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Serial Review: Heme Oxygenase in Human Disease
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Heme oxygenase and the cardiovascular–renal system ☆

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Abstract

Heme oxygenase (HO) has been shown to be important for attenuating the overall production of reactive oxygen species (ROS) through its ability to degrade heme and to produce carbon monoxide (CO), biliverdin/bilirubin, and the release of free iron. Excess free heme catalyzes the formation of ROS, which may lead to endothelial cell (EC) dysfunction as seen in numerous pathological conditions including hypertension and diabetes, as well as ischemia/reperfusion injury. The upregulation of HO-1 can be achieved through the use of pharmaceutical agents, such as metalloporphyrins and some HMG-CoA reductase inhibitors. Among other agents, atrial natriretic peptide and donors of nitric oxide (NO) are important modulators of the heme–HO system, either through induction of HO-1 or the biological activity of its products. Gene therapy and gene transfer, including site- and organ-specific targeted gene transfer, have become powerful tools for studying the potential role of HO-1/HO-2 in the treatment of various cardiovascular diseases as well as diabetes. HO-1 induction by pharmacological agents or gene transfer of human HO-1 into endothelial cells (ECs) in vitro increases cell-cycle progression and attenuates Ang II, TNF-, and heme-mediated DNA damage; administration in vivo acts to correct blood pressure elevation following Ang II exposure. Moreover, site-specific delivery of HO-1 to renal structures in spontaneously hypertensive rats (SHR), specifically to the medullary thick ascending limb of the loop of Henle (mTALH), has been shown to normalize blood pressure and provide protection to the mTAL against oxidative injury. In other cardiovascular situations, delivery of human HO-1 to hyperglycemic rats significantly lowers superoxide (O_2^-) levels and prevents EC damage and sloughing of vascular EC into the circulation. In addition, administration of human HO-1 to rats in advance of ischemia/reperfusion injury considerably reduces tissue damage. The ability to upregulate HO-1 through pharmacological means or through the use of gene therapy may offer therapeutic strategies for cardiovascular disease in the future. This review discusses the implications of HO-1 delivery during the early stages of cardiovascular system injury or in early vascular pathology and suggests that pharmacological agents that regulate HO activity or HO-1 gene delivery itself may become powerful tools for preventing the onset or progression of certain cardiovascular pathologies.

Abbreviations: AA, arachidonic acid; Ang II, angiotensin II; ARF, acute renal failure; β-AR, β-adrenergic receptor; BP, blood pressure; cdk, cyclin-dependent kinases; CEC, circulating endothelial cell; cGMP, cyclic guanosine monophosphate; CKI, cyclin kinase inhibitors; CO, carbon monoxide; CoPP, cobalt protoporhyrin; CORM, carbon monoxide-releasing molecule; COX, cyclooxygenase; CPE, chronic pulmonary emphysema; cTAL, cortical thick ascending limb; CYP450, cytochrome P450; EC, endothelial cell; EC-SOD, extracellular superoxide dismutase; EETs, epoxyeicosatrienoic acids; EGF, endothelial growth factor; eNOS, endothelial nitric oxide synthase; GFR, glomerular filtration rate; GSH, glutathione; HDGF, hepatic-derived growth factor; HETE, hydroxyeicosatetraenoic acid; HIF-1α, hypoxia inducible factor-1-alpha; HO, heme oxygenase; HPH, hypoxic pulmonary hypertension; HPVSR, hypoxic pulmonary vascular structural remodeling; IMCD, inner medullary collecting duct; iNOS, inducible nitric oxide synthase; JGA, juxtaglomerular apparatus; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MSC, mesenchymal stem cells; mTALH, medullary thick ascending limb of the loop of Henle; NOD, nonobese diabetic; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; PETN, pentaesithrityl tetranitrate; PG, prostaglandin; PKC, protein kinase C; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; SMC, smooth muscle cell; SnMP, tin mesoporphyrin; SnPP, tin protoporphyrin; STZ, streptozotocin; TALH, thick ascending limb of the loop of Henle; TNF, tumor necrosis factor; TX, thromboxane; VEGFRI, vascular endothelial growth factor receptor I; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell; ZnDPBG, zinc deuteroporphyrin 2,4-bisglycol; ZnPP, zinc protoporhyrin.

☆ This article is part of a series of reviews on Heme Oxygenase in Human Disease. The full list of papers may be found on the home page of the journal.

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Contents

Introduction

The heme–heme oxygenase (HO) system is a regulator of endothelial cell (EC)[1] integrity and oxidative stress. Heme is the prosthetic group of numerous enzymes and is important to EC function through regulation of the activity of soluble guanylate cyclase (sGC), nitric oxide synthase (NOS), cytochrome P450 (CYP450) monooxygenases, cyclooxygenase (COX), and catalase [1]. HO exists as the constitutive isoenzymes HO-1, HO-2, and HO-3 [2]. HO-3 has no activity and is not expressed in humans (G. Scapagnini, personal communication). HO-2 is a constitutive isoform [3–5]. HO-1 is an inducible isoform that may be induced through the use of various pharmaceutical agents. We have shown that overexpression of HO can be successfully achieved through site- and organ-specific gene delivery by means of adenoviral, retroviral, and liposome-based vectors [6–11].

HO-1 and HO-2 are both viewed as playing a major role in heme breakdown [1,12] and are alike in terms of mechanism of heme oxidation, cofactor, and substrate specificities, and susceptibility to inhibition by synthetic metalloporphyrins, in which the central iron atom is replaced, for example, by zinc, tin, or other elements (reviewd in [1]). Synthetic heme analogues have been used in animals and in humans to suppress or induce HO activity and to inhibit development of severe hyperbilirubinemia in newborns [13–15]. The recognition that HO-1 is strongly induced by oxidant stress and its substrate, heme, in conjunction with the robust ability of HO-1 to protect against oxidative insult [7,16–19] has led to examination of the antioxidant nature of HO-1 and HO-2 activities [1,7,18,20]. Antioxidant effects arise from the capacity of HO-1 to degrade the heme moiety from destabilized heme proteins [21] and from biliverdin and bilirubin, products of HO with potent antioxidant properties (Fig. 1) [22,23].

Carbon monoxide (CO), an HO product as well, is not an antioxidant [24], but can cause induction of antioxidant genes; it also decreases superoxide (O_2^-) levels [25,26], increases GSH levels [27], and has an antiapoptotic effect [28]. Further, CO is a vasodilator, which has been shown to play an important role in the regulation of basal and constrictor-induced vascular tone in blood vessels [29–34]. Upregulation of bilirubin and CO, through the induction of HO-1, has shown promising results in protection against oxidative stress and injury whereas the absence of HO-1, for example, in mice, results in accelerated atherosclerotic lesion formation and vein-graft disease as well as elevated blood pressure, cardiac hypertrophy, and acute renal failure. In addition, the occurrence of organ damage and mortality are more frequent in HO-1 null mice [35]. Induction of HO-1 also prevents cell death, which is attributed to its augmentation of ion efflux and exportation of iron-binding protein [36]. The protective actions of HO-1 or CO are not confined to overtly oxidant processes, but rather extend widely to such pathalogic processes. Conners et al. [37] were the first to demonstrate that induction of HO-1 has an anti-inflammatory effect. This finding was substantiated by studies from other laboratories [38,39]. Further-

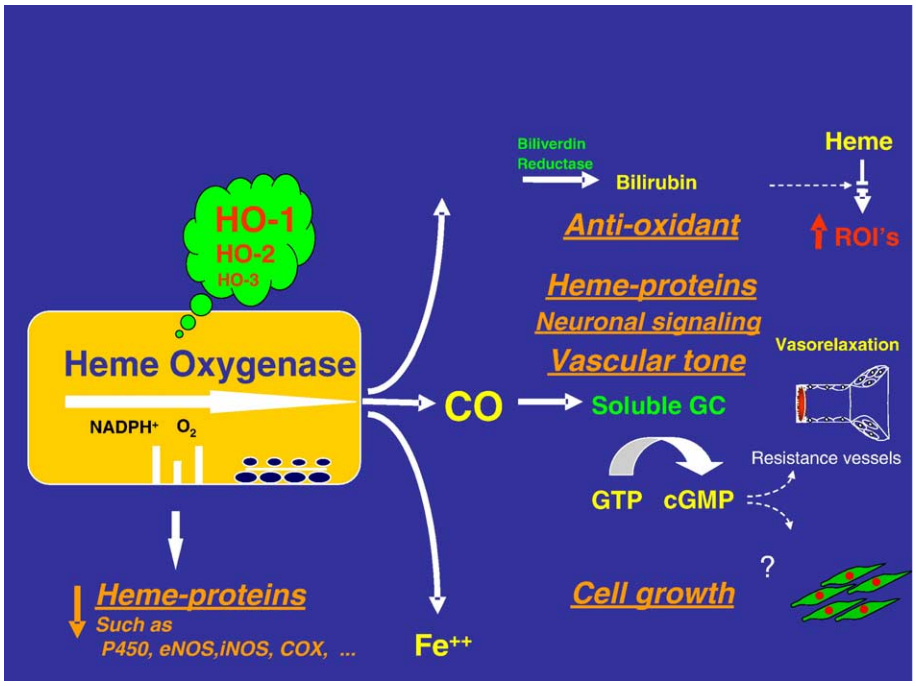

Fig. 1. The heme–HO system: proposed mechanism for HO function in cellular protection following oxidative injury.

more, HO-1 induction has been shown to be cytoprotective in atherosclerosis [40–42,40] sepsis [24], diabetes [26,43], lung injury [44], occlusive vascular disease, and ischemia [45–47].

HO-derived bilirubin and its antioxidant effect

HO-1 catalyzes the rate-limiting step in heme degradation to biliverdin. Biliverdin is, in turn, converted into bilirubin by the cytosolic enzyme biliverdin reductase at the expense of NADPH. Unconjugated bilirubin efficiently scavenges singlet oxygen and serves as a reducing agent for certain peroxidases, including horseradish peroxidase and prostaglandin H (PGH) synthase, in the presence of hydrogen peroxide or organic hydroperoxides. Biliverdin and bilirubin are reducing species and hence potential antioxidants.

An adult human produces about 300 mg of bilirubin per day [48,49]. Some 80 to 85% of the bilirubin produced in vivo is derived by heme catabolism of hemoglobin released by aging or damaged erythrocytes [48,49]. The biologic actions of bilirubin may be particularly relevant to prevention of the oxidant-mediated vasoconstrictive actions of tumor necrosis factor (TNF) and angiotensin II (Ang II) [50,51]. Bilirubin, in low concentrations, scavenges reactive oxygen species (ROS) in vitro, reduces oxidant-induced cellular injury, and attenuates oxidant stress in vivo [22,52–61]. Mazza et al. [62] have provided evidence of the antioxidant and cytoprotective effects of bilirubin in attenuating Ang II-mediated EC DNA damage and cell death. Morita et al. [63] showed that Ang II significantly stimulates O_2^- formation in monocytes, and that exogenously applied bilirubin suppresses not only O_2^- formation but also Ang II-enhanced chemotactic activity in monocytes.

Bilirubin has been shown to inhibit NADPH oxidase [64] and protein kinase (PKC) activity [65]; both have been shown to mediate Ang II-induced vascular injury [66,67]. Recently, biliverdin and bilirubin have been shown to preserve EC integrity [68] and prevent EC death and sloughing, to enhance vascular reactivity in diabetic rats [26,69], and to prevent restenosis [70,71]. Bilirubin is also implicated in reducing oxidative stress in experimental diabetes, in part, by increasing the bioavailability of nitric oxide (NO) needed for EC integrity. Bilirubin-mediated inhibition of PKC and NADPH oxidase may be one mechanism by which HO-1 attenuates the diabetes-mediated generation of oxidants and the uncoupling of EC nitric oxide synthase (eNOS). Glucose enhances EC O_2^- production, leading to increased vascular formation of the NO/O_2^- reaction product, peroxynitrite. Peroxynitrite oxidizes the active NOS cofactor, tetrahydrobiopterin, to cofactor inactive molecules, such as dihydrobiopterin. This uncouples the enzyme, which then preferentially increases O_2^- production over NO production [72]. Functional eNOS expression, rather than dysfunctional uncoupled eNOS (Fig. 2), may be increased by HO-1 gene expression. Peroxynitrite stimulates HO-1 in nondiabetic pathological conditions [73] but in diabetes [25,26], due to the glucose-suppressive effect on HO-1 gene expression [74]. Thus, the HO-1 gene expression-mediated increase in extracellular superoxide dismutase (EC-SOD) may protect eNOS from uncoupling, which in turn attenuates the diabetes-mediated generation of oxidants [75,76].

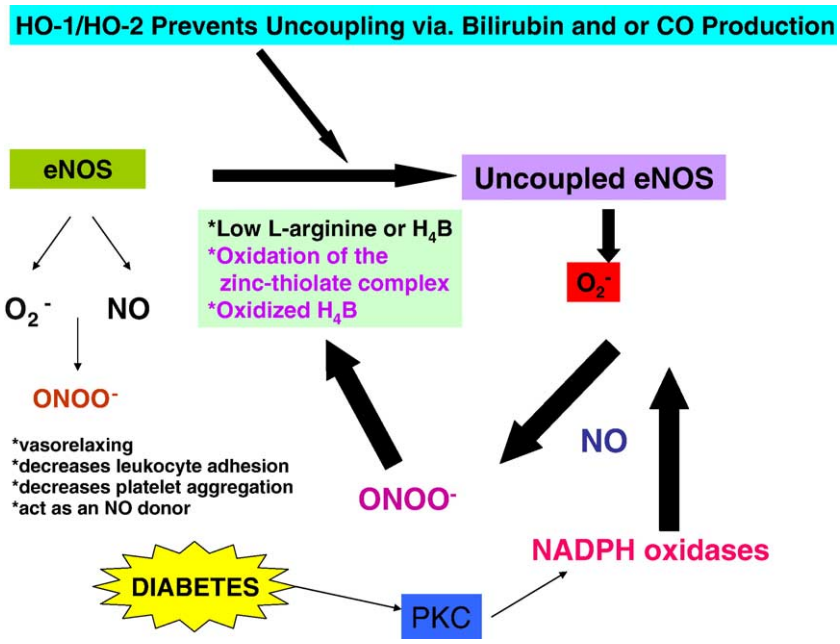

Fig. 2. Schematic representation of the possible interaction of HO/CO in the prevention of eNOS uncoupling.

HO-1-derived bilirubin has also been shown to display cytoprotective properties in the cardiovascular system [61,77]. Numerous reports indicate that a higher serum bilirubin level is associated with a decrease in the risk for coronary artery disease in humans. Free and albumin-bound bilirubin has been shown to inhibit oxidation of low-density lipoprotein (LDL) [54]. In renal vessels from rats pretreated with biliverdin, the lipopolysaccharide (LPS)-induced expression of P- and E-selectin was shown to be significantly attenuated, confirming that biliverdin or its metabolite, bilirubin, might account for the anti-inflammatory properties of HO [78]. Additionally, Wang et al. [79], employing a rat model of endotoxemia, examined the hypothesis that bilirubin is a key mediator of HO-1 cytoprotection. Bilirubin treatment improved survival of cells and attenuated liver injury in response to LPS infusion. Serum levels of NO and TNFα, key mediators of endotoxemia, and hepatic inducible nitric oxide synthase (iNOS) expression were significantly lower in bilirubin-treated rodents versus control animals. In addition, bilirubin treatment induced a threefold increase in LPS-mediated prostaglandin (PG) synthesis in the absence of significant changes in COX expression or activity, suggesting that bilirubin enhances substrate availability for eicosanoid synthesis.

HO-derived CO, vasodilation and antiapoptotic effects

Almost all CO produced in vivo comes from the degradation of heme by HO. Before CO was found to have an antihypertensive effect, Sacerdoti et al. [80] discovered that induction of HO-1 served to attenuate hypertension in spontaneously hypertensive rats (SHR). This finding provided a potentially crucial lead in defining the potential therapeutic benefits of regulating HO activity in the treatment of hypertension. Later, it was discovered that CO and NO have similar properties [81–83]; both behave as messenger and signaling molecules. Both CO and NO are capable of inducing the relaxation of blood vessels through vasodilation and inhibiting the proliferation of vascular smooth muscle cells (VSMC) [84]. Like NO, HO-derived CO influences the sGC and cyclic guanosine monophosphate (cGMP) pathways, which serve to regulate both blood pressure and vascular contractility [85]. sGC acts to increase cGMP, which in turn serves as a vasodilator to lower blood pressure levels. It has been demonstrated that by upregulating the HO system in young (8-week-old) SHR, coincidently, sGC and cGMP levels rise, which leads to a significant reduction in blood pressure [85,86]. On the other hand, by using an inhibitor of HO-1 activity, the blood pressure of rats undergoing HO-1 inhibition significantly increases [87]. Moreover, CO has been shown to inhibit platelet aggregation and to stimulate angiogenesis [88]. Interestingly, a study done by Ushiyama et al. [89] indicates that CO regulates blood pressure cooperatively with NO in hypertensive rats and by impairing the function and production of NO, HO gene expression is increased and the effect of CO in the regulation of blood pressure is, in turn, augmented.

HO-derived CO, bilirubin, and balloon angioplasty

Apart from being a vasodilator, CO has also been shown to protect against ischemic tissue damage through anti-

apoptotic mechanisms [46]. By inducing HO-1 during postischemic myocardial dysfunction, myocardial function is improved after total ischemia and reperfusion [90,91]. Kukoba et al. [92] injected hemin into rats before inducing ischemia in order to upregulate HO-1. They demonstrated that, during ischemia and reperfusion, left ventricular pressure decreased while end diastolic pressure, coronary perfusion pressure, and coronary resistance increased. The products of HO action are protective in rodent models of ischemia-reperfusion injury, allograft and xenograft survival, intimal hyperplasia following balloon injury, or chronic graft rejection [91]. HO-1 expression is increased in human atherosclerotic lesions [42,93], as well as in vascular EC and smooth muscle cells (SMC) exposed to oxidized LDL [41].

Moreover, Togane et al. [90] have reported that CO generated through HO inhibits mitogen-induced proliferation of VSMCs, which in turn inhibits neointimal formation. According to Tulis et al. [94], the pharmacological induction of HO-1 also attenuates neointima formation after balloon angioplasty-induced injury. Furthermore, they showed that HO-1 gene transfer attenuated the remodeling response to vascular injury by stimulating medial wall SMC apoptosis and inhibiting medial wall DNA replication [94]. Others have shown that adenovirus-mediated HO-1 overexpression inhibits the growth of SMCs in vitro and in vivo, as well as the upregulation of $p21^{Cip1}$ [95], suggesting the importance of HO-1 in vascular wall remodeling. Overexpression of human HO-1 in rabbit and rat ECs renders the cells resistant to hemoglobin toxicity and highlights not only the important metabolic and cytoprotective roles of the HO-1 gene [7,17], but also its importance in cell-cycle progression [7,19,96].

Discovery of the differential effects of HO-derived products on ECs and SMCs by Li Volti et al. [96] opens an avenue toward understanding the basic and clinical problems of neointimal formation. Three key findings substantiate this development. The key finding was that induction of HO activity in both ECs and SMCs [50,96,97], as well as other mammalian cells [98,99] regulates cell-cycle progression. The second was that, in SMCs, upregulation of HO-1 gene expression is associated with a decrease in cell-cycle progression. In ECs, unlike SMCs, overexpression of HO-1 increases cell-cycle progression and DNA distribution in the S and G_2/M phases [25,96]. The third key finding was that inhibition of HO activity with SnMP resulted in the enhancement of cell-cycle progression in SMCs. In contrast, cell-cycle progression was reduced by SnMP in ECs. The use of vinblastine demonstrated that it is possible to distinguish cells with DNA content at different phases of the cell cycle, i.e., G_2/M cells of low DNA ploidy [100]. The addition of vinblastine to EC and SMC cultures caused cell arrest in mitosis. The percentage of cells entering mitosis thus increases with time, and the rate of the increase reflects the kinetics of cell-cycle progression [101]. HO-1 inhibition increased DNA content in vinblastine-treated SMCs in the G_2/M phase, suggesting an increased rate of cell proliferation [25,96]. In contrast, in ECs, HO-1 inhibition decreased DNA content in the G_2/M phase, thus providing evidence of its activating effect on cell-cycle progression [25,102].

In a similar study, McClung et al. [70] also concluded that HO-1 has a differential effect on cell-cycle proliferation in ECs and VSMCs. In their study, inhibition of HO-1 led to VSMC proliferation, thus, causing stenosis in the lumen (Fig. 3B) while induction of HO-1 stimulated neointima

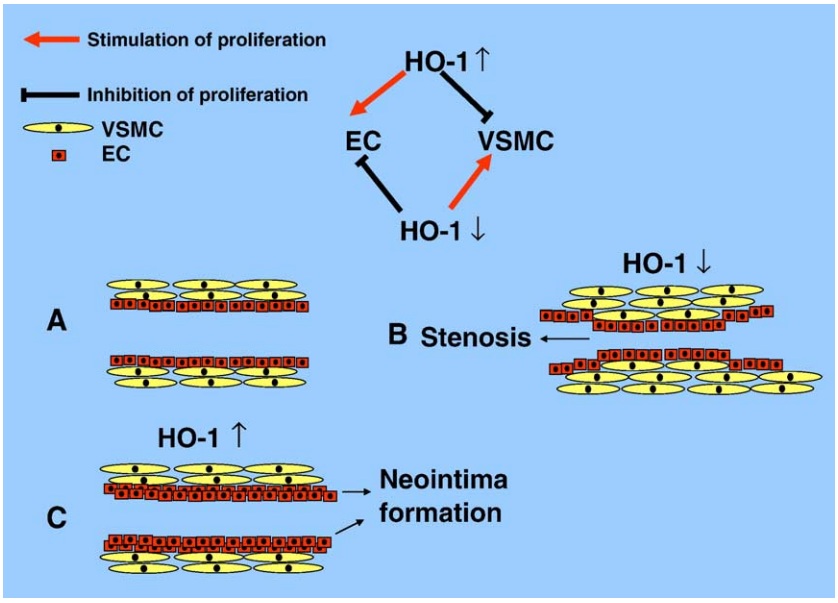

Fig. 3. The possible role of HO/CO in stenosis and neointima formation: a decrease in HO-1 produces vascular smooth muscle cell proliferation and causes stenosis (B). By increasing HO-1, vascular smooth muscle cell proliferation is inhibited and prevents neointima formation (C). This in turn produces healthy tissue (A).

formation (Fig. 3C), compared to control (Fig. 3A). Kong et al. have more recently shown that transplantation of circulating endothelial progenitor cells overexpressing eNOS and HO-1 to balloon-induced injured vessels served to enhance the vasculoprotective properties of the reconstituted endothelium, which in turn led to inhibition of neointimal hyperplasia [70,103]. These findings are of particular relevance since the differential role of HO-1 could be a key factor in vascular wall remodeling in different clinical situations, such as occurs in hypertension or VSMC hyperplasia, or in the recovery of the vascular wall after mechanical injury, such as restenosis.

HO-1 promoter polymorphism

A series of studies have examined HO-1 polymorphism in human disease. Yamada et al. [93] examined the polymorphism of the HO-1 gene in patients with chronic pulmonary emphysema (CPE). Specifically they examined a (GT)*n* dinucleotide repeat in the 5′-flanking region of the human HO-1 gene and reported that the larger the size of the (GT)*n* repeat in the HO-1 gene promoter the greater the chance of reducing HO-1 inducibility by reactive species in cigarette smoke, resulting in the development of emphysema. Kanai et al. [104] examined the polymorphic (GT)*n* repeat in German and Japanese newborns in an attempt to explain the higher incidence and severity of hyperbilirubinemia in the latter population. A significant difference in the allele frequency of the (GT)*n* repeats between the two populations was found, but they found no relationship between those polymorphisms and neonatal hyperbilirubinemia. Ono et al. [105] reported that the AA genotype of HO-1 is associated with an increased incidence of hypertension in Japanese women. They spectulated that CO can attenuate NO-induced vasodilation; thus, a high expression of HO-1 may result in hypertension in women. Kanai et al. [106] found no association of HO-1 polymorphism to susceptibility to Kawaski disease in Japanese children. Chang et al. [107] examined polymorphism in the HO-1 promoter in relation to the risk of oral squamos cell carcinoma (OSCC) in Asian male areca chewers. They examined the allelotypic frequency of (GT)*n* repeats in 83 controls, 147 OSCC patients, and 71 oral submucous fibrosis patients. Longer (GT)*n* repeat alleles in the HO-1 promoter were associated with the risk of areca-related OSCC, while a shorter (GT)*n* allele may have protective effects, thus confirming the observation of Yanada et al. that the longer the (GT)*n* repeat, the greater the susceptibility to disease. The results above indicate that, in certain disease conditions, the ability of an individual to upregulate HO-1 may be an important protective factor.

The recent elegant work by Funk et al. investigating the association of HO-1 repeat polymorphism and gene expression showed that short GT repeats were associated with upregulation of HO-1 and therefore influenced susceptibility to ischemic injury [108]. Short repeats (<25 GT repeat) in the HO-1 gene promoter confer reduced risk for a cereberovascular event in individuals with normal plasma lipid levels [108]. This may explain controversial findings in differing populations. Further, this may also explain the different results of some investigators in different strains of animals. In support of these findings, the work of Schillinger et al. showed that the HO-1 promoter genotype that controls the degree of HO-1 upregulation in responses to stress stimuli is associated with the postintervention inflammatory response and restenosis risk after balloon angioplasty [109].

Cytoprotective effects of HO-derived CO and bilirubin following myocardial infarction

The induction of HO-1 may also have therapeutic benefits during chronic heart failure. Gu et al. [110] recently demonstrated that upregulation of HO-1 during heart failure serves to mitigate pathologic left ventricular remodeling and reduce myocardial hypertrophy, oxidative stress, and inflammatory activation. Upregulating HO-1 also has the potential of attenuating cardiac hypertrophy in genetically hypertensive rats [111]. More recently, studies by Ooi et al. [112] in mice suggest that HO-1 confers significant antioxidant and cytoprotective effects in the heart. They showed that HO-1 is induced by β-adrenergic receptor (β-AR) stimulation and may protect against β-AR-mediated apoptosis. Further, it has been demonstrated that induction of HO-1 increases adult cardiomyocyte tolerance to ischemia after in vivo transplantation [113]. Autologous atrial cardiomyocytes are a readily available cell source for infarct repair; however, they readily undergo apoptosis, precluding their use as cellular repair grafts. This study demonstrates that preconditioning with HO-1 acts to retain functional viability in vivo in adult cardiomyocyte cellular grafts after implantation. This, in turn, may prove effective in repairing infarcted myocardium. In addition, Tang et al. [114] have performed similar studies with mesenchymal stem cells (MSC). They demonstrated that hypoxia-regulated HO-1 vector modification enhances the tolerance of engrafted MSCs to hypoxia-reoxygenation injury in vitro and improves their viability in an ischemic milieu. These findings may also make cell therapy more effective in infarct repair.

Carbon monoxide-releasing molecules

The discovery, by Motterlini et al. [115], that transitional metal carbonyls have the ability to bind and release CO has furthered the understanding of CO in vascular biology. Such pharmacological inducers have been termed carbon monoxide-releasing molecules (CORMs) and have contributed greatly to our understanding of the role each HO product

might play in vascular cytoprotection [115,116]. Water-soluble CORM-3 (Ru(CO)3Cl(glycinate)) has been recently developed and has been used to confirm the vasodilatory and cardiovascular benefits of CO. CORM-3 (Fig. 4) has the capability of generating aortic vasodilation ex vivo and reduces blood pressure in vivo [115–118]. Guo et al. showed that the administration of CORM-3 could reduce infarct size in vivo when given in a clinically relevant manner at the time of reperfusion [46]. The results indicate that infarct size was dramatically smaller in treated mice. This experiment shows that CORM-3 can be used not only to bypass HO-1 induction but also to provide the beneficial effect needed as evidenced by a decrease in injury during myocardial ischemia–reperfusion [46]. Although the cardioprotective effect of CORMs occurs through the release of CO and may also occur through the induction of HO, it is by means of HO that the degradation of heme releases CO and produces favorable cardiovascular benefits. In a study conducted by Stanford et al. [84], pulmonary artery SMCs showed an elevation of HO-1 in the presence of oxidants. Through the use of the CORM tricarbonyldichlororuthenium (II) dimer, the proliferation of pulmonary artery SMCs was shown to profoundly decrease. It is apparent that the upregulation of CO through HO-1 has a profound opposing effect to hypertension. Rodella et al. (personal communication). have shown that CORM-3 renders ECs resistant to oxidative stressors even when HO-1/HO-2 are inhibited with SnMP.

CO produced from heme metabolism in blood vessels is reported to elicit relaxation [32,119] through the elevation of cGMP levels [120]. Ndisang et al. [121] studied the administration of hemin, an inducer of HO, and its effect on the pulmonary artery in young SHR and demonstrated that hemin decreases blood pressure (BP) by inducing the HO/CO-sGC/cGMP system. Impairment of this system in the pulmonary artery may be indicative of the pathogenesis and development of hypertension. Sacerdoti et al. [80] and Levere et al. [122] have shown that upregulating the HO/CO system lowers BP in young SHR but not in adults. In young SHR, blood pressure rises and continues to increase with aging whereas adult SHR have an established hypertension

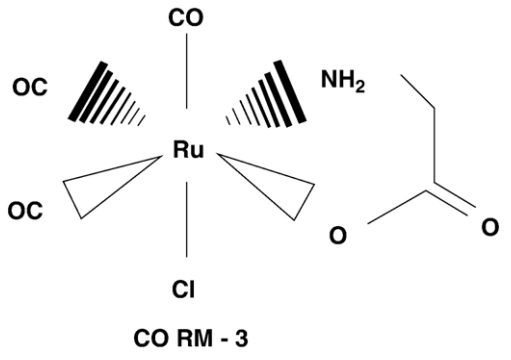

Fig. 4. Schematic representation of the structure of CORM-3 [115].

[80,121]. It was hypothesized that if the HO/CO system is defective in young SHR, then HO-1 inducers could enhance the activity of this system.

In assessing the effect of CO-releasing molecules, such as CORM-3, it is essential to use a pharmacological dose. Motterlini's group has shown that a physiological dose of CO has a powerful vasodilator effect and attenuates the phenylephrine-contracting effect [118,123,124]. However, nonphysiological levels of CO-releasing molecules will result in less relaxation or contraction [R. Motterlini, personal communication]. This is essentially similar to the effect of acute and chronic HO-1 inducers on cell survival and on heme-dependent enzymes [125,126]. For example, moderate induction of HO-1 increases cGMP, but extreme levels of HO-1 lead to a decrease in cGMP [125], which has been attributed to the stripping of heme from the sGC protein. This is analogous to the roles of COX-2 and NOS; in cell function, for example, moderate levels of COX-2 are beneficial while an extreme elevation in COX-2 can have harmful effects. A similar situation exists with inhibitors of HO. At low levels, these inhibitors can decrease CO and bilirubin; however, at a higher concentration, they can inhibit other heme proteins, including CYP450, NOS, or COX [127].

HO-1/HO-2, renal function and hypertension

HO is present in the kidney as the constitutive isoform, HO-2, and as the inducible isoform, HO-1. The discovery that atrial natriuretic peptide is an inducer of HO-1 in renal and endothelial cells [128] opens a new direction in the immunoprotection of renal function during the use of cyclosporin [129]. Cyclosporin is an immunomodulator and provides cytoprotection during organ transplant; however, due to the cyclosporine-suppressive effect on HO-1, its use became limited. Rifampin is another immunomodulator and its beneficial effect has been [130] attributed to its ability to enhance HO-1 gene expression. Atrial peptide-mediated upregulation of HO-1 [128,129,131] has been shown to ameliorate the effect of cyclosporine on renal function, presumably via a cGMP-dependent mechanism [131].

Induction of HO-1, by retroviral delivery of the human HO-1 gene into human microvessel endothelial cells, has been shown to attenuate TNFα-mediated cell death [50,51]. HO-1 gene upregulation has also been shown to attenuate Ang II-mediated DNA damage in endothelial and kidney cells [27,62]. CO, in addition to activating the cGMP pathway and eliciting vasodilation [132], has also been shown to function as an anti-inflammatory and cytoprotective molecule in renal tissue. Further, CO has also been shown to participate in the regulation of ion channels, including the apical 70-pS K-channel activity of the thick ascending limb of the loop of Henle (TALH) [133]. It is now believed that CO-induced NO may have a differential effect

on cGMP due to the low affinity of sGC for CO; however, it may cause the robust production of cGMP [134].

Abnormalities in kidney function are responsible, in part, for the elevation of blood pressure in SHR. Blood pressure elevation in SHR has been shown to decrease through renal transplantation from normotensive donors [135]. Renal abnormalities leading to hypertension consist of reduced excretion of sodium and water, decreased renal blood flow, and decreased glomerular filtration. Such abnormalities can be corrected by inducing HO-1 activity. HO-1 induction has also been shown to exert a protective effect on renal function in animal models of rhabdomyolysis [136], cisplatin nephrotoxicity [98], and nephrotoxic nephritis [137]. Further, the products of HO provide a protective role in acute renal failure (ARF) and hypertension. Arregui et al. [138] have demonstrated that HO-1-derived CO increased blood carboxyhemoglobin levels, renal blood flow, glomerular filtration, and urinary cGMP excretion. In a similar study, it was verified that the renal vascular bed of hypertensive animals exhibits a greater propensity to upregulate the HO system, which may serve to lower blood pressure in hypertension [139]. In another study [140], it was established that heme-induced renal vasodilation, which increases renal blood flow, is a COX-dependent response whereas heme-induced diuresis and natriuresis are HO-dependent responses, involving inhibition of tubular reabsorption of water and sodium.

A well-known source of oxidative stress in the kidney is the inflammatory cytokine, Ang II. Ang II is systemically or locally elevated in many forms of hypertension [141] and is associated with increased vascular O_2^- production [142] which contributes to renal injury [143,144]. Moreover, increased O_2^- has been shown to contribute to the vascular and renal effects of Ang II [145]. Ang II induces renal oxidant stress and HO activity, suggesting that upregulation of HO-1 in renal proximal tubules is essential in the amelioration of oxidant-mediated injury [143]. In addition, HO-1 is reported to counteract aldosterone-elicited arterial injury through the inhibition of oxidative stress as well as inflammatory reactions [112]. Thus, it is apparent that HO-1 and its products play a critical protective role against cardiovascular injury. Fig. 5 illustrates the roles of bilirubin and CO in preventing DNA degradation, decreasing p21 and p27 levels, and enhancing cell-cycle progression [50,97].

In a rat model of radiation-induced nephropathy, elevated glomerular HO-1 expression was prevented by treatment with AT_1-receptor antagonists, which block the upregulation of HO-1 expression. This suggests that Ang II may be a mediator of HO-1 induction [146]. Furthermore, Haugen et al. [143] demonstrated elevated levels of HO-1 in the renal proximal tubule of rats treated with Ang II, which was associated with increased HO activity. These data demonstrate that Ang II directly induces HO-1 in renal proximal tubular epithelial cells in vitro. In another study, Ang II infusion decreased glomerular filtration rate (GFR) and increased proteinuria, which led to hypertension. HO-1 upregulation naturally followed and provided a cytoprotective effect [144]. Once again, the evidence suggests that HO-1 has a protective effect against the development of hypertension.

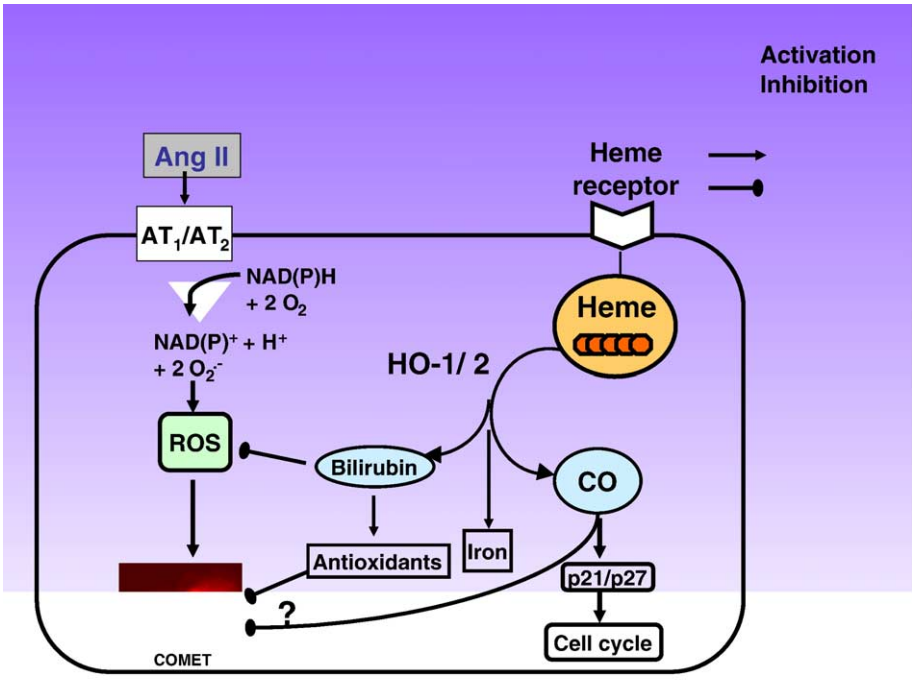

Fig. 5. Schematic representation of the hypothesis that overexpression of HO-1 attenuates the cytostatic effects of Ang II and DNA degradation as measured by COMET assay. CO inhibits p21 and p27 and increases cell-cycle progression while bilirubin inhibits ROS-mediated DNA degradation.

In contrast, Ishizaka and Griendling have shown that treatment of rat VSMCs with Ang II decreased HO-1 mRNA levels and this decrease was blocked by losartan, a selective AT_1-receptor antagonist [147]. It is conceivable then that Ang II-mediated upregulation of HO-1 subserves mechanisms that counteract the action of Ang II. The recent findings that HO-1 gene transfer ameliorated oxidative tissue injury [9,28] and that oxidant-induced cellular injury was increased in HO-1 knockout mice [148] and in human HO-1 deficiency [149] provide further evidence that HO-1 acts favorably against oxidative stress. Moreover, Ang II, delivered by an osmotic minipump, provoked systemic hypertension, which was associated with an elevation of vascular and renal HO-1 [143,144,150]. Importantly, in animal models, Ang II-induced O_2^- production and elevated BP were prevented by preadministration of other antioxidant genes, such as SOD [151] or the SOD mimetic tempol [152].

Yang et al. [10] proposed that overexpression of HO-1, in rats transduced with retroviruses containing the human HO-1 gene, would significantly attenuate pressor responsiveness to Ang II and ameliorate Ang II-induced hypertension in rats chronically infused with Ang II (Fig. 6). The postulated mechanisms by which overexpression of HO-1 improves vascular function and ameliorates both genetic and experimental (Ang II-induced) forms of hypertension include the following: the ability of CO to inhibit constrictor responsiveness to myogenic stimuli and attenuate the sensitivity of arterial vessels to vasoconstrictors [32,132]; the capacity of biliverdin and bilirubin, as antioxidants, which may downregulate the activity of redox mechanisms involved in the vascular actions of Ang II and in the development of hypertension [153]; the potential that HO-1 overexpression minimizes oxidative stress as a result of lowering cellular heme; and the ability of HO activity to affect the expression of COX and CYP450 [154,155]. The production of constrictor eicosanoids, including endoperoxides and thromboxanes as well as 20 HETE [156–159], would be regulated by the levels of HO-1 [80,155,160–162]. The fact that Ang II administration rapidly increases HO-1 expression and HO activity in several tissues [67,144,150] further suggests that the heme–HO-1 system serves as a control mechanism to the pressor activity of Ang II.

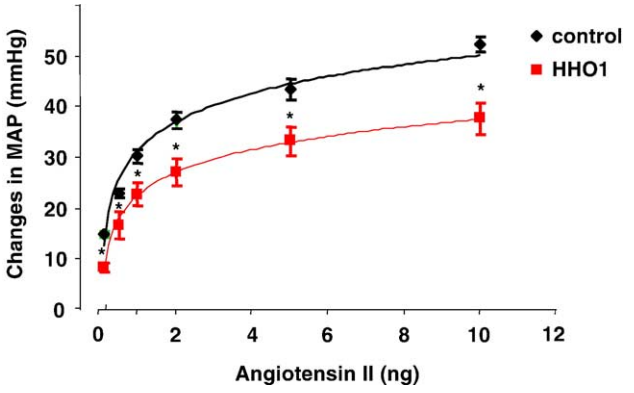

Fig. 6. Angiotensin II-induced blood pressure increase is attenuated in rats expressing the human HO-1 gene using retrovirus gene transfer [10].

HO-arachidonic acid metabolism: Role of CYP-AA metabolism

The metabolites of arachidonic acid (AA) metabolism by CYP450-dependent enzymes have been associated with the development and maintenance of hypertension (Fig. 7). When compared to control groups, CYP content and activity are increased in the kidneys of SHR [80,163]. CYP enzymes catalyze the metabolism of AA to four epoxyeicosatrienoic acids (EETs), ω/ω-1 alcohols (20-HETE and 19-HETE), and six regioisomeric *cis–trans*-conjugated monohydroxyeicosatetraenoic acids (HETEs) [164,165]. Some of these metabolites (e.g., 5,6-EET and 20-HETE) can be processed further by COX to products having biological activities [166,167]. Metabolites of AA, via the CYP450 pathway, are endowed with biological activities most relevant to the vascular and renal mechanisms of blood pressure regulation. 20-HETE acts as a vasoconstrictor whereas 19-HETE functions to increase sodium retention by acting as a potent sodium-potassium-ATPase stimulator. Furthermore, 20-HETE can stimulate contraction of VSMC [168], inhibit Na^+-K^+-ATPase [169], and reduce the activity of potassium channels in arterial smooth muscle and renal tubular cells [170–172]. 20-HETE also affects the movement of ions, constricts blood vessels, participates in tubuloglomerular feedback, and acts as mitogen [173,174], effects that are prohypertensive. Together, both monooxygenases serve to increase blood pressure. In 1990, studies from this laboratory described that hypertension could be attenuated in SHR through administration of heme arginate, a potent inducer of HO-1 [122]. As CYP450 is a heme-containing protein, CYP450 levels are regulated in part by the availability of heme. These experimental data indicate that the increase in HO activity is associated with a parallel decrease in CYP450 content and in the activity of CYP450 ω/ω-1 arachidonate hydroxylases in SHR kidneys.

HO-derived CO has been reported to inhibit the activity of CYP450 [175] and the generation of vasoconstrictive substances, such as 20-HETE, thus ameliorating the development of hypertension. Sacerdoti et al. [80] used $SnCl_2$, a specific inducer of renal HO, to demonstrate that increased HO activity, which resulted in depletion of the CYP-AA metabolites, 20-HETE and 19-HETE, was associated with reduction in BP. In addition, Sabaawy et al. [176] have shown that a single intracardiac injection of retroviral vector containing the human HO-1 gene attenuated the development of hypertension in 5-day-old SHR. Others have shown that administration of heme arginate caused a rapid decrease in BP in young SHR [122,162,177] and that pretreatment with ZnDPBG, a potent HO inhibitor [178], greatly attenuated the antihypertensive response to heme arginate. Thus, it appears

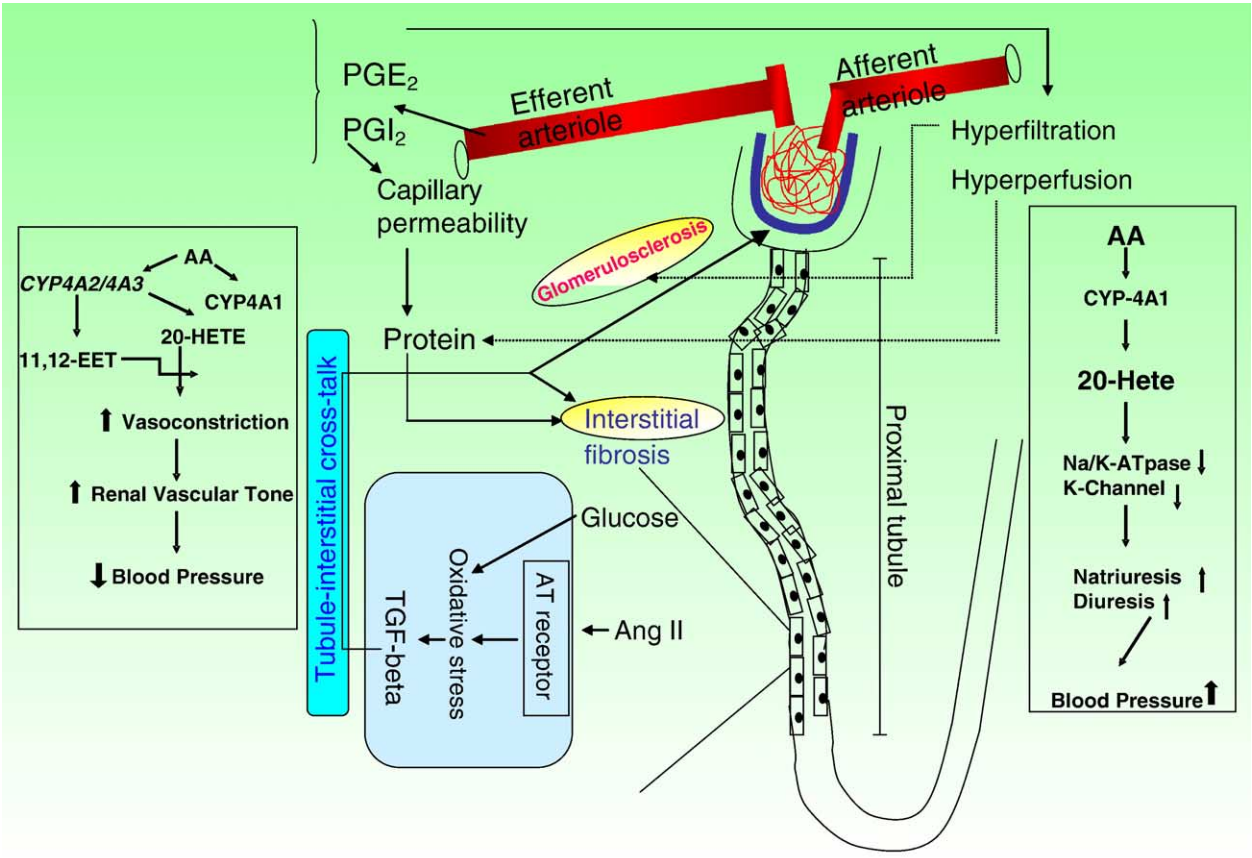

Fig. 7. Schematic presentation of HO-1 interaction to CYP450 and COX metabolites.

that the antihypertensive effect of HO enhancement may be due, in part, to blunting the vasoconstrictor action of 20-HETE formed via CYP [157,170,173].

More recently, HO inhibitors have been shown to decrease renal blood flow acutely, implying that the renal HO system supports the renal circulation via formation of CO [140,179,180]. HO-1/HO-2 and CYP450 are expressed in the renal medulla [173,181,182], in the arterial and preglomerular arteries [157], and in ECs [96,155,183,184]. Further studies have been performed in which the human HO-1 gene was delivered to SHR using a concentrated infectious viral particle [185]. Treated rats demonstrated a functional expression of the human HO-1 gene, which was associated with a significant decrease in BP in young SHR. These rats also showed a significant reduction in urinary 20-HETE, which is believed to be partially responsible for this decrease in BP [185].

HO-arachidonic acid metabolism: Role of cyclooxygenases

COX-1 and COX-2, heme-dependent enzymes, are responsible for the conversion of AA to PGH_2, which is further metabolized by thromboxane (TX) synthase, PGE synthase, and prostacyclin synthase to TXA_2, PGE_2, and PGI_2, respectively, and to other prostaglandins ($PGF_{2\alpha}$ and PGD_2) by other proteins (Fig. 8). The two COX isoforms are expressed in the rat kidney. COX-1 is constitutively expressed and has been localized to arteries and arterioles,

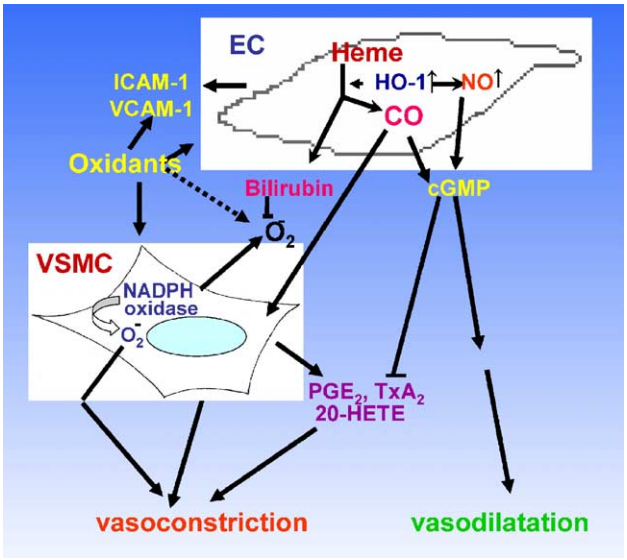

Fig. 8. Schematic presentation of interaction of HO/CO in endothelial cells and vascular smooth muscle cells.

glomeruli, and collecting ducts [186]. Although COX-2 is considered to be the inducible form, it is constitutively expressed in the TALH and in the region of the macula densa, primarily the cortical structure [187,188], where it is involved in the stimulation of renin release from the juxtaglomerular cells [189]. Studies have demonstrated that, in cells overexpressing HO-1, COX isoform expression and activity are impaired, and induction of HO-1 by $SnCl_2$ significantly reduced the expression of COX-2 in the rat kidney. Other studies have shown that eicosanoid-dependent mechanisms participate in the regulation of ion and water movement in the mammalian nephron [165]. COX-derived eicosanoids may be responsible for either prohypertensive or antihypertensive mechanisms. PGI_2 is a potent vasodilator and early studies have documented that endogenous PGE_2 production increases in response to volume depletion to help maintain GFR by dilating the afferent arteriole [190].

In the kidney, COX-2 expression is restricted to macula densa-containing segments, the cortical thick ascending limb (cTAL) [187], and, at significantly lower levels, the inner medullary collecting duct (IMCD). The level of COX-2 in these segments is regulated by dietary salt intake. In rats on a low-salt diet, cortical COX-2 increases while it decreases in rats on a high-salt diet, suggesting a role in the regulation of glomerular circulation and renin release. On the other hand, medullary COX-2 expression increased in rats on a high-salt diet and decreased in rats on a low-salt diet, suggesting a role in the regulation of salt excretion [187]. Traynor et al. [189] have recently demonstrated that COX-2 inhibition abolished the increase in renin release stimulated by low luminal NaCl concentration, supporting the role of macula densa COX-2 in the regulation of renin release. Other convincing evidence was provided by studying COX-$2^{-/-}$ null mice; these mice have attenuated renin expression in response to either a low-salt diet [191] or ACE inhibition [192].

COX may convert the CYP-AA metabolite, 20-HETE, to a vasoconstrictor in rat aortic rings [156,167]. Sessa et al. [160] have also showed that upregulation of HO by $SnCl_2$ resulted in corresponding decreases in renal heme content, CYP450 content, and renal thromboxane A_2 synthase activity in young SHR. These results also demonstrated that renal HO-1 induction via $SnCl_2$ administration affected COX-2 protein expression, leading to a marked decrease in its levels in the cortex as well as in the inner and outer medulla [154]. Haider et al. have shown that upregulation of HO activity elicits a decrease in PGE_2 levels in rabbit coronary microvessel ECs [193]. Several reports [187,189,191] have shown that macula densa COX-2 is involved in the regulation of renin release from the juxtaglomerular apparatus (JGA) in the afferent arteriole. These support the notion that HO activity, through regulation of heme content and CO generation, may modulate COX expression and/or activity on the molecular or biochemical levels. Whether the HO system regulates renin release, directly or indirectly via COX-2, remains to be investigated.

HO and endothelial cell-cycle progression in diabetes

An expanding body of information has shown that progression through the mammalian cell cycle is orchestrated by distinct, multiple holoenzymes composed of catalytic subunits, cyclin-dependent kinases (cdk), whose activities depend upon a regulatory protein called cyclin [194–196]. It is well established that cdk levels remain high throughout the cell cycle. Their activity is mainly regulated by cyclin binding, with the levels of cyclin fluctuating at different stages of the cell cycle. Cell-cycle transition from G_1 to the S phase is controlled by three types of cyclins, D, E, and A. Different cyclins are expressed at specific stages of the cell cycle and bind to a particular cdk. In addition to the cdk-specific positive regulation of the cell cycle, cdk activities are also controlled by a new class of small proteins, the so-called cyclin kinase inhibitors (CKI); the latter bind to cyclin-cdk complexes and inhibit their kinase activity. CKI inhibitor proteins are specific for cdk4 and cdk6 [197,198]. Overexpression of these inhibitory molecules leads to cell-cycle arrest in G_1.

High glucose conditions facilitate the oxidative-mediated inhibition of EC proliferation [199]. Overexpression of human HO-1 in ECs may have the potential to provide protection against a variety of agents that cause oxidative stress. The role of endogenously produced CO in the prevention of EC apoptosis has been addressed by examining the effect of conditions that affect HO activity and expression [200]. The antiapoptotic effect of HO-1-derived CO release has been shown to be mediated via the activation of p38 of mitogen-activated PKC [61,201]. Increased HO-derived CO has been shown to significantly shift, to the left, the concentration–cell death curve. This effect, however, can be reversed by the inhibition of HO [73]. These observations support the idea that the vascular HO system involves mechanisms that decrease apoptosis mediated by oxidative stress-inducing factors, including hypoxia or inflammatory molecules, such as TNF [50,51,202].

Using retrovirus-mediated human HO-1 gene transfer, it was demonstrated that HO-1 attenuates glucose-mediated cell growth arrest and apoptosis in human microvessel ECs [25]. In this study, incubation of ECs in a high-glucose medium resulted in a decrease of HO activity and a decrease in HO-1 and HO-2 proteins compared with cells exposed to low glucose or cells exposed to mannitol. As shown in Fig. 9A, apoptosis was induced in both control ECs and ECs transduced with HO-1 antisense. In contrast, cells transduced with HO-1 in the sense orientation were not affected by glucose. However, the addition of tin mesoporphyrin (SnMP), an inhibitor of HO activity, reversed the cytoprotective effect of HO-1 overexpression against the glucose (Fig. 9B).

Overexpression of HO-1 was coupled with an increase in HO activity and CO synthesis, decreased cellular heme, and acceleration in all phases of the cell cycle. The rate of the

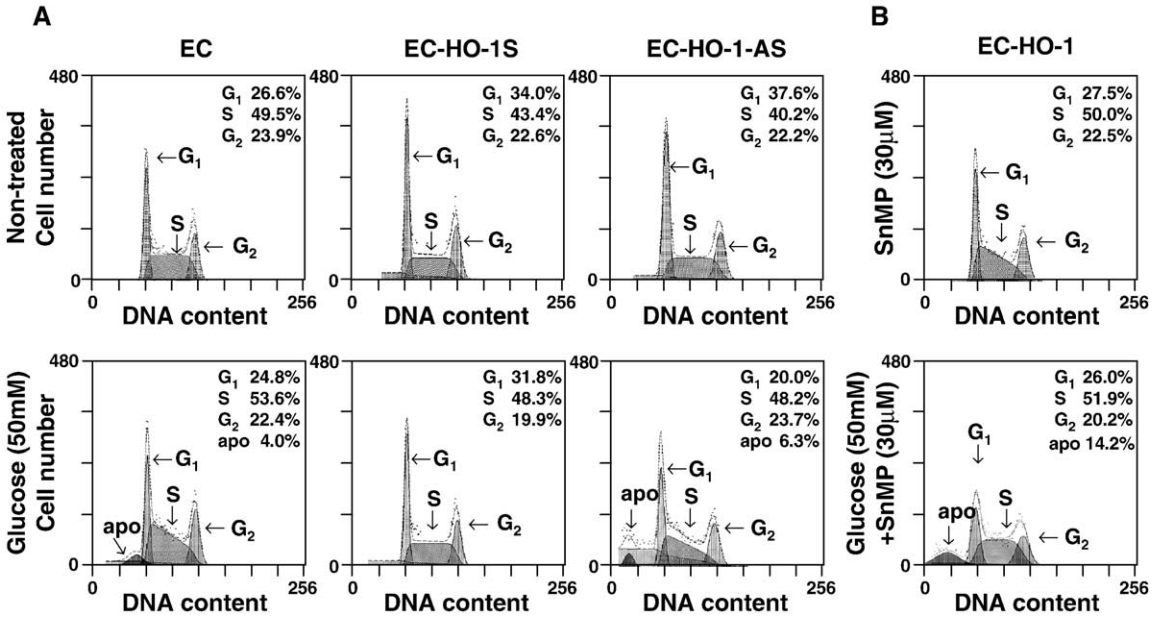

Fig. 9. The effect of high glucose on DNA distribution: (A) Control cells, nonexposed cells, and glucose-exposed control cells and cells transduced with the HO-1 sense or HO-1 antisense were stained with DAPI and analyzed by flow cytometry. Representative DNA distributions are shown. (B) Cells transduced with HO-1 and treated with SnMP while exposed to high glucose were stained with DAPI and analyzed by flow cytometry. Representative DNA distributions are shown. EC, control endothelial cells; EC-HO-1S (sense), endothelial cells transduced with the HO-1 in the sense orientation; EC-HO-1AS (antisense), endothelial cells transduced with the HO-1 in the antisense orientation [25].

cell cycle or cell birth rate was increased in cells overexpressing HO-1, but was decreased in cells underexpressing HO-1 compared with control cells. Exposure to high glucose significantly decreased cell-cycle progression in control cells and in cells underexpressing HO-1, but did not decrease cell-cycle progression in cells overexpressing HO-1. The protein levels of the cdk inhibitors, p21and p27, were greatly affected by the level of expression of HO-1. High glucose induces p21 and p27 in control cells, but not in cells overexpressing HO-1. The addition of SnMP reversed the HO-1-mediated decrease of p21 and p27 in cells overexpressing HO-1. As seen in Fig. 10, the basal and glucose-induced p21 levels were decreased in cells overexpressing HO-1 compared to control cells. In contrast, in HO-1-deficient cells, basal and glucose-induced p21 levels were increased. Likewise, basal and glucose-induced p27 levels were significantly reduced in cells overexpressing HO-1 and increased in HO-1-deficient cells compared to control cells. These findings identify a novel effect of HO-1 on EC growth and indicate that heme metabolism and HO-1 expression regulate signaling systems in cells exposed to high glucose, which control cell-cycle progression.

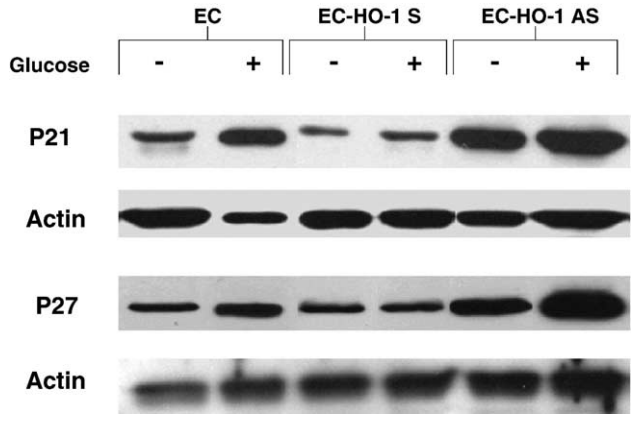

Fig. 10. Effect of high glucose on p21 and p27 protein levels in cells overexpressing (HO-1 sense) and underexpressing HO-1 (HO-1 antisense). Cells were exposed to glucose. The levels of p21 and p27 were visualized by immunoblotting with antibodies against p21 and p27. Representative blots are shown [25].

The view that HO-1 and HO-2 contribute to antiapoptotic mechanism(s) has also received support from studies on the effect of HO inducers on protection from oxidative stress-mediated cell death [95]. Another study was performed in which a retroviral vector was used to investigate the physiological significance of human HO-1 overexpression on cell-cycle progression in the presence and absence of oxidants, such as pyrrolidine dithiocarbamate [97]. This was suggested to be a potential therapeutic means for attenuating the effects of oxidative stress-causing agents [97]. The role of human HO-1 in cell-cycle progression following exposure to heme or human HO-1 gene transfer, for the identification of target genes associated with human HO-1-meditated increases in cell-cycle progression, was examined using cDNA microarray technology [102]. The results showed the upregulation of several genes associated with cell-cycle progression, including cyclin E and D; downregulation of the cdk inhibitors p21 and p27, the cdk 2, 5, and 6, and monocyte chemoattractant protein-1; and the upregulation of growth factors, including vascular endothelial growth factor (VEGF), vascular endothelial growth

factor receptor I (VEGFRI), endothelial growth factor (EGF) and hepatic-derived growth factor (HDGF). These findings identify an array of gene responses to overexpression of human HO-1 and elucidate new aspects of human HO-1 signaling involved in cell growth.

More recently, Colombrita et al. [203] reported that expression of HO-1 in human ECs in the G_0/G_1 phase, in the presence of Ang II, might be a key player in attenuating DNA damage during cell-cycle progression. Exposure of ECs to Ang II causes a complex response involving the generation of O_2^-, which may be involved in DNA damage. Thus, upregulation of HO-1 ensures the generation of bilirubin and CO in the G_0/G_1 phase to counteract the Ang II-mediated oxidative DNA damage. Inducibility of HO-1 in the G_0/G_1 phase is essential for protecting cells from DNA damage and ensuring cell-cycle progression.

HO-1 and endothelial cell dysfunction in diabetes

In type 1 diabetes mellitus, insulin deficiency provokes high blood glucose levels and lipid metabolism alterations. Evolution of the disease may be associated with the development of premature micro- and macrovascular complications, the pathogenesis of which may be linked in part to oxidative stress. O_2^- is vasoconstrictive through the removal of vasodilators and the stimulation of vasoconstrictors; e.g., O_2^- can convert NO to peroxynitrite, thereby consuming the endogenous vasodilator in the vasculature [204,205]. Additionally, O_2^- can induce assorted vasoconstrictors, such as endothelin, PDGF, TxA_2, and isoprostanes [204,205]. Reports have indicated that oxidative stress, as a result of hyperglycemia, plays an important role in the development and progression of diabetic vascular complications, such as nephropathy [206]. Hyperglycemic rats have also shown an increase in urinary 8-epi-isoprostane PGF_2, O_2^- formation, and the number of circulating endothelial cells and fragments [26].

As seen in Fig. 11. studies from these laboratories [25,26] imply that it is the products of HO-1-mediated heme degradation (i.e., CO and bilirubin) that produce the antiapoptotic effect of HO-1. In collating the different effects of bilirubin and CO, we have hypothesized that the products of HO metabolism of heme serve as a countervailing influence on Ang II-mediated damage in ECs and in the vasculature by actions that include decreasing EC sloughing, inhibition of O_2^-, and the expression of inflammatory molecules (i.e., TNF, Ang II, ICAM), and a reduction in constrictor mechanisms.

In a study conducted by Koya et al. [206], diabetic glomeruli showed a 16-fold increase in the expression of HO-1 mRNA and protein. Further, they showed that through the use of vitamin E and other antioxidants, HO-1 levels are eventually normalized, indicating that antioxidants could be a potential therapeutic treatment for diabetes-related nephropathy. In addition, animals preconditioned with HO-1

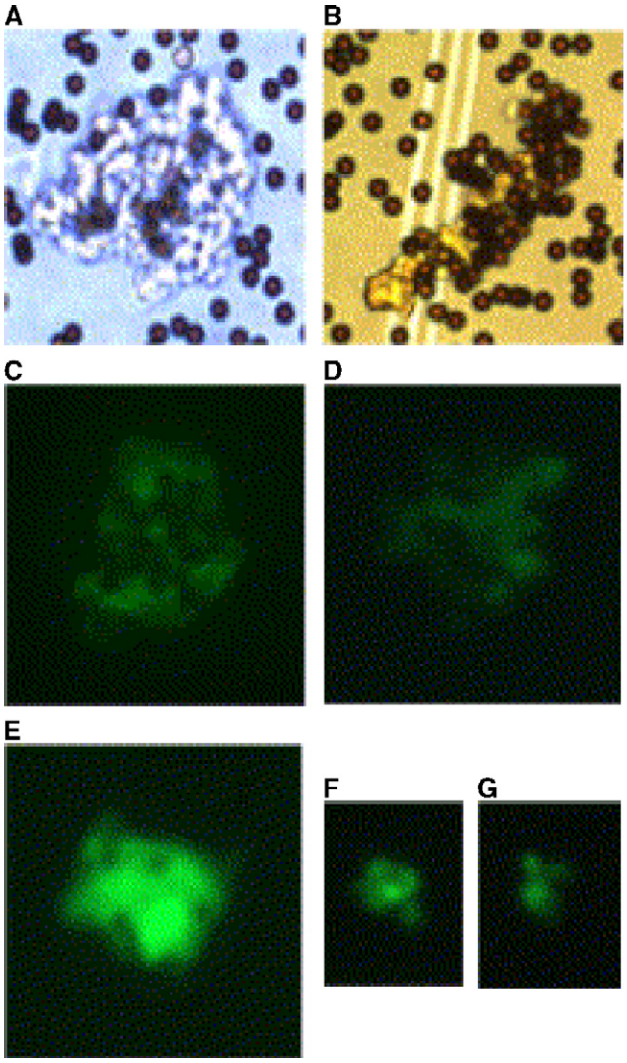

Fig. 11. Morphology of circulating endothelial cells with Dynabeads attached in STZ-induced diabetic rats. Typical circulating endothelial cells and cell debris under light microscope (A, B) or fluorescent microscope (C–G) [207].

show lower levels of O_2^- formation [25,207], vasoconstriction, and circulating ECs and fragments [70]. McClung et al. [70] concluded that HO-1 upregulation decreases neointimal vessel size associated with a reduction in O_2^- production and a decrease in CECs in balloon-injured diabetic rats, suggesting that it may have a role in the treatment of vascular restenosis.

HO and pharmacologic/genetic interventions in cardiovascular disease

A. Cardiovascular drugs and HO-1 induction

A variety of drugs have been developed to reduce the complications of cardiovascular disease. The most common of these is aspirin, which has been reported to decrease platelet COX-2 levels and thus reduce cardiovascular risk.

Grosser et al. [208] have reported that aspirin increased HO-1 protein levels and activity in a dose-dependent manner in cultured ECs derived from human umbilical vein. Pretreatment of cells with aspirin or bilirubin protected ECs from hydrogen peroxide-mediated toxicity. The authors concluded that aspirin targets HO-1, via NO-dependent pathways similar to NOS blockers, such as L-NAME, preventing aspirin-dependent HO-1 induction. Oberle et al. [209] expanded these studies using the NO donor, pentaesithrityl tetranitrate (PETN). They showed that the active PETN metabolite, pentaerithyrityl trinitrite (PETriN), increased HO-1 mRNA and protein levels. This was accompanied by an increase in enzyme activity as measured by increased CO and bilirubin production. Polte et al. [210] demonstrated that HO-1 is a cGMP-sensitive endothelial gene and established a causal relationship between HO-1 induction and endothelial protection by the cGMP/NO system. Aspirin has also been shown to increase ferritin synthesis in ECs, presumably as a result of HO-1 induction and iron release, suggesting a role in the prevention of endothelial injury during atherogenesis. Grosser and coworkers [209,211,212] have examined the mechanism of statins with respect to their antioxidation action. In cultured ECs derived from human umbilical vein, simvastatin and lovastatin increased HO-1 mRNA levels [211,213]. Increased transcriptional expression in statin-mediated HO-1 was associated with elevated HO-1 protein levels and a reduction in free radical formation. Lee et al. also reported that simvastatin activates HO-1 in VSMCs in vitro and in vivo and suggested the involvement of p38 and the p13K-Akt pathway in HO-1 induction. The authors concluded that the anti-inflammatory and antiproliferative effects of simvastatin are largely mediated through the induction of HO-1 [212].

B. Rationale for gene transfer

Pharmacological agents such as SnMP and cobalt protoporphyrin (CoPP) can inhibit or induce HO activity significantly and effectively [13–15,127]. However, such agents can exert only transient control of HO activity. Current pharmacological agents effectively treat cardiovascular diseases such as essential vascular hypertension, but may not prevent end organ damage or influence the propensity for other diseases. Changes in CO and bilirubin formation and heme content as a result of HO-1 protein expression with genetic interventions are modest and less abrupt or volatile than those obtained after bolus administration of chemical inducers. Genetic interventions result in a steady change in HO activity and heme content, which is regulated by an increase in the rate of heme synthesis [11]. Human gene therapy for cardiovascular disease has the potential to provide important advances in therapeutic angiogenesis, myocardial protection, myocardial regeneration and repair, restenosis, prevention of bypass graft failure, and risk-factor management.

Gene transfer is a powerful tool that can be used to insert specific genes into cells otherwise deficient or that underexpress the gene. Overexpression of the HO gene by targeted gene transfer has become a powerful tool for studying the role of the human HO-1 enzyme. The delivery of the human HO-1 gene to rats has been a successful approach for inducing the long-term overexpression of human HO-1. Delivery of human HO-1 into SHR has been shown not only to attenuate hypertension but also to enhance somatic body growth and cell proliferation [176]. Successful and functional HO gene transfer requires two essential elements [11]. First, the HO gene must be delivered by a safe vector, and second with the exception of HO gene delivery to ocular or cardiovascular tissue via catheter-based interventions, HO gene delivery must be site and organ specific.

Abraham et al. [6,214] described the first adenoviral construct used to deliver the human HO-1 gene. It was found that the human HO-1 gene could be introduced into rabbit ocular tissues by microinjection of a recombinant replication-deficient adenovirus human HO-1 cDNA [6,214]. Microinjection of the Adv-HHO construct into the vitreous resulted in HO-1 mRNA expression in the corneal endothelium, iris, lens, and retina. After intracameral injection, human HO-1 mRNA was detected in the corneal epithelium and endothelium, ciliary body, lens, and iris, and acted to decrease oxidant-mediated injury [6,214]. Regardless of the injection site, transfected human HO-1 mRNA was site-specific and undetectable in tissues outside the eye. The ability to transfect the human HO gene and to demonstrate its expression may offer a new therapeutic strategy for treating pathological disorders in humans.

C. Gene transfer to correct for cardiovascular disorders

The ultimate goal in the treatment of cardiovascular disease is the timely delivery of the best therapeutic agents, which would be able to protect the heart from the deleterious effects of prolonged ischemia or the effects of repeated bouts of ischemia [215]. Ischemia and reperfusion represent major mechanisms of tissue injury and organ failure. In addition, myocardial ischemia may be asymptomatic and repeated. As a result, patients may not receive timely treatments [215]. Pachori et al. [216] have developed a preemptive strategy for tissue protection using an adeno-associated vector system, containing erythropoietin hypoxia response elements, for ischemia-regulated expression of the therapeutic human HO-1 gene. They demonstrated that a single administration of this vector several weeks in advance of ischemia/reperfusion injury to the heart produced a rapid and timely induction of human HO-1 during ischemia, which resulted in a dramatic reduction in tissue damage. In addition, they also showed that overexpression of the therapeutic transgene prevented long-term pathological tissue remodeling and normalized tissue function. A similar study done by Coito et al. [217], with regards to ischemia-

induced myocardial injury, demonstrated that rats having undergone successful HO-1 gene administration had a dramatic reduction in left ventricular myocardial infarction after coronary artery ligation and release. In addition to the reduction in infarct size, there was a decrease in myocardial lipid peroxidation, proapoptotic Bax, and proinflammatory interleukin-1 beta (IL-1β) protein abundance, as well as an increase in antiapoptotic Bcl-2 protein levels. These data demonstrate that HO-1 gene transfer produces therapeutic, cardioprotective benefits by reducing oxidative stress and associated inflammation and cell death.

According to Vassalli et al. [218], current methods of heart transplantation are limited by incomplete effectiveness, significant toxicity, and failure to prevent chronic rejection. Genetic manipulation of the donor heart at the time of removal offers a unique opportunity to introduce a therapeutic molecule within the graft itself while minimizing systemic effects. Cytoprotective approaches, including HO-1 gene transfer, have reduced ischemia/reperfusion injury and delayed cardiac allograft rejection in small animals. However, despite major experimental advances, gene therapies for heart transplantation have not yet entered the clinical arena due to unanswered questions regarding the most suitable vector, the best gene, and safety issues. Gene therapy is being further considered for allograft repair. Braudeau et al. [219] analyzed the effects of specific overexpression of HO-1 following adenovirus-mediated HO-1 gene transfer in an acute cardiac allograft rejection model. These results indicate that HO-1 overexpression prolongs the survival of vascularized allografts by promoting tolerogenic mechanisms acting on allogeneic cellular immune responses.

Others such as Tang et al. [220] believe, however, that the constitutive overexpression of human HO-1 may lead to unwanted side effects. As a result, they designed a hypoxia-regulated human HO-1 gene therapy system that can be switched on and off. Their vigilant plasmid system is composed of a myosin light-chain-2v promoter and a gene switch that is based on an oxygen-dependent degradation domain from the hypoxia-inducible factor-1-alpha (HIF-1α). This vector can sense ischemia and switch on the human HO-1 gene system, specifically in the heart. Subsequently, its expression can then turn off, after sensing that the tissue is adequately oxygenated, once proper blood flow is restored to the heart. Hearts from HO-1 gene therapy-treated mice showed improved recovery of contractile and diastolic performance after myocardial infarction compared with saline controls. This study documents the therapeutic potential of vigilant plasmid-mediated human HO-1 gene transfer, which may provide cardiac-specific protection from repeated bouts of ischemic injury.

D. Gene transfer and hypertension

Gene transfer for the management of hypertension has considerable clinical potential, especially if the gene is delivered to the vascular system before hypertension becomes sustained. Goodman et al. [185] and others [10] performed a study using a retroviral vector in which they examined the effects of human HO-1 gene delivery on cellular heme in renal tissues. Analysis indicated an increase in human HO-1 distribution and activity throughout the renal tissue, which was accompanied by a significant reduction in blood pressure. Sabaawy et al. [176] performed a similar experiment in which they gave a single intracardiac injection of concentrated infectious viral particles expressing human HO-1 to SHR. These results, which were similar to those of Goodman et al., demonstrated functional expression of human HO-1 and attenuation of development of hypertension.

It was recently shown that delivery of the HO-1 gene, in sense or antisense orientation, alters HO-1 expression and subsequently regulates BP [10]. A retrovirus carrying the human HO-1 gene in its sense orientation (LSN-HHO-1) or the rat HO-1 in its antisense orientation (LSN-RHO-AS) was administered to newborn Sprague-Dawley (SD) rats. Two months later the rats were exposed to Ang II. Rats exposed to the human HO-1 gene in its antisense orientation demonstrated decreased HO-1 expression and showed an increase in BP after exposure to Ang II. In contrast, rats given the human HO-1 gene in its sense orientation demonstrated human HO-1 expression and a decrease in BP after being exposed to Ang II. These data demonstrate that overexpression of HO-1 brings about a reduction in pressor responsiveness to Ang II, which is most likely due to the increased generation of an HO-1 product, presumably CO, with the ability to inhibit vascular reactivity to constrictor stimuli.

E. Gene transfer and diabetes

Diabetes is another example of a metabolic disturbance in which HO-1 gene transfer in the experimental animal has been shown to be beneficial. Although oxidative stress is induced under diabetic conditions and causes various forms of tissue damage, acute diabetes does not result in an increase HO-1 protein [25,26]. Abraham et al. [26] compared the basal levels of HO-1 and HO-2 proteins, as well as the effect of heme on these levels, in aorta from control and hyperglycemic rats using immunohistochemistry. In hyperglycemic rats, HO-1 immunoreactivity was reduced in the intima, but not in the media or adventitia. In contrast to control rats, HO-1 immunoreactivity in the intima and media was less intense in hyperglycemic rats treated with heme (Figs. 12C and D; left panel). HO-2 immunoreactivity was evident in the intima, media, and adventitia, and was not affected by heme or hyperglycemia. Moderate HO-1 immunoreactivity was observed in the intima, media, and adventitia of the aorta from control rats. Heme administration resulted in strong immunoreactivity in the intima (thin arrow) and in the media (thick arrow) (Fig. 12B, right upper panel). This study and other suggested that

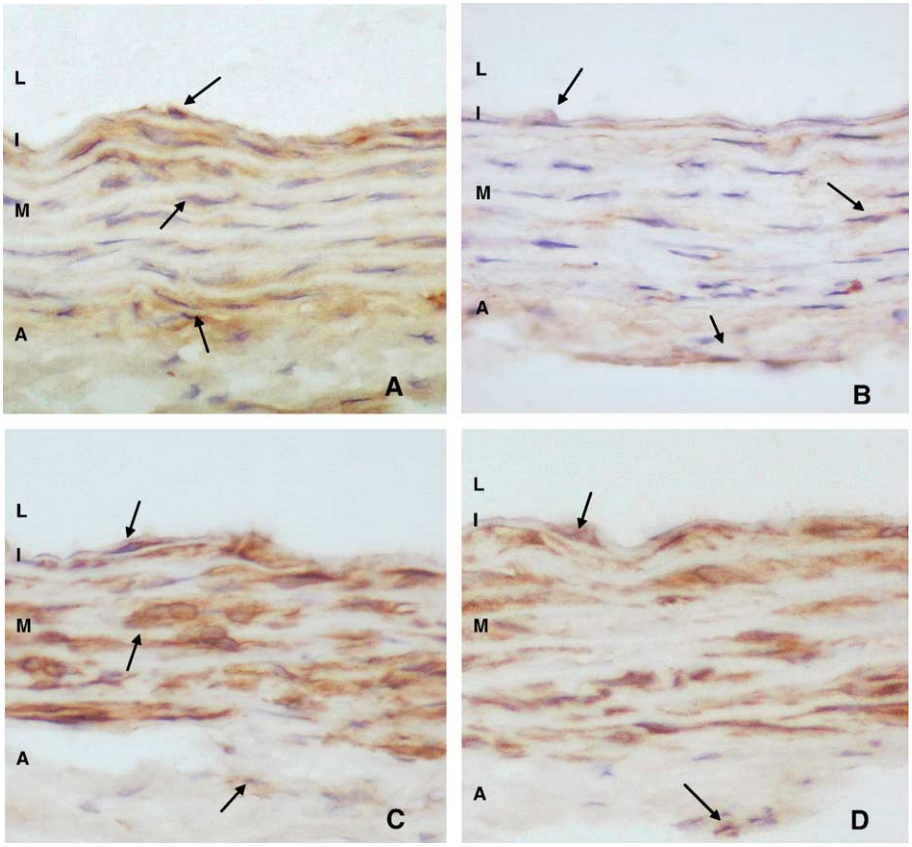

Fig. 12. HO-1 immunohistochemistry in aorta from control (A), heme-treated (B), STZ-treated (C), and STZ plus heme-treated rats (D). Heme was given prior to sacrifice. In STZ animals, HO-1 staining of intima was decreased with respect to controls whereas in the media, as in controls, some focal areas of positivity are present. Administration of heme restored HO-1 staining. Immunopositivity was a dark brown stain in the cytoplasm of cells. Nuclei are counterstained with hematoxylin [26].

delivery of the human HO-1 gene, or HO-1 preconditioning, may provide cytoprotection to the endothelium [26].

Abraham et al. [26] also assessed the functional expression of human HO-1 gene transfer as determined by the levels of human HO-1 protein in the aorta of rats receiving LSN-HHO-1 compared to controls receiving empty retrovirus vector (LSXN) [26]. Western blot analysis of aorta obtained from LSN-HHO-1 rats demonstrated human HO-1 protein expression [26]. In contrast, no human HO-1 protein was expressed in aorta from rats injected with the empty retrovirus vector. These data confirm the functional expression of the human HO-1 gene on HO-1 protein levels after intracardiac delivery of LSN-HHO-1. More importantly, neither rat HO-2 nor actin were affected by expression of the human HO-1 gene as indicated by comparable expression of HO-2 and actin in rats receiving human HO-1 gene transfer or in LXSN-treated rats. The effect of hyperglycemia on HO activity in rats overexpressing (LSN-HHO-1) and underexpressing HO-1 (LSN-rHO-1 AS) was assessed [26]. Streptozotocin (STZ)-induced hyperglycemia resulted in significantly reduced HO activity in both control rats and rats transduced with LSN-rHO-1-AS (Fig. 13).

The effect of HO overexpression and underexpression on the generation of 8-epi-isoprostane $PGF_{2\alpha}$, which is considered as a reliable index of oxidative stress status in vivo in normal and hyperglycemic rats, was also studied [26]. Urinary excretion of 8-epi-isoprostane $PGF_{2\alpha}$ was increased in hyperglycemic rats compared to control rats, but not in rats overexpressing human HO-1 when compared to controls. Rats overexpressing HO-1 (LSN- HHO-1) showed a significant decrease in urinary excretion of 8-epi-isoprostane $PGF_{2\alpha}$. In contrast, rats underexpressing HO-1 excreted higher levels of 8-epi-isoprostane $PGF_{2\alpha}$, which were further increased as a result of hyperglycemia. Underexpression of HO-1 exacerbated excretion of 8-epi-isoprostane $PGF_{2\alpha}$, increasing its output significantly in LSN-rat-HO-1 AS rats. The mechanism by which human HO-1 gene transfer into rats exerts antioxidant effects was further studied by measuring O_2^- in control and diabetic rat vessels overexpressing and underexpressing HO activity (Fig. 13). Production of O_2^- was increased in diabetic rats compared to controls. The report also examined whether the preventive vascular protection induced by human HO-1 gene transfer would decrease vascular O_2^-. HO-1 gene transfer attenuated the hyperglycemia-mediated increase in O_2^-. In contrast, diminished HO-1 expression potentiated the hyperglycemia-mediated increase in O_2^- seen in transgenic HO-1 AS rats. These results provide additional

evidence that pharmacological induction of HO-1 or delivery of the HO-1 gene to the vascular system can provide vascular protection and subserves an antioxidant defense mechanism against pathological conditions, in which decreasing O_2^- formation is an important goal.

The effect of hyperglycemia on EC damage and sloughing into the circulation in control and hyperglycemic rats overexpressing or underexpressing HO-1 was also examined [26]. The basal levels of circulating EC in HO-1 transgenic and control rats before the onset of diabetes were not substantially different, but were significantly elevated in transgenic HO-1-AS rats as a result of diminished HO activity. As seen in Fig. 14, the number of circulating EC in blood obtained from control rats was low. However, a large increase in the number of circulating EC was observed in blood obtained from hyperglycemic rats, similar to that seen in other pathologic circumstances. The number of circulating EC in hyperglycemic rats over-expressing HO-1 was decreased. These results demonstrate that the vascular system may develop an adaptive response to hyperglycemia-mediated stress as a result of transduction of the HO-1 gene and the decreased detachment of EC. In contrast, the number of circulating endothelial cells in hyperglycemic rats underexpressing HO-1 was significantly higher than that in control hyperglycemic rats, suggesting that the extent and severity of the increase in circulating endothelial cells were not predetermined at the onset of diabetes, but could be modified by the level of HO-1 gene

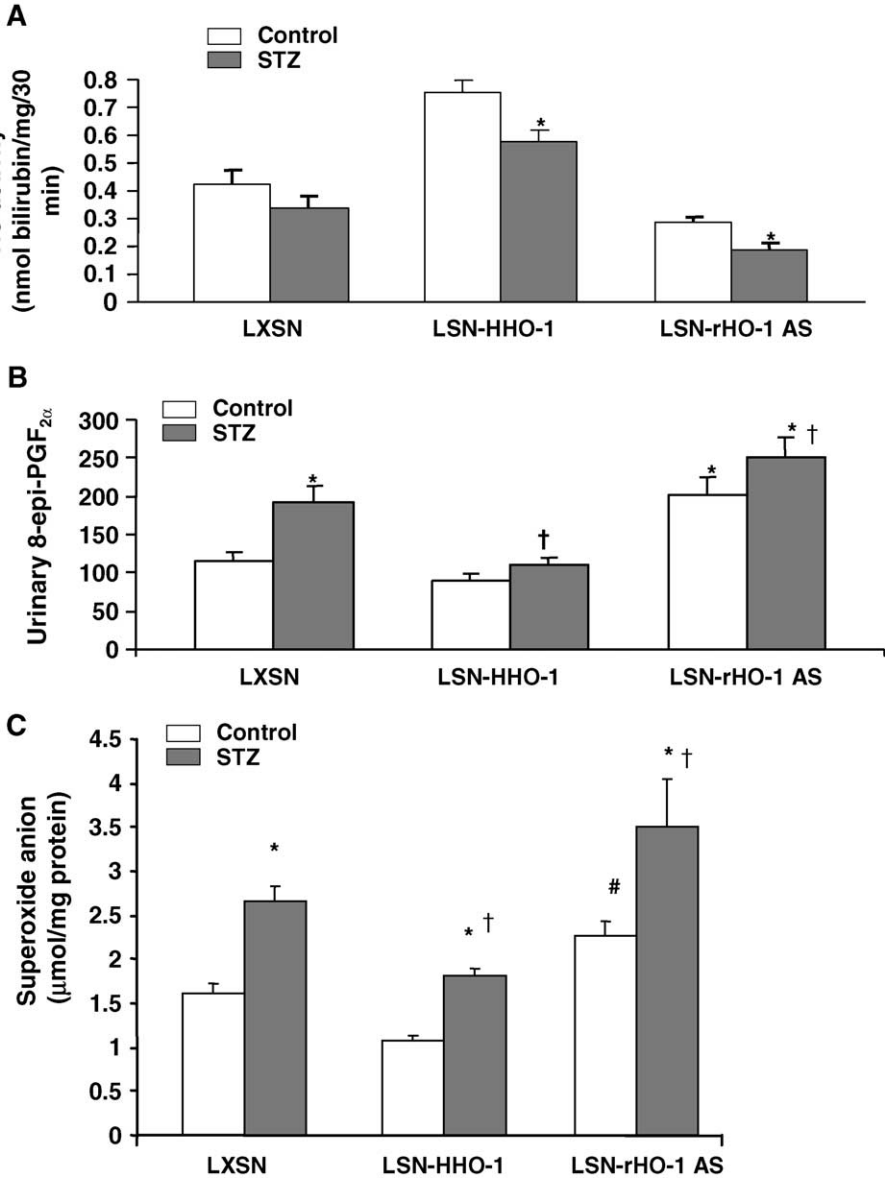

Fig. 13. Effect of hyperglycemia on (A) HO activity; (B) rats transduced with in aorta from rats transduced with LXSN, LSN-HHO-1, and LSN-rHO-1 AS. HO activity and urinary 8-epi-isoprostane PGF_{2a} were measured as described in the text. *$p < 0.05$ vs. the corresponding control (not treated with STZ); ‡$p < 0.05$ vs. STZ-treated LXSN transduced rats. (C) Aortic O_2^- production in STZ rats transduced with LXSN, LSN-HHO-1, and LSN-rHO-1 AS. O_2^- production was measured as described in the text. *$p < 0.05$ vs. the corresponding control (not treated with STZ); #$p < 0.05$ vs. STZ-treated LXSN transduced rats.

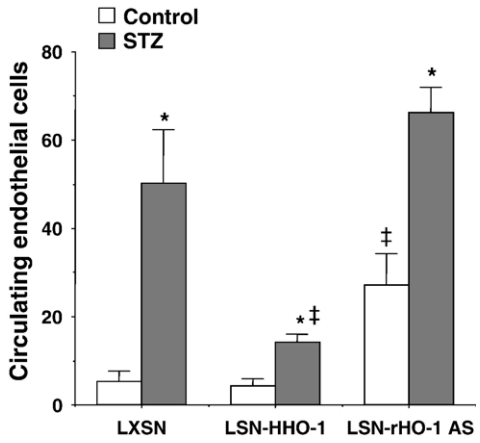

Fig. 14. Effect of hyperglycemia on the number of circulating endothelial cells. Diabetic rats demonstrated a significant increase in the number of circulating endothelial cells when compared to nondiabetic control rats. Furthermore, diabetic rats preconditioned with the HO-1 gene showed a significant decrease in the number of circulating endothelial cells when compared to untreated diabetic rats. The upregulation of HO-1 has a cytoprotective effect, which prevents endothelial cell sloughing [26].

expression. These data demonstrate that HO-1 gene transfer into normal rats at an early stage during the development of the vascular system may attenuate cardiovascular complications during the development of diabetes [26,207].

F. Gene targeting

Site- and tissue-specific transcriptional regulating elements represent an alternative strategy for restricting adenoviral transgene expression to specific cell lineages or tissues in vivo [221,222]. This strategy has been recently applied using a SMC-specific SM22α promoter to direct expression of recombinant gene products to vascular or visceral SMCs in vivo [222]. A few examples of cell lineage-specific promoters are listed in Table 1.

The mTALH is situated in a site of markedly diminished oxygen tension and is highly vulnerable to ischemic insult involving Ang II-mediated increases in COX-2 activity. Quan et al. [27] demonstrated that the site-specific delivery of HO-1 to renal structures, specifically the mTALH, in SHR, using the Na^+-K^+-Cl^- cotransporter (NKCC2 promoter), normalized BP and provided protection to the mTALH against oxidative stress caused by Ang II. Western blot and RT-PCR revealed that human HO-1 was selectively expressed in primary cultured TALH cells following infection with Ad-NKCC2-HO-1. In TALH cells infected with Ad-NKCC2-HO-1, Ang II-stimulated PGE_2 levels were significantly reduced. Ang II caused a marked decrease in reduced glutathione (GSH) levels and this decrease was greatly attenuated in TALH cells upregulated with HO-1. This is the first demonstration that HO-1 overexpression increases reduced GSH [27]. Others have shown that upregulation of HO-1 may decrease cellular heme availability for the synthesis gp91 (phox) and related NOX oxidase and the generation of O_2^- [227].

Concluding remarks

The heme oxygenase (HO) system plays an important role in many aspects of human physiology, as well as in pathological circumstances associated with myocardial ischemia/reperfusion, hypertension, cardiomyopathy, organ transplantation, endotoxemia, and pulmonary disorders among others. Active agents in these situations include not only heme itself, but its metabolic products, CO, and bile pigments as well as those agents derived from the wide array of genetic and metabolic processes which respond when heme metabolism is perturbed [102]. The use of pharmacological and genetic interventions for regulating heme oxygenase has already provided important new insights into the relation of the heme–HO system to biological and pathological events and offers the potential for development of new therapeutic strategies directed against recalcitrant disease processes. It is, for example, possible to envisage the use of a single drug or gene intervention using site-specific expression to induce long-term prophylaxis against certain pathological cardiovascular events or to promote and enhance repair processes in individuals who have experienced, or are at high risk for, cardiac injury. The use of stable gene integration may also lead to the development of more effective, perhaps long-lasting, therapies which can moderate other chronic diseases.

The HO system is on the one hand a seemingly simple biochemical axis and on the other, the hub of a complex of coupled processes whose actions and products exert a wide array of diverse and potent metabolic effects. Down regulating the HO system by pharmacological or genetic means has already led to new approaches for managing old clinical problems [15] and up-regulating the system offers even more promise for new approaches to experimental work in cell biology and new methods for moderating some of the consequences of clinical disorders. The use of pharmacological and genetic interventions for upregulation of HO-1 in the managements of cardiovascular diseases appears to be especially promising. In the future, it may be possible to utilize a single administration of a drug or gene therapy using site-specific expression of HO-1/HO-2 to promote long-term prophylaxis against secondary coronary

Table 1

Promoter	Specific cell cineage	Transgene
SM22α early marker genefor SMCs	SMCs [222]	HO/CYP/COX-2
GGT (α-glutamyl transpeptidase)	Proximal tubules [223]	HO/CYP/COX-2
KAP-HAGT (kidney androgen-regulated protein promoter)	Proximal tubules [224]	HO/CYP4AS
Tie (tyrosine kinase receptor)	Endothelium/ bloodvessels [225]	HO/CYP4AS
NKcc2/Slc12 a1 (Na-K-Cl cotransporter)	TALH [226]	COX-2/HO

events and to promote myocardial repair in patients who have experienced an infarct as well as in those at high risk of myocardial injury. In addition, using stable gene integration, it may be possible to develop more effective and long-sustanied therapies for chronic disorders such as diabetes and other metabolic diseases. These pharmacologic or genetic strategies to regulate the heme–HO system could, as we have earlier suggested [13], open up new therapeutic approaches for the effective management of a number of clinical disorders.

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