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FORUM REVIEW ARTICLE

A Central Role of Heme Oxygenase-1 in Cardiovascular Protection

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Abstract

The intrinsic defense mechanisms of the body are critical in protecting tissues from injury in response to pathological stress. Heme oxygenase-1 (HO-1), a stress response protein, is induced in response to various pathological stimuli to serve a cytoprotective function. By degrading the oxidant heme and generating the antioxidant bilirubin and anti-inflammatory molecule carbon monoxide, HO-1 may protect cell from injury due to oxidative and pathological stress. Oxidative stress in the heart caused by ischemia and reperfusion leads to cardiomyocyte death and subsequent myocardial infarction. Vascular diseases including atherosclerosis, graft failure, and restenosis are all associated with reactive oxygen species-induced injury and inflammation. Given that cardiovascular disease is the leading cause of death worldwide, there is considerable interest in developing new strategies for preventing and treating cardiovascular disease. Since HO-1 is induced in the heart and blood vessels in response to various stresses, a role of HO-1 has been implicated in cardiovascular homeostasis. Numerous studies using pharmacological method or genetic approach have since demonstrated the cardiovascular protective function of HO-1. Importantly, a number of studies have associated human HO-1 gene promoter polymorphisms with risk for vascular diseases. Taken together, HO-1 has a great therapeutic potential for cardiovascular disease. *Antioxid. Redox Signal.* 15, 1835–1846.

Introduction

In response to pathophysiological stress, the body elicits an endogenous adaptive mechanism to protect cells and tissues from injury. Heme oxygenase-1 (HO-1) upregulation is one such intrinsic defense system. HO-1 is a member of the HO family of proteins, which include three isoforms, HO-1, HO-2, and HO-3. HO-1, also known as heat shock protein 32, is the inducible isoform. HO-2 is constitutively expressed; whereas HO-3 has lower enzymatic activity and is less well characterized. HO degrades the prooxidant heme to carbon monoxide (CO), biliverdin (subsequently reduced to bilirubin), and ferrous iron (56). By degrading the prooxidant heme and generating antioxidant bilirubin, HO-1 may protect cells against oxidative stress. CO is a gas molecule that increases intracellular levels of cGMP, which regulates vascular tone and smooth muscle cell (SMC) development. CO has been recently shown to have antiproliferative and anti-inflammatory properties. Ferrous iron can induce ferritin expression for iron sequestration. By generating these biologically active molecules and its upregulation in response to stress conditions, HO-1 may, thus, protect cells and tissues from oxidative stress-induced injury.

Cardiovascular disease is a leading cause of death worldwide. Vascular diseases including atherosclerosis, graft failure, and restenosis are all associated with reactive oxygen species-induced injury. In addition, oxidative stress in the heart caused by ischemia and reperfusion leads to cardiomyocyte death and subsequent myocardial infarction. Therefore, increased expression of antioxidant enzymes and stress proteins might protect against the development of cardiovascular diseases. Since HO-1 is induced in the heart and blood vessels in response to various stresses, a role of HO-1 has been implicated in cardiovascular homeostasis. Numerous studies using pharmacological method or genetic approach have since demonstrated the cardiovascular protective function of HO-1. A number of studies have associated human HO-1 gene promoter polymorphisms with risk for vascular diseases (11, 22, 81, 83). Importantly, the clinical significance of HO-1 in human

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cardiovascular disease has been highlighted in a child with HO-1 deficiency (45, 92).

HO-1 in the Development of Atherosclerosis

Atherosclerosis is the leading cause of mortality and morbidity in the United States and many other countries. When low-density lipoprotein (LDL) particles in individuals with hypercholesterolemia become trapped in an artery, they undergo oxidation and are then internalized by macrophages, resulting in foam cell formation (75). Removal and sequestration of oxidized LDL are the initial, protective role of macrophages in the inflammatory response and minimize the damaging effects of modified LDL on endothelial and SMCs. However, the inflammatory response may be further amplified, and atherosclerotic lesions develop by accumulating macrophage-derived foam cells in the intima (32, 75). Subsequent migration and proliferation of SMCs from media into intima lead to formation of advanced complicated lesions (76). Recognizing the important contribution of inflammation in atherogenesis, Ross has proposed that atherosclerosis is an inflammatory disease (76).

The detection of HO-1 expression in endothelium, foam cells, and macrophages of advanced lesions from both humans and experimental apolipoprotein E-deficient ($apoE^{-/-}$) mice indicates a role of HO-1 in atherogenesis (89). Supporting a function of HO-1 in atherosclerosis, induction of HO-1 inhibits the monocyte transmigration induced by mildly oxidized LDL (38). Pharmacological inhibition of HO-1 activity by Sn-protoporphyrin IX (SnPP) in Watanabe heritable hyperlipidemic rabbits significantly increases atherosclerotic lesions, thus suggesting that HO-1 has antiatherogenic properties *in vivo* (39). Similarly, HO-1 inhibition by SnPP in LDL-receptor knockout mice fed high-fat diets results in increased lesion size (40). Evaluation of plasma lipid levels shows that HO-1 inhibition increases plasma and tissue lipid peroxide levels without affecting plasma lipid composition, thus suggesting that the antiatherogenic properties of HO-1 might be conducted through the prevention of lipid peroxidation (39, 40).

Since the effect of metalloporphrins might have nonselective effects on HO-1 (31), to demonstrate unequivocally the role of HO-1 in atherosclerosis, we used a loss-of-function approach by first generating mice deficient in both HO-1 and apoE ($HO\text{-}1^{-/-}apoE^{-/-}$) and then subjected mice to Western diet (93). In response to hypercholesterolemia, although total plasma cholesterol levels are similar between $HO\text{-}1^{-/-}apoE^{-/-}$ and $apoE^{-/-}$ mice, $HO\text{-}1^{-/-}apoE^{-/-}$ mice develop larger and more advanced lesions than mice deficient in apoE alone (93) (Fig. 1A, B). Further, Sudan IV staining revealed greater lipid accumulation in mice deficient in both HO-1 and apoE (93) (Fig. 1C, D). Our genetic study clearly demonstrates that an absence of HO-1 exacerbates atherosclerotic lesion formation (93).

Orozco *et al.* explored whether HO-1 expression in macrophages contributes to the antiatherogenic effect of HO-1 (66). Using peritoneal macrophages from wild type, HO-1 heterozygous, or $HO\text{-}1^{-/-}$ mice, they found that HO-1 expression in macrophages reduces inflammatory responses against oxidized LDL (66). Further, $HO\text{-}1^{-/-}$ macrophages exhibit increased foam cell formation. Bone marrow transplantation experiments reveal that lack of HO-1 in the reconstituted bone marrow results in an increase in the inflammatory component of atherosclerotic lesions in LDL-

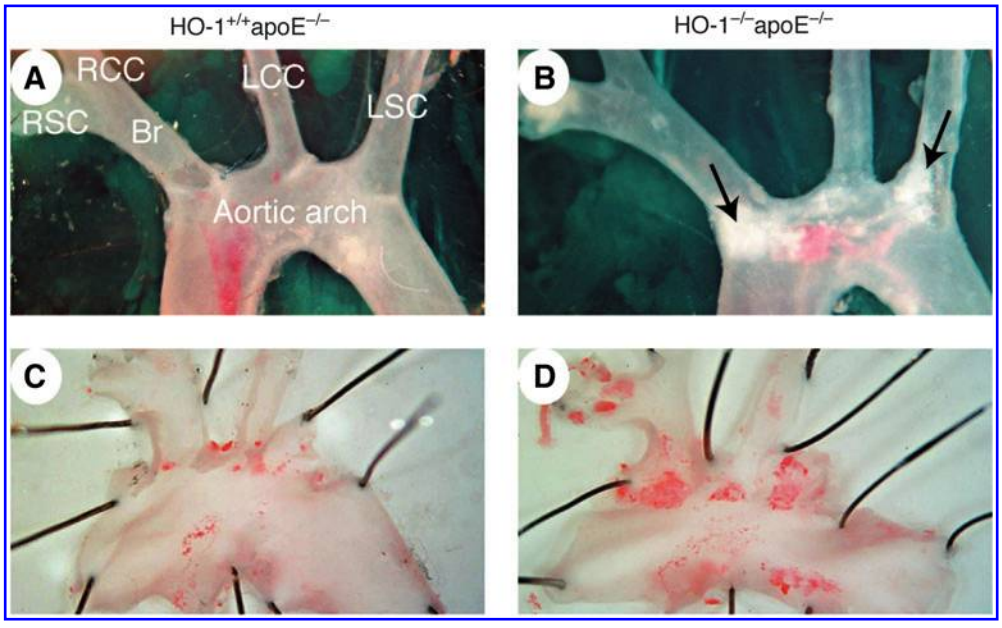

FIG. 1. Absence of HO-1 exacerbates lipid accumulation and lesion formation in arteries. $HO\text{-}1^{+/+}apoE^{-/-}$ and $HO\text{-}1^{-/-}apoE^{-/-}$ mice were fed Western diet starting at 4 weeks for a total of 8 weeks. The aortic arch and its branches were then harvested and analyzed. Photographs of representative arteries from $HO\text{-}1^{+/+}apoE^{-/-}$ **(A)** or $HO\text{-}1^{-/-}apoE^{-/-}$ **(B)** mice. Lesions are shown as white areas and indicated by *arrows*. **(C, D)** Sudan IV staining (red) of lipid in the arteries of $HO\text{-}1^{+/+}apoE^{-/-}$ **(C)** or $HO\text{-}1^{-/-}apoE^{-/-}$ **(D)** mice. Original magnification ×15 **(A** through **D)**. Reproduced from Yet *et al.* (93) with permission. $apoE^{-/-}$, apolipoprotein E-deficient; Br, brachiocephalic artery; HO-1, heme oxygenase-1; LCC, left common carotid artery; LSC, left subclavian artery; RCC, right common carotid artery; RSC, right subclavian artery. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

receptor knockout mice. These results indicate that HO-1 expression in macrophages contributes to antiatherogenic effect by decreasing the inflammatory component of atherosclerotic lesions (66).

Given that lack of HO-1 exacerbates atherosclerosis, the next question is whether overexpression of HO-1 would attenuate atherosclerotic lesion formation. HO-1 induction by chemical inducers (hemin or hemin and desferrioxamine) significantly attenuates atherosclerotic lesions in LDL-receptor knockout mice fed high-fat diets (40). Similarly, adenovirus-mediated HO-1 gene transfer attenuates the development of atherosclerosis in apoE$^{-/-}$ mice (42). Further, the iron deposition as well as tissue iron content was much less in aortic tissue of mice treated with adenovirus expressing HO-1, indicating that overexpression of HO-1 in vascular cells facilitates iron metabolism and attenuates development of atherosclerosis in apoE$^{-/-}$ mice (42). These gain-of-function experiments demonstrate a critical protective role of HO-1 in atherosclerosis.

HO-1 and Plaque Stability

As the atherosclerotic lesions progress, the lesions may become unstable and vulnerable with thinning of the fibrous cap and a large necrotic core with lipid accumulation, leading to plaque rupture, acute thrombus formation, and subsequent myocardial infarction. Accumulating evidence indicates that matrix metalloproteinases (MMPs) and their inhibitors tissue inhibitor of metalloproteinase (TIMP) are important mediators in intimal thickening and atherosclerotic plaque rupture (19, 63, 64). Early studies show that increased expression and matrix degrading activity of MMPs in vulnerable regions of human atherosclerotic plaques might contribute to plaque rupture (27, 35). On the other hand, expression of TIMPs, particularly TIMP-3, might counteract MMP activity in atheroma and increase plaque stability (23). Further, analysis of human carotid endarterectomy tissues revealed a differential distribution of MMPs and TIMPs over atherosclerotic plaques (15).

Although it is well accepted that MMPs are critically involved in atherosclerotic plaque instability; however, the molecular mechanisms regulating plaque stability remain unclear. Recently, Cheng *et al.* (13) explored the role of HO-1 in the progression of atherosclerotic lesions from stable toward vulnerable plaque. Analysis of atheretomy biopsy from patients with clinical carotid artery disease revealed that HO-1 expression levels correlate closely with features of vulnerable human atheromatous plaque, including increased thrombogenicity and increased expression levels of MMP-9, but inversely correlate with intraplaque vascular SMCs and collagen deposition (13). The increased MMP-9 expression is consistent with previous findings that MMP-9 is very abundant in segments of human carotid endarterectomy tissues with intraplaque hemorrhage (15). The correlation of HO-1 and MMP-9 levels suggested a role of HO-1 in regulating MMP-9 expression. This idea is corroborated by recent findings that HO-1 inhibits human breast carcinoma cell invasion and migration through suppressing MMP-9 expression (49). Further supporting this notion, transplantation of HO-1-overexpressing mesenchymal stem cells (MSCs) into rat hearts after myocardial infarction significantly reduced MMP-9 expression in the infarcted myocardium when compared with transplantatios of MSCs expressing vector only (80).

The upregulation of HO-1 in advanced atherosclerotic plaques with a vulnerable phenotype might be an adaptive endogenous defense mechanism. Thus, further induction of HO-1 expression could aid in the stabilization of the atheromatous plaque. This hypothesis was tested in an apoE$^{-/-}$ mouse model for vulnerable plaque formation. HO-1 inhibition by zinc protoporphyrin augments lipid accumulation and increases necrotic core size. In contrast, HO-1 induction by cobalt protoporphyrin or adenovirus-mediated gene transfer prevents plaque progression into vulnerable lesions by increasing fibrous cap thickness and intimal vascular SMC (VSMC) accumulation, whereas the necrotic core area and intraplaque lipid depostion is reduced (13). Supporting these findings, HO-1 has been shown to protect VSMCs from oxidative stress-induced apoptosis/cell death (6, 93). Taken together, it is conceivable that by suppressing MMP-9 expression and by preventing VSMC death in the lesion, HO-1 may, thus, promote plaque stability.

HO-1 in Arterial Injury

The proliferation and migration of VSMCs from the media into the intima contribute to arterial intimal thickening (24, 41). Interestingly, Morita and Kourembanas (59) show that VSMC-derived CO, a product of HO-1, inhibits mitogen endothelin-1 and platelet-derived growth factor (PDGF)-B expression of endothelial cells, which, in turn, inhibits VSMC proliferation. On the other hand, increasing CO production or exposing cells to exogenous CO leads to a markedly attenuated growth response of VSMCs (60), implicating HO-1 and its reaction product CO in the response to vascular injury. Indeed, the *in vivo* protective role of HO-1 in vascular remodeling is demonstrated in a pig model of arterial injury (21). HO-1 gene transfer significantly reduced cellular proliferation in the intima and media. The decrease in cell proliferation in HO-1 transduced arteries is associated with a significant reduction in intimal lesion formation, thus indicating that HO-1 confers protection against vascular injury through its effects on proliferation (21). Further supporting this notion, in response to femoral artery wire injury, HO-1$^{-/-}$ mice develop larger intimal lesions than wild-type mice (21). Moreover, VSMCs from HO-1$^{-/-}$ mice display enhanced DNA synthesis and proliferation compared with wild-type VSMCs (21).

After carotid artery balloon injury, wild-type Wistar rats (HsdBlu: GUNNJ/J) develop maximum neointimal hyperplasia with proliferating VSMC in the neointima (65). In contrast, the artery of hyperbilirubinemic Gunn rats (HsdBlu: GUNNj/j) shows very few proliferating cells and minimal neointimal hyperplasia compared with the control (65), suggesting bilirubin and its precursor biliverdin might have an antiproliferative effect during vascular remodeling. Supporting this notion, biliverdin is found to ameliorate neointimal hyperplasia associated with balloon injury and that bilirubin/biliverdin inhibits VSMC proliferation through cell cycle arrest (65). Thus, in addition to CO, bilirubin/biliverdin—another reaction product of HO-1—contributes to the reduction of neointima formation after varterial injury by its antiproliferative effect on VSMCs.

In addition to proliferation, SMC migration contributes to the development of neointima after injury (24, 41, 67). PDGF, by inducing VSMC chemotaxis, is one of the growth factors

involved in this process (41). Interestingly, generation of H_2O_2 is required for PDGF signal transduction (82). Therefore, reactive oxygen species (ROS) and HO-1 might be involved in regulating VSMC migration after arterial injury. In a recent study, Rodriguez *et al.* showed that overexpression of HO-1 by adenovirus or chemical inducer cobalt protoporphyrin results in decreased VSMC migration (74). They further show that CO is responsible for the antimigratory effects seen with increased HO-1 expression. Since nicotinamide adenine dinucleotide phosphate oxidase is an important source of ROS generation (84), it is not surprising that the effect of HO-1/CO on inhibiting PDGF-induced VSMC migration is mediated by inhibition of nicotinamide adenine dinucleotide phosphate oxidase (74).

HO-1 in In-Stent Restenosis and Vein Graft Stenosis

Percutaneous coronary angioplasty to restore blood flow is a routine procedure to treat coronary artery disease; however, recurrent stenosis remains a major drawback in percutaneous transluminal angioplasty. Although stenting provides an additional benefit by decreasing reocclusion (25), an acute coronary syndrome presentation in patients with in-stent restenosis is associated with a higher incidence of recurrent adverse cardiovascular events and angiographic restenosis (3). Drug-eluting stents with antiproliferative drugs reduce the rates of restenosis and neointimal hyperplasia and associated clinical events (61, 69). However, questions regarding the long-term safety of drug-eluting stents have been raised (33, 44). To reduce in-stent restenosis without compromising re-endothelization, Hyvelin *et al.* recently investigated the potential of HO-1 induction in this regard (37). In rat and rabbit models of in-stent stenosis, treatment with hemin, a potent HO-1 inducer, reduces neointima growth without compromising re-endothelialization of the stented arteries (37). Hemin increases HO-1 expression and limits the early inflammatory, apoptotic, and proliferative cellular events that are associated with in-stent stenosis (37). SnPP, an HO-1 inhibitor, abolished the benefical effect afforded by hemin. Further, a CO donor also reduces in-stent stenosis in rat aorta, suggesting that CO might contribute to the protection. Their results suggest that HO-1 plays an important role in limiting in-stent stenosis and might serve as a novel target after endovascular therapies (37). This conclusion is supported by a previous report that patients with long (guanosine thymidine dinucleotide [GT]) repeat length polymorphism in the HO-1 promoter (see the session: HO-1 and microsatellite polymorphism in humans) have lower transcriptional activity and have a greater risk for angiographic restenosis as well as adverse cardiac events after coronary stenting (10).

Although coronary artery stenting is being increasingly used to treat patients with obstructive atherosclerotic lesions, stenting is associated with a greater need for repeated procedures in comparison with coronary artery bypass graft surgery (CABG) (79). A recent study showed that for patients with multivessel disease, CABG continues to be associated with lower mortality rates than does treatment with drug-eluting stents and is also associated with lower rates of death or myocardial infarction and repeat revascularization (33). Therefore, CABG remains an important treatment for multivessel disease, and autologous vein grafts provide important and convenient conduits for bypass graft surgery (1, 70). However, vein graft occlusion does develop due to intimal thickening (73). Given that HO-1 is induced in the neointima of the vein grafts in a mouse jugular vein/carotid artery autograft model (93), which mimics the vein graft used in bypass surgery, we hypothesized that HO-1 may play a role in the adaptation of VSMCs to hemodynamic stress in vascular wall remodeling. Consistent with the notion that HO-1 and its product CO inhibit VSMC proliferation (60), in response to similar hemodynamic stress 10 days after grafting, HO-$1^{-/-}$ mice have much larger neointima than wild-type mice (93). Surprisingly, instead of developing even larger lesions 14 days after surgery, there is massive cell death in the neointima of HO-$1^{-/-}$ mice (93), suggesting that lack of HO-1 might render VSMCs more prone to oxidant (generated by hemodynamic stress)-induced cell death. Indeed, HO-$1^{-/-}$ VSMCs are more sensitive to H_2O_2-induced cell death, indicating that susceptibility to oxidative stress is one of the mechanisms leading to cell death in the absence of HO-1 (93).

HO-1 in Transplant Arteriosclerosis

Chronic allograft rejection is mainly manifested by progressive arteriosclerosis; thus, reducing transplant arteriosclerosis would improve graft survival. Interestingly, in an aortic allotransplant model, Allotrap peptide RDP58 therapy markedly inhibits vascular intimal thickening, media necrosis, and adventitial cellular inflammation. The attenuation of arteriosclerosis is associated with the induction of HO-1 expression (48). Using a rat aorta chronic rejection model, Bouche *et al.* show that specific HO-1 overexpression after gene transfer of HO-1 inhibits chronic rejection by reducing leukocyte and VSMC infiltration of the aorta intima (5). In another study using a rat aortic transplant model, adenoviral gene transfer of HO-1 results in a significant reduction in leukocyte infiltration, a decreased number of VSMCs in the intima, significantly lower levels of NF-κB, and fewer apoptotic cells in the aortas (20). The protective role of HO-1 in inhibiting graft arteriosclerosis seems to correlate with reduced levels of NF-κB and inhibition of apoptosis in the grafts (20). A study showed that donor HO-1 expression has a direct influence on the recipient immune response. Endothelial cells overexpressing HO-1 significantly inhibit proliferation and interferon-γ production in allogeneic CD8(+) T cells, both of which are important in chronic rejection (16). Their data suggest that donor HO-1 expression may be useful to augment other immunosuppressive therapies to prolong graft survival and inhibit intimal hyperplasia (16). Cheng *et al.* (14) further demonstrated the important role of HO-1 in the regulation of vascular alloimmune response elicited by dendritic cells. In a murine model for transplantation arteriosclerosis, adoptive transfer of HO$1^{-/-}$ dendritic cells before allograft transplantation is associated with pronounced intragraft CD4(+) T cell infiltration and increased IgG deposition, suggestive of an accelerated development of vasculopathy toward the chronic phase (14).

HO-1 in Thrombosis

Thrombosis is induced after vascular injury and leads to several cardiovascular diseases associated with stroke, myocardial infarction, and venous thromboembolic disorders (26). Thrombus formation involves the interaction of injured vessels and platelets. Tissue factors, von willebrand factor, and

fibrinogen trigger platelet activation and aggregation. Several lines of evidence suggest a role of HO-1 in thrombosis. Using *ex vivo* blood clotting perfusion chamber, induction of HO-1 expression has been found to inhibit platelet-dependent thrombosis (71). In a mouse-to-rat cardiac transplant model, inhibition of HO-1 activity by SnPP causes graft rejection, which is associated with platelet aggregation and thrombosis of coronary arterioles (77). Exogenous CO reverses the SnPP effect, including inhibition of platelet aggregation and thrombosis, suggesting CO generated by HO-1 suppresses graft rejection by inhibiting platelet aggregation and vascular thrombosis (77). HO-1 gene transfer into injured vessels of apoE$^{-/-}$ mice immediately after angioplasty results in earlier thrombolysis and restoration of blood flow (12). Further, CO mediates this thrombolytic effect through inhibiting plasminogen activator inhibitor-1 expression and fibrin deposition (12). Bilirubin, another byproduct of HO-1, also contributes to the antithrombotic property of HO-1, because bilirubin was found as effective as HO-1 induction in delaying ferric chloride-induced thrombus formation (52). To investigate the direct function of HO-1 on thrombosis, True *et al.* used photochemical-induced vascular injury in wild-type and HO-1$^{-/-}$ mice. HO-1 deficiency leads to accelerated, occlusive arterial thrombus formation compared with wild-type mice (87). In addition, inhaled CO and biliverdin administration rescue the prothrombotic phenotype in HO-1$^{-/-}$ mice (87). Confirming the role of HO-1/CO in protection against vascular arterial thrombosis, adoptive transfer of HO-1-expressing platelets rescues HO-1$^{-/-}$ mice from arterial thrombosis in allogeneic aortic transplantation (8). In addition to arterial thrombosis, HO-1 also has a role in venous thrombosis. In a model of stasis-induced thrombosis created by ligation of the inferior vena cava, HO-1 deficiency impairs thrombus resolution and exaggerates the inflammatory response to thrombus formation (86). The clinical relevance is demonstrated in a prospective cohort study that patients with long (GT) repeat alleles in HO-1 gene promoter have an increased risk of recurrent venous thromboembolism (62).

HO-1 and Microsatellite Polymorphism in Humans

A number of studies associate human HO-1 gene promoter polymorphisms with risk for vascular diseases (11, 22). A microsatellite DNA with dinucleotide (GT) repeat in the promoter region of the HO-1 gene shows a length polymorphism that modulates the level of HO-1 gene transcription (11, 22). Patients with short (<25 GT) dinucleotide repeats in the HO-1 gene promoter on either allele have higher expression levels of HO-1 and significantly less often restenosis than patients with longer (≥ 25 GT) dinucleotide repeats (22) and coronary atherosclerosis (7). In a Hisayama cohort study, HO-1 expression is found to be intimately associated with atherogenesis and may play an important role as an adaptive molecule in the inflammatory/repair process (81). The significance of (GT)n polymorphism in the HO-1 promoter was further demonstrated in a recent study that (GT)n allelic variants of the promoter directly modulate HO-1 expression levels and the proangiogenic and antiinflammatory functions of HO-1 in human endothelium (83). Thus, the capacity to upregulate HO-1 expression may be genetically regulated, and reduced ability to induce HO-1 may be involved in the mechanism of coronary atherosclerosis (7, 11, 22, 83).

HO-1 and Hypoxia in the Heart

Reduced blood flow due to lumen narrowing from arterial thrombosis or atherosclerotic lesions leads to myocardial hypoxia. A potential function of HO-1 in cardiomyocytes was implicated when primary rat neonatal cardiomyocytes respond to hypoxia with increased expression levels of HO-1 mRNA and protein while under normal physiological conditions, HO-1 is expressed at low levels in cardiomyocytes (4). Hypoxia causes pulmonary hypertension and induces right ventricular hypertrophy. To gain insight into the role of HO-1 in cardiac adaptation to hypoxic stress, we generated HO-1$^{-/-}$ mice and subjected the mice to chronic hypoxia in an early study (94). Although the myocardium in HO-1$^{-/-}$ mice appears to be normal under normoxic conditions, under hypoxic conditions, severe right ventricular dilatation and infarcts with mural thrombi develop in HO-1$^{-/-}$, but not in wild-type mice, despite similar degree of pulmonary hypertension after hypoxia exposure (94) (Fig. 2). In addition to lipid peroxidation and oxidative damage in the right ventricular cardiomyocytes of HO-1$^{-/-}$ mice, we also detected apoptotic cardiomyocytes surrounding areas of infarcted myocardium. These data suggest that the absence of HO-1 in cardiomyocytes leads to an accumulation of ROS that cause cardiomyocyte death and the high percentage of right ventricular mural thrombi in HO-1$^{-/-}$ mice maybe due to increased platelet aggregation (94).

HO-1 in Cardiac Ischemia and Reperfusion Injury

The results from hypoxia studies led us to test the hypothesis that HO-1 may play a central role in cardiac homeostasis (95). For gain of function experiments, we generated cardiac-specific transgenic mice overexpressing different levels of HO-1 (95). To determine whether overexpression of HO-1 protects against postischemic injury and to exclude the involvement of inflammatory components on reperfusion, we used an isolated perfused heart preparation by subjecting hearts to 30 min of global ischemia, followed by 40 min of reperfusion (95). Compared with wild-type hearts, postischemic recovery of cardiac function in all three lines of HO-1 transgenic hearts improved in an HO-1 dose-dependent manner (95) (Fig. 3). In a myocardial infarction model, in contrast to the large infarcts in wild-type mouse hearts, HO-1 transgenic mouse hearts show small infarcts (95) (Fig. 4A, B). Despite a similar percentage of left ventricle at risk between wild-type and transgenic mouse hearts, the infarct size is significantly reduced in transgenic mice compared with wild-type mice (95) (Fig. 4C). Our study clearly demonstrates that overexpression of HO-1 in the cardiomyocyte protects against ischemia and reperfusion injury, thus improving the recovery of cardiac function (95).

To explore the therapeutic potential of HO-1 gene transfer in long-term myocardial protection, Melo *et al.* took advantage of an adeno-associated virus (AAV)-mediated delivery method (58). AAV-mediated transfer of the HO-1 gene into normal rat hearts 8 weeks before ischemia/reperfusion injury leads to a dramatic reduction in left ventricular myocardial infarction (58), supporting the findings of our transgenic studies (95). The HO-1-mediated protection from myocardial ischemia/reperfusion injury is associated with a decrease in oxidative stress and proapoptotic and proinflammatory protein levels (58). These findings demonstrate the therapeutic potential of AAV-mediated HO-1 gene transfer for sustained cardiac

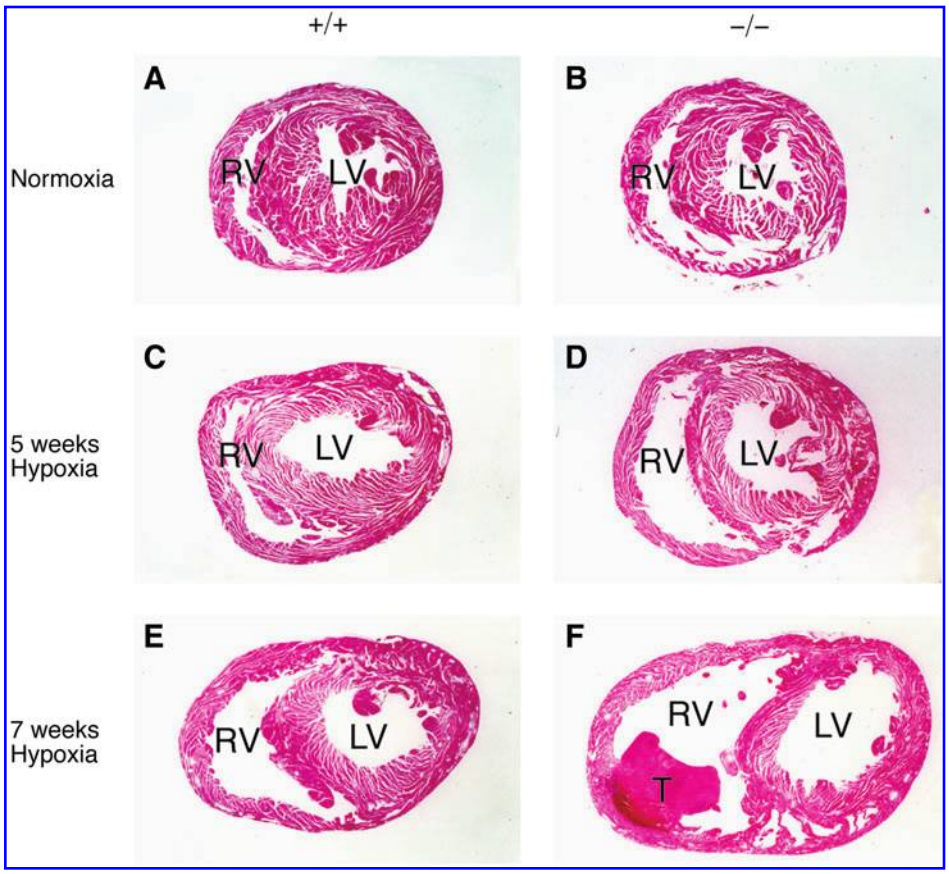

FIG. 2. Right ventricular dilatation and thrombus formation in HO-1$^{-/-}$ mice in response to hypoxia. Heart cross-sections (at the papillary muscle level) from wild-type (+/+) and HO-1 null (−/−) mice were stained with hematoxylin and eosin (n = 5–6 in each group). Mice were exposed to normoxia (**A,** +/+; **B,** −/−), 5 weeks of hypoxia (**C,** +/+; **D,** −/−), or 7 weeks of hypoxia (**E,** +/+; **F,** −/−). Original magnification, 15×. Reproduced from Yet *et al.* (94) with permission. RV, right ventricle; LV, left ventricle; T, thrombus. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

protection from ischemic injury and introduce the concept of pre-event gene therapy in protection against future injury (58).

After showing that predelivery of HO-1 by AAV to the heart can markedly reduce injury after acute myocardial infarction (58), Liu *et al.* investigated the effect of HO-1 gene delivery on postinfarction recovery in a follow-up study (53). Interestingly, AAV-mediated HO-1 gene transfer normalizes postinfarction echocardiographic left ventricular function and chamber dimensions 3 months after myocardial infarction by preventing ventricular remodeling (53). When the effect of HO-1 gene delivery was assessed 1 year after acute myocardial infarction, mortality was markedly reduced in the HO-1-treated animals compared with the LacZ-treated animals (54). The therapeutic efficacy of preemptive AAV-HO-1 delivery is further demonstrated by the findings that chronic recurrent myocardial ischemic injury is significantly attenuated by AAV-HO-1 delivery (68). These results suggest that preemptive HO-1 gene delivery may be useful as a therapeutic strategy to reduce postmyocardial infarction left ventricular remodeling and heart failure.

HO-1 in Diabetic Heart

Myocardial infarction and heart disease is one of the chronic complications of diabetes. Given the cardioprotective role of HO-1 against ischemia/reperfusion injury, we tested the role of HO-1 in the settings of diabetes (55). Although an absence of HO-1 significantly increases infarct size in normoglycemic mice, diabetes exacerbates myocardial infarction in the setting of HO-1 deficiency (55). In addition to exaggerated infarct size, mortality is twofold higher in diabetic HO-1$^{-/-}$ than wild-type mice after ischemia/reperfusion injury. Interestingly, the mortality rate of patients with diabetes suffering from myocardial infarction due to ischemia/reperfusion injury is twofold higher than that of patients who are nondiabetic. Importantly, we observed 55% of diabetic HO-1$^{-/-}$ mice that survived ischemia/reperfusion developed left ventricular thrombi (55) (Fig. 5). Intriguingly, it has been suggested that increased mortality rate in patients with diabetes is, in part, due to the tendency toward thrombosis (2). Corroborating our findings, HO-1 inhibition in streptozotocin-induced diabetic rat further enhances infarct size during ischemia/reperfusion (18). It was found that hyperglycemia predisposed the heart to produce high levels of both the cytokines IL-1β and CXCL8 and subsequent ischemia/reperfusion further increases the cytokine production (18).

In humans, patients with type 2 diabetes with longer (GT) repeats of the HO-1 gene promoter (with lower HO-1 inducibility) are shown to have higher oxidative stress and increased susceptibility to coronary artery disease (11). In a subsequent study, by measuring serum bilirubin and ferritin levels, Chen *et al.* links HO-1 gene promoter polymorphism and the susceptibility to coronary artery disease in diabetic patients (9). Hisayama study examining HO-1 expression in coronary atherosclerotic lesions of Japanese autopsies reveals that HO-1 is intimately associated with intraplague in patients with diabetes (81).

HO-1 in Re-Endothelialization, Angiogenesis, and the Heart

Endothelial cell damage is an important pathophysiological step of atherosclerosis and restenosis after angioplasty

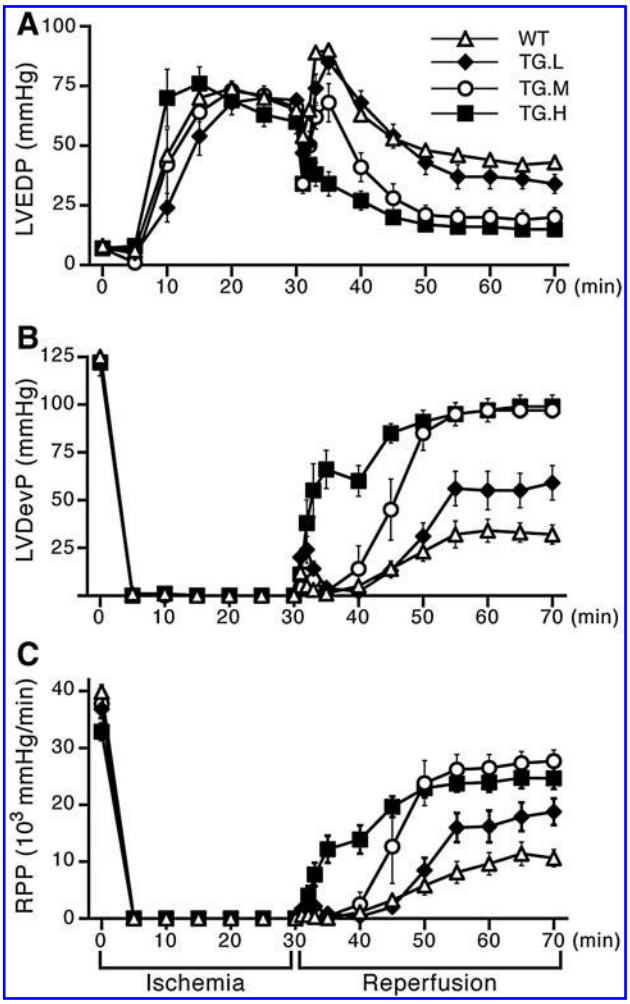

FIG. 3. HO-1 dose dependently improves postischemic cardiac performance in isolated perfused hearts. Hearts from mice were stabilized for 30 min, then subjected to 30 min of global ischemia followed by 40 min of reperfusion. Cardiac contractile performance is shown during ischemia and reperfusion. WT: open triangles, $n = 12$; transgenic TG.L: filled diamonds, $n = 12$; TG.M: open circles, $n = 5$; TG.H: filled squares, $n = 9$. **(A)** Left ventricular end diastolic pressure at the end of ischemia was 65 ± 2, 69 ± 2, 66 ± 3, and 63 ± 5 mmHg for WT, TG.L, TG.M, and TG.H, respectively. **(B)** Left ventricular developed pressure. **(C)** Rate pressure product. Error bars indicate standard errors. Reproduced from Yet *et al.* (95) with permission. LVDevP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; RPP, rate pressure product; WT, wild type.

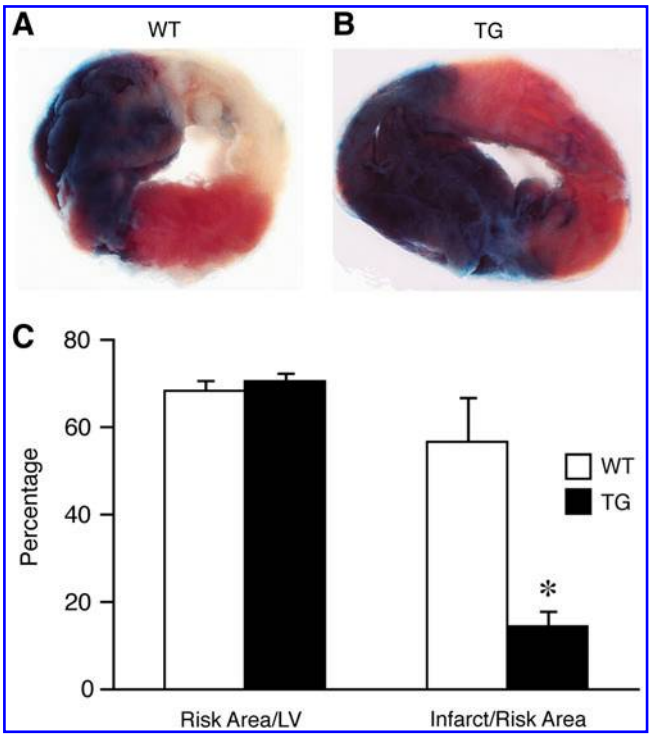

FIG. 4. HO-1 protects against myocardial infarction in transgenic mice. Myocardial infarcts from **(A)** WT ($n = 6$) and **(B)** TG.H transgenic (TG, $n = 8$) mice were assessed by Evans blue and TTC staining after 1 h ischemia and 24 h reperfusion. The Evans blue perfused area, which is not at risk, stained blue; viable myocardium stained red and infarcted myocardium appeared pale. Representative wild-type and transgenic heart sections were shown and oriented anterior side up in **A** and **B**, respectively. Original magnification: ×15. **(C)** Myocardial infarcts are reduced in HO-1 transgenic mice. WT (open bars, $n = 6$) and TG.H transgenic (filled bars, $n = 8$) mice were subjected to 1 h ischemia and 24 h reperfusion as in **A**. Risk Area/LV, percentage of LV at risk; infarct/risk area, infarcted area as percentage of risk area. Error bars indicate standard errors. *$p = 0.001$ *versus* infarct/risk area of WT animals. Reproduced from Yet *et al.* (95) with permission. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

and, thus, endothelial regeneration is critical in repairing injured vessels and protecting them from the development of an intimal lesion (34). Increasing evidence suggests that endothelial progenitor cells (EPCs) derived from bone marrow augment neovascularization of tissue after ischemia and contribute to re-endothelialization after endothelial injury, thereby providing a novel therapeutic option (88).

Since human HO-1 deficiency had elevated oxidative stress with severe endothelial cell damage (45, 92), it is conceivable that HO-1 may affect mobilization and homing of circulating EPCs. As demonstrated by Lin *et al.* (51), systemic overexpression of HO-1 with adenovirus in mice leads to an acceleration of re-endothelialization of denuded vessels due to enhanced EPC mobilization; conversely, lack of HO-1 in mice results in significant attenuation of EPC mobilization and re-endothelialization. Additionally, exposing mice to CO before carotid injury is also able to increase EPC mobilization and enhance re-endothelialization (51). Using a rabbit model of aortic balloon injury and pharmacological induction of HO-1, another study came to the same conclusion that HO-1 contributes to vascular repair by increasing the number and maturation of circulating EPCs derived from the bone marrow (91). Consistent with these findings, bone marrow cells from HO-$1^{-/-}$ mice, compared with wild-type mice, generate fewer endothelial colony-forming cells (91).

In addition to a role in promoting re-endothelialization, overexpression of HO-1 in coronary microvessel endothelial cells enhances endothelial cell proliferation and capillary formation, linking HO-1 to angiogenesis (17). Given the role of HO-1 in cardioprotection and its angiogenic potential, HO-1 may enhance myocardial angiogenesis in ischemic myocardium. The

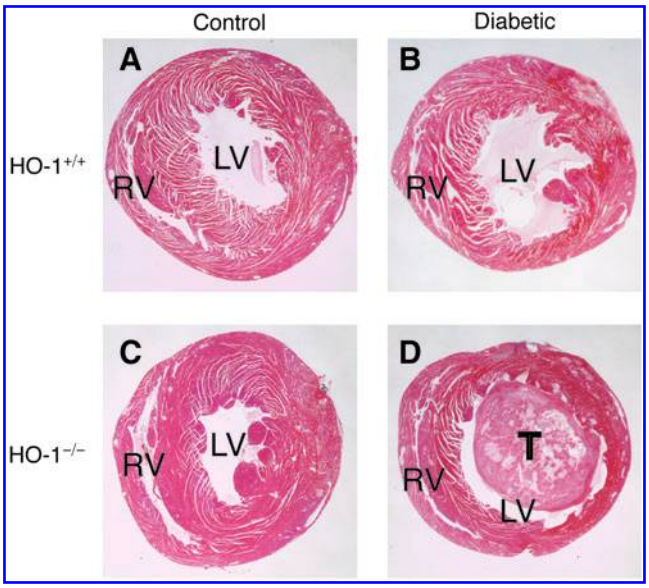

FIG. 5. Ischemia/reperfusion injury induces left ventricular mural thrombi formation in diabetic HO-1$^{-/-}$ mice. Heart cross-sections at the papillary muscle level after ischemia/reperfusion were stained with H&E. Sections from control **(A)** and diabetic **(B)** HO-1$^{+/+}$ mouse hearts. **(C)** Ventricular section from control HO-1$^{-/-}$ mice. **(D)** Thrombus formation in the left ventricle of HO-1$^{-/-}$ diabetic mouse heart. Original magnification: ×15. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

role of HO-1 in improving myocardial angiogenesis was supported by a resveratrol study. Resveratrol, a polyphenolic compound, enhances angiogenesis in the infarcted rat myocardium by induction of vascular endothelial growth factor (VEGF), and the induction of VEGF is mediated by thioredoxin-1 and HO-1 (43). In a hypercholesterolemic myocardial infarction model, secoisolariciresinol diglucoside, a compound isolated from omega-3 fatty acids-rich flaxseed, increases capillary and arteriolar density and improves left ventricular function (72). The cardiac functional improvement might be due to increased HO-1, VEGF, and p-endothelial nitric oxide synthase (NOS) expression (72). These studies emphasize that HO-1 mediates ischemic myocardial angiogenesis after myocardial infarction. Using heterozygous Flt-1 mice with reduced expression of Flt-1, Thirunavukkarasu *et al.* found that reduced expression of Flt-1 results in less ventricular functional recovery compared with the wild-type mice. The reduced functional recovery paralleled with increased myocardial infarction and apoptosis (85). Interestingly, heterozygous Flt-1 mice have pronounced inhibition of the expression of HO-1, inducible NOS, p-AKT and p-endothelial NOS after ischemia/reperfusion injury (85).

HO-1 gene transfer has been shown to promote neovascularization and ventricular function in the ischemic heart of mice receiving HO-1, due to higher cardiac levels of VEGF and stromal cell-derived factor-1 (SDF-1) (50). Concomitant treatment with both VEGF and SDF-1 neutralizing antibodies attenuates the protective effect of HO-1 to a greater extent than treatment with either neutralizing antibody alone (50). This emphasizes the cooperative roles of these two factors in HO-1-mediated protection. Further, increased recruitment of circulating c-kit^{+} stem/progenitor cells to the infarct area in mice receiving HO-1 gene transfer suggests that EPCs participate in the angiogenic effect (50). A follow-up study showed that EPC mobilization and re-endothelialization are significantly attenuated in HO-1$^{-/-}$ mice after vascular injury, which is rescued by exposing mice to CO before carotid injury (51). The role of CO in myocardial angiogenesis is further supported by that pretreatment of CO-donor increases accumulation of c-kit+ stem/progenitor cells in the infarct areas after myocardial infarction (46). These c-kit+ cells are able to differentiate into VSMCs and contribute to the formation of new coronary arteries in infarct areas. The induction of proangiogenic factors hypoxia inducible factor-1α, SDF-1, and VEGF-B was evident in CO-donor pretreated hearts. Thus, HO-1 and CO promote neovascularization after myocardial infarction by modulating the expression of hypoxia inducible factor-1α, SDF-1, and VEGF-B (46). Therapeutic angiogenesis is a novel strategy for treatment of patients with ischemic heart disease. Since HO-1-mediated angiogenesis protects ischemic myocardium, HO-1 and its product, CO, could serve as targets for the treatment of heart disease.

Therapeutic Potential of HO-1

Given the cardiovascular protective function of HO-1, HO-1 offers a great therapeutic potential for cardiovascular disease. Overexpression *via* gene delivery methods has proved the beneficial effects of HO-1 in many animal disease models; however, therapeutic gene delivery methods in humans are not readily available. Therefore, targeted induction of HO-1 by pharmacological means might be a more promising alternative. For example, in addition to the cholesterol-lowering effects, various statins, hydroxymethylglutaryl coenzyme A reductase inhibitors, also have antiinflammatory and antiproliferative effects. Intriguingly, these effects of statins are largely mediated through induced HO-1 expression in vascular cells (29, 30, 47). Further, atorvastatin strongly induces angiogenesis likely *via* HO-1 induction, indicating a potential for therapeutic angiogenesis in ischemic diseases (57). Other than statins, another cholesterol-lowering drug probucol reduces cardiovascular disease incidence in patients with hypercholesterolemia (78) and interestingly, HO-1 is the molecular target of probucol (90). Moreover, induction of HO-1 by aspirin may be a novel mechanism by which aspirin prevents cellular injury under inflammatory conditions and in cardiovascular disease (28). An alternative for HO-1 induction is to inhibit its degradation by increasing mRNA stability. Indeed, simvastatin-dependent upregulation of HO-1 was found to be mediated by stabilization of HO-1 mRNA *via* a PI3K/Akt-dependent signaling pathway (36). Taken together, pharmacological induction of HO-1 holds a promise as a therapeutic intervention for treating cardiovascular disease. For a more refined therapy, time-dependent and tissue-specific induction of HO-1 is needed and, thus, challenges remain for the development of time- and tissue-specific pharmacological agents for HO-1 induction.

Conclusion

Collective evidence from the past two decades has demonstrated the central role of HO-1 in protection against disease. We have highlighted the protective function of HO-1 against cardiovascular diseases. Various stresses, including hypoxia, ischemia/reperfusion injury, diabetes, hypercholesterolemia, and so on, lead to the production of ROS in the

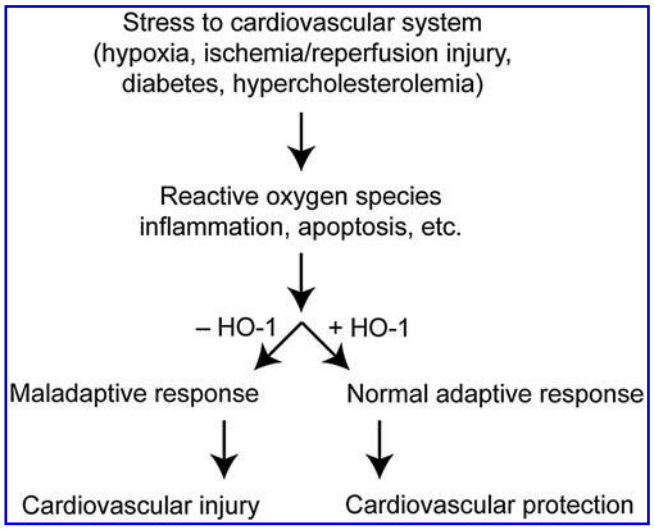

FIG. 6. HO-1 in cardiovascular system. Various stresses lead to the production of ROS in the cardiovascular system. In the absence of HO-1, perhaps through the excessive accumulation of ROS, cardiovascular cells have a maladaptive response, resulting in cell death. In the presence of the buffering effect of HO-1, normal adaptive response provides protection to the cardiovascular system. This implies that overexpression of HO-1 may play a protective role in cardiovascular diseases caused by ROS. ROS, reactive oxygen species.

cardiovascular system (Fig. 6). Increased ROS, in turn, induces inflammation and apoptosis, among others. In the absence of HO-1, perhaps through the excessive accumulation of ROS, cardiovascular cells have a maladaptive response, resulting in cell death. In the presence of the buffering effect of HO-1, normal adaptive response provides protection to cardiovascular system (Fig. 6). As such, overexpression of HO-1 may protect against cardiovascular diseases caused by ROS. The clinical significance of HO-1 in human cardiovascular disease was highlighted in a child with HO-1 deficiency (45, 92). Although the therapeutic potential of HO-1 is clear, developing HO-1 therapeutics would greatly benefit patients suffering from cardiovascular disease.

Acknowledgments

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Abbreviations Used

AAV = adeno-associated virus
apoE$^{-/-}$ = apolipoprotein E-deficient
Br = brachiocephalic artery
CABG = coronary artery bypass graft surgery
CO = carbon monoxide
EPCs = endothelial progenitor cells
GT = guanosine thymidine dinucleotide
HO-1 = heme oxygenase-1
LCC = left common carotid artery
LDL = low-density lipoprotein
LSC = left subclavian artery
LV = left ventricle
LVDevP = left ventricular developed pressure
LVEDP = left ventricular end diastolic pressure
MMPs = matrix metalloproteinases
NOS = nitric oxide synthase
PDGF = platelet-derived growth factor
RCC = right common carotid artery
ROS = reactive oxygen species
RPP = rate pressure product
RSC = right subclavian artery
RV = right ventricle
SDF-1 = stromal cell-derived factor-1
SMC = smooth muscle cell
SnPP = Sn-protoporphyrin IX
T = thrombus
TIMP = tissue inhibitor of metalloproteinase
VEGF = vascular endothelial growth factor
VSMC = vascular smooth muscle cell
WT = wild type

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