type cells, mutant Hepa cell lines deficient in HIF-1β did not exhibit HO-1 mRNA accumulation in response to hypoxia [121]. Interestingly, hypoxic activation of the *ho-1* gene in CHO cells occurred independently of HIF-1 as demonstrated in mutant CHO cells deficient in HIF-1α [132]. Taken together, these results suggest that while HIF-1 mediates the hypoxic induction of HO-1 in some cell types (i.e. VSMC), it may not be the sole factor involved.

Hyperoxia, or high O_2 tension, used clinically for critical care applications, also activates a stress response *in vitro* and *in vivo*. Hyperoxia causes oxidative injury to the lung, associated with increased production of mitochondrial ROS [133]. Hyperoxia (>95% O_2) increased HO-1 mRNA, protein, and enzymatic activity in the adult rat lung [120], and increased HO activity in neonatal rat lung [134]. Hyperoxia activated *ho-1* transcription *in vitro* in cultured cells of lung origin (epithelial cells, fibroblasts, macrophages, and smooth muscle cells) [120]. In human cell lines the activation of HO-1 by hyperoxia could be augmented by iron loading and diminished in the presence of iron-chelators [89, 135]. Thus, iron appears to represent a general requirement for the activation of *ho-1* gene expression under either high or low O_2 tension.

Regulation of HO-1 expression by heat shock

The rat HO-1 protein classifies as a heat shock protein (HSP-32) since it responds to transcriptional regulation by heat

(42°C); and the 5′ regulatory region of its gene contains heat shock elements (HSEs) resembling those described in the promoter regions of heat shock genes (i.e. HSP70) [136–138] HO-1 mRNA and protein accumulate to a high degree after whole-body hyperthermia (42°C) in rat organs, including the liver, heart, and kidney, and brain [138–139, 140–141]. Heat shock (42°C) increases the transcriptional rate of HO-1 mRNA in cultured rat glioma cells [136].

The rat *ho-1* gene contains two HSEs, HSE1 (–290/–276) and HSE2 (–222/–212) which contain inverted repeats of the core element 5′NGAAN3′ [142]. The rat, mouse, and human *ho-1* genes differ in the number, position, and configuration of HSEs in their 5′ regulatory regions [142–144]. Both the human and rat HSEs formed complexes with heat inducible nuclear proteins, and conferred heat responsiveness to reporter gene constructs in respective transient transfection assays [136, 145]. Human cell lines, however, generally failed to induce HO-1 in response to heat [101, 145–146].

Signal transduction and transcriptional regulation of ho-1

The signal transduction pathways that operate *ho-1* gene activation under the multiplicity of inducing conditions remain only partially understood. Existing studies often report contradictory data, or cell type-specific and inducer-dependent variations, which are based on known specificities of chemical inhibitors. Mitogen activated protein kinase (MAPK) pathways, including extracellular regulated kinases (ERK) [113, 147] and/or p38 MAPK [113, 147–148], participate in the activation of *ho-1* by inducing xenobiotics. For example, the $CdCl_2$ induction of *ho-1* transcription in murine MCF-7 cells, could be abolished by the p38 MAPK inhibitor (SB203580) and by dominant negative mutants of p38α, but not by an ERK kinase (MEK1) inhibitor (PD98059) [148]. Similar MAPK inhibitor studies have demonstrated the requirement for both ERK and p38 MAPK pathways in the $NaAsO_2$-dependent transcriptional activation of the chicken *ho-1* promoter [147]. In this system, the overexpression of dominant negative forms of Ras, MEK1, and p38 MAPK inhibited transcriptional activation of *ho-1* in response to $NaAsO_2$ treatment [147]. Both p38 MAPK and ERK pathways participated in *ho-1* activation in HeLA cells following exposure to NO donors [113]. In contrast, *ho-1* activation by $NaAsO_2$, heme, or $CdCl_2$ in HeLA cells required tyrosine kinase activity but not ERK or p38 MAPK pathways [149].

The regulation of *ho-1* under hypoxia required p38 MAPK, but not ERK or tyrosine kinase dependent pathways in cardiomyocytes [150]. To the contrary, the p38 MAPK inhibitor SB203580 activated HO-1 mRNA expression under hypoxia in rat PAEC, whereas a MEK1/2 inhibitor (UO126) strongly activated HO-1 under normoxic conditions in the absense of stimuli; indicating that MAPK inhibitors alone may activate *ho-1* transcription under certain conditions [151]. The over-expression of MAPK kinase kinases (MEKK1, TAK1, and ASK1) induced *ho-1* in HEPG2 cells [152].

The murine *ho-1* gene 5′ flanking sequence contains two transcriptional enhancer sequences located at –4kb (E1; formerly SX2) and –10 kb (E2; formerly AB1) of the transcriptional start site [144, 153–155]. These elements maintain basal promoter activity and mediate the induction of *ho-1* by many xenobiotics, including $CdCl_2$, 12-*O*-tetradecanoylphorbol-13-acetate, endotoxin, heme, and H_2O_2 [144, 153–156]. Both E1 and E2 consist of repeated essential *cis*-acting DNA motifs designated as stress responsive elements (StRE) with the consensus sequence (T/CGCTGAGTCA). Intrinsic to the StRE appears several overlapping consensus sequences for transcription factor binding sites: AP-1, v-maf oncoprotein, and the Cap'n'collar/basic-leucine zipper family of proteins (CNC-bZIP). The latter sequence resembles the antioxidant responsive element (GCNNNGTCA) [157].

The StRE elements of E1 are critical for the *ho-1* transcriptional response to $CdCl_2$ [158]. Transfection studies in L929 cells with candidate transcription factors demonstrated that only members of the CNC/bZIP family of proteins effectively activate an E1 reporter construct, with nuclear regulatory factor-2 (Nrf2) displaying the strongest activity. The over-expression of the dominant negative mutant form of Nrf2 inhibited E1 enhancer activity (and endogenous *ho-1* induction) in response to $CdCl_2$ and other inducing agents in L929 and MCF-7 cells [148, 157]. Transcription factor ATF4 has recently been identified as the possible binding partner of Nrf2 in regulating *ho-1* transcription, by yeast two-hybrid analysis [159].

The hyperoxia-mediated induction of *ho-1* in RAW 264.7 cells requires E1 and the participation of E2 enhancer regions. The response is mediated by the intrinsic AP-1 elements acting in cooperation with STAT (signal transducer and activator of transcription) elements located within the proximal promoter region [160]. In contrast, the hypoxic activation of *ho-1* in VSMC requires a sequence at –9 kb (hypoxia responsive element) distinct from E1, that contains two functional binding sites for HIF-1 [121].

Heme oxygenase confers protection against oxidative stress *in vitro* and *in vivo*

An increasing body of evidence supports the general hypothesis that HO-1 acts as an inducible mediator of cellular and systemic defenses against oxidative stress, in models of inflammation, ischemia-reperfusion, hypoxia, and hyperoxia-mediated injury. For example, induction of endogenous HO-1 protein with hemoglobin infusion increased survival in a rat model of LPS-induced inflammatory lung injury [161]. Pre-

induction of HO-1 with either LPS or hemoglobin infusion conferred protection in a rat model of renal injury (glycerol-induced rhabdomyolysis) [162–164].

Homozygous *ho-1* null mice (*ho-1*$^{-/-}$) displayed increased mortality in a model of lung ischemia-reperfusion (I/R). Inhalation CO (0.2%) compensated entirely for the *ho-1* deficiency in *ho-1*$^{-/-}$ mice, and restored survival following I/R to that of the wild-type mice [165]. The proposed mechanism involved the CO/ cGMP-dependent inhibition of plasminogen activator inhibitor-1 (PAI-1) leading to enhanced fibrinolysis [165]. Adenoviral mediated overexpression of HO-1 (AdHO-1) in pigs inhibited vascular cell proliferation and lesion formation in a model of arterial injury. Conversely, HO-1$^{-/-}$ mice subjected to arterial injury displayed increased vascular cell proliferation, and developed hyperplastic lesions in comparison to HO-1$^{+/+}$ controls [166].

Chronic hypoxia treatment (10% O_2) increased right ventricular dilation and caused right myocardial infarction in *ho-1*$^{-/-}$ mice relative to wild-type mice that withstood the treatment [167]. In this model wild-type or *ho-1*$^{-/-}$ mice did not differ in their development of pulmonary hypertension following chronic hypoxia [167]. The induction of HO-1 protein by chemical inducers (i.e. $NiCl_2$ or hemin) however, prevented the development of pulmonary hypertension in the rat lung as a consequence of chronic hypoxia treatment [168]. Transgenic mice with a lung-specific HO-1 overexpression phenotype, resisted the inflammatory and hypertensive effects of hypoxia [169].

Both HO-1 and HO-2 potentially contribute to pulmonary defenses against high O_2 levels. The adenoviral mediated gene transfer of HO-1 into rat lungs protected against the development of lung apoptosis and inflammation during hyperoxia [5]. Heme oxygenase-2 null mice (*ho-2*$^{-/-}$), displayed increased sensitivity to the lethal effects of hyperoxia relative to wild-type mice, despite compensatory increases in HO-1, and accumulated iron in their lungs [170]. On the other hand *ho-1*$^{-/-}$ mice had low serum iron anemia, yet accumulated non-heme iron in the kidney and liver, suggesting that iron recycling by HO-1 is critical in maintaining blood iron levels [46]. The mechanism by which HO-1 deficiency resulted in accumulation of tissue iron is unclear. These studies have indicated that animals deficient in either HO-1 and HO-2 display enhanced sensitivity to oxidative stress conditions, and aberrations in the distribution of intra- and extracellular iron [8, 46, 170].

HO-1 also confers protection in animal models of arteriosclerosis, where it may be found in atherosclerotic lesions [171]. The adenoviral-mediated transduction of HO-1 into ApoE deficient mice inhibited the formation of arteriosclerotic plaques relative to control virus transduced mice [172]. Induction of endogenous HO-1 by chemical treatment (hemin) reduced the formation of atherosclerotic lesions in LDL-receptor knockout mice fed high fat diets, relative to untreated or SNPPIX treated controls [173].

Evidence from *in vitro* studies also supports protective roles of HO-1. For example, the overexpression of HO-1 in endothelial cells conferred protection against heme and hemoglobin-mediated toxicity [3]. Cultured cerebral granular neurons overexpressing HO-1 displayed resistance to glutamate toxicity relative to wild-type cells [174]. Embryo fibroblasts with the *ho-1*$^{-/-}$ genotype displayed hypersensitivity to heme and H_2O_2 treatment and generated increased intracellular ROS production in response to these agents [8]. Overexpression of HO-1 in lung epithelial cells or rat fetal lung cells conferred resistance against the cytotoxic effects of hyperoxia, associated with growth arrest [4, 9]. The conditional overexpression of HO-1 in cultured L929 fibroblasts inhibited TNFα–induced apoptosis, a phenomenon that could be blocked by inhibitors of HO activity (SnPPIX), and mimicked by exogenous CO (250 ppm) [7]. Finally, the administration of HO-1 antisense oligonucleotides inhibited the cytoprotective effect of UVA-preconditioning against subsequent lethal UVA exposures in human skin fibroblasts [10].

On the other hand, not all model systems support a protective role for HO-1. Pro-oxidant effects of HO activity have been reported in over-expression systems, related to transient iron overload [24, 175–176]. For example, the susceptibility of HeLa cells to UVA radiation was increased in HO-2 overexpressing strains, when the UVA was applied in combination with a substrate load (heme), in a fashion dependent on heme iron release [175].

Functional significance of carbon monoxide released from the HO reaction

Carbon monoxide

Carbon monoxide is a low molecular weight diatomic gas that occurs ubiquitously in nature as an air pollutant. Environmental CO arises from the oxidation or combustion of organic matter (i.e. wood, coal, gasoline, natural gas, tobacco). Ambient CO concentrations in the lower atmosphere occur in the range of 0.4–1.0 μl/L or <1 ppm; which may reach 1–20 ppm in urban areas, and still higher in heavily polluted areas [177–178]. CO is a major component of cigarette smoke, reaching yields of up to 20 mg per cigarette [179]. In man, endogenous CO arises principally from heme degradation (>86%). The remainder arises from other sources that may include lipid peroxidation, and xenobiotic metabolism [177].

Physiological roles for CO involving cGMP-dependent signaling

The field of small gas signal transduction was born with the realization that an endothelial derived relaxing factor responsible for the paracrine regulation of vascular smooth muscle tone, was identical to the diatomic free radical gas NO. The

nitric oxide synthase (NOS) enzymes generate NO during the conversion of L-arginine to L-citrulline. The effects of NO on vasodilation involve the activation of soluble guanylate cyclase (sGC), increasing the production of guanosine 3′,5′-cyclic monophosphate (cGMP) [180]. This paradigm led to the proposal that CO, a small gas of similar structure, released directly from heme during HO activity, may function as a soluble messenger molecule in a similar fashion [12, 14–18, 40, 181]. Unlike NO however, CO is not a radical, and therefore is relatively inert by comparison. Both CO and NO stimulate sGC activity *in vitro* by binding to the ferrous heme moiety of the enzyme [12, 182–183]. While NO forms a pentacoordinate complex with the heme of sGC, CO may initially form a hexacoordinate complex [12, 182]. CO has a relatively lower affinity for the heme-iron of sGC than NO, and is one-thousandfold less potent than NO with respect to vasodilation and the *in vitro* activation of sGC [12, 183]. CO signaling may become relevant under oxidative stress or pathophysiological conditions where HO-1 is dramatically induced, and/or where the bioavailability of NO is reduced. Little is known about how CO is mobilized for signaling, apart from two intuitive mechanisms (I) the availability of substrate heme for enzymatic degradation, and (II) the availability of active HO enzymes, a process which in turn may be regulated by the transcriptional activation of the *ho-1* gene by stress, and the possible modulation of *ho-2* by glucocorticoids [18]. Transient fluxes in the free heme pool have been reported following oxidative stress conditions such as UVA (320–380 nm) radiation treatment [184]. Paradoxically, CO may inhibit HO activity in reconstituted microsomal systems, implying that the production of CO may be limited by negative feedback regulation [185].

Physiological roles for CO, which directly involve modulation of cGMP levels, include neurotransmission, vasodilation, the inhibition of platelet aggregation, and anti-proliferative effects on smooth muscle [12, 14–18, 40, 181]. In brain slices, *in situ* hybridization studies demonstrated that the distribution of HO-2 matches that of NADPH cytochrome P-450 reductase and guanylate cyclase [17]. The induction of guanylate cyclase in cultured olfactory neurons by olfactory stimulants can be inhibited by metalloporphyrin inhibitors of HO such as ZnPPIX, but not inhibitors of NOS [17].

Recent studies point to the involvement of CO in cardiovascular signaling. In the rat, both whole body hyperthermia (42°C), or renal I/R triggered the elevation of cGMP levels in the heart in parallel with the transcriptional induction of HO-1 [141, 186]. In VSMC, an elevation of cGMP occurred following exogenous CO treatment [14]. cGMP increased also following hypoxia in association with HO-1 elevation, an effect that could be inhibited by SnPPIX, and the CO scavenger hemoglobin, but not inhibitors of NOS [14]. VSMC derived CO had paracrine effects on endothelial cells in co-culture, stimulating the production of endothelial cGMP, and suppressing the expression of endothelial-derived mitogens (PDGF, endothelin 1) [15]. Both exogenously applied CO, or hypoxia induced CO had antiproliferative effects on VSMC, associated with elevation of cGMP, and inhibition of transcription factor E2F, a regulator of cell cycle control [16]. AdHO-1 infection in VSMC stimulated cGMP production, and inhibited cell proliferation *in vitro* by G_1/G_o arrest, which required the G1 cyclin dependent protein kinase inhibitor $p21^{cip1}$ [166].

The involvement of endothelial derived CO in NO-independent vasodilation has been suggested in inhibitor studies. In the presence of the NOS inhibitor Nω-nitro-L-arginine-methylester, (L-NAME), the HO inhibitor SnPPIX further inhibits vasorelaxation elicited by acetylcholine in porcine aortic rings [40]. Conversely, the endothelium-dependent contractile response to phenylephrine in thoracic aortic rings was more pronounced in the presence of both ZnPPIX and Nω-nitro-L-arginine (NNA); than in the presence of NNA alone [187]. In this system, exogenously applied CO relaxed the aortic rings in a cGMP-dependent fashion. Overexpression of HO-1 by AdHO-1 infection in pigs inhibited phenylephrine-dependent vasoconstriction in isolated aortic rings. Furthermore, AdHO-1 infection induced cGMP production in VSMC. The effects of HO-1 expression on vasoconstriction and cGMP production were subject to inhibition by ZnPPIX; but occurred in the presence of NOS inhibitors (i.e. L-NNA, L-NAME) [166]. Thus, these effects are dependent on heme degradation and independent of NOS activity or NO generation.

Exogenous CO or heme treatment dilated pig cerebral artioles, the latter effect which could be blocked by chromium mesoporphyrin [188]. ZnPPIX, but not NOS inhibitors, inhibited smooth muscle relaxation in the opposum internal anal sphincter produced by nonadrenergic noncholinergic (NANC) nerve stimulation [189]. In isolated perfused rat liver, ZnPPIX diminished CO levels detectable in the effluent, and increased the perfusion pressure under the constant flow conditions. These effects were reversed by the addition of CO or cGMP analogues in the perfusate [190].

These studies support the existence of CO/cGMP signal transduction cascades and their possible regulation by heme oxygenases, as potential pathways governing physiological processes. It remains possible, however, that a fraction of endogenous CO originating from non-heme sources may contribute to a corresponding fraction of cGMP production. More discussion on the significance of CO in the cardiovascular system under normal and pathophysiological states appears in other recent reviews [13, 191].

Carbon monoxide (CO): An anti-inflammatory mediator

HO-1 exerts a novel anti-inflammatory effect mediated by carbon monoxide (CO) generated in the HO reaction [6]. The

effectiveness of bacterial lipopolysacharide (LPS) (heretofore 1 μg/ml), to stimulate the production of the pro-inflammatory cytokine TNFα, was inhibited in transfected RAW 264.7 macrophage cells overexpressing HO-1, compared to that in control transfectants. Exogenously administered CO (heretofore 250 ppm) inhibited the production of TNFα in wild-type RAW 264.7 cells after LPS treatment, indicating that CO can substitute for HO activity in mediating these effects. The treatment of RAW 264.7 cells with exogenous CO prior to LPS treatment inhibited the expression of additional pro-inflammatory cytokines (i.e. IL-1β, and the macrophage inflammatory protein-β, MIP-1β), whereas increased the production of the anti-inflammatory cytokine interleukin-10 (IL-10). The LPS mediated stimulation of pro-inflammatory cytokines in macrophages involves the activation of MAPK signaling pathways [192–195]. LPS treatment activated the p38, ERK1/ERK2 and c-JUN N-terminal kinase, (JNK) pathways in RAW 264.7 macrophages. In the presence of LPS, CO increased p38 MAPK activation, but did not modulate ERK1/ERK2 and JNK. Of the MAP kinase kinases (MKK): (MKK3, MKK4, and MKK6) that activate p38 MAPK [196–197], CO enhanced the LPS-mediated stimulation of MKK3 and MKK6 in RAW 264.7 cells. CO treatment did not significantly modulate cGMP production in RAW 264.7 macrophages, but dramatically increased cGMP levels in control smooth muscle cells. Pretreatment of the RAW 264.7 macrophages with a non-hydrolysable cGMP analog or L-NAME did not compromise the ability of CO to inhibit LPS-inducible TNFα production.

These anti-inflammatory effects of CO were substantiated *in vivo*, in experiments where mice received injections of LPS (heretofore 1 mg/kg) with or without CO pretreatment (heretofore 250 ppm). CO dose-dependently inhibited LPS-inducible serum TNFα levels and increased LPS-inducible IL-10 production. The responsiveness of TNFα to LPS treatment appeared downregulated in MKK3$^{-/-}$ mice compared to wild-type mice. CO failed to further downregulate TNFα levels or upregulate IL-10 levels in LPS treated MKK $^{-/-}$ mice. In IL-10$^{-/-}$ mice, CO inhibited TNFα levels within the first hour of LPS treatment to a similar extent than in wild-type mice, excluding a role for IL-10 in the early anti-inflammatory effects of CO [6].

These results, taken together, demonstrate that CO exerts anti-inflammatory effects by inhibiting the synthesis of the pro-inflammatory cytokines under inducing conditions, by a mechanism that involves stimulation of the MKK3/p38 MAPK pathway, but excludes sGC/cGMP, iNOS, or NO-dependent signaling. The direct physical target of CO in initiating this pathway remains obscure. Various intracellular hemoproteins (i.e. cytochrome p-450, cytochrome c oxidase, NAD(P)H: oxidase, peroxidases, and others) may serve as targets for CO binding [19–22, 198–199]. Future research may focus on elucidating the functional significance (with respect to cell signaling) of CO-hemoprotein interactions *in vivo*.

Cytoprotective and anti-inflammatory effects of carbon monoxide in oxidative lung injury: Involvement of the MKK3/p38 MAPK pathway

CO, through anti-inflammatory action, protects the lung in a model of hyperoxia-induced lung injury [11], which evokes symptoms in mice similar to human acute respiratory distress syndrome (ARDS) [200]. Mice subjected to continuous hyperoxia treatment (heretofore >95% O_2), displayed signs of lung injury by 64–72 h, and all died within 90–100 h of exposure. The presence of CO (heretofore, 250 ppm) initiated prior to the hyperoxia, prolonged the survival of mice in the hyperoxic environment, increasing the LD_{50} to 128 h exposure. CO inhibited the appearance of markers of lung injury associated with hyperoxia (i.e. hemorrhage, fibrin deposition, edema, and protein accumulation in the airway), as well as markers of oxidative damage (i.e. lung lipid peroxidation) [11]. CO also inhibited the influx of neutrophils into the airways associated with hyperoxia treatment, as measured in bronchoalveolar lavage fluid.

Hyperoxia induced the expression of proinflammatory cytokines including TNFα, IL-1β, and IL-6, by 84 h of exposure and activated stress kinases in lung tissue including ERK1/2, JNK, P38/MAPK and MKK3/MKK6. The protection afforded by CO treatment against the lethal effects of hyperoxia correlated with the inhibited expression of the pro-inflammatory cytokines, TNFα, IL-1β and IL-6.

MKK3$^{-/-}$ mice, or wild-type mice injected with the selective inhibitor of p38 α/β MAPK (SB203580), displayed the accelerated manifestation of tissue damage markers (with the exception of neutrophil influx) and increased sensitivity to the lethal effects of hyperoxia, relative to untreated wild-type mice. Cytokine mRNA (TNFα, IL-1β and IL-6) expression in response to hyperoxia appeared earlier in the MKK3$^{-/-}$ mice relative the wild-type mice exposed to continuous hyperoxia. CO failed to inhibit the expression of the pro-inflammatory cytokines in the MKK3 $^{-/-}$ mice, and furthermore failed to confer protection or extend survival against hyperoxia in MKK3$^{-/-}$ mice or in wild-type mice injected with SB203580. On the other hand, JNK$^{-/-}$ mice behaved as wild-type mice with respect to the anti-inflammatory effects of CO [11]

The CO treatment of A549 lung epithelial cells *in vitro* increased MKK3 activation, and specifically the β-isoform of p38. The presence of CO increased the survival of A549 cells grown in continuous hyperoxia, relative to cells exposed to hyperoxia alone. Treatment with the inhibitor of p38 α/β MAPK or transient transfection with dominant negative mutants of p38β or MKK3 abolished the cytoprotective effect of CO against hyperoxia. Currently no studies support the selective activation of antioxidant enzymes or stress proteins as an underlying mechanism for the anti-inflammatory properties of CO *in vivo*. However, the treatment of endothelial cells *in vitro* with exogenous CO (100 ppm) stimulated the expression of

manganese superoxide dismutase (MnSOD) and HO activity [201]. In summary, these experiments demonstrate that CO protects against the lethal and inflammatory effects of hyperoxia *in vivo* and *in vitro*, by downregulating the expression of pro-inflammatory cytokines, through a mechanism dependent on activation of the p38β/ MKK3 pathway [11].

Summary

The functional significance of heme oxygenase-1, which provides the rate-limiting step in heme degradation, and whose induction represents a general response to cellular stress, has remained a subject of debate for decades [23–24, 37, 202]. The overwhelming evidence described above supports the conclusion that HO-1 expression confers protection in animal models of oxidative stress. These studies taken together, suggest that HO-1 expression may have therapeutic value in gene therapy approaches.

Attempts to explain the cytoprotective action of HO-1 have implicated possible roles for all the products of HO-activity including redox active iron and bile pigments [23–24]. CO, formerly regarded as a toxic elimination product of the HO reaction has taken on a new significance as a possible autocrine and paracrine signaling molecule. CO regulates vascular and neural processes by modulation of cGMP production [18]. Recent work has identified anti-inflammatory and anti-apoptotic properties of HO-derived CO [6, 11]. In animal models of lung oxidative stress, including hyperoxia and ischemia/ reperfusion, exogenously applied CO may apparently substitute for HO-1 expression with regard to protection [6, 165]. Such studies point to a possible therapeutic use of inhalation CO in inflammatory disease states.

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