Heme Oxygenase and Atherosclerosis

Toshisuke Morita

Abstract—Overproduction of reactive oxygen species under pathophysiologcal conditions, including dyslipidemia, hypertension, diabetes, and smoking, is integral in the development of cardiovascular diseases (CVD). The reactive oxygen species released from all types of vascular cells regulate various signaling pathways that mediate not only vascular inflammation in atherogenesis but also antioxidative and antiinflammatory responses. One such protective and stress-induced protein is heme oxygenase (HO). HO is the first rate-limiting enzyme in heme breakdown to generate equimolar quantities of carbon monoxide, biliverdin, and free ferrous iron. Accumulating evidence has shown that inducible HO (HO-1) and its products function as adaptive molecules against oxidative insults. The proposed mechanisms by which HO-1 exerts its cytoprotective effects include its abilities to degrade the pro-oxidative heme, to release biliverdin and subsequently convert it bilirubin, both of which have antioxidant properties, and to generate carbon monoxide, which has antiproliferative and antiinflammatory as well as vasodilatory properties. Herein, I highlight the relationship of HO and cardiovascular disease, especially atherosclerosis, gene-targeting approaches in animal models, and the potential for and concern about HO-1 as a novel therapeutic target for cardiovascular diseases.

Key Words: heme  reactive oxygen species  inflammation  cytoprotection

Well-established cardiovascular risk factors, such as dyslipidemia, hypertension, diabetes, and smoking, can initiate endothelial dysfunction by altering the cell redox state in the vessel wall and thus induce atherosclerosis. In this paradigm, a common mechanism by which cardiovascular risk factors initiate the disease process is oxidative stress, which leads to vascular inflammation. Excessive production of reactive oxygen species (ROS) has been implicated in many pathophysiologcal conditions such as oxidative stress.

The oxidative modification of plasma low-density lipoproteins (LDL), mainly formed in the arterial wall, markedly increases their atherogenicity, and deposition of oxidized LDL in arterial walls is central to the development of an atherosclerotic lesion. LDL, which is initially accumulated in the extracellular subendothelial space in arterial walls, is mildly oxidized to minimally modified LDL, which in turn induces monocyte chemotactic protein 1 and macrophage colony-stimulating factor, both of which stimulate monocyte recruitment and differentiation to macrophages in arterial walls. The accumulation of monocytes/macrophages in arterial walls generates ROS, resulting in the conversion of minimally modified LDL into oxidized LDL. Because oxidized LDL-elicited dysfunction of endothelial cells is one of the earliest steps in the development of atherosclerosis, the adaptive and/or protective responses of the vasculature to oxidative stress are important in preventing atherogenesis.

One such protective and stress-induced protein is heme oxygenase (HO). HO is the rate-limiting enzyme in heme degradation. For much of the time, HO was studied within the confines of heme metabolism. After HO-1, an inducible form of HO, was found to be readily upregulated by diverse factors associated with various cardiovascular diseases, however, many studies have been reported and accumulating evidence suggests that HO-1 induction confers protection against injuries associated with ROS.

In this article, I review the enzymatic aspects of HO, the biological properties of HO reaction products, the possible involvement of HO in atherosclerosis, and the therapeutic application of HO for atherosclerosis in humans by directly delivering or pharmacologically activating the HO-1 gene.

Heme and Heme Oxygenase

Heme is a crucial substance that is absolutely required for aerobic life. Free heme can be quite cytotoxic, however, particularly in the presence of oxidants or activated phagocytes. Of all sites in the body, the vasculature may be at greatest risk of exposure to free heme, as erythrocytes contain heme at a concentration of 20 mmol/L and are prone to undergoing unexpected lysis. Extracellular hemoglobin is easily oxidized and readily releases heme. Given the hydrophobic nature of heme, it can rapidly intercalate with membranes and cause severe damage. Exposure of microsomal membrane fractions to free heme in the presence of \(H_2O_2\) leads to a dose-dependent peroxidation of unsaturated lipids in vitro. Heme also catalyzes the protein peroxidation of susceptible groups in membranes, serum, or cytosolic pro-
teins, leading to the formation of nonreducible covalent cross-linkages or protein fragmentation. Heme has been shown to be involved in the pathogenesis of atherosclerosis, which is considered to be an inflammatory disease characterized by the accumulation of lipids and fibrous material in arterial walls. Hemoglobin-derived heme has been demonstrated to act as a catalyst for the oxidation of LDL, which in turn causes atherosclerosis.6 Wagener and colleagues7 observed heme-induced expression of adhesion molecules, such as intercellular adhesion molecule-1, vascular adhesion molecule-1, and E-selectin, in cultured endothelial cells.

HO is a rate-limiting and microsomal enzyme that participates in heme breakdown to generate equimolar amounts of biliverdin IXa, free ferrous iron, and carbon monoxide (CO). Subsequently, biliverdin is rapidly converted to bilirubin by biliverdin reductase, and free iron is sequestered by ferritin8,9 (Figure 1). This reaction requires oxygen and occurs in association with NADPH-ferrihemoprotein reductase (cytochrome reductase), which supplies electrons to reduce heme iron or to activate molecular oxygen.10 Three isoforms of HO that are the products of separate genes have been identified in mammals.11–13 HO-1 is transcriptionally upregulated as a sensitive antiinflammatory protein by various types of oxidative stress, such as oxidized LDL,14 ultra-violet radiation,15 thiol scavengers,16 and hypoxia,17,18 as well as substrate heme19 in the cardiovascular system. A common feature of these HO-1 inducers is their ability to regulate the intracellular redox state. HO-1 is also transcriptionally activated through several regulatory mechanisms. Studies on the promoter region of HO-1 have revealed transcriptionally responsive elements, including activator protein I, activator protein II, nuclear factor-kB, interleukin-6-responsive elements, and an antioxidant response element (ARE).20–22 HO-2 is constitutively expressed in many organs throughout the body, although it is particularly high in the brain and testes, but is unresponsive to any of the inducers of HO-1.13 The third isoform, HO-3, is nearly devoid of catalytic activity;13 however, Hayashi and colleagues23 recently reported that HO-3 is a pseudo gene derived from HO-2 transcripts.

**Potential Roles of HO Reaction Products on Vascular Function**

HO and the subsequent metabolites of heme catabolism seem to play vital roles in regulating important biological responses, including inflammation, oxidative stress, cell survival, and cell proliferation. The proposed mechanisms by which HO-1 exerts its biological effects include its ability to release biliverdin with its subsequent conversion to bilirubin and to generate ferritin and CO, as well as to decompose the pro-oxidative heme.

**Biliverdin and Bilirubin**

Biliverdin and bilirubin have been considered to be waste products of heme degradation. These products can be toxic at high concentrations, especially in neonates.24 Little attention had been paid to the physiological roles of these products until the observation that bilirubin suppresses the oxidation of chemically induced peroxyl radicals to a greater extent than α-tocopherol, which provides powerful protection against lipid peroxidation in vitro.25 After bilirubin was recognized as an antioxidant, many epidemiological studies demonstrated that higher serum levels of bilirubin are inversely associated with the incidence of coronary arterial disease.26–29 The antiatherogenic properties of bilirubin may include inhibitory effects against LDL oxidation and scavenging of oxygen radicals, which are generated from phospholipids, triglycerides, and cholesterol esters.30 Indeed, Ishikawa and colleagues31 demonstrated that HO inhibition resulted in enhanced formation of lipid peroxides in aortic tissue in Watanabe heritable hyperlipidemic (WHHL) rabbits. They speculated that HO reaction products like biliverdin and bilirubin might reduce the formation of plasma and tissue lipid peroxidation products.

The mechanism by which bilirubin reacts with ROS is not completely understood, although its hydrophobic tetrapyrrole structure has been reported to inhibit the activation of superoxide-producing NADPH oxidase.32 Bilirubin has also been shown to have an inhibitory effect on protein phosphorylation33 and protein kinase C activity.34 Both of which lead to the inactivation of various proatherogenic factors, such as lipid oxidation,35 immune reactions,36 and inflammatory processes.37 Oxidative metabolites of bilirubin have recently been detected in atherosclerotic lesions.38 The physiological role of neonatal hyperbilirubinemia has been assumed to be the result of the inhibitory action of bilirubin on lipid and protein peroxidation.39 Oxidized metabolites of bilirubin (biopyrrins) have been reported to be excreted in urine after exposure to oxidative stress.40 The antioxidant actions of bilirubin have been demonstrated in vivo in a variety of diseases, including Alzheimer disease, ischemia-reperfusion injury, and coronary artery diseases.41,42 These observations suggest that increased bilirubin production is an adaptive response against oxidative insults.

**Carbon Monoxide**

CO is another product of the HO reaction in vivo, and the similarities between nitric oxide (NO) and CO have suggested that CO may have a physiological role.43 Earlier studies have suggested that CO might bind to and activate guanylate cyclase, thereby increasing intracellular levels of cyclic guanine monophosphate (cGMP), as has been demonstrated for NO.4,18,44 However, the physiological relevance of CO as a vasodilator is controversial. NO is a potent activator
of guanylate cyclase and increases cGMP production in vitro
≈130 fold, whereas the increase in cGMP induced by CO is
≈4.4 fold.45,46 Because CO is more chemically stable than
NO and there appears to be no enzymatic pathway catalyzing
CO degradation in vivo, the biological availability of NO and
CO may differ.1 CO has been suggested to act as a partial
antagonist for soluble guanylate cyclase (sGC) activation.47–49
It has been posited that overproduction of CO might
impair NO-elicited activation of sGC, resulting in suppres-
sion of the cGMP increase in the aortas of transgenic mice
that overexpressed HO-1 in vascular smooth muscle cells.49
HO activity, as measured by CO production, has been
detected in arteries of rodents and humans in vivo.50 Some
reports have suggested that CO may act as a physiological
regulator of vascular tone through cGMP-mediated responses
in large vessels (eg, aorta),46,51 whereas CO may dilate the
smaller renal arteries or pial arterioles via activation of
Ca2+-activated potassium channels.52,53 Other reports have
hypothesized that CO may play a role in blood pressure
regulation in acute hypertension.54 In addition to relaxing
vascular smooth muscle, CO has been shown to have a
regulatory effect on smooth muscle cell proliferation and
death,55–58 which may play an important negative feedback
role in limiting the development of vascular diseases.

Recently, CO has been reported to exert cGMP-
dependent actions on Ca-dependent potassium channels53,59
and on the mitogen-activated protein (MAP) kinase.60,61 CO
directly activates Ca-dependent potassium channels, leading
to a regulatory relaxation through Ca desensitization. Another
cGMP-independent action of CO is the activation of the p38
MAP kinase pathway. The inhibitory effect of CO against
lipopolysaccharide-induced proinflammatory cytokine pro-
duction in macrophages and its antiproliferative effect on
vascular smooth muscle cells after balloon injury are medi-
ated by the p38 MAP kinase pathway, including MAP kinases
3 and 6. The effect of CO on p38 MAP kinase and caspase 3
has been reported in pulmonary artery endothelial cells,
indicating that CO has antiapoptotic effects and reduces
ischemia-reperfusion lung injury.62 In addition, p38 MAP
kinase pathway activation by CO has been observed to
protect endothelial cells from tumor necrosis factor-α-mediated
apoptosis.63 Thus, the actions of CO via cGMP-
dependent and cGMP-independent pathways may explain a
number of the potential actions of CO regarding the patho-
genesis of cardiovascular diseases.

**Ferritin and Iron**

Ferritin is a ubiquitous and highly conserved iron-binding
protein. In vertebrates, the cystosolic form consists of 2
subunits, termed H and L. Twenty-four ferritin subunits
assemble to form the apoferritin shell.64 In the ferritin shell,
the proportion of H and L depends on the iron status in
the cell or tissue and varies among organs and species. Ferritin H
catalyzes the oxidation of ferrous iron to ferric iron under
aerobic conditions to allow intracellular iron storage in
biological systems. The content of cytoplasmic ferritin is
regulated by the translation of ferritin H and L mRNAs in
response to the content of intracellular free iron.65,66 When
iron levels are low, ferritin synthesis decreases; conversely,
when iron levels are high, ferritin synthesis increases. Al-
though under certain circumstances there is an increase in
ferritin mRNA in response to iron,67 the regulatory response
of ferritin to iron is largely posttranscriptional68 and is due to
the recruitment of stored mRNA from monosomes to poly-
somes in the presence of iron.66 The induction of ferritin has
been shown to provide marked antioxidant cellular protection
by rapidly sequestering free cytosolic iron, the crucial catalyst
of oxygen-centered radical formation via the Fenton reaction
in biological systems.

HO-1 releases Fe2+ from the core of the heme molecule,
leading to the rapid expression of the protein ferritin in tandem
with HO-1.69 Ferritin H is induced after HO-1
induction in vascular smooth muscle cells.70 Furthermore, the
enhancement of intracellular ferritin protein through HO-1
has been reported to reduce the cytotoxic effects of hemin and
hydrogen peroxide in vascular endothelial cells.71 Addition-
ally, increased expression of ferritin mRNA has been shown
in a rat model of brain ischemia/reperfusion injury,72 in
pericardial fluid of patients with coronary disease,73 and in
human atherosclerotic aortas with abdominal aneurysm.74
Hoekstra and colleagues75,76 revealed that aortic endothelial
cells in a strain of atherosclerosis-susceptible Japanese quail
were more susceptible to oxidative stress than those from an
atherosclerosis-resistant strain, which coincided with higher
HO activity, HO-1 expression, ferritin, and glutathione levels.
These findings suggest that the cytoprotective effect of
ferritin production after HO-1 induction on ROS-induced
cellular injury may act to limit intracellular-free iron.

**HO-1 Induction by ROS**

Well-established cardiovascular risk factors, such as dyslip-
idemia, hypertension, diabetes, and smoking, increase ROS
production in arterial wall cells. In endothelial cells as well as
in vascular smooth muscle cells and monocytes/macrophages,
all of which constitute atherosclerotic lesions, ROS generated
via several enzymatic reactions initiate and promote the
atherogenic process. Xanthine oxidase,77 NAD(P)H oxida-
dase,78 uncoupled NO synthase,79 and myeloperoxidase80 are
well-known ROS-generating enzymes expressed in athero-
sclerotic lesions. Among these enzymes, NAD(P)H oxidase is
a key player in generating ROS in the vasculature, and it
produces superoxide anion, one of the important reactive
oxygen species, by catalyzing a 1-electron reduction of
oxygen using NAD(P)H as the electron donor. ROS affect
many proatherogenic processes such as lipoprotein oxidation,
endothelial apoptosis, and matrix metalloproteinase expres-
sion and cause atherosclerosis, restenosis, and diabetes mel-
litus vasculopathy. ROS production activates several tran-
scription factors such as nuclear factor-κB, activator protein
1, and early growth response gene 1 (Egr-1) in vascular walls,
leading to the initiation of proinflammatory processes.81,82 By
contrast, cells exposed to ROS also induce a group of
antioxidative genes via the ARE and Nrf2, a transcription
factor for ARE.83,84

Stimulation of the HO-1 gene is primarily controlled at the
transcriptional level, which is governed by responsive ele-
ments localized in the promoter 5′-flanking region of the
HO-1 gene. Studies of the promoter region of HO-1 have
revealed transcriptionally responsive elements, including nuclear factor-kB, activator protein 1, activator protein 2, interleukin-6–responsive elements and ARE. Although considerable information is available on the inducible responsive elements of the HO-1 gene, much less is known about the transcriptional factors that actually mediate this induction. The transcriptional factor Nrf2, which belongs to the family of Cap’n’Collar basic leucine zipper proteins, has previously been shown to be essential for gene induction mediated by ARE and serves as a key transcription factor in the cytoprotection of tissues against oxidative stress. Nrf2 acts in a wide-ranging metabolic response to oxidative stress and constitutes a cellular sensor for oxidative stress through a nuclear shuttling mechanism with the cytosolic regulator protein Keap 1. Alam and colleagues have demonstrated that a DNA sequence containing an activator protein 1 consensus site is regulated by Nrf2 and that Nrf2 mediates the induction of the HO-1 gene. The important role of Nrf2 in the stress-dependent induction of HO-1 has been confirmed by the finding that HO-1 is not inducible in Nrf2 null mice. More recently, Ishii and colleagues have demonstrated that oxidized LDL and 4-hydroxynonenal increased HO-1 expression in murine macrophages, whereas they failed to increase it in Nrf2-deficient macrophages.

**Oxidized LDL Accumulation and HO-1 Expression in Atherosclerotic Lesions**

As noted earlier, numerous studies have shown that oxidized LDL plays a pivotal role in the development of atherosclerosis. Development of fatty-streak lesions is initiated with the production of adhesion molecules, monocyte chemotactic factors, and vascular smooth muscle growth factors. As the atherosclerosis lesions progress, migration and proliferation of smooth muscle cells and deposition of fibrous tissue lead to an advanced, complicated lesion. HO-1 in vascular endothelial cells, vascular smooth muscle cells, and macrophages is markedly upregulated by oxidized LDL, whereas HO-1 is not increased in vascular endothelial cells or smooth muscle cells when exposed to native LDL. The component in oxidized LDL responsible for HO-1 induction appears to be oxidized arachidonic acid-containing phospholipids, such as 1-palmitoyl-2-isoprostanoyl-sn-glycero-3-phosphorylcholine and linoleyl hydroperoxide, but not lysophosphatidylcholine. HO-1 expression was observed throughout the development of the lesions, from an early fatty streak to an advanced complex atherosclerotic lesion; that is, HO-1 is expressed in vascular endothelial cells and macrophages in the early stages of atherosclerotic lesion formation and in foam cells and smooth muscle cells residing in the necrotic core of advanced lesions. In all these atherosclerotic lesions, HO-1 has been found to be colocalized with oxidized phospholipids, strongly suggesting that HO-1 is induced by oxidized phospholipids in vivo. The fact that HO-1 was detected in early atherosclerotic lesions suggests that the changes may be the cause rather than a consequence.

**Antiatherogenic Properties of HO-1**

**In Vitro Study**

Atherosclerosis is thought to be accelerated when the antioxidative defense is not sufficiently induced in the vascular wall under oxidative and inflammatory stress. Accumulating evidence suggests that HO-1 contributes to the balance of pro-oxidant and antioxidant factors in the vascular wall through multiple mechanisms. Taille et al explored the role of HO-1 in the expression and activity of NADP(H) oxidase, the key player in generating ROS in the vasculature. They revealed that HO-1 expression by HO-1–encoded plasmid transfection in macrophages resulted in a decrease of NADP(H) oxidase activity and expression of gp91(phox), its heme-containing catalytic component, because reduction of the heme content due to HO-1 activation limited heme availability for maturation of the gp91(phox) subunit and assembly of the functional NADP(H) oxidase. Endothelial dysfunction caused by ROS is a key step in the initiation of lesion formation. Abraham et al demonstrated that increased expression of HO-1 attenuated ROS-mediated cell growth and apoptosis of endothelial cells. The protective effect of the HO-1 signaling mechanism, which attenuated glucose (ROS)-mediated endothelial cell apoptosis, was maintained through suppression of p21 and p27 cyclin kinase inhibitors or, perhaps, through its powerful antioxidant effect. Monocyte transmigration into the vessel wall is an essential event for the development of atherosclerosis. Ishikawa et al demonstrated that HO-1 induction significantly inhibited monocyte transmigration induced by oxidized LDL, whereas HO inhibition had the opposite effect. The inhibitory effect of HO-1 on monocyte transmigration could be mediated by biliverdin and bilirubin, as these compounds can directly inhibit monocyte transmigration. We confirmed the role of bilirubin in monocyte transmigration. My colleagues and I also revealed that angiotensin II increased CC chemokine receptor 2 (CCR2) expression in monocytes and increased ROS production, leading to enhanced chemotactic activity. However, HO-1–induced monocytes with increased accumulation of bilirubin exhibited enhanced CCR2 expression, as well as ROS production and chemotactic activity induced by angiotensin II. Similar inhibitory action of bilirubin on leukocyte adhesion to activated vascular endothelial cells has been reported.

Smooth muscle cell proliferation and monocytes recruitment are essential steps for the development of atherosclerosis. My colleagues and I have previously investigated the role of HO-1 in hypoxia-induced growth factor expression in vitro. My colleagues and I demonstrated that HO-1 was induced by hypoxia in vascular smooth muscle cells, and that the smooth muscle cells–derived CO inhibited the endothelial cells production of endothelin 1, platelet-derived growth factor-B, and vascular endothelial growth factor. The inhibition of these factors by CO led to a decrease in vascular smooth muscle cells proliferation. In addition, smooth muscle cell–derived CO directly decreased vascular smooth muscle cells growth by inhibiting E2F-1, a transcription factor that participates in the control of cell cycle progression from G1 to S phase. These reports suggest that CO, by limiting vascular smooth muscle cell growth, may be a key mediator in the body’s compensatory response to vascular remodeling associated with hypoxia.
In Vivo Animal Study
Several investigators have also demonstrated that endogenous HO-1 plays a role in atherosclerotic lesion formation based on the fact that chronic inhibition of the HO system using metalloporphyrins promoted exaggerated lesion formation in a rat model of balloon injury, LDL-receptor knockout mice, and Watanabe heritable hyperlipidemic rabbits. My colleagues and I previously demonstrated that HO-1 was upregulated in rat carotid artery after endothelial denudation using a balloon catheter and that inhibition of endogenous HO-1 by metalloporphyrins promoted neointimal formation, whereas HO induction by hemin resulted in a reduction of neointimal formation. In LDL receptor knockout mice, HO-1 is abundantly expressed in atherosclerotic lesions after dietary cholesterol feeding. In this mouse model, HO overexpression resulted in a reduction of plaque formation, whereas HO inhibition by Sn-protoporphyrin IX promoted lesion development. In the WHHL rabbit model, inhibition of HO-1 by Sn-protoporphyrin IX increased plasma and tissue lipid peroxidation levels. These studies provided insight regarding the role of endogenous HO enzymes and their therapeutic application against atherosclerotic lesion formation; however, confirmation of this response in HO-1-deficient mice is critical because the effects of metalloporphyrins are not selective for HO-1 or even the HO system. Metalloporphyrins have been reported to inhibit NOS and sGC in addition to heme oxygenase. Other nonspecific effects of metalloporphyrins include the inhibition of muscle relaxation and suppression of cyclic adenosine monophosphate and cGMP. These non-HO activity-related inhibitory effects may be consequent to the interaction of metalloporphyrins with membrane receptors or their downstream signal transduction pathways. Heme oxygenase inducers are also known to exert an adverse effect when used especially for chronic upregulation of HO-1. The disadvantage of using SnCl2, a synthetic substance with unique selectivity for renal heme oxygenase, is its nephrotoxicity. The application of CoCl2 was limited by its excessive toxicity and high mortality found in the animal study. Hemin, the most prominent and frequently used HO inducer, increases oxidative stress. Heme arginate increases not only the production of CO from HO-1, but also that of NO, as arginate is a derivative of t-arginine.

Recent studies, therefore, have explored the strategy of administering the HO gene with a vector and generating transgenic mice to investigate the long-term and specific protective effects of HO-1 against atherosclerosis and vascular injury. Abraham et al demonstrated that retrovirus-mediated HO-1 overexpression reduced the number of detached endothelial cells in a rat model of diabetes, indicating that endothelial HO-1 protects against ROS-mediated endothelial damage. Overexpression and underexpression of human HO in endothelial cells can be modulated on a long-term basis by the introduction of the HO-1 gene with a retroviral vector. The findings that overexpression of the HO-1 gene in the rat carotid artery reduced neointimal formation and inhibited medial-wall DNA synthesis after balloon injury further suggested that adenovirus-mediated HO-1 gene delivery has a potential therapeutic application. Juan and colleagues demonstrated that selective overexpression of HO-1 was also able to decrease lesion formation in apolipoprotein-E (ApoE)–deficient mice using adenovirus-mediated gene transfer of HO-1. Duckers et al showed that adenovirus-mediated HO-1 introduction to arterial walls directly reduced vascular cell proliferation in a pig model of vascular injury. They revealed that the vasorelaxation response was mediated by guanylate cyclase and cGMP, whereas growth inhibition and cell-cycle arrest in cells expressing the HO-1 transgene were associated with the induction of p21. Yet et al generated mice deficient in both HO-1 and ApoE. Despite similarly elevated total plasma cholesterol levels in response to a Western diet, mice deficient in both HO-1 and ApoE developed larger and more advanced atherosclerotic lesions than mice deficient in ApoE alone.

In a mouse model of vein graft stenosis, vein grafts from HO-1 knockout mice showed a robust neointima formed by α-actin–positive vascular smooth muscle cells as compared with that in vein grafts from wild-type mice. Further studies revealed that vascular smooth muscle cells from HO-1 knockout mice were more susceptible to oxidant H2O2-induced cell death than those isolated from wild-type mice, suggesting that increased susceptibility to oxidative stress may lead to cell death in HO-1 knockout mice.

To confine HO-1 gene delivery to vascular smooth muscle cells, my colleagues and I generated transgenic mice which overexpress HO-1 under the control of the SM22α promoter, which is specifically expressed in vascular smooth muscle cells. Using these transgenic mice, my colleagues and I investigated the pathophysiological role of HO-1 in vascular smooth muscle cells after arterial injury induced by angiotensin II combined with high salt. We demonstrated that wild-type mice exhibited increased production of ROS and inflammatory changes such as expression of inflammatory cytokines and macrophage infiltration into and around the coronary artery, whereas HO-1 transgenic mice did not show any changes in ROS production or inflammatory responses. Interestingly, in vascular smooth muscle cells, HO-1 reduced ROS production not only in vascular smooth muscle cells but also in adjacent endothelial cells and cardiomyocytes.

Human Cases
The importance of HO-1 in vascular biology was highlighted by the discovery of a child with HO-1 deficiency. In this HO-1–deficient child, both intravascular hemolysis and endothelial cell injury were prominent. Importantly, oxidation of hemoglobin to methemoglobin occurred in the plasma, and iron was accumulating in his LDL. Iron-induced oxidative modification of lipoproteins is cytotoxic and causes endothelial damage, leading to the development of fatty streaks and fibrous plaques in the aorta. Endothelial cells of this child were susceptible to oxidative insults because of heme-mediated oxidation of LDL and an associated lack of adaptive responses, suggesting that HO-1 plays a crucial role in protecting vessels from oxidative insults. To support this concept in humans, Exner et al carried out a cohort study patients to evaluate HO-1 gene promoter polymorphisms and their risk for restenosis. They found that a dinucleotide repeat in the promoter region of the HO-1 gene showed a length...
polymorphism that modulate the level of gene transcription. In their study, patients with short (<25 GT) dinucleotide repeats in the HO-1 gene promoter on either allele had restenosis significantly less often that patients with longer (≥25 GT) dinucleotide repeats. These data imply that up-regulation of HO-1, associated with shorter dinucleotide repeats, may be a protective factor after balloon angioplasty. More recently, a study that also assessed microsatellite polymorphisms in the HO-1 gene promoter showed that type 2 diabetic patients carrying longer (≥2 GT) repeats had higher oxidative stress and increased susceptibility to the development of atherosclerosis and coronary artery disease. Although the results of these studies on genetic polymorphism of the HO-1 gene may indicate the potential importance of HO-1 in the pathogenesis of cardiovascular diseases, direct measurement of HO-1 activity in the patients is necessary, as varying oxidative stress may elicit the HO-1 response in endothelial cells, smooth muscle cells, and inflammatory cells to different degrees. Development of a serial monitoring system of HO-1 activity for use in clinical setting is desirable.

**HO and Pharmacological Interventions**

HO may have potential in the treatment of atherosclerosis and vascular injury in humans by being directly delivered or by pharmacological activation of the HO-1 gene. However, most of the pharmacological inducers of HO-1 used in experimental studies, such as hemin and heavy metals, exhibit cellular and tissue toxicity, and the long-term and adverse effects of HO-1 gene delivery must be elucidated before its application in humans. Recent studies, however, revealed that some well-used drugs modulate HO-1 expression in vascular cells. Aspirin is known to reduce the incidence of thrombotic occlusive events such as myocardial infarction and stroke by inhibiting platelet activity. Recently, more direct effects of aspirin on the integrity of the vascular wall, free radical scavenging, and the capacity to protect endothelial cells from the deleterious effects of hydrogen peroxide have been reported. Grosser et al reported that aspirin increased HO-1 protein levels in a concentration-dependent fashion via NO dependent pathways in cultured endothelial cells derived from the human umbilical vein. They concluded that induction of HO-1 activity is a novel mechanism by which aspirin prevents cellular injury under inflammatory conditions and in cardiovascular disease. Statins, widely used lipid-lowering agents, substantially decrease cardiovascular morbidity and mortality in patients with and without coronary disease via not only a cholesterol-lowering effect but also their pleiotropic cholesterol-independent effects. The precise mechanisms of those pleiotropic effects, however, remain unclear. Grosser and colleagues explored the role of HO-1 as a target and mediator of statins. They revealed that statins, including simvastatin, lovastatin, and rosuvastatin, increased the transcription of HO-1 mRNA in human umbilical vein endothelial cells, which coincided with a reduction of free radicals formation. These results may explain the pleiotropic antioxidant, antiinflammatory, and antiatherogenic actions of statins. Rapamycin, a macrolide antibiotic, blocks cell cycle progression at the G1 phase and rapamycin-coated coronary stents have been shown to reduce coronary restenosis. Visner and colleagues reported that rapamycin induced HO-1 and suppressed platelet-derived growth factor-dependent vascular smooth muscle cell growth. It has been suggested that HO-1 induced by rapamycin in smooth muscle cells shows an antiproliferative effect, resulting in the reduction of restenosis rate.

**Conclusion and Perspectives**

Ample evidence has been provided to support HO-1 as a key player in various cardiovascular diseases, especially those in
which ROS have been implicated (Figure 2). With regard to the possible relevance of HO-1 in atherosclerosis, it should be noted that HO-1 is induced by most of the well-established cardiovascular risk factors in vascular cells and circulating macrophages. These HO-1 responses appear to have a protective role in the vascular wall against atherogenesis through multiple pathways. Interventions aimed at modulating the levels of HO in the vascular wall, therefore, might be a novel target to treat or prevent atherosclerotic diseases. Some studies, however, have clearly demonstrated the surprising role of HO-1 in promoting endothelial cell apoptosis, endothelial dysfunction, and vasoconstriction. Akin et al. reported that bilirubin (10 to 100 mol/L) induced apoptosis in bovine brain microvascular endothelial cells. Thom and colleagues showed that biochemical effects of CO occur at environmentally relevant concentrations and that exposure to relatively high concentrations of CO causes apoptotic cell death in bovine pulmonary artery endothelial cells. Johnson et al. revealed that exogenous as well as endogenous CO induces vasoconstriction in isolated gracilis muscle arterioles, most likely through the inhibition of endothelial NO synthesis, and that elevated HO-1 levels and activity in arteries contribute to endothelial dysfunction, leading to hypertension in deoxycorticosterone acetate rats and Dahl-S rats.

Furthermore, reduction of heme and heme proteins caused by HO-1 may impair the function of various crucial enzymes, such as mitochondrial cytochromes, microsomal cytochromes, cyclooxygenases, NO synthase, and NAD(P)H oxidase. HO-1 may impair the function of various crucial enzymes, such as mitochondrial cytochromes, microsomal cytochromes, cyclooxygenases, NO synthase, and NAD(P)H oxidase. HO-1; the intracardiac injection of the HO-1 gene alters other organ responses in endothelial cells are mediated by cyclic adenosine monophosphate. J Clin Invest. 1993;92:471–478.


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