Basic Science for Clinicians

Heme Oxygenase-1
A Novel Drug Target for Atherosclerotic Diseases?

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Schmid and coworkers[1] were the first to report on the presence of heme oxygenase (HO) in liver microsomes capable of degrading heme to bilirubin, and this activity was subsequently dissociated from cytochrome P-450.[2,3] HO catalyzes the first and rate-limiting step in the oxidative degradation of heme (Fe-protoporphyrin-IX) to carbon monoxide (CO), ferrous iron (Fe^{2+}), and biliverdin-IX (Figure 1). The enzyme binds heme in a 1:1 molar complex, and HO-bound heme acts as prosthetic group and substrate. The reaction requires 3 molecules of molecular oxygen (O_2) per heme molecule oxidized and reducing equivalents derived from nicotinamide adenine dinucleotide phosphate or nicotinamide adenine dinucleotide (reduced form) and transferred to the oxygenase via nicotinamide adenine dinucleotide phosphate-:cytochrome P-450 reductase. Regiospecific oxidation of heme is achieved in a stepwise reaction, with α-meso-hydroxyheme and verdoheme as intermediates, and the dissociation of CO followed by that of Fe^{2+}.[4] The release of biliverdin from HO is accelerated by biliverdin reductase, which reduces the green pigment to bilirubin-IX,[4] which is then excreted into bile as the glucuronic acid conjugate.

Originally, the interest in HO was related to its well-established function in heme catabolism and the turnover of erythrocytes. For many years, CO and bilirubin were regarded as toxic waste byproducts of the HO reaction, but in 1987, a potential beneficial role of bilirubin was proposed[5] based on the in vitro antioxidant activities of the pigment. Over the last decade, however, the interest in HO has shifted greatly from a metabolic to a protective function of the enzyme in a variety of conditions associated with cellular stress and pathologies, and this has been the subject of excellent recent reviews.[6,7]

The relationship of HO to atherosclerotic vascular disease was suggested initially in 1994 by an observational study reporting that low serum concentrations of bilirubin are associated with increased risk of coronary artery disease.[8] Since then, the implied cardioprotective role of HO has been developed and substantiated significantly in experimental models of atherosclerotic vascular disease, including atherosclerosis,[9] intimal hyperplasia,[10] and myocardial infarction.[11] There is now good evidence that induction of the inducible isozyme HO-1 by a broad spectrum of physical and chemical agents leads to several vascular cell-specific protective activities in the setting of inflammatory atherosclerotic diseases. Despite this recent work, however, the precise relationship between HO-1 and atherosclerosis remains unknown. In the present study, we review recent progress in our understanding of the protective mechanisms of HO-1 in atherosclerotic vascular disease and highlight areas of insufficient knowledge that require additional work in the future.

HO-1: Critical Contribution to Iron Homeostasis

A discussion of the role of HO-1 in atherosclerotic vascular disease should begin with a brief review of the established role of the enzyme in iron homeostasis. HO-1 in macrophages of the reticuloendothelial system plays a key role in the reuse of the iron essential for erythropoiesis. In adult humans, the majority of Fe-protoporphyrin-IX degraded by HO-1 is derived from hemoglobin, resulting in a daily production of $\approx$28 mg Fe^{2+} or nearly 1% of the total body iron store.[12] This iron is returned almost quantitatively to the circulation where it is bound tightly by the plasma glycoprotein transferring, which transports the iron to the bone marrow where it is used to synthesize hemoglobin in developing erythroid cells. Indeed, mice lacking functional HO-1 develop an anemia that is associated with abnormally low levels of serum iron and the accumulation of iron in liver and kidneys.[13] This function of HO-1 in iron homeostasis implies that at least in macrophages, HO-1 activity is associated with the release of cellular iron.

In the context of atherosclerotic vascular diseases, it is not clear whether a role of HO-1 in iron reuse extends to macrophages, or indeed to other cells, in the vessel wall and/or to heme derived from sources other than hemoglobin. Ferris et al[14] reported decreased iron efflux from fibroblasts deficient in HO-1 compared with control cells and transfection of an epithelial cell line with HO-1 to augment iron release without changes to the cellular iron storage protein ferritin. In apparent contrast, an earlier study reported Ultraviolet A radiation induced HO-1 activity to co-induce ferritin and that ferritin to store released iron.[15] Homeostasis of cellular iron is accomplished by coordinated regulation of iron import and export proteins.[16] Export of iron to transferrin

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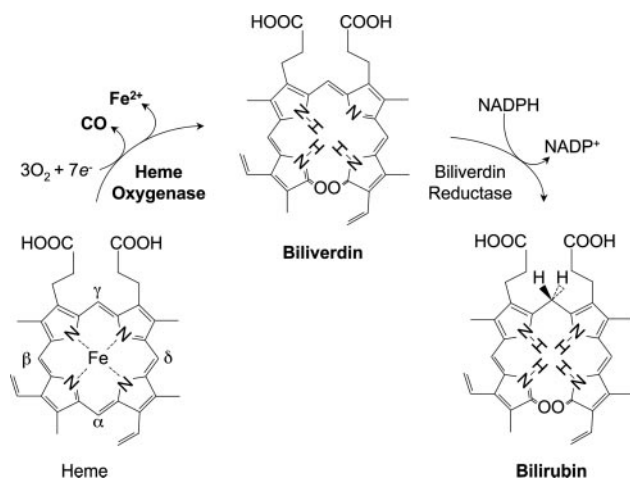**DOI: 10.1161/CIRCULATIONAHA.105.598698**

Figure 1. Oxidative metabolism of heme by HO and biliverdin reductase, giving rise to CO, iron, biliverdin, and bilirubin.

is generally thought to occur via the plasma membrane transporter ferroportin, and this requires oxidation of Fe^{2+} to Fe^{3+} by ceruloplasmin.[16] In vitro vascular endothelial cells express ferroportin in response to high glucose[17] and proinflammatory cytokines.[18] It is not known, however, whether this extends to endothelial and/or vascular smooth muscle cells in vivo, whether ferroportin expression is responsive to increased cellular HO-1 activity, or whether ceruloplasmin is present in atherosclerotic vessels. What is known is that arteries with atherosclerotic lesions express HO-1[19] and contain more iron than corresponding healthy vessels.[20] Because iron can conceivably affect atherosclerotic disease, investigations of the role of HO-1 on iron content in diseased arteries are warranted. In addition, the possibility that non–bone marrow HO-1 supplies iron for growth of nonerythroid stem cells, such as endothelial progenitor cells implicated in the repair of vascular injury relevant to atherosclerotic vascular disease, deserves investigation.

HO-1: Functions Beyond Iron Homeostasis

There are 2 genetically distinct isozymes of HO: the inducible HO-1 and a constitutively expressed form, HO-2. As indicated, HO-1 is expressed most strongly in tissues involved in erythrocyte or hemoglobin metabolism, whereas in most other tissues, HO-1 typically occurs at low basal levels but responds rapidly by transcriptional activation to diverse stimuli. In contrast, HO-2 is strongly expressed in testes but also is present ubiquitously in other tissues, including the vasculature, and it does not generally respond to transcriptional activation. HO-1 is classically characterized as a protein associated with the endoplasmic reticulum,[1] although recent evidence suggests that the enzyme also is present in caveolae of the plasma membrane of endothelial cells[7] and in liver mitochondria of heme-treated rats.[21] A function of nonmicrosomal HO-1 currently is not established, although the reported colocalization of biliverdin reductase[21] suggests that the enzyme is also metabolically active at these sites and hence may contribute to a localized regulation of heme protein turnover.

HO-1: Protection Against Atherosclerotic Disease

HO-1 is a cytoprotective enzyme, and its induction commonly occurs in the setting of increased cellular stress to help maintain physiological homeostasis. HO-1 is induced by a number of stressors,[7] and one may predict that risk factors for the development of coronary heart disease and other cardiovascular disease processes will mediate HO-1 expression. In fact, this is the case because increased blood pressure[22] and altered laminar flow in blood vessels,[23] advanced glycation end products,[24] cigarette smoke,[25] oxidized lipids,[26] and a multitude of systemic inflammatory processes[7] lead to increased cellular HO-1 expression. Moreover, this constellation of processes that lead to HO-1 induction may suggest a role for HO-1 in mediating the cardiovascular disease processes associated with obesity and metabolic syndrome,[27] which have become significant public health problems. Pragmatically determining that upregulation of HO-1 plays a protective role in atherosclerotic disease would require investigation into patients who either lack HO-1 or do not robustly express HO-1 in the presence of risk factors for coronary heart disease. With the availability of these patients, investigations into the prevalence of coronary heart disease would provide critical insight into the physiological role of HO-1.

HO-1 deficiency is very rare in humans; however, an autopsy report from a 6-year-old boy with HO-1 deficiency revealed hyperlipidemia associated with fatty streaks and fibrous plaques in the aorta.[28] The concept that HO-1 may be causally related to cardiovascular diseases in humans also has been suggested by studies assessing polymorphisms in the 5′-flanking sequence of the human HO-1 gene.[29] Different polymorphisms have been identified in the HO-1 promoter, with the most studied being (GT)n dinucleotide-length polymorphism. In general, the belief is that compared with longer (GT)n repeats, shorter (GT)n repeats have higher transcriptional activity and thus higher expression levels. For example, in a Chinese population of type II diabetic patients, long (GT)n repeats ($\geq$32 GTs) were associated with increased risk for coronary artery disease.[30] Conversely, in a Japanese population of patients with significant risk factors (hyperlipidemia, diabetes, and smoking) for coronary artery disease, shorter (GT)n repeats ($<$27 GTs) were associated with less disease.[31] However, this concept does not appear to hold for white patients with myocardial infarctions or stable coronary artery disease.[32] In contrast, studies assessing the association of length of (GT)n repeats with the risk of restenosis after percutaneous transluminal angioplasty have been more consistent across ethnic backgrounds.[29,33] In addition to (GT)n dinucleotide-length polymorphism, a single nucleotide polymorphism in the HO-1 promoter, T(-413)A, correlated with a reduced incidence of ischemic heart disease in a Japanese population.[34] In this study, the AA genotype (A on each allele) at position −413 was less likely to be associated with myocardial infarction and angina pectoris, and cell culture experiments suggested the AA genotype to have significantly higher basal promoter activity that was independent of the length of (GT)n repeats.[34] These studies advocate the potential role of HO-1 gene regulation in atherosclerotic disease processes. Truly understanding HO-1 as a modulator of

Support for a Protective Role of HO-1 Against Atherosclerosis and Related Diseases

Evidence	Reference
Presence of HO-1 in human and experimental atherosclerotic lesions	19
High serum bilirubin levels associated with decreased risk of coronary artery disease	8
Low serum bilirubin levels associated with increased risk of coronary artery disease	7
HO-1 induction/upregulation decreases experimental atherosclerosis	36–38
HO-1 inhibition/downregulation increases experimental atherosclerosis	36, 37
Increased atherosclerosis in ApoE$^{-/-}$HO-1$^{-/-}$ vs ApoE$^{-/-}$HO-1$^{+/+}$ mice	9
Increased vein graft stenosis in ApoE$^{-/-}$HO-1$^{-/-}$ vs ApoE$^{-/-}$HO-1$^{+/+}$ mice	9
HO-1 overexpression decreases intimal thickening in experimental restenosis	10, 40, 46
HO-1 deficiency/downregulation increases intimal thickening in experimental restenosis	10, 120
Cardiac-specific HO-1 overexpression protects against experimental ischemia/reperfusion injury	11, 85
Lung-specific overexpression of HO-1 ameliorates pulmonary vascular hypertension in a model of chronic hypoxia	86

atherosclerosis, however, requires further in-depth investigation into this specific disease process.

Atherosclerosis is an inflammatory disease in which lipid deposition in the arterial wall, resulting from elevated levels of plasma cholesterol, is central to lesion development. This process involves the uptake of modified low-density lipoprotein (LDL) by macrophages and is associated with a state of heightened oxidative stress and damage.[35] As atherosclerotic lesions progress, migration and proliferation of smooth muscle cells and deposition of fibrous tissue lead to an advanced, complicated lesion. Wang and colleagues[19] previously demonstrated that expression of HO-1 is prominent in endothelial cells, macrophages, and foam cells in human and animal atherosclerotic lesions. Ishikawa and colleagues[36,37] showed that inducers of HO-1 reduce lesion size in Watanabe heritable hyperlipidemic rabbits[36] and LDL-receptor–deficient mice.[37] Using adenovirus-mediated gene transfer of HO-1, Juan and colleagues[38] demonstrated that selective overexpression of HO-1 decreases lesion size in apolipoprotein E–deficient (ApoE$^{-/-}$) mice.

There are additional lines of evidence in support of HO-1 playing a protective role in atherosclerotic lesion formation (the Table). Long-term inhibition of the HO activity using metalloporphyrins promotes lesion formation in LDL-receptor–deficient mice[37] and rabbits.[36] A potentially serious limitation of these studies, however, is that metalloporphyrins are not selective for HO-1 or even the HO system,[39] particularly when used at high doses. Thus, the use of HO-1–deficient (HO-1$^{-/-}$) mice was important to specifically establish a protective role of endogenous HO-1 in atherosclerosis. For this, Yet et al[9] subjected ApoE$^{-/-}$ mice and mice deficient

in ApoE and HO-1 (ApoE$^{-/-}$HO-1$^{-/-}$) to a Western diet for 8 weeks and then analyzed them for the development of atherosclerosis. Despite similarly elevated total plasma cholesterol, ApoE$^{-/-}$HO-1$^{-/-}$ mice had larger and more advanced lesions than ApoE$^{-/-}$ mice. The lesions in ApoE$^{-/-}$HO-1$^{-/-}$ mice were complicated with fibrous caps, comparable to plaques seen in ApoE$^{-/-}$ mice on a Western diet for longer periods of time (12 weeks). These results, in conjunction with the HO-1 gene transfer studies, provide strong evidence for a beneficial effect of HO-1 on experimental atherosclerosis.

When evaluating the potential for HO-1 as a therapeutic target, we should also assess the ability of HO-1 to ameliorate vascular complications after coronary artery bypass surgery or percutaneous transluminal angioplasty, namely vein graft failure and restenosis. Although coronary artery stenting is being used increasingly to treat patients with obstructive atherosclerotic lesions, coronary artery bypass graft surgery remains an important treatment for multivessel disease. Autologous vein grafts provide a convenient conduit for bypass graft surgery, and although early grafts occlude as a result of thrombotic events, late-onset graft occlusion is the result of intimal thickening and superimposed atherosclerosis. In experimental vein graft stenosis, HO-1$^{-/-}$ mice develop more robust neointima consisting of smooth muscle cells 10 days after surgery than wild-type mice,[9] suggesting that HO-1 plays a protective role in the pathophysiology of not only atherosclerosis but also vein graft failure.

As indicated, studies assessing human HO-1 polymorphisms in disease suggest that higher levels of HO-1 expression are associated with a reduced risk for restenosis after percutaneous transluminal angioplasty.[29] This concept has been confirmed in animal models of restenosis. Thus, adenovirus-mediated transfer of the HO-1 gene reduces intimal thickening in balloon-injured pig femoral[10] and rat carotid arteries.[40] Conversely, arterial lesions induced by wire injury are more severe in HO-1$^{-/-}$ than wild-type mice,[10] again establishing a protective role for HO-1 in models of injury-induced vascular disease. Together, the above results strongly support the notion that HO-1 plays a protective role in experimental atherosclerotic vascular disease. Although the relevance of these findings to the human disease has yet to be established, the overall outcome of the preclinical studies carried out to date clearly points to HO-1 as a potential novel target for therapy.

Induction of HO-1

Signaling Pathways

The gene coding for HO-1 is highly regulated, and in most cell types, HO-1 is expressed in response to numerous stimuli.[7] Interestingly, the overwhelming majority of stimuli cause a rapid and temporary induction of the HO-1 gene; only a few mediators that suppress HO-1 are known.[41] Regulation of the HO-1 gene is predominantly at the transcriptional level.[7] Multiple enhancer regions have been identified in the 5′-flanking sequence of the HO-1 gene, and depending on the specific stimulus and the cell type involved, various transcription factors will interact with their cognate DNA binding domains in the HO-1 promoter to regulate gene transcription.

One of the first pathways to link extracellular stimuli to activation of HO-1 is the mitogen-activated protein kinase (MAPK) pathway.[7] The MAPKs are a family of serine-threonine protein kinases that regulate many cellular events, including responses to environmental stimuli. The MAPK pathway encompasses 3 signaling cascades (ie, extracellular signal regulated kinases 1/2, c-Jun-N-terminal kinase, and p38-MAPK) that phosphorylate downstream targets. In the case of HO-1 transcriptional events, downstream targets of these kinase cascades are transcription factors involved in HO-1 gene regulation. Beyond the MAPKs, other kinases that have emerged as mediators of HO-1 gene regulation include phosphatidylinositol 3-kinase and protein kinases A, C, and G.[7]

We have already mentioned that genetic polymorphisms in the human HO-1 gene promoter modulate the level of transcriptional activity and the magnitude by which HO-1 responds to a pathophysiological stimulus. These polymorphisms are associated with an altered risk profile for cardiovascular disease.[29] To date, little is known about the interaction of nuclear proteins within these polymorphic regions. However, a number of excellent reviews have covered the complex topic of HO-1 gene regulation by transcriptional events involving classic DNA binding domains and their associated transcription factors, as well as cell type and species specificity (see Reference 7 and references therein). Therefore, for the purposes of the present review, we focus on selected transcriptional events activated by proatherogenic stimuli.

There are several critical regulatory domains present in a 10-kb region of the 5′-flanking sequence of the HO-1 gene. Two of the most highly studied enhancer regions, called E1 and E2, contain stress-response elements (StRE) that are conserved between human, mouse, and rat genes and that structurally resemble the antioxidant response element (ARE) and the AP-1/TPA, Maf, and cyclic adenosine monophosphate (cAMP) response elements.[7] Nuclear proteins that bind to these elements belong to the basic-leucine zipper (bZIP) superfamily of transcription factors and include AP-1 (Fos/Jun), CREB/ATF, Maf, Cap'n'collar-bZIP (Nrf, Bach), and biliverdin reductase.[42] These nuclear proteins bind to StRE as homodimers or heterodimers (within or between families). Because of the similarity of StRE with consensus AP-1 binding sites, complexes such as c-Fos/c-Jun heterodimers were the focus of initial HO-1 gene regulation studies.[43] However, more recent studies have emphasized additional bZIP family members as important mediators of the StRE response. For example, during exposure to heme or heavy metals, cytosolic Nrf2 is stabilized and then translocates to the nucleus, where it binds to consensus StRE/ARE binding sites and forms heterodimers with Maf proteins. Because Maf proteins do not have transactivation domains, Nrf2 drives transcriptional activity. The heme binding protein Bach1 is another binding partner for Maf proteins at StRE/ARE sites. Because Bach1 also lacks a transactivation domain, its heterodimerization with Maf proteins results in repression of HO-1 expression. Heme, the substrate for HO-1 enzyme activity, abrogates the repression of Bach1 by inhibiting its binding to DNA.[44] This inhibition of Bach1 binding allows activators of the HO-1 gene such as Nrf2 to bind with Maf

proteins at StRE/ARE sites[45] and thus provides a feedback loop for the regulation of HO-1 expression. The potential importance of Bach1 in cardiovascular disease is supported by the recent finding that mice deficient in Bach1 have increased expression of HO-1 and less intimal proliferation after vascular (cuff) injury.[46]

In addition, enhancer regions outside E1 and E2 also have been described in the transcriptional regulation of HO-1.[7] For example, a balance of Ets protein family members, including transcriptional activators such as Ets-1 and Ets-2, and the repressor Elk-3 regulates the overall transcriptional response of HO-1 during an inflammatory stimulus by binding to domains far downstream from the E1 and E2 enhancer regions.[47] This demonstrates the complexity of the system and underscores the importance of differences in HO-1 gene regulation by different stimuli and in different cell types.

Response to Physical Stress

Increased synthesis of HO-1 protein in response to physical and chemical stress occurs commonly and in most tissues examined.[7] This is not surprising when we consider that HO-1 belongs to a larger family of stress proteins in which transcriptional regulation responds to altered environmental conditions. In fact, HO-1 was initially referred to as 32-kDa heat shock protein because of its transcriptional responsiveness to hyperthermia,[48] although the protein shares little amino acid homology with heat shock proteins, nor does it display protein chaperone activity. More recently, it has been reported that in cultured vascular smooth muscle cells, endoplasmic reticulum stress increased HO-1 mRNA and protein via the ARE, and this was associated with cell survival.[49] The "endoplasmic reticulum stress response" constitutes a general response to endoplasmic reticulum–associated stress such as unfolded proteins, glucose starvation, and disruption of intracellular calcium homeostasis. Endoplasmic reticulum–initiated cell death pathways are increasingly recognized as playing important roles in several diseases, including ischemia/reperfusion injury, heart disease, and diabetes.

In addition to these general types of physical stress, HO-1 responds directly to vascular injury such as that which occurs during angioplasty.[10] This response appears to be biphasic, with an initial decrease in endogenous HO-1 followed by an increase to above baseline and then a return to background levels of HO-1 expression.[10,50] Furthermore, the temporal and spatial pattern of HO-1 expression appears to be similar to that of the G_1 cyclin-dependent kinase inhibitors p27^{Kip1} and p21^{Cip},[10] consistent with the notion that HO-1 functions upstream of and participates in the regulation of smooth muscle cell proliferation in injured vessels.

Response to Chemical Stress

As reviewed previously,[7] HO-1 regulation responds to a large and broad spectrum of chemical stresses, including agents that cause oxidative stress or diminish oxygen, thiol-reactive agents, heavy metals, electrophilic polyphenolic compounds, inflammatory mediators, growth factors, hormones, and environmental pollutants. The following discussion is limited to agents directly relevant to atherosclerotic vascular disease.

As we have learned already, atherosclerotic vascular disease is characterized by a state of heightened oxidative stress that includes the presence of oxidized lipids,[35] and HO-1 is expressed in atherosclerotic lesions.[19] In vitro studies have demonstrated that hydrogen peroxide,[51] linoleic acid hydroperoxides,[52] oxidized phospholipids[53] and LDL[54] induce HO-1 in different cell types. When investigated, this induction was shown to be mediated via StRE/ARE in murine and human cells.[53] However, whether increased levels of hydrogen peroxide and/or oxidized lipids directly induce HO-1 in atherosclerotic lesions is unclear at present. Hepatic HO activity is unaltered in mice deficient in glutathione peroxidase-1 or selenoprotein P (ie, enzymes involved in the cellular metabolism of hydrogen peroxide and lipid hydroperoxides) but increased in mice deficient in thioredoxin reductase,[55] an enzyme that affects several redox-related cellular processes.[35]

Response to Therapeutic Agents

An increasing number of therapeutic agents have been reported to induce HO-1, in direct support of the notion that HO-1 may represent a novel drug target for atherosclerotic disease. These therapeutic agents include statins, rapamycin, paclitaxel, nitric oxide (NO), aspirin, and probucol. At micromolar concentrations, several statins dose dependently induce HO-1 in the human epithelial cell line ECV304[56] and vascular smooth muscle cells.[57] In addition, oral administration of statins at 100 mg/kg body weight resulted in a statin- and tissue-specific increase in HO-1 mRNA and protein in mice.[58] Because this induction was associated with beneficial cellular effects such as increased resistance to oxidative stress and inhibition of smooth muscle cell proliferation,[57] it was speculated that this novel activity may contribute to the pleiotropic and antiatherogenic actions of statins. While attractive, additional studies are required to establish whether statins at concentrations pharmacologically relevant to humans induce HO-1 and, if so, whether this indeed results in significant protection.

Rapamycin (sirolimus) is a macrolide antibiotic with potent immunosuppressive properties that inhibits cell proliferation by blocking the progression of cells from the G_1 to the S phase of the cell cycle. In experimental models, rapamycin has antiproliferative properties against vascular endothelial and smooth muscle cells,[59] and it reduces the fibroproliferative response to vascular injury in vivo.[60] This has led to its successful clinical application in the form of sirolimus-eluting stents for the treatment of in-stent restenosis. Rapamycin induces HO-1 expression in primary human endothelial and smooth muscle cells,[61] an activity not shared by other immunosuppressive agents such as cyclosporin A. In addition, inhibition of HO activity by tin protoporphyrin resulted in a loss of the antiproliferative activity of rapamycin,[61] and smooth muscle cells from HO-1$^{-/-}$ mice were refractory to growth inhibition by rapamycin.[62] Similarly, rapamycin was recently reported to induce HO-1 in the lungs of rats and to inhibit the development of monocrotaline-induced pulmonary hypertension, with the protective effect being blocked by co-treatment of the animals with tin protoporphyrin.[62] Collectively, these findings suggest that the antiproliferative action of rapamycin may be modulated, at least in part, by its actions on HO-1. Similar to rapamycin, paclitaxel, which induces apoptosis, interferes with normal function of microtubule growth, and is used to treat in-stent restenosis, induces HO-1 in vascular smooth muscle cells.[63]

Nitroglycerin and long-acting nitrates are used commonly in cardiovascular medicine for various anginal syndromes and congestive heart failure and in patients with left ventricular dysfunction. The mechanisms for relief of myocardial ischemia by nitrates are intimately linked to the formation of NO within vascular smooth muscle cells, where it stimulates the enzyme guanylate cyclase, resulting in increases in guanosine $3',5'$-cyclic monophosphate (cGMP) and vasodilation. In addition to regulating vascular tone, it is now increasingly recognized that NO modulates a variety of important physiological activities related to vascular homeostasis, including platelet aggregation, leukocyte trafficking, cell signaling, and the migration and growth of endothelial and smooth muscle cells. NO donors[64] and pure, gaseous NO[65] induce HO-1. This induction is due, in part, to the increased stability of HO-1 mRNA[65] and extends to many different forms of NO (eg, NONOates, *S*-nitrosothiols, sodium nitroprusside, and pentaerythritol tetranitrate) and vascular cells.[7] Activation of soluble guanylate cyclase and enhanced formation of cGMP have commonly been associated with HO-1 induction by NO and related compounds.[7] However, this is not the case in all situations, just as not all NO-related compounds induce HO-1. Perhaps most notably, induction of HO-1 in human embryonic lung fibroblasts by gaseous NO was reported to be independent of the guanylate cyclase/cGMP pathway,[65] and isosorbide dinitrate, unlike pentaerythritol tetranitrate, was unable to induce HO-1 in endothelial cells.[66] The cellular generation of NO also may be responsible for, or contribute to, the ability of therapeutically used drugs such as aspirin[67] to induce HO-1.

Probucol, a rarely used cholesterol-lowering drug, has been reported to inhibit atherosclerosis in carotid artery[68] and neointimal proliferation after coronary angioplasty with[69] and without[70] stent deployment in humans and to retard atherosclerosis[71] and intimal thickening after balloon injury in animals.[72] Probucol has been reported to upregulate HO-1 mRNA, protein, and activity in smooth muscle cells in vitro after exogenous addition and in vivo after oral administration to animals undergoing vascular injury induced by balloon angioplasty.[50] Blockade of HO-1 upregulation by siRNA and HO activity by tin protoporphyrin inhibited proliferation of vascular smooth muscle cells in vitro[50] and in vivo, respectively.[73] Interestingly, blocking the ability of probucol to induce HO-1 in vivo completely prevented the ability of the drug to inhibit intimal hyperplasia, promote reendothelialization,[73,74] and restore endothelium-dependent relaxation of the injured blood vessel.[73] Together, these findings indicate that induction of HO-1 is the mode of action of probucol and results in several concerted protective activities against atherosclerotic disease. Furthermore, as a result of in vivo structure-function studies, Wu et al[73] proposed novel probucol analogs as potential new antiatherosclerotic therapeutics that act via HO-1 induction. Probucol has largely been withdrawn as a therapeutic drug because it failed to inhibit

femoral atherosclerosis[75] and because it has QT-prolonging and high-density lipoprotein cholesterol–lowering activities.[69] However, the monosuccinate ester of probucol (AGI-1067) is presently being tested in phase 3 clinical trials as an antiatherosclerotic agent. Like probucol, AGI-1067 inhibits neointimal proliferation after coronary stenting in humans.[69] Unlike probucol, however, AGI-1067 does not appear to prolong the QT interval, and it has comparatively less high-density lipoprotein cholesterol–lowering activity.[69] Interestingly, we observed recently that AGI-1067 also induced HO-1 in injured arteries of experimental animals (B.J. Wu and R. Stocker, unpublished observation, 2006).

Collectively, the above results are consistent with the notion that targeting HO-1 is a promising and therapeutically feasible approach against atherosclerotic[66,73,76,77] and cardiovascular-renal disease.[6] A potential problem with this approach, however, is that the chemical upregulation of HO-1 commonly involves Nrf2/ARE, as we learned earlier. The importance of this is that the Nrf2/ARE signaling pathway also causes induction of a phase II response,[78] an undesirable effect in relation to the development of novel drugs. Given the complexities involved in HO-1 regulation, an alternative therapeutic may be the application of a single metabolic product of HO activity such as the CO-releasing molecules[79] or biliverdin and bilirubin. Preclinical trials suggest that this is a promising approach,[80–82] although there are concerns about using the bile pigments in intervention studies such as their poor chemical characterization and water solubility[83] and their potential toxicity.

Prolonged and High Levels of HO-1 Expression

After reviewing the regulation of HO-1, from the basic level of gene transcription to the in vivo level during disease, we must raise the question of whether prolonged induction or high levels of HO-1 expression have adverse consequences. As HO-1 degrades heme, long-term increases in HO-1 or induction of HO-1 to high levels of expression could conceivably lead to heme depletion and associated adverse consequences such as loss of cellular heme proteins required for normal cell function. Contrary to such an expectation, however, the prolonged induction of high levels of HO-1 seen in genetically mutant mice with hemolytic anemia does not lead to changes to microsomal heme content or heme biosynthesis.[84] In fact, it has been proposed that in vivo the extent of heme degradation and heme synthesis is carefully balanced by corresponding long-term adaptive responses.[84] Several independent studies have demonstrated a beneficial effect of targeted overexpression of HO-1 in experimental models of cardiovascular disease. Cardiac-specific overexpression of HO-1 in mice and rats provides improved recovery of contractile performance and long-term myocardial protection after ischemia/reperfusion in an HO-1 dose-dependent manner, with the greatest protection at the highest level of HO-1 expression.[11,85] In addition, in a mouse model of chronic hypoxia, sustained increases in the human HO-1 transgene targeted to the lung ameliorated pulmonary vascular hypertension, vessel wall hypertrophy, and inflammation.[86]

Furthermore, targeting HO-1 to vascular smooth muscle cells, resulting in long-term increases in HO-1 expression, decreased angiotensin II–induced production of reactive oxygen species and inflammatory responses in transgenic mice.[87] Despite this, however, further studies are needed to unambiguously rule out the possibility of adverse effects of prolonged HO-1 expression because the biological effects of HO-1 are different in different cell types and in different pathological settings. In this context, it is also important to point out that there may be differences between situations of long-term versus short-term HO-1 induction, eg, with regard to the impact on heme content and activities of heme-containing enzymes. Thus, short-term induction of HO-1 expression and HO activity in rats by treatment with hemin is associated with decreased mitochondrial heme content and activities of cytochrome oxidase and NO synthase.[21] Similarly, in vitro overexpression of HO-1 in endothelial cells can result in decreased cyclooxygenase activity.[88]

Proposed Mode of Protection

To date, studies investigating the mode of protection offered by HO-1 induction have been limited mostly to research examining the effects of the products derived from HO activity. For example, the ability of CO and biliverdin/bilirubin to prevent neointimal development after vascular injury has been examined in several independent studies. CO was reported to prevent balloon angioplasty–induced arteriosclerotic lesion formation in rats.[89] Interestingly, this study showed that 1 hour of exposure to inhaled CO (250 ppm) before injury ameliorated lesion formation 14 days later.[89] In addition, Öllinger and colleagues[82] demonstrated neointimal formation after balloon injury to be reduced in hyperbilirubinemic Gunn rats and in wild-type rats treated with biliverdin, the precursor to bilirubin, compared with control animals. This section discusses our present knowledge of how HO-1 and the products of its catalytic activity are thought to protect against atherosclerotic vascular pathology.

Inflammation

There is overall support for the concept that HO-1 is important in regulating inflammation in vivo. Compared with wild-type mice, HO-1$^{-/-}$ mice develop chronic inflammation with increasing age, characterized by enlarged spleens (due in part to follicular hyperplasia) and lymph nodes, hepatic inflammatory infiltrates, and high peripheral white blood cell counts.[13] Notably, HO-1$^{-/-}$ mice also show hallmarks of vascular injury, indicated by monocytes adhering to vessel walls.[13] Interestingly, death of the only person diagnosed to date with HO-1 deficiency was reported to be due to an inflammatory syndrome associated with vulnerability of endothelial cells to oxidative stress–induced injury.[28] These reports suggest that HO-1 contributes to physiological homeostasis and that in the absence of HO-1, the potential for a proinflammatory environment develops.

It has been suggested that CO contributes significantly to the antiinflammatory properties of HO-1.[76,90] For example, overexpression of HO-1 or the administration of CO suppresses proinflammatory cytokines and chemokines such as

tumor necrosis factor-α, interleukin-1β, interleukin-6, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1β,[76,85,86,90] and increases the expression of the antiinflammatory mediator interleukin-10.[91] This antiinflammatory effect of CO occurs in part via induction of peroxisome proliferator–activated receptor-γ and blocking expression of Egr-1.[92] Other beneficial properties of HO-1 and CO that may mediate the inflammatory response and blood flow during vascular injury include their ability to prevent platelet activation[93] and subsequent thrombosis,[94] to suppress plasminogen activator inhibitor type-1 expression,[95] and to prevent endothelial cell death.[96] In vitro, overexpression of HO-1 has been reported to decrease the expression of the adhesion molecules E-selectin and vascular cell adhesion molecule-1.[97]

Smooth Muscle Cell Proliferation

In addition to altering the inflammatory response, HO-1 and products of its enzymatic activity can prevent neointimal formation by inhibiting the proliferation of smooth muscle cells. Morita and colleagues[98,99] originally provided some insight into the regulation of smooth muscle cell proliferation by HO-1 and CO using hypoxic conditions in vitro. These studies demonstrated that smooth muscle cell–derived CO was an important mediator of cell proliferation and that inhibiting CO formation or scavenging CO increased the growth of smooth muscle cells in response to endothelial mitogens such as endothelin-1 and platelet-derived growth factor-β.[98,99] It was also noted that CO had effects independent of the endothelium, increasing cGMP and suppressing E2F-1 (a transcription factor involved in cell cycle progression) in vascular smooth muscle cells.[99] Advancing this biological activity into a model of vascular injury, Duckers and colleagues[10] showed that HO-1 directly stimulated vascular relaxation and inhibited vascular cell proliferation in a model of vascular injury in pigs using adenoviral HO-1 gene transfer. Vessel relaxation was mediated via NO-independent activation of soluble guanylate cyclase and resulting increases in cGMP, whereas growth inhibition and cell cycle arrest were associated with induction of the cell cycle inhibitor p21^{Cip1}.[10] Further studies using a wire injury model in the femoral arteries showed that lesions in HO-1$^{-/-}$ mice were more severe than lesions in wild-type mice.[10] Vascular smooth muscle cell proliferation was greater in lesions from HO-1$^{-/-}$ mice compared with HO-1$^{+/+}$ mice, confirming the antiproliferative effect of HO-1 on arteries in vivo. In addition to activation of guanylate cyclase, CO may affect vascular tone by stimulating calcium-activated potassium channels in smooth muscle cells.[100] More recent studies have shown that beyond CO, biliverdin decreased neointimal formation after balloon injury in rats compared with control animals.[82] In this model, decreased smooth muscle cell proliferation was reported to be due, in part, to impaired activation of the p38-MAPK signaling pathway, resulting in the inhibition of cyclins D1, A, E, and cdk2 and thus a reduction in retinoblastoma protein phosphorylation.[82] The culmination of these events is the arrest of cell cycle progression at the G_1 phase and consequently inhibition of vascular smooth muscle cell growth.

Besides inhibiting smooth muscle cell proliferation, it has been suggested that HO-1 may contribute to cell death. This appears to occur in a cell-specific manner. HO-1–derived CO prevents apoptosis of endothelial cells,[96] whereas in cultured vascular smooth muscle cells, transfer of the HO-1 gene or administration of biliverdin/bilirubin promotes apoptosis.[101] The authors speculated that viral transfer of HO-1 gene might be a therapeutic means to treat occlusive vascular diseases. Given the importance of the growth of endothelial versus smooth muscle cells in the context of atherosclerotic vascular disease, these divergent responses of the 2 cell types to HO-1 and products of its catalytic activity emphasize the need to understand the cell-specific effects of HO-1 in disease. Probucol may serve as a useful example of this cell-specific action of HO-1. As mentioned, this drug inhibits smooth muscle cell proliferation while promoting endothelial cell growth and re-endothelialization in vivo,[74] and both processes are completely blocked by Sn-protoporphyrin.[73] In vitro, probucol induces HO-1 in smooth muscle but not endothelial cells.[50]

Vasodilation

Early suggestions of a dilatory action of HO-1 were attributed to an HO-mediated decrease in cytochrome P-450 content and activity,[102] in part via binding of CO to cytochrome P-450. Subsequently, HO-1–mediated vessel relaxation was increasingly linked to CO-modulated physiological responses involving the NO-cGMP pathway.[103] Indeed, overexpression of HO-1 in arteries was reported to stimulate vascular relaxation, mediated by guanylate cyclase and cGMP, independently of NO.[10] More directly, experiments with CO-releasing molecules further validated a role for CO in vasodilation, although in this case, modulation of cGMP and ATP-dependent Ca^{2+} channels was thought to be involved.[104] These studies provide strong support for the notion that high concentrations of CO can cause vasodilation. This is consistent with the overall increasing body of evidence confirming CO-mediated relaxation of different large and small vessels in different animals. CO likely acts via multiple mechanisms, including direct modulation of cGMP levels and K^+ channels in smooth muscle cells and indirect effects via modulation of endothelium-dependent vasoconstrictors and via myogenic factors.[7] It remains to be established however, how precisely this relates to HO-1 induction in vivo and to what extent CO is to be considered a signaling molecule in analogy to NO. With regard to vasodilation and other activities attributed to HO-1–derived CO, a missing piece of information is how much CO is actually produced in vivo in situations when HO-1 is induced. This is important because CO is 1000-fold less potent than NO as a relaxing agent.[105] The situation is complicated further by the fact that depending on the situation, HO-2 also has the potential to significantly affect vessel relaxation.[106] Furthermore, the ability of HO-1 and CO to dilate blood vessels is not a universal finding. In fact, emerging studies indicate that in some vascular beds, HO-1 and CO promote vasoconstriction by inhibiting the activity of endothelial NO synthase,[107] which may lead to endothelial dysfunction and hypertension.[108]

Antioxidant Protection

Induction of HO-1 is commonly considered to enhance the antioxidant activity of cells and to provide in vivo protection against conditions associated with oxidative stress. Several lines of arguments support such a notion. For example, genetic evidence demonstrates that cells derived from HO-1$^{-/-}$ mice that lack functional HO-1 are less capable to withstand an oxidative challenge than the corresponding cells obtained from HO-1$^{+/+}$ mice.[9,109] Cultured HO-1$^{-/-}$ embryonic fibroblasts demonstrated elevated levels of reactive oxygen species when exposed to pro-oxidants such as hydrogen peroxide and paraquat, and they were hypersensitive to cytotoxicity caused by hemin and hydrogen peroxide.[109] In addition, young adult HO-1$^{-/-}$ mice were vulnerable to mortality and hepatic necrosis when challenged with endotoxin,[109] a condition associated with increased oxidative stress. Conversely, cells overexpressing HO-1 have been reported to be more resistant to oxidant-induced toxicity than the corresponding control cells.[110]

The underlying mechanism(s) for the increased antioxidant protection provided by HO-1 are less clear, although several lines of evidence point to an important role of a coordinated upregulation of the cytoprotective iron-binding protein ferritin.[15,111] This may help to explain the surprising observation that after acute hyperoxic exposure, HO-1$^{-/-}$ mice had significantly decreased markers of lung oxidative injury than wild-type controls, which was reversed by transduction of human HO-1 in the knockout animals.[112] There is also strong support for a role of HO-1–derived bile pigments in the enhanced antioxidant protection. Thus, it is now well established that biliverdin and bilirubin are efficient in vitro scavengers of different types of oxidants,[113] and there is increasing evidence that this translates into cellular and possibly in vivo antioxidant activity. For example, when added at micromolar concentrations to cell culture media, bilirubin protects various cells, including endothelial and smooth muscle cells, against toxicity induced by hydrogen peroxide.[114] In cells in which HO-1 is induced and exogenous hemin is provided as a substrate, increased resistance to oxidant-mediated toxicity is observed only while bilirubin formation takes place.[114] Evidence for an in vivo antioxidant function of bilirubin is limited. Using Gunn rats as an experimental model of hyperbilirubinemia, however, Dennery et al[115] showed that plasma of jaundiced rats exposed to hyperoxia showed decreased signs of oxidative damage than plasma from corresponding control animals.

It is not clear, however, how these apparently enhanced antioxidant activities provided by HO-1 upregulation result in increased protection against atherosclerotic disease. As discussed earlier for CO, a key unresolved question concerns the extent to which increased HO-1 activity augments the concentration of bile pigments in vivo. In this context, it is worth remembering that high concentrations of bilirubin are toxic. In addition, cultured cells are artificially depleted of several antioxidants (eg, vitamins C and E) that both protect cells from oxidant-induced damage and interact with bilirubin, so it is difficult to extrapolate results from in vitro cellular studies to the in vivo situation. In addition, although atherosclerosis is undoubtedly associated with a state of heightened
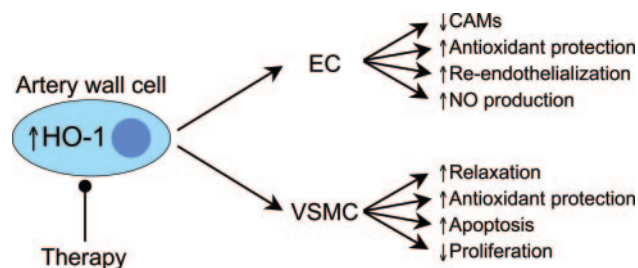

Figure 2. Scheme representing proposed modes of protection against atherosclerotic vascular disease by HO-1 induction. HO-1 may be modulated in vascular cells by cell type–specific gene therapy or therapeutic means. In endothelial cells (EC), protective functions resulting from altered HO-1 activity include (1) decreased expression of cell adhesion molecules (CAMs) that itself leads to decreased numbers of inflammatory cells in the vessel wall; (2) increased antioxidant protection; (3) promotion of cell growth and re-endothelialization; and (4) increased production of NO resulting from the increased numbers of endothelial cells. In smooth muscle cells (VSMC), increased HO-1 provides protection by (1) increased relaxation, (2) increased antioxidant protection, (3) increased apoptosis, and (4) decreased proliferation.

oxidative stress, a causal link of this to disease and its outcome remains an unproven hypothesis, and there is a need to consider the possibility that oxidative damage within the diseased vessel wall is a bystander process of the disease.[35] HO-1 has been identified repeatedly as a genetic factor in atherosclerosis that acts at the level of vessel wall endothelial cells. In these studies, the extent of induction of HO-1 by oxidized LDL has been reported to be associated with mice of a genetic background that predisposes to atherosclerosis,[116] whereas one might have predicted the opposite outcome if HO-1 induction increased antioxidant activity and protected against atherosclerosis. An alternative interpretation is that endothelial cells from atherosclerosis-prone mice are more responsive to oxidized LDL, thus requiring HO-1 to be induced. In mice with a genetic background not predisposed to atherosclerosis, induction of HO-1 may not be needed.

Unifying Mechanisms

In the preceding sections, we have reviewed HO-1 induction and proposed resulting modes of protection against atherosclerotic disease (Figure 2). Given the multitude of different conditions and agents that have been reported to induce HO-1, as well as the various protective activities derived from HO-1 activity and its metabolic products, this section focuses on potential unifying principles involved in these processes. With regard to HO-1 induction, three general hypotheses have been proposed as unifying mechanism: a transient increase in cellular heme content, a transient increase in cellular reactive oxygen species levels, and an alteration in cellular thiol status.[7] Although there is evidence in support of each of the 3 hypotheses, none of them singularly provide a satisfactory explanation. Thus, although the first hypothesis is important for HO-1 regulation induced by heme, the translocation of Nrf2, and the relief of transcriptional repression, many inducers such as thiol-reactive agents do not cause an initial increase in cellular heme. Similarly, the list of HO-1 inducers includes several phenolic antioxidants that are un-

likely to cause cellular oxidative stress. On the other hand, there is a likely link between alterations in cellular thiol status and the level of reactive oxygen species, so thiol redox–mediated modification of the cytoplasmic factor Keap1 and related alterations in the nuclear translocation of Nrf2 could provide a general underlying mechanism for HO-1 regulation that involves redox processes.

A remarkable facet of the list of known HO-1 inducers is that these agents represent both stimulators and inhibitors of a particular disease process. For example, both proinflammatory (eg, tumor necrosis factor-α[117]) and antiinflammatory cytokines (eg, interleukin-10[91]) have been reported to induce HO-1 in different cell types. Similarly, and more directly related to vascular disease, HO-1 inducers include pro-atherogenic stimuli (eg, hypoxia[118]) and agents with proven ability to attenuate vascular disease such as NO[119] and probucol.[50,73] The simplest interpretation of this apparent dichotomy is that induction of HO-1 represents both an endogenous protective response to and a therapeutic defense against atherosclerotic disease.

The knowledge we have gained over the last 5 to 10 years supports HO-1 as a protein that, when expressed, exhibits many activities that preserve vascular homeostasis. As such, HO-1 has clearly emerged as a therapeutic target that offers protection against atherosclerotic vascular disease (Figure 2). Indeed, Bach and colleagues[76,77] have presented the view that HO-1 acts as a "therapeutic amplification funnel". In this model, the therapeutic effect of several molecules such as rapamycin, NO, and statins is mediated, in part, by one or more of the products of HO activity as a result of induction of HO-1.[76,77] Amplification can be achieved in several ways, eg, by one of the HO-1-derived products inducing the formation of a molecule that upregulated HO-1 in the first place.[76,77] Using HO-1 as a therapeutic means against atherosclerotic disease raises additional questions. How can regulating a single protein provide so many different modes of protection, and are all of these necessarily linked to the metabolic activity of the enzyme? Is a therapeutic approach aimed at modulating HO-1 activity superior to an approach based on the administration of a metabolic product? As experimental work continues, answers to these important questions will likely be found.[120]

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Disclosures

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