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Targeting Heme Oxygenase-1 in Vascular Disease

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Abstract

Heme oxygenase-1 (HO-1) metabolizes heme to generate carbon monoxide (CO), biliverdin, and iron. Biliverdin is subsequently metabolized to bilirubin by biliverdin reductase. HO-1 has recently emerged as a promising therapeutic target in the treatment of vascular disease. Pharmacological induction or gene transfer of HO-1 ameliorates vascular dysfunction in animal models of atherosclerosis, post-angioplasty restenosis, vein graft stenosis, thrombosis, myocardial infarction, and hypertension, while inhibition of HO-1 activity or gene deletion exacerbates these disorders. The vasoprotection afforded by HO-1 is largely attributable to its end products: CO and the bile pigments, biliverdin and bilirubin. These end products exert potent anti-inflammatory, antioxidant, anti-apoptotic, and anti-thrombotic actions. In addition, CO and bile pigments act to preserve vascular homeostasis at sites of arterial injury by influencing the proliferation, migration, and adhesion of vascular smooth muscle cells, endothelial cells, endothelial progenitor cells, or leukocytes. Several strategies are currently being developed to target HO-1 in vascular disease. Pharmacological induction of HO-1 by heme derivatives, dietary antioxidants, or currently available drugs, is a promising near-term approach, while HO-1 gene delivery is a long-term therapeutic goal. Direct administration of CO via inhalation or through the use of CO-releasing molecules and/or CO-sensitizing agents provides an attractive alternative approach in targeting HO-1. Furthermore, delivery of bile pigments, either alone or in combination with CO, presents another avenue for protecting against vascular disease. Since HO-1 and its products are potentially toxic, a major challenge will be to devise clinically effective therapeutic modalities that target HO-1 without causing any adverse effects.

Keywords

heme oxygenase-1; carbon monoxide; biliverdin; bilirubin; atherosclerosis; thrombosis; myocardial infarction; hypertension

Introduction

Heme oxygenase-1 (HO-1) is the inducible rate-limiting enzyme in the oxidative degradation of heme yielding equimolar amounts of carbon monoxide (CO), biliverdin, and ferrous iron (Figure 1). This reaction requires molecular oxygen, nicotinamide adenine dinucleotide phosphate, and the concerted action of cytochrome p450 reductase [1]. This catabolic pathway is inhibited by various metalloporphyrins, including zinc and tin protoporphyin-IX. Subsequently, biliverdin is metabolized to bilirubin by biliverdin reductase, and free iron is sequestered by ferritin and either excreted by cells or recycled for heme synthesis. HO-1 is a ubiquitously distributed, highly inducible enzyme. The expression of HO-1 is upregulated by

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numerous stimuli, including its substrate (heme), oxidants, heavy metals, cytokines, growth factors, gases, homocysteine, hormones, dietary antioxidants, radiation, hemodynamic forces, and by specific therapeutic agents (Figure 1). The control of HO-1 expression occurs primarily at the transcriptional level and is mediated by multiple signaling pathways and transcription factors. However, stress-activated transcription factors such as nuclear factor E2-related factor-2 (Nrf2), activator protein-1, and nuclear factor-κB play predominant roles and mediate the potent induction of HO-1 by agents that cause cellular stress [see 2,3]

Although interest in HO-1 initially focused on the heme degrading properties of the enzyme, research in the past two decades has shifted and currently center on its protective functions. It is now well established that the induction of HO-1 provides a fundamental cellular defense mechanism against tissue injury. The cytoprotection afforded by HO-1 is mediated by several different mechanisms, including the catabolism of pro-oxidant heme to the antioxidant bile pigments biliverdin and bilirubin; the co-ordinate induction of ferritin, which chelates free iron; and the liberation of CO, which exerts significant anti-inflammatory and anti-apoptotic effects [see 4–6]. Compelling evidence indicates that HO-1 also protects against the development of cardiovascular disease. Genetic deficiency of HO-1 is associated with oxidative tissue damage, anemia, chronic inflammation, thrombosis, and increased susceptibility to atherosclerosis in both mice and humans, while overexpression of HO-1 improves vascular dysfunction in numerous animal models [7–12]. In addition, functional polymorphisms in the promoter region of the HO-1 gene that are linked to impaired inducibility are associated with several cardiovascular pathologies [see 13]. Further evidence for a beneficial role for HO-1 is provided by clinical studies demonstrating that low serum concentrations of the heme metabolite, bilirubin, are correlated with an increased risk of coronary and peripheral artery disease [14–16].

This article will review the effects of HO-1 in the circulation and discuss cellular and molecular mechanisms that contribute to the vasoprotective properties of this enzyme. In addition, it will highlight potential therapeutic strategies targeting HO-1 or its end products in the treatment or prevention of vascular disease.

HO-1 and Atherosclerosis

Considerable evidence suggests that HO-1 plays a beneficial role in atherosclerosis. HO-1 is highly expressed in the endothelium, macrophage, and foam cells of atherosclerotic plaques in both humans and animals [17]. HO-1 expression is observed throughout the development of lesions from early fatty streaks to advanced complex atherosclerotic lesions. Atherectomy biopsy samples of patients with coronary artery disease reveals that HO-1 expression closely correlates with features of the vulnerable plaque: HO-1 is specifically upregulated in human vulnerable atherosclerotic lesions with lipid and macrophage accumulation and low collagen and vascular smooth muscle cell content [18]. Topographical distribution of HO-1-positive cells shows a significantly higher density of cells in the shoulder region and fibrous cap of complex lesions [19]. Interestingly, HO-1 expression is more prevalent in carotid atherosclerotic plaques obtained from asymptomatic compared with symptomatic patients [20], suggesting a possible role for HO-1 in blocking plaque rupture. Significantly, the only identified human case of HO-1 deficiency displayed hyperlipidemia and early development of fatty streaks and fibrous plaque in the aorta [9,10]. Moreover, studies assessing polymorphisms in the 5'-flanking region of the human HO-1 gene suggests a beneficial role for HO-1 in atherosclerosis. In particular, a long (GT)*n* microsatellite polymorphism in the human HO-1 promoter that is linked to reduced expression is associated with susceptibility to coronary artery disease in some patient populations [21–23]. Furthermore, a single nucleotide polymorphism in the HO-1 promoter, T(−413)A, that elevates basal promoter activity correlates with a reduced frequency of ischemic heart disease in a cohort of Japanese subjects [24].

The induction of HO-1 also co-localizes with oxidized phospholipids in atherosclerotic lesions [25,26]. Given that oxidized lipids are potent inducers of HO-1, they may contribute to the induction of HO-1 in vascular lesions [17,27]. However, numerous other atherogenic molecules, including peroxynitrite, homocysteine, inflammatory cytokines, growth factors, and hypochlorous acid, have been implicated in stimulating HO-1 expression in atherosclerotic lesions [28–33]. Interestingly, the induction of HO-1 by many of these atherogenic agents is mediated through Nrf2 but the upstream signaling pathways that trigger the activation of Nrf2 differ between stimuli. Protein kinase C, mitogen-activated protein kinases, and phosphatidylinositol-3-kinase have all been implicated in the direct activation of Nrf2 while oxidants and peroxynitrite may indirectly mobilize Nrf2 by interfering with its Keap1-mediated sequestration and/or degradation. Thus, atherogenic factors are capable of activating multiple signaling pathways that stimulate Nrf2-mediated HO-1 gene transcription.

Animal studies provide further proof for the protective role of HO-1 in atherosclerosis. Long-term inhibition of HO activity by metalloporphyrins promotes lesion formation in atherogenic mice and rabbits [26,34]. Similarly, deletion of HO-1 in apolipoprotein E (apoE)-deficient mice fed a western diet results in larger and more advanced lesions despite comparable increases in circulating cholesterol [8]. Alternatively, pharmacological induction of HO-1 decreases lesion size in low-density lipoprotein (LDL)-receptor-knockout mice fed a high fat diet [26]. In addition, intraventricular delivery of an HO-1 adenovirus significantly retards lesion formation in apoE-deficient mice [12]. Similarly, adenoviral-mediated gene transfer of HO-1 inhibits graft arteriosclerosis in both rat aortic and cardiac transplants [35–37]. More recent work indicates that HO-1 may also regulate plaque phenotype. Using a vulnerable plaque model, Cheng et al [18] demonstrated that pharmacological induction or gene delivery of HO-1 prevents vulnerable plaque formation in apoE-deficient mice resulting in lesions with a diminished necrotic core, increased fibrous cap thickness, reduced lipid levels, and elevated intimal smooth muscle cell content. In contrast, inhibition of HO-1 by zinc protoporphyrin-IX elicits an opposite effect and triggers plaque destabilization. The specific expression of HO-1 in macrophages also plays a beneficial role in atherosclerosis by decreasing the inflammatory component of vascular lesions [38]. Bone marrow transplantation experiments performed in lethally irradiated LDL-receptor-null mice indicate that animals reconstituted with bone marrow from HO-1-deficient mice exhibit atherosclerotic lesions with greater macrophage content compared to animals reconstituted with bone marrow from wild-type mice. Collectively, clinical and experimental studies strongly support the hypothesis that HO-1 protects against the development of coronary artery disease by reducing plaque size and stabilizing plaque phenotype.

There are several potential mechanisms by which HO-1 prevents the development of atherosclerosis. Since increases in iron deposition are closely associated with the progression of atherosclerosis [39], the ability of HO-1 to reduce iron overload in aortic lesions of apoE-knockout mice is highly significant and may contribute to the anti-atherogenic properties of this protein [12]. Although the precise mechanism whereby HO-1 decreases iron deposition in atherosclerosis is not known, HO-1-mediated increases in iron efflux from cells may be involved [40]. In addition, the increase in ferritin that accompanies HO-1 induction may chelate intracellular free iron and maintain it in a less reactive form. Other beneficial effects of HO-1 may derive from the antioxidant property of this enzyme. Consistent with this proposal, induction of HO-1 decreases plasma hydroperoxide levels in LDL-receptor knockout mice and hyperlipidemic rabbits while HO inhibition increases circulating and tissue hydroperoxide concentrations [26,34]. Bile pigments are likely involved in the antioxidant actions of HO-1 since bilirubin oxidative metabolites are detected in atherosclerotic lesions [41]. However, CO may also contribute to the antioxidant actions of HO-1 by stimulating the expression of antioxidant genes and inhibiting the activity of pro-oxidant enzymes [42,43].

HO-1 may also protect against atherosclerosis by preserving vascular cell function and survival. Endothelial cell dysfunction manifested by impaired endothelium-dependent vasodilation and endothelial nitric oxide (NO) synthesis is one of the earliest changes associated with the development of atherosclerosis. Interestingly, overexpression of HO-1 improves endothelium-dependent vascular relaxation and restores endothelial NO synthase expression in various animal models [44,45]. Both bilirubin and CO have been implicated in this protective response [45,46]. Apoptosis of endothelial cells also plays a pivotal role in the progression of atherosclerosis. Many of the classical pro-atherogenic stimuli are potent inducers of endothelial cell apoptosis, and apoptotic endothelial cells have been directly detected in human atherosclerotic plaques [47]. Significantly, HO-1 gene delivery protects cultured endothelial cells from apoptosis in response to both extrinsic and intrinsic pathways of apoptosis and affords endothelial protection in preclinical models of transplant arteriosclerosis [48,49]. The anti-apoptotic action of HO-1 in endothelial cells appears to be predominantly mediated by CO which modulates multiple steps of the apoptotic cascade [48, 50]. However, we recently demonstrated that biliverdin and bilirubin can also inhibit endothelial cell apoptosis by preserving mitochondrial membrane potential [29]. Interestingly, the induction of HO-1 also prevents apoptosis of vascular smooth muscle cells [51,52]. In this case, the anti-apoptotic action of HO-1 appears to be mediated exclusively by CO [51]. Since vascular smooth muscle cell apoptosis has recently been identified as a critical process in mediating plaque rupture [53], the ability of HO-1 to inhibit smooth muscle cell apoptosis in the vulnerable shoulder region of plaques may provide an important mechanism by which HO-1 promotes plaque stability.

Another important mechanism by which HO-1 exerts an anti-atherogenic effect is by arresting inflammation. HO-1 inhibits monocyte chemotaxis and leukocyte adhesion to activated vascular endothelium [26,54]. HO-1 overexpression also attenuates the production of inflammatory cytokines from activated endothelial cells and macrophages [55]. These anti-inflammatory actions of HO-1 are largely mediated through the formation of CO and bile pigments. In this respect, CO elicits divergent regulatory effects on the production of cytokines: CO inhibits the generation of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1, and macrophage inflammatory protein-1β while increasing the synthesis of the anti-inflammatory cytokine interleukin-10 [55]. Furthermore, CO downregulates the inflammatory response by blocking inducible NO synthase activity and the expression of granulocyte-macrophage colony stimulating factor, which is known to promote the production of inflammatory mediators and the differentiation of hematopoietic progenitor cells into macrophages and neutrophils [56,57]. Similarly, the HO-1 product biliverdin stimulates the production of interleukin-10 while suppressing the synthesis of inflammatory cytokines [58]. In addition, bilirubin diminishes monocyte chemotaxis and the expression of adhesion receptors on endothelial cells, leading to inhibition of leukocyte rolling, adhesion, and infiltration into the vessel wall [26,54,59]. Thus, HO-1 is able to alleviate vascular inflammation in a multifold manner through the production of CO and biliverdin/bilirubin.

HO-1 and Vascular Occlusion

HO-1 may also exert a salutary effect on the pathologic remodeling response that frequently occurs following percutaneous transluminal angioplasty or coronary artery bypass surgery. These surgical procedures are commonly used to treat atherosclerotic disease; however, they are prone to early occlusion due to thrombosis and late-onset occlusion as a result of vascular smooth muscle cell proliferation and intimal thickening. Interestingly, HO-1 protects against both of these negative clinical outcomes. In an experimental vein graft model where a segment of jugular vein is patched into a defect created in the autologous carotid artery of the mouse, HO-1-knockout mice develop large smooth muscle cell-rich intimal lesions ten days after surgery compared to wild-type animals, suggesting an essential protective role for HO-1 in

graft stenosis [8]. Prior induction of HO-1 by hemin also retards neointima formation following balloon injury of rat carotid arteries, whereas inhibition of HO activity by metalloporphyrins augments lesion formation [60–62]. In addition, localized adenovirus-mediated HO-1 gene delivery immediately after arterial injury attenuates neointimal hyperplasia in rat carotid and pig femoral arteries [63,64]. Furthermore, deletion of the HO-1 gene exacerbates neointima formation following wire injury of mouse femoral arteries [63] and following the creation of an arteriovenous fistula in mice [65]. Importantly, HO-1 may also influence the remodeling response following arterial injury in humans. Patients with the long (GT)*n* repeat in the HO-1 promoter exhibit a significantly enhanced risk of restenosis following peripheral percutaneous transluminal angioplasty [66,67]. The presence of long (GT)*n* repeats is also associated with stenosis-related arteriovenous fistula failure in a cohort of Chinese hemodialysis patients [68]. However, conflicting data has been presented relating this (GT)*n* length polymorphism to the risk of restenosis following coronary stenting [69,70].

The beneficial actions of HO-1 following arterial injury are mediated, in part, by its ability to suppress vascular smooth muscle cell proliferation. Induction or gene delivery of HO-1 in cultured vascular smooth muscle cells blocks cell growth and DNA synthesis [63,71]. In addition, vascular smooth muscle cells derived from HO-1-null mice display enhanced growth and DNA synthesis compared to cells obtained from wild-type animals. Moreover, greater smooth muscle cell proliferation is observed in arterial lesions from HO-1-deficient mice compared to wild-type animals, confirming the anti-proliferative effect of HO-1 *in vivo* [63]. The anti-proliferative action of HO-1 is mediated through the soluble guanylate cyclase/cGMP pathway since inhibition of soluble guanylate cyclase or protein kinase G restores cell growth [63]. Flow cytometry experiments indicate that HO-1 arrests smooth muscle cells in the G_0/G_1 phase of the cell cycle and this is associated with a pronounced increase in p21 expression [65]. The exogenous administration of CO or bilirubin also inhibits vascular smooth muscle cell proliferation, cell cycle progression, DNA synthesis, and the expression of cell cycle regulatory proteins, suggesting a role for both these products in the anti-proliferative action of HO-1 [71–74]. More recently, HO-1 has also been demonstrated to inhibit vascular smooth muscle cell migration via the CO-mediated inhibition of Nox1 enzyme activity and downstream redox-sensitive pro-migratory pathways [75]. The ability of HO-1 to inhibit the migration of vascular smooth muscle cells from the media to the intima may provide another possibility by which HO-1 limits neointima formation.

Interestingly, HO-1 stimulates cell cycle progression and proliferation in vascular endothelium. Transduction of the HO-1 gene into endothelial cells promotes their growth and the development of capillary-like tube structures while inhibition or deletion of HO-1 blocks endothelial cell growth [76]. HO-1 also facilitates endothelial cell proliferation, migration and capillary sprout formation in response to specific angiogenic factors [77,78]. In addition, HO-1 promotes the re-endothelialization of injured arteries. Pharmacological induction of HO-1 or systemic administration of an HO-1 adenovirus accelerates the re-endothelialization of denuded blood vessels while HO-1 deletion impairs this process [79–81]. Aside from stimulating the proliferation and migration of endothelial cells from the injured border zone or from branching vessels adjacent to the site of injury, HO-1 enhances re-endothelialization by promoting the mobilization and homing of endothelial progenitor cells to sites of injury [79–81]. HO-1 increases circulating levels of endothelial progenitor cells and elevates the number of endothelial progenitor cells detected in cultures of mononuclear cells. Moreover, the expression of HO-1 by endothelial progenitor cells is required for their incorporation into blood vessels. Notably, endothelial progenitor cells lacking HO-1 are unable to re-endothelialize the retinal vasculature following ischemic injury [77]. The HO-1-mediated increase in endothelial regrowth following arterial injury is dependent on the induction of stromal cell-derived factor-1 and vascular endothelial growth factor, and is mimicked by the inhalation of CO, suggesting a key role for this gas in repairing endothelium-denuded areas of blood vessels [80,81]. The

ability of HO-1 to restore endothelial cells at sites of arterial injury provides another mechanism to blunt intimal expansion since the re-endothelialization of the vessel wall assists in maintaining the underlying smooth muscle in a quiescent, non-proliferative, and non-migratory state.

HO-1 may also preserve blood vessel patency by inhibiting thrombosis. HO-1 deficiency accelerates arterial thrombus formation following photochemical injury and increases thrombus size in a murine model of deep vein thrombosis [11,82]. The absence of HO-1 in mice also leads to arterial thrombosis following allogeneic aortic transplantation [83]. On the contrary, induction of HO-1 retards micro and macrovascular thrombus formation following endothelial injury [84,85]. Interestingly, there are no significant differences in bleeding time, platelet counts, or prothrombin times between HO-1-knockout and wild-type mice, suggesting that abnormalities in primary or secondary hemostasis are not responsible for the accelerated thrombosis [12]. The anti-thrombotic actions of HO-1 are likely mediated by CO and biliverdin since the exogenous administration of either compound rescues HO-1-deficient animals from thrombosis [11,83]. Both these HO-1 products may prevent intravascular thrombosis by ameliorating endothelial cell damage, which is a crucial factor in thrombus formation. In addition, HO-1-derived CO may block thrombosis by inhibiting platelet aggregation and the expression of plasminogen activator inhibitor-1 and tissue factor [11,87].

HO-1 and Myocardial Infarction

Substantial data support a protective role for HO-1 against myocardial ischemia/reperfusion-induced injury. Pharmacological induction of HO-1 significantly attenuates infarct size and the incidence of reperfusion arrythmias following ischemia/reperfusion, while HO inhibition aggravates cardiac tissue damage [88–90]. Studies in transgenic animals also confirm the importance of HO-1 in ischemic cardiac injury. Hearts from heterozygous HO-1 knockout mice are more prone to ischemia-reperfusion injury whereas cardiac-specific HO-1 overexpressing animals suffer less damage [91–93]. A maladaptive response consisting of increased ventricular dilation, infarction, and thrombosis has also been reported in HO-1-deficient mice during chronic hypoxia [94]. Significantly, human HO-1 gene transfer using adeno-associated virus several weeks prior to acute coronary artery ligation and release results in sustained myocardial protection from ischemia-reperfusion injury in rats [95]. In addition, overexpression of the transgene prevents long-term pathologic tissue remodeling and normalizes tissue function. Moreover, the improvement in cardiac function is accompanied by decreases in oxidative stress, inflammation, and interstitial fibrosis. These findings support the utility of using a pre-emptive HO-1 gene transfer approach to protect tissues from future episodes of injury and may provide a potential preventative therapy for individuals at risk for developing coronary ischemic events.

Both bilirubin and CO contribute to the protective actions of HO-1 in the heart. Exogenously administered bilirubin significantly improves cardiac function and decreases myocardial infarct size and mitochondrial damage upon reperfusion insult [96]. Similarly, treatment of isolated cardiac cells or hearts with a CO-releasing molecule preserves cell viability and myocardial performance against hyperoxia-reoxygenation damage [97]. In addition, administration of a CO-releasing agent at the time of reperfusion reduces infarct size in a murine model of coronary occlusion [98]. Interestingly, brief exposure to CO produces sustained, long-term cardioprotection that mimics the late phase of ischemic preconditioning [99]. Inhalation of CO gas prior to myocardial ischemia-reperfusion injury likewise reduces infarct area in mice and this is associated with diminished migration of macrophages and monocytes into the infarcted zone, and with reduced expression of tumor necrosis factor-α [100]. Inhalation of CO also improves cardiac energetics and safeguards the heart during reperfusion after cardiopulmonary bypass in pigs, and attenuates ischemia-reperfusion injury following cardiac

transplantation in mice [101,102]. Indeed, protective actions of CO against an ischemic insult have been reported following the transplantation of other organs [see 103].

HO-1 and Hypertension

HO-1 is an important modulator of blood pressure and alterations in the expression or activity of this enzyme has been linked to the pathogenesis of hypertension. However, divergent regulatory effects on blood pressure have been reported in different experimental models of hypertension. Chemical induction of HO-1 or the administration of HO substrates attenuates hypertension in spontaneously hypertensive rats (SHR) and this effect is abolished when HO inhibitors are introduced prior to HO-1 induction [104–106]. In addition, gene transfer of HO-1 in SHR results in a significant decline in blood pressure [107]. More recently, Wang et al [108] demonstrated that administration of hemin for 3 consecutive weeks to 12-week old SHR rats normalizes systolic blood pressure. Importantly, this normalization persists for 9 months after the discontinuation of hemin. The mechanism underlying this dramatic and sustained reversal in hypertension is not known but may be related to the persistent elevation in vascular HO-1 expression and the reversal of eutrophic inward remodeling of small resistance arteries. Notably, the antihypertensive effect of hemin is not associated with any changes in body weight or hepatotoxicity, raising the possibility that hemin administration may be a viable treatment option in humans.

The antihypertensive effect is likely mediated by CO, since it is the only HO-1 product able to decrease blood pressure in SHR [104]. There are several potential mechanisms by which CO may lower blood pressure. In particular, CO can reduce peripheral resistance by directly dilating blood vessels via the activation of soluble guanylate cyclase and/or calcium-activated potassium channels [109,110]. Beyond its direct vasodilating effect, CO may also decrease vascular tone by regulating the production of vasoactive molecules. In this regard, CO blocks the synthesis of the potent vasoconstrictor endothelin-1 and the cytochrome P450-mediated generation of vasoconstrictors [105,106,111]. CO may also exert an antihypertensive effect by stimulating the release of NO from intracellular stores, by depressing central sympathetic outflow, and by promoting sodium excretion by the kidney [112–114].

A beneficial role for HO-1 has also been demonstrated in other experimental models of hypertension. Systemic induction of HO-1 by cobalt protoporphyrin-IX lowers blood pressure in angiotensin II-treated hypertensive mice and this is associated with a pronounced decline in renal superoxide production [115]. Similarly, a single intraventricular injection of a retroviral vector containing human HO-1 results in widespread transgene expression and blunts the angiotensin II-mediated pressor response in rats [116]. Surprisingly, kidney-specific induction of HO-1 is sufficient to attenuate angiotensin II-dependent hypertension, suggesting a critical role for this organ in mediating the antihypertensive effect of HO-1 in this experimental model [117]. HO-1 also affords protection in the one kidney-one clip model of renovascular hypertension [118]. In this model, HO-1-deficient mice exhibit more severe hypertension and renal injury compared to wild-type mice. Furthermore, HO-1-null mice exhibit a sustained and significant elevation in systemic arterial pressure when subjected to deoxycorticosterone acetate (DOCA)-salt whereas wild-type animals remain normotensive [119]. Significantly, the antihypertensive action of HO-1 is not limited to the systemic circulation. Overexpression of HO-1 or inhalation of CO inhibits the development of pulmonary hypertension in response to hypoxia or monocrotaline [120–123]. In addition, injection of an adeno-associated virus expressing HO-1 through the portal vein markedly diminishes portal hypertension in carbon tetrachloride-treated rats [124].

Surprisingly, HO-1 promotes hypertension in some animal models. Dahl salt-sensitive rats placed on high-salt diet for 4 weeks develop severe systemic hypertension that is associated

with a significant increase in vascular HO-1 protein and CO production [125,126]. In addition, administration of the HO inhibitor, zinc deuteroporphryin 2,4-*bis* glycol, selectively lowers blood pressure in hypertensive Dahl rats but not in control animals fed a low-salt diet. Obese Zucker rats, a well established genetic model of metabolic syndrome, also develop hypertension that is coupled to an increase in respiratory CO production [127]. However, treatment with a HO inhibitor lowers CO excretion and normalizes blood pressure in these obese animals. Interestingly, endothelium-dependent vasodilation is impaired in both obese and salt-sensitive rats, and acute treatment of blood vessels with a HO inhibitor fully restores endothelial function in these animals. Furthermore, the co-application of CO prevents the restoration of endothelial function by HO inhibition. Taken together, these findings suggest that elevated CO production contributes to endothelial dysfunction and hypertension in these animals. Consistent with this notion, exogenously applied or endogenously derived CO inhibits endothelial NO release and vasodilation in several vascular beds [112,128,129]. Moreover, transgenic mice that selectively overexpress HO-1 in vascular smooth muscle cells are also hypertensive and display impaired nitrovasodilator-mediated vasodilation and cGMP production [130].

In summary, HO-1 lowers blood pressure in most experimental forms of hypertension but pro-hypertensive effects have also been reported in some animal models. Although reasons for the divergent effects of HO-1 on blood pressure are not known, the complex interaction between the HO-1 and NO synthase enzymes can lead to alterations in gaseous monoxide production and competition between CO and NO for specific target proteins that impact blood pressure regulation (see 6).

Therapeutic Strategies Targeting HO-1 in Vascular Disease

HO-1 has recently emerged as a promising therapeutic target in the treatment of vascular disease. Numerous mechanisms contribute to the vasoprotective action of HO-1 (Figure 2). In particular, HO-1 exerts a direct antioxidant effect by degrading pro-oxidant heme and preventing intracellular iron accumulation. In addition, the HO-1 end products CO and bile pigments possess potent anti-inflammatory, antioxidant, anti-apoptotic, and anti-thrombotic actions. Moreover, CO and bile pigments act to preserve vascular homeostasis at sites of arterial injury by influencing the proliferation, migration, and adhesion of vascular smooth muscle cells, endothelial cells, endothelial progenitor cells, or leukocytes.

Several strategies can be employed to target HO-1 in vascular disease (Table 1). One promising approach involves the use of pharmacological inducers. Heme and its synthetic analogues are potent inducers of HO-1 and have been shown to protect against the development of vascular disease in numerous animal models. In addition to upregulating HO-1 expression, heme is a substrate for HO-1 and this may serve to further enhance the synthesis of CO and bilirubin since HO-1 activity may be substrate-limited in vascular cells [131]. Hemin is already approved by the United States Food and Drug Administration for the treatment of acute porphyria and heme compounds have been used to treat thalassemia intermedia, myelodysplastic syndrome, and liver allograft failure following erythropoietic protoporphyria [132–135]. Interestingly, a recent small clinical study affirmed the efficacy of a single intravenous infusion of hemin to stimulate plasma HO-1 protein expression and activity in healthy subjects without any adverse effects [136], illustrating the potential of using heme derivatives to elevate HO-1 expression. Clearly, further clinical studies employing larger subject populations are needed to establish optimal safe and effective dosing regimens for these compounds.

Since free heme possess pro-oxidant and pro-inflammatory properties [137], a potentially less toxic approach to inducing HO-1 gene expression may involve the use of dietary antioxidants. The dietary antioxidants *tert*-butylhydroquinone, quercetin, caffeic acid phenethyl ester, and

curcumin are all potent inducers of HO-1 [138,139]. Similarly, catechins, the major polyphenolic ingredient in green tea, and α-lipoic acid, a thiol-containing antioxidant found in certain vegetables, are strong inducers of HO-1 [140,141]. Furthermore, resveratrol, a phytoalexin found in high concentration in red wine, stimulates the expression of HO-1 in vascular cells raising the possibility that HO-1 contributes to the cardioprotection associated with the consumption of red wine [142]. In addition, a host of other dietary antioxidants have been found to enhance HO-1 expression, including the coffee diterpenes cafestol and kahwoel, carnosol, and sulphoraphane [138]. Interestingly, phenolic antioxidants which fail to stimulate HO-1 do not confer protection against vascular disease, underscoring the crucial role of HO-1 in mediating the atheroprotective effects of these compounds [79]. Aside from antioxidants, amino acids such as methionine, alanine, and glutamine have also been reported to induce HO-1 expression [143–145]. Thus, a variety of dietary approaches may be used to stimulate HO-1 expression. However, careful clinical studies are needed to establish the efficacy and safety of any nutritional approach.

There is a growing appreciation that many frontline drugs used in the treatment of cardiovascular disease exert their therapeutic effect, at least in part, through the induction of HO-1. Statins are widely prescribed lipid lowering agents that decrease mortality in patients with coronary artery disease. Several statins are capable of elevating HO-1 expression in cultured vascular cells [146,147]. In addition, oral administration of statins results in statin- and tissue-specific increases in HO-1 that is associated with increased resistance to oxidative stress and inhibition of smooth muscle cell proliferation, suggesting that HO-1 may contribute to the pleiotropic and anti-atherogenic actions of statins [148]. However, the induction of HO-1 by statins is not universally observed and it remains unclear whether statins at concentrations pharmacologically relevant to humans elevate HO-1 expression, and whether this induction contributes to protection against coronary artery disease [149].

Probucol is another cholesterol lowering drug that reduces the risk of postangioplasty restenosis. The protective effect of probucol depends not only on its ability to suppress lipid peroxidation, but also on the induction of HO-1 [79]. Probucol inhibits macrophage accumulation and smooth muscle cell proliferation, and stimulates endothelial cell regrowth following arterial injury in a HO-1-dependent fashion. Aspirin is another drug used in the treatment of cardiovascular disease that induces HO-1 expression. Indeed, pharmacologically relevant concentrations of aspirin stimulate HO-1 expression and this may contribute to the drugs antioxidant and anti-thrombotic profile [150]. Interestingly, the induction of HO-1 is unique for aspirin and is not mimicked by other non-steroidal anti-inflammatory agents. The organic nitrate ester, pentaesithrityl tetranitrate (PETN) has also been observed to stimulate vascular HO-1 expression and this may, in part, mediate its antioxidant and anti-atherogenic actions [151]. Moreover, the induction of HO-1 by PETN prevents the development of tolerance by eliminating nitrate-induced generation of reactive oxygen species [152]. Similarly, sildenafil, the phosphodiesterase-5 inhibitor used for the treatment of erectile dysfunction, upregulates HO-1 expression both in cultured endothelial cells and *in vivo*, and this may contribute to its biological actions in cavernous tissue [153,154]. Finally, presently employed drug eluting stents may prevent coronary artery restenosis through the induction of HO-1. Both rapamycin and paclitaxel stimulate HO-1 expression in vascular smooth muscle cells and HO-1 contributes to the anti-proliferative action of these compounds [155–157].

Collectively, the above results validate the use of pharmacological approaches in targeting HO-1 in vascular disease. However, there are some challenges associated with the deployment of pharmacological inducers. One concern relates to possible non-specific effects of HO-1 inducers that can potentially negate the beneficial effects associated with the upregulation of HO-1 [137,158). Furthermore, the induction and duration of HO-1 expression must be carefully titrated since prolonged, high level HO-1 expression can adversely affect cell viability [159,

160]. In addition, the presence of polymorphisms in the promoter that restrains the induction of HO-1 may limit the efficacy of HO-1 inducers in certain patient populations. Thus, assessment of patient genotype prior to pharmacological intervention may be necessary. Increasing HO-1 gene expression via viral-mediated delivery obviates this problem and allows for the specific induction of this gene in all patients. Gene therapy approaches with HO-1 have proven highly effective in animal studies and the recent development of inducible and cell-specific vectors allows for selective and temporal patterns of gene expression [161,162]. However, current limitations in human gene therapy are well recognized and will require additional improvement and documentation of clinical safety and efficacy.

As an alternative to direct targeting of HO-1, products of the HO-1 reaction can be administered to treat vascular disease. As outlined above, CO is effective in several preclinical models of cardiovascular disease. Moreover, a growing number of studies suggest that acute, periodic inhalation of low concentrations of CO (50–500 parts per million) is sufficient to elicit protection in animal models of pulmonary hypertension, intimal hyperplasia, sickle cell disease, ischemia-reperfusion injury, and postoperative ileus [72,122,163–165]. Significantly, no deleterious effects on heart rate, blood chemistry, serum electrolytes, and arterial oxygen saturation are noted with this low-dose CO inhalation strategy (158,159). A number of clinical trials are currently exploring the pharmacokinetics, safety, and efficacy of acute, episodic CO inhalation regimens in human subjects for the treatment of lung inflammation, chronic obstructive pulmonary disease, and renal transplantation. Initial reports indicate that inhalation of CO (95–500 parts per million) for one or two hours are well-tolerated, safe, feasible, and of potential benefit to patients with chronic obstructive pulmonary disease [166,167].

The use of CO-saturated solutions and prodrugs provides another vehicle for the administration of CO. In this respect, we recently reported that brief local delivery of a saturated solution of CO blocks the pathophysiological response to arterial injury [168]. Alternatively, intraperitoneal injection of CO-saturated solutions provides a simple procedure for the systemic delivery of CO. Indeed, a single intraperitoneal dose of CO–saturated Ringer's lactate solution ameliorates post-operative ileus in mice [169]. Systemic delivery of CO can also be achieved using the prodrug dichloromethane which is readily metabolized in the liver by cytochrome P450 isozymes to CO. Oral ingestion of dichloromethane results in a marked increase in CO production that is associated with a significant decrease in aortic intimal thickening in rodent model of chronic allogeneic rejection, demonstrating the ability of this approach to generate biologically relevant concentrations of CO [170]. However, there are serious concerns with toxicity related to the use of this compound [171). More promising, are the recently developed CO-releasing compounds (CORMs) that liberate CO under physiologic conditions. Several CORMs have been synthesized with various solubility and release kinetics and their biological activity verified both *in vitro* and *in vivo* [172]. These compounds may allow for a more controlled and targeted delivery of CO and are good candidates for therapeutic development.

As a complementary approach, CO-sensitizing compounds such as 3-(−5'-hydroxymethyl-2'-furyl)-1-benzyl indazole, or YC-1, can be utilized to potentiate the biological activity of exogenously administered CO. This benzyl indazole derivative augments CO-mediated activation of soluble guanylate cyclase and markedly enhances the anti-aggregatory and vasodilator potency of CO [173,174]. Moreover, we recently demonstrated that YC-1 also induces the expression of HO-1 in vascular cells (175). The ability of YC-1 to sensitize soluble guanylate cyclase to CO and simultaneously stimulate the production of CO through the induction of HO-1 may provide an important mechanism by which YC-1 amplifies the vascular actions of CO. Thus, co-administration of YC-1 may increase the efficacy of CO and lessen the risk of CO-mediated toxicity by reducing CO dosing regimens.

Systemic or local application of the bile pigments biliverdin and bilirubin may provide another approach in treating vascular disease. The administration of biliverdin has salutary effects following transplantation or ischemia-reperfusion in several organs, including the heart [96]. In addition, the delivery of biliverdin prevents intimal thickening following arterial injury in rodents [73,74]. Surprisingly, the protective effects associated with the administration of bile pigments in animals occur with modest increases in the levels of circulating bilirubin. This finding is in-line with clinical studies showing that mild increases in serum bilirubin are sufficient to reduce the risk of coronary artery disease [14], suggesting that a suitable therapeutic window for these compounds exist. In this respect, a preliminary clinical report found that traditional biliverdin-containing Chinese medicines are safe and effective in treating chronic liver disease [176]. However, given the potential neurotoxicity associated with bile pigments [177], further pharmacological studies are needed to establish safe and effective treatment regimens. Since the cytoprotective properties of HO-1 may be mediated through the synergistic effects of its reaction products, dual therapy with CO and biliverdin may confer greater benefit than the singular application of either HO-1 product [178].

Circulating levels of bilirubin may also be elevated by blocking the conjugation and excretion of bilirubin into bile salts. The hepatic enzyme uridine-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) is responsible for conjugating bilirubin and genetic mutations in the UGT1A1 gene in humans (Crigler-Najjar or Gilbert's syndrome) that reduce the activity of the enzyme lead to increased levels of unconjugated bilirubin. Significantly, individuals with this syndrome exhibit a diminished risk for coronary artery disease [see 179]. Similarly, the homozygous Gunn rat which lacks the counterpart enzyme has elevated serum bilirubin levels and is also protected against vascular disease [73,119]. Thus, development of specific pharmacological inhibitors that target UGT1A1 may provide a unique approach to raise endogenous bilirubin levels. However, inhibition of UGTA1A must be carefully calibrated to avoid the development of liver dysfunction.

In some instances inhibition of HO-1 may be therapeutically desirable. Metalloporphyrins such as tin protoporphyrin, zinc protoporphyrin, and chromium mesoporphyrin are well-recognized and widely used non-selective inhibitors that block both HO-1 and HO-2 activity. They reversibly compete with heme for binding to HO and block HO activity both acutely and chronically. However, metalloporphyrins are not selective for HO and can regulate the activity and expression of numerous other proteins (180,181). Paradoxically, metalloporphyrins can induce HO-1 expression further confounding interpretations when using these inhibitors. Recently, several non-porphyrin imidazole-dioxalane derivatives have been isolated that are more selective for HO (see 182). Their inhibitory activity has been validated both in isolated organs and rodents, and certain compounds exhibit isoform selectivity for HO-1. However, additional pharmacokinetic and pharmacodynamic studies are needed to establish the therapeutic potential of these second generation HO-1 inhibitors. Finally, molecular approaches using antisense or small interference RNA (siRNA) technology have been successfully employed to knockdown HO-1 expression both *in vitro* and *in vivo* (183–185). Although siRNA shows much promise, current difficulties in delivery and potential off-target effects limit the clinical utility of this approach.

Conclusion

Studies in the past decade indicate that HO-1 is a promising candidate for the development of therapies to treat vascular disease. Several approaches can be used to target HO-1. The use of pharmacological inducers represents an attractive near-term strategy. Numerous inducers of HO-1 have been identified and quite a few are already in clinical use. Indeed, several vanguard drugs used to treat cardiovascular disease may exert their clinical effects, in part, via the induction of HO-1. The use of dietary supplements, either alone or in combination with HO-

inducing drugs, provides another viable avenue to target HO-1. However, the occurrence of polymorphisms in the promoter that minimizes the induction of HO-1 may limit the effectiveness of pharmacological and nutritional approaches in some patients. In this case, HO-1 gene therapy or direct application of HO-1 end products may be more efficacious. Inhalation of CO has proven highly effective in preclinical animal models of vascular disease and has recently been demonstrated to be well tolerated in phase I clinical trials. Furthermore, recently developed CO-releasing and CO-sensitizing molecules offer an appealing alternative or complement to inhalational gas therapy. Given recent experimental and epidemiological studies showing beneficial effects of biliverdin and bilirubin, the delivery of bile pigments may also be clinically useful. Since HO-1 and its end products are potentially toxic, a major challenge will be to develop clinically effective therapeutic regimes that target HO-1 without causing any detrimental effects.

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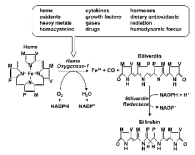

Figure 1.
Regulation of heme metabolism by heme oxygenase-1 (HO-1). HO-1 is induced by numerous biochemical and biophysical stimuli and catalyzes the oxidative degradation of heme into equimolar amounts ferrous iron (Fe^{2+}), carbon monoxide (CO), and biliverdin. Biliverdin is subsequently metabolized to bilirubin by biliverdin reductase. M, P, and V represent methyl, propionyl, and vinyl groups, respectively; NADPH, nicotinamide adenine dinucleotide phosphate.

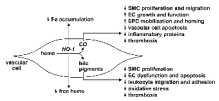

Figure 2.

Vasoprotective effects of heme oxygenase (HO-1) and its end products. HO-1 exerts a direct antioxidant effect by degrading pro-oxidant heme and by preventing intracellular iron (Fe) accumulation. In addition, the HO-1 end products, CO and the bile pigments biliverdin and bilirubin, possess potent anti-inflammatory, antioxidant, anti-apoptotic, and anti-thrombotic actions. Moreover, CO and bile pigments act to preserve vascular homeostasis at sites of arterial injury by influencing the proliferation, migration, and adhesion of vascular smooth muscle cells (SMC), endothelial cells (EC), endothelial progenitor cells (EPC), or leukocytes.

Table 1

Therapeutic Strategies Targeting HO-1 in Vascular Disease

A.	Enhancing Endogenous HO-1 Expression and/or Biological Activity
	• pharmacological or dietary induction of HO-1
	• gene delivery of HO-1
	• increasing substrate (heme) availability
	• administration of CO-sensitizing compounds
B.	Exogenous Delivery of HO-1 End Products
	• inhalation of CO
	• administration of CO-containing solutions
	• administration of prodrugs or CO-releasing compounds
	• administration of bile pigments
	• administration of CO and bile pigments
	• administration of CO and CO-sensitizing compounds
C.	Elevating Endogenous Bilirubin Levels
	• administration of uridine-diphosphate-glucuronyltransferase 1A1 inhibitors
D.	Inhibiting HO-1 Expression or Activity
	• administration of metalloporphyrins or imidazole-dioxolane compounds
	• use of HO-1 antisense technology
	• use of HO-1 small interference RNA technology