Heme Oxygenase-1 and Carbon Monoxide in the Heart
The Balancing Act Between Danger Signaling and Pro-Survival

Leo E. Otterbein,* Roberta Foresti,* Roberto Motterlini*

Abstract: Understanding the processes governing the ability of the heart to repair and regenerate after injury is crucial for developing translational medical solutions. New avenues of exploration include cardiac cell therapy and cellular reprogramming targeting cell death and regeneration. An attractive possibility is the exploitation of cytoprotective genes that exist solely for self-preservation processes and serve to promote and support cell survival. Although the antioxidant and heat-shock proteins are included in this category, one enzyme that has received a great deal of attention as a master protective sentinel is heme oxygenase-1 (HO-1), the rate-limiting step in the catabolism of heme into the bioactive signaling molecules carbon monoxide, biliverdin, and iron. The remarkable cardioprotective effects ascribed to heme oxygenase-1 are best evidenced by its ability to regulate inflammatory processes, cellular signaling, and mitochondrial function ultimately mitigating myocardial tissue injury and the progression of vascular-proliferative disease. We discuss here new insights into the role of heme oxygenase-1 and heme on cardiovascular health, and importantly, how they might be leveraged to promote heart repair after injury. (Circ Res. 2016;118:1940-1959. DOI: 10.1161/CIRCRESAHA.116.306588.)

Key Words: carbon monoxide ■ heme oxygenase-1 ■ inflammation ■ mitochondria ■ myocardial ischemia

Self-preservation is a fundamental tenet exhibited by all organisms and is perhaps most apparent when the organism is confronted by various threats to survival. This concept also holds true at the most basic cellular level where the cell coordinates a series of responses evolved to ensure the best chance of defense and survival. The ability of cells and tissues to mount an adaptive response to stress, which is ultimately responsible for protecting against damage and restoring homeostasis, is a powerful intrinsic strategy that depends on the induction of several beneficial defensive systems. Among these, the stress protein heme oxygenase-1 (HO-1, encoded by the Hmox1 gene) plays a prominent role, which has been recognized in different organs and tissues, as well as different pathological scenarios.1,2 The main function of HO-1 is to degrade heme and generate carbon monoxide (CO) and biliverdin while simultaneously releasing iron, which is stored within the iron-binding protein ferritin.3,4 One common factor is that many of these molecular patterns (DAMPs) that when released can initiate pro-survival, and perpetuate a sterile, noninfectious immune response. They include mitochondrial DNA, ATP, formyl peptides, HMGB1, and the serum amyloid protein family.10,11 Recent evidence suggests that also heme could be considered a DAMP or an alarmin.12,13 One common factor is that many of these molecular patterns of cytoprotective genes that exist solely for self-preservation processes and serve to promote and support cell survival. Although the antioxidant and heat-shock proteins are included in this category, one enzyme that has received a great deal of attention as a master protective sentinel is heme oxygenase-1 (HO-1), the rate-limiting step in the catabolism of heme into the bioactive signaling molecules carbon monoxide, biliverdin, and iron. The remarkable cardioprotective effects ascribed to heme oxygenase-1 are best evidenced by its ability to regulate inflammatory processes, cellular signaling, and mitochondrial function ultimately mitigating myocardial tissue injury and the progression of vascular-proliferative disease. We discuss here new insights into the role of heme oxygenase-1 and heme on cardiovascular health, and importantly, how they might be leveraged to promote heart repair after injury. (Circ Res. 2016;118:1940-1959. DOI: 10.1161/CIRCRESAHA.116.306588.)

Key Words: carbon monoxide ■ heme oxygenase-1 ■ inflammation ■ mitochondria ■ myocardial ischemia

Review

Heme Oxygenase-1 and Carbon Monoxide in the Heart
The Balancing Act Between Danger Signaling and Pro-Survival

Leo E. Otterbein,* Roberta Foresti,* Roberto Motterlini*

Abstract: Understanding the processes governing the ability of the heart to repair and regenerate after injury is crucial for developing translational medical solutions. New avenues of exploration include cardiac cell therapy and cellular reprogramming targeting cell death and regeneration. An attractive possibility is the exploitation of cytoprotective genes that exist solely for self-preservation processes and serve to promote and support cell survival. Although the antioxidant and heat-shock proteins are included in this category, one enzyme that has received a great deal of attention as a master protective sentinel is heme oxygenase-1 (HO-1), the rate-limiting step in the catabolism of heme into the bioactive signaling molecules carbon monoxide, biliverdin, and iron. The remarkable cardioprotective effects ascribed to heme oxygenase-1 are best evidenced by its ability to regulate inflammatory processes, cellular signaling, and mitochondrial function ultimately mitigating myocardial tissue injury and the progression of vascular-proliferative disease. We discuss here new insights into the role of heme oxygenase-1 and heme on cardiovascular health, and importantly, how they might be leveraged to promote heart repair after injury. (Circ Res. 2016;118:1940-1959. DOI: 10.1161/CIRCRESAHA.116.306588.)

Key Words: carbon monoxide ■ heme oxygenase-1 ■ inflammation ■ mitochondria ■ myocardial ischemia

S

Original received February 16, 2016; revision received April 14, 2016; accepted May 2, 2016.
From the Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA (L.E.O.); INSERM, U955, Equipe 12, Créteil, France (R.F., R.M.); and University Paris Est, Faculty of Medicine, Créteil, France (R.F., R.M.).
*All authors equally contributed to the article.

Correspondence to Leo Otterbein, PhD, Beth Israel Deaconess Medical Center, Center for Life Sciences, #630 Boston, MA. E-mail lotterbe@bidmc.harvard.edu; Roberta Foresti, PhD, and Roberto Motterlini, PhD, INSERM U955 - University Paris Est, 8 Rue du General Sarrai, Créteil 94010, France. E-mails roberta.foresti@inserm.fr; roberto.motterlini@inserm.fr

© 2016 American Heart Association, Inc.
Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.306588
pattern molecules have been shown to increase the activity of HO-1. Thus, HO-1 is positioned as a crucial arbiter of oxidative stress and inflammatory responses. This review will discuss the participation of HO-1 and its products in protection and modulation of function in cardiac and vascular tissues. We will begin by recalling the most salient discoveries that have shaped our understanding of the HO-1 system in physiology and disease.

**Activity and Function of Heme Oxygenases: A Closer Look at the History of HO-1**

Heme oxygenases are ubiquitous and evolutionary conserved proteins found in both the plant and the animal kingdoms. In mammals, they are endoplasmic reticulum–anchored enzymes that catalyze the rate-limiting step in the degradation of heme. The oxidation of heme by heme oxygenases involves a series of redox reactions and the participation of cytochrome P450 reductase that ultimately generate stoichiometric amounts of CO, iron, and biliverdin, which is then converted to bilirubin by the cytosolic biliverdin reductase. The characterization of this enzymatic activity was performed in 1969 by Tenhunen who demonstrated that the system has an absolute requirement for molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) reducing equivalents (Figure 1).

Interestingly, and before the discovery of heme oxygenase, Sjostrand observed 20 years earlier that CO was produced constantly in the human body and was considerably increased in conditions accompanied by abnormal red blood cell decomposition. These data were confirmed in 1966 by Coburn et al., who demonstrated an augmented endogenous CO production in patients with hemolytic anemia. For the ensuing 15 years, the degradation of heme was viewed primarily as a catabolic pathway performed by specialized organs such as the spleen and the liver to regulate heme levels and iron recycling from senescent erythrocytes or oxidant-damaged hemoglobin. However, the true biological role for the heme oxygenase enzymes started to emerge during the mid-1980s, when Maines et al. demonstrated the existence of two major isoforms of heme oxygenase: a constitutively expressed HO-2 present primarily in liver, spleen, and testes, and an HO-1 protein that could be highly induced in various tissues by a host of chemicals including the substrate heme and heavy metals. It was also found that HO-2 was refractory to all these potential inducers, and that HO-1 was encoded by a distinct gene, thus indicating a different regulation and function for the two proteins. In 1989, Keyse and Tyrrell reported that human skin fibroblasts exposed to UVA radiation, hydrogen peroxide, and arsenite displayed high levels of a 52-kD stress protein. The same group proposed later on that induction of this protein, identified as HO-1, represented a general and adaptive response to oxidative stress in mammalian cells. These studies were a turning point in the field of heme oxygenase as they inspired other laboratories to investigate the role of HO-1 as an endogenous defense mechanism against cellular injury. But how could a protein fundamental in heme catabolism contribute to protect tissues under conditions of cellular stress?

Some answers to this question emerged when scientists began to analyze the intrinsic biochemical and bioactive properties of the substrate and the products of HO-1/HO-2 enzymatic activities. The findings by Stocker et al. showing that low micromolar concentrations of biliverdin and bilirubin in vitro efficiently scavenge peroxyl radicals and decrease peroxidation of low-density lipoproteins provided the first clue that these bile pigments act as endogenous chain-breaking antioxidants. Verma et al. also proposed that heme oxygenase–derived CO could function as a neurotransmitter, despite the renowned poisonous effect of this gas at high concentrations. The authors described colocalization in the brain of a heme-dependent guanylate cyclase and HO-2 and that inhibition of HO-2 with zinc-protoporphyrin abrogated the production of the second messenger cGMP by guanylate cyclase. Therefore, they concluded that, similar to nitric oxide (NO), CO was functioning as a signaling molecule and neurotransmitter. Although the proof that endogenous CO-activated cGMP in neurons emerged later, application of exogenous CO gas in vitro was already known to exert a series of cGMP-dependent vascular effects including inhibition of platelet aggregation, smooth muscle cell proliferation, and vasodilatation. Importantly, in 1995, Suematsu et al. demonstrated that blockade of HO-2 activity in the perfused liver reduced CO flux in the venous effluent, thus increasing hepatic vascular resistance and reducing sinusoidal flow rate. It was then confirmed that induction of HO-1 in vascular tissues under stress conditions also resulted in increased endogenous CO, which in the absence of NO activity acted as a major regulator of vasomotor tone and blood pressure. The different pharmacological actions of CO, including its ability to modulate the immune response, are highlighted in recent investigations by the groups of Motterlini, Otterbein, Soares, Choi, and Pinsky. In fact, CO possesses the ability to modulate...
inflammation and apoptosis to the point that nontoxic doses of CO gas or CO-releasing molecules (CO-RMs) provide therapeutic benefit in a variety of disease models. Importantly, the effects of exogenously applied CO mimicked that observed with HO-1 induction. These studies raised the concrete possibility of using pharmacological approaches to deliver controlled amounts of CO for the treatment of vascular and ischemic heart disease as well as inflammatory disorders.

From a historical perspective, the production of bile was observed centuries ago by the ancient Egyptians and Greeks who both considered it as one of four humors that reflect human health and allowed diagnoses to be made. This is not surprising because the process of heme degradation is probably the only example of enzymatic reactions that can be visibly observed in the human body. Essentially, the evolution of a bruise in the skin is a real-time spectrophotometric assay of heme oxygenase and biliverdin reductase at work as the rupture of capillaries and consequent red blood cell lysis results in the development of a red-brown color (hemoglobin/met-hemoglobin), which then turns green (biliverdin) and subsequently into a yellow pigment (bilirubin). Although CO is also produced, it is colorless and thus cannot be seen. However, in the 19th century, Haldane already realized that CO was physiologically important in respiration and affected the oxygen-carrying capacity of hemoglobin in man. The questions remained, however, as to why the body generates CO and the bile pigments, which at that time were perceived as waste products and detrimental to survival.

**HO-1 and Cardiovascular Protection**

**HO-1 in Myocardial IR**

During its early characterization, HO-1 was defined as heat-shock protein-32 (HSP-32) and was shown to be expressed in primary cultures of rat cardiomyocytes and rat hearts under conditions of oxidative stress. The findings on the isolated rat hearts are interesting in that induction of HO-1...
occurred only during the reperfusion phase but not during the preceding period of global ischemia. Furthermore, application of catalase or superoxide dismutase at reperfusion nearly abolished the increase in HO-1.\textsuperscript{59} Therefore, HO-1 was responding to oxidative signals produced by pathological events such as ischemia-reperfusion (IR) or other stimuli, which we now suggest could have been heme released into the environment as a result of tissue injury. We note that HO-1 was upregulated only at reperfusion, but it is possible that the period of ischemic time (5 or 20 minutes) was insufficient to induce HO-1. Indeed, prolonged exposure to hypoxia increases HO-1 expression in primary cultures of cardiomyocytes and cardiac cell lines.\textsuperscript{60,61} The explanation for hypoxia-induced HO-1 could be related to effects on cellular bioenergetics involving mitochondria, where complex I is in a low activity state during cardiac ischemia and is reactivated by reperfusion, generating superoxide and hydrogen peroxide, which cause cell damage and death.\textsuperscript{62} HO-1 induction during IR suggested either a role for the protein as a marker of oxidative stress or its active participation in the tissue adaptation to stress. Several additional studies have since confirmed the cytoprotective action of HO-1 in the heart. Overexpression of HO-1 before IR reduces cardiac ischemic damage in the isolated rat heart, whereas inhibition of HO-1 activity abrogated the protective effects and in many instances enhanced IR damage.\textsuperscript{63,64} Transgenic mice with cardiac-specific overexpression of HO-1 or enhanced cardiac HO-1 levels after gene therapy or pharmacological approaches exhibit markedly lower infarct size and improved cardiac function after IR both in the short term\textsuperscript{65–70} and even after 1 year from the ischemic event.\textsuperscript{71} Remarkably, cardioprotection was observed also when HO-1 gene transfer with an adenoviral vector was performed 1 year before myocardial infarction in mice,\textsuperscript{72} indicating the long-term potential of this strategy as a sort of immunization against infarction. In contrast, HO-1 null mice displayed increased myocardial damage after IR.\textsuperscript{73} In another interesting approach, Ma et al\textsuperscript{74} generated a recombinant HO-1 protein that crossed cell membranes resulting in prolonged preservation of heart grafts and reduced IR after cardiac transplant. The enzymatic activity of HO-1 was crucial for this beneficial outcome because treatment with a mutant HO-1 protein lacking enzymatic function did not afford protection.\textsuperscript{74} Consistent with this finding, the products of heme metabolism were as effective as HO-1 induction in decreasing IR injury. Bilirubin given in the low nanomolar range before IR was initially established as a candidate for cardioprotection and hearts from heme-treated animals contained higher levels of bilirubin.\textsuperscript{63} However, a role for CO was also documented by demonstrating that one of the first CO-releasing molecules synthesized in our laboratory (CORM-3), prevented the damage induced by IR in ex vivo isolated rat heart preparations and in vivo models of myocardial infarction.\textsuperscript{46,75–77} The reduction in infarct size in vivo was comparable with that observed with ischemic preconditioning.

![Figure 2. Landmarks in the history of heme oxygenase research.](http://circres.ahajournals.org/). This is not an exhaustive list as additional important findings have been published over the years by scientists working in the field and are not reported here because of space limitation. BACH-1 indicates BTB and CNC homology 1 transcription factor; CO-RM, carbon monoxide-releasing molecules; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; and Nrf2, nuclear factor erythroid 2 (NFE2)-related factor 2 transcription factor.
the most powerful intervention that reproducibly and consistently diminishes cardiac injury after IR.76

Besides acute ischemic events, HO-1 is also able to counteract cardiac dysfunction caused by chronic heart failure. In this case, enhanced HO-1 expression in cardiomyocytes of transgenic mice or mice deficient in BTB and CNC homology 1 transcription factor (BACH-1), a repressor of HO-1 gene transcription, prevented left ventricular remodeling and hypertrophy caused by coronary artery ligation.68,77 In cardiomyocytes in vitro HO-1 significantly diminished hypertrophy induced by endothelin-1.79 Diverse mechanisms seem to mediate the protective activities of HO-1 in the heart, including reduction in oxidative stress and inhibition of apoptotic cell death caused by decreased proapoptotic proteins such as p53 and increased antiapoptotic factors like Bcl-2 and inhibitor of nuclear factor-kB and activator protein-1 (AP-1).66,67,73,78,80,81

In addition, changes in the function of mitochondria, which are known to be critical during IR injury and control signaling, metabolic and cell death pathways involved in cellular adaptation to stress, have been associated with HO-1 overexpression and prevention of damage.63,78,82–84 The production of free radicals or heme metabolites such as biliverdin and bilirubin as well as iron chelation with ferritin have also been studied and show protective effects in preventing allograft dysfunction.94–96 The mechanisms have been dissected and include many signaling cascades involved in the effects including modulation of the mitogen-activated protein kinases, nuclear factor-kB, NO, cGMP, and likely changes in bioenergetics that lead to increased cell survival led by mitochondrial biogenesis.97–99

What remains unclear is whether the molecular target is the same regardless of the cell type. In addition, it is still unclear whether CO simply modulates the function of existing heme-proteins or additional effects may be exerted by CO on gene expression patterns directly via heme-containing transcription factors such as Npas2 or Bach-1 or indirectly through generation of ROS that trigger redox activation of nuclear factor erythroid 2 (Nfe2)-related factor 2 transcription factor (Nrf2) and Maf proteins. Regardless of direct or indirect mechanisms of action, CO has evolved into a novel therapeutic in transplantation. Furthermore, the use of CO and biliverdin or bilirubin mimics the positive effects observed with HO-1.

Treatment with CO by inhaled gas or administration of a CO-RM essentially recapitulates that observed with HO-1 induction.95 CO treatment of the donor, graft, and recipient has been extensively studied, and there is no doubt that CO imparts remarkable beneficial effects in preventing transplant vascular stenosis, thrombosis while promoting cardiac graft function. The addition of CORM-3 to the preservation solution resulted in significant improvements in systolic and diastolic function and coronary flow when compared with hearts treated with an inactive CORM-3. This improved cardiac function correlated with lower cardiac enzyme levels of creatine kinase and lactate dehydrogenase.95 In a model of arterial thrombosis, Hmox1−/− mice exhibited a prothrombotic phenotype that could be ameliorated with administration of either CO or biliverdin. The mechanisms involved specific effects on the regulation of cell cycle, coagulation, thrombosis, and reactive oxygen species (ROS).93 Both biliverdin and bilirubin as well as iron chelation with ferritin have also been studied and show protective effects in preventing allograft dysfunction.94–96 The mechanisms have been dissected and include many signaling cascades involved in the effects including modulation of the mitogen-activated protein kinases, nuclear factor-kB, NO, cGMP, and likely changes in bioenergetics that lead to increased cell survival led by mitochondrial biogenesis.97–99

What remains unclear is whether the molecular target is the same regardless of the cell type. In addition, it is still unclear whether CO simply modulates the function of existing heme-proteins or additional effects may be exerted by CO on gene expression patterns directly via heme-containing transcription factors such as Npas2 or Bach-1 or indirectly through generation of ROS that trigger redox activation of nuclear factor erythroid 2 (Nfe2)-related factor 2 transcription factor (Nrf2) and Maf proteins. Regardless of direct or indirect mechanisms of action, CO has evolved into a novel therapeutic in transplantation that resulted in the first clinical trial for inhaled CO in transplantation (www.clinicaltrials.gov).

**HO-1 in Pulmonary Hypertension and Right Heart Failure**

Pulmonary arterial hypertension (PAH) is a disease with an unknown cause that results from progressive increases in pulmonary vascular resistance that leads to right heart failure. PAH is characterized as a disease of small pulmonary arteries...
that exhibit uncontrolled smooth muscle proliferation, a narrowing of vessel lumen, and a thickening of right heart muscle necessary to counter the continuous rise in resistance as the heart strives to deliver adequate blood supply to the lungs. HO-1 is induced in models of PAH likely as a stress-dependent compensatory mechanism in attempts to maintain vascular homeostasis after inflammatory insults that include monocrotaline, platelet activation, and hypoxia. When HO-1 is blocked the beneficial effects of known therapeutic agents including simvastatin, IL-10, or hydrogen sulfide (H2S) are lost. Indeed, there is a clear interrelationship between the gasotransmitters H2S, CO, and NO, where each contributes toward the maintenance and restoration of vascular function. Induction of HO-1 with hemin, injection of mesenchymal stromal cells overexpressing HO-1, or administration of exogenous CO can not only prevent hypoxia-induced PAH but also reverse established disease. Interestingly, studies in dogs and sheep show that both endogenous and exogenous CO reduce pulmonary artery vasoconstriction likely involving increased cGMP and blockage of endothelin-1. This offers explanations and insight into potential mechanisms of action even with the knowledge that CO is known as a poor vasodilator compared with NO. Mechanistically, HO-1 and CO have been shown to exhibit multiple mechanisms of action in the vasculature including early anti-inflammatory effects with reduced EC activation, thrombosis, leukocyte infiltration, and cytokine production all of which reduce arterial injury that otherwise contributes to vessel remodeling. From a therapeutic standpoint, however, the above events are already ongoing and established at the time the patients present with symptoms, making therapeutic interventions challenging. There are currently no therapeutic options for patients having PAH. Administration of inhaled CO, however, at the time of peak right heart hypertrophy, targets the endothelium to generate increased NO via endothelial nitric oxide synthase (eNOS) that in turn activates cell death programs in the hyperproliferative smooth muscle cells as measured by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) positivity. The reversal of the intimal thickening restored normal arterial pressures and retro remodeling of the right heart to normal size. A similar effect can be observed in a model of carotid artery intimal hyperplasia where treatment with CO mediates regression of intimal lesions. In addition, treatment with CO activates large-conductance voltage and Ca++-activated K+ channels that attenuate remodeling of the right heart to normal size. A similar effect can be observed in a model of carotid artery intimal hyperplasia where treatment with CO mediates regression of intimal lesions. HO-1 induction, biliverdin/bilirubin, and CO have been shown to inhibit the expression of adhesion molecules in the endothelium and reduce leukocyte rolling and adhesion as early control of inflammation. In addition, CO liberated by CORM-3 inhibits myeloperoxidase activity, which participates in the crucial events that govern the transition between tissue damage and initiation of repair processes. Although never intensely explored, it was already observed in the first HO-1 overexpression animal studies that the protection against IR damage correlated with a decreased infiltration of neutrophils and macrophages in the heart. A recent and elegant article by Hinkel et al examined in detail the influx of postischemic inflammatory cells in mice and pigs overexpressing human HO-1 in cardiac tissue after treatment with a recombinant adenovirus. They showed that the number of neutrophils and proinflammatory monocytes recruited within the first 24 hours after reperfusion is significantly lower in transgenic animals compared with controls. This inhibited inflammation was associated with decreased infarct size and better functional recovery and, interestingly, was similar whether IR was performed in ubiquitously transgenic or regional (intracardiac) HO-1 overexpressing animals. Hence, it seems that local HO-1 is sufficient to provide protection against IR injury, possibly by modulating intracellular events that cause the consequent recruitment of inflammatory cells and induce damage. If heme is a DAMP that initiates and propagates inflammation, we suggest that HO-1 overexpressing cardiomyocytes are better equipped at the onset of IR to deal locally with excessive heme released during cell death and can therefore dampen in the early reperfusion phase heme-mediated proinflammatory cell recruitment and damage. This makes sense because the first few minutes after reperfusion will determine the response of the tissue to the insult that causes long-term damage and dysfunction. The metabolic activity of HO-1 that provides CO, biliverdin, and bilirubin would then amplify these protective and anti-inflammatory functions in multiple ways. For example, HO-1 induction, biliverdin/bilirubin, and CO have been shown to inhibit the expression of adhesion molecules in the endothelium and reduce leukocyte rolling and adhesion as early control of inflammation. In addition, CO liberated by CORM-3 inhibits myeloperoxidase activity, which
produces in leukocytes strong oxidizing compounds like hypochlorous acid, which cause endothelial oxidative stress and dysfunction. HO-1, biliverdin, and CO also inhibit proinflammatory molecules (such as tumor necrosis factor-α) and stimulate the production of IL-10, the anti-inflammatory molecule that is produced by macrophages exhibiting a pro-healing phenotype. Viable cardiomyocytes surrounding the infarcted area could be directly responsive to heme released by injured cells and play a fundamental role both in regulating inflammation and for initiating the reparative response that is dependent, in part, on tissue macrophages. In fact, cardiomyocytes situated in the border zone of the damaged cardiac area have been recently described to actively modulate macrophage trafficking that is essential for heart healing. It is likely that other DAMPs liberated over the course of reperfusion will synergize with heme to induce HO-1, even though only heme can be used as a substrate by the enzyme. In general, the mechanisms underlying the regulation of the innate immune response and acute inflammation by HO-1 as well as how DAMPs are implicated in the activation of the stress response are still poorly defined and necessitate focused investigations.

The vascular endothelium and smooth muscle are also compromised by IR injury in the heart and the reparative processes to restore myocardial function involve angiogenesis and endothelial progenitor cells. HO-1 and its products have not been directly examined in the regeneration of heart vessels after IR but can confer protection against vascular injury and inflammation in models of atherosclerosis and vessel injury and significantly contribute to neangiogenesis and neovascularization (see below). Therefore, we can foresee a multifunctional implication of the HO-1 system in IR: (1) as a sensor of cardiac injury and DAMPs; (2) as a modulator of inflammation and the immune response; and (3) as a mediator that aids the repair and reconstruction of cardiac tissue.

**HO-1 and Vascular Dysfunction**

Circulating heme leads to vascular dysfunction, in part, by damaging the endothelium, which likely involves its ability to increase the levels of oxidized low-density lipoproteins that contribute to EC death. In the setting of elevated hemoglobin and iron such as after vessel trauma or intravascular hemolysis there is induction of HO-1 in both endothelial and smooth muscle cells. The importance of this regulation is evidenced in the Hmox1−/− mice that exhibit exaggerated injury under similar conditions and likely reflect an inability to modulate inflammation and subsequent repair processes. Indeed, animals lacking HO-1 show elevated mean arterial pressures basally. In contrast, in mice in which HO-1 was selectively overexpressed in the heart, there is improved cardiac function, a reduced number of myocardial infarctions, and an overall reduction in inflammatory and oxidative injury after coronary artery ligation and reperfusion. This phenotypic outcome has been recently confirmed both in mice and in a population-based cohort where length polymorphisms of the Hmox1 promoter region were assessed. The authors found that Hmox1−/− mice infused with angiotensin II, treated with streptozotocin to induce diabetes mellitus or during aging, exhibited increased vascular dysfunction that was inversely correlated with heme oxygenase activity. Endothelial inflammation and infiltration of proinflammatory monocytes and neutrophils were also exacerbated in Hmox1−/− mice after angiotensin II treatment. Likewise, in hypertensive subjects, the expression of Hmox1 mRNA in monocytes was positively correlated with flow-mediated vasodilation and inversely with proinflammatory monocytes. The authors also found that an unfavorable Hmox1 length polymorphism (>30 GT repeats in the promoter region, which results in lower HO-1 expression) potentially increases the risk of arterial hypertension.

HO-1 expression and activity when induced can impact the same cell type differently depending on the environmental conditions, the type of stressor, and likely even the location, eg, conducting vessels versus capillaries or sterile versus pathogen-mediated immune responses. In general, most cellular stressors, which include changes in oxygen tension, cytokines/chemokines, shear stress, DAMPs, and pathogen-associated molecular patterns, all increase HO-1 in vascular endothelial and smooth muscle cells, as well as tissue leukocytes and fibroblasts. One of the earliest reports demonstrating the role of HO-1 showed that its induction before tumor necrosis factor-α–induced apoptosis prevented cell death of ECs via specific activation of the p38 MAP kinase signaling pathway. This was one of the seminal papers to define HO-1 as an antia apoptotic gene. Similarly, HO-1 overexpression in VSMC was first defined as antiproliferative and critical in preventing intimal expansion and vascular stenosis after angioplasty. Again, the Hmox1−/− mice supported these findings showing enhanced stenosis and VSMC proliferation in response to vessel trauma or transplant vascular stenosis leading to cardiac allograft rejection. Further studies demonstrated that the effects of HO-1 can be attributed to more than one of the products which, in certain instances, could rescue tissue function in the absence of HO-1. In recent years, it has become apparent that although HO-1 is induced under numerous conditions, it would be inaccurate to conclude that HO-1 is only antia apoptotic and only antiproliferative. Studies by Grochot-Przeczek, Deshane, and Wegiel clearly show that HO-1 and CO act to promote EC growth in models of wound healing, peripheral artery disease, and vessel repair and can also promote dysregulated smooth muscle cell death to remove unwanted and unnecessary cell mass found in stenotic or overly muscularized vessels as in intimal hyperplasia and pulmonary artery hypertension, respectively. In prostate and lung cancer models, the role of HO-1 is complicated and heavily debated. On the one hand, there is strong evidence that HO-1 prevents endothelial proliferation and tumor angiogenesis resulting in inhibition in tumor growth. Importantly, there are numerous reports that blockade of HO-1 results in anticancer and antiangiogenic effects strongly arguing a pro-cancer role for HO-1. Whether the differences are cancer specific is unclear as the mechanisms that have been described range from changes in intracellular HO-1 protein localization that influences proliferation, to an angiogenic switch with Sp1-dependent increases in vascular endothelial growth factor (VEGF), to inhibition in apoptosis and immune cell function. CO, unlike NO, is a poor vasodilator. This only holds true however in large vessels that control central...
pressure. In the microcirculation, HO-1 and CO do exert effects on vasomotor tone, acting to vasodilate and encourage small capillary recruitment. These data suggest different regulatory mechanisms at work that are cGMP independent. The reports described above would suggest that HO-1 and CO act in a manner that befits the needs of the tissue and restricting their function to one cellular process or another such as anti-apoptotic or anti-inflammatory does not fully and accurately define their role in pathological settings. Induction of HO-1 and CO generation provides the cell, and organism with the optimal chance at maintaining function to ensure survival. It is important to note in the context of cardiovascular physiology the complex interaction among the bioactive gases that include NO, H₂S, and even CO and oxygen. The interplay of these gases and the cellular targets they are known to modulate, eg, cyclooxygenase, HO-1, NOS, and arachidonic acid metabolites must be considered as we seek to understand how best to interfere and treat cardiovascular disease.

**Interaction of CO With Cellular Targets**

**CO and Cytosolic Hemoproteins**

Unlike other gasotransmitters including oxygen, CO does not undergo any physical or chemical changes in the cell or body. Its high diffusivity permits it to traverse into essentially all cellular compartments where it binds principally to iron-containing heme moieties in proteins. The CO molecule carries a negative charge on the carbon but is neutral because of oxygen having a positive charge. However, the C is electropositive and seeks to let go of this stress of carrying the negative charge, which is why it is attracted to positively charged iron atoms. Under basal conditions, CO is continuously generated during heme turnover and occupies ≈1% of the heme sites in hemooglobin and myoglobin. SO, how does CO impart such a diverse set of effects spanning ever-increasing and diverse areas of biology and medicine? The answer is complex and continues to be actively explored although it is unlikely that one target is solely responsible for the multifaceted set of effects of CO. An important question to raise is whether the effects of exogenous CO are similar to that generated endogenously by HO-1 (and HO-2). Based on theoretical algorithms, Levitt and Interest suggested in a recent review that CO production by tissue heme oxygenase must be sufficiently rapid to at least temporarily maintain a concentration of 0.1 μmol/L in the presence of diffusion into the local blood sink. Although interesting, what is not considered in detail here is how much CO is truly present at the cell surface when in proximity to HO-1 activity, what amount enters the cell, and perhaps more importantly how much CO is necessary at the cell surface and within the cell to elicit a response. One might argue that this is a fail-safe mechanism to control the amount of CO present in and around the cell. CO primarily diffuses away, drawn into the blood sink by partial pressure differences, and is therefore unavailable to cause toxic effects on the cell and tissue until homeostasis is achieved and HO-1 is turned off.

In quiescent cells, any CO produced will target hemo-proteins that are necessary for basal function such as soluble guanylate cyclase, oxidases, NO synthases, and the heme-containing transcription factors including BACH-1 and NPAS2. In the cardiovascular tissues, these heme-based proteins sensitive to gaseous molecules differ in prominence between cell types. For instance, guanylate cyclase is highly prevalent in smooth muscle cells while virtually absent in macrophages. eNOS is constitutively active in the endothelium while in most other cells is absent. Mitochondria are present among all cell types, but differ in number per cell type. Cardiac myocytes contain many more mitochondria per cell then smooth muscle and ECs. In each cell type, the function of the hemoproteins can be increased or blocked by binding CO. When CO binds soluble guanylate cyclase or eNOS, it activates the enzyme generating more cGMP or NO, respectively. As described below, when bound to cytochrome c oxidase in mitochondria, CO inhibits their activity resulting in increased superoxide ions that rapidly provoke signaling cascades as ROS leading to changes in gene regulation and ultimately influence cellular behavior. The consequences that have been observed include the modulation of many nonheme proteins including the p38 and extracellular signal-regulated kinase MAP kinases, peroxisome proliferator-activator receptor-γ, heat-shock proteins, adenosine receptors, and hypoxia inducible factor-1α (HIF-1α). By increasing ROS, there is activation of peroxisome proliferator-activator receptor-γ and HIF-1α that in turn regulates the gene expression toward a more tolerant anti-inflammatory phenotype that prevents TLR4 expression, MAP kinase activation, ion channel activation/inhibition, NADPH oxidase complex formation.

In the vasculature, the soluble guanylate cyclase present in VSMC is a constant target for CO and thus influences vasomotor tone albeit to a lesser extent than NO and involves soluble guanylate cyclase–induced activation of protein kinase G that controls VSMC relaxation and protein kinase B that regulates Ca²⁺ flux. Some of the cellular targets described above are likely affected by CO indirectly as they do not contain transition metals to which CO would bind and modulate their activity. Proteins such as guanylate cyclase, NO synthase, ion channels, and NADPH oxidase all bind CO that results in altered activity. Others are activated by ROS such as peroxisome proliferator-activator receptor-γ, whereas a third set of targets relates to the oxygen sensors that result in a pseudohypoxia resulting in stabilization of HIF-1α.

However, the literature comprised alternative accounts of CO effects in the body particularly in the heart as it relates to ion channel status. How CO interacts with specific ion channels is unclear, but the speculation is that it is a combination of the channel itself and associated cellular heme. The myocardium possesses 3 primary ion channels. Two channels, the L-type Ca²⁺ and the voltage-gated sodium channels (Na 1.5), are located primarily in sinoatrial and atrioventricular nodes of the heart, as well as in blood vessels (carotid body). CO inhibits the L-type Ca²⁺ channels, reducing Ca²⁺ influx into the cell. Inhibition of these channels decreases heart rate, atrioventricular node conduction, and ultimately reduces myocardial contractility resulting in cardiac vasodilation. Such an effect on the heart would be important in patients with unstable angina; however, it should be noted that there are no clear controlled studies demonstrating this effect.
In contrast to cells or animals that are in a basal state of activity, exposure of cells or animals to CO in the presence of an ongoing stress now presents a different set of heme-based sensors. Inducible proteins such as iNOS, NADPH oxidase, or other heme-dependent proteins may become targets for CO to bind to and modulate their function. If the purpose of the research is clinical relevance, there are few indications that would warrant prophylactic therapy. Balloon angioplasty of a stenotic vessel and organ transplantation are among the only scenarios where administering CO before tissue manipulation would be clinically relevant. In each model, CO is effective at blocking the ensuing insult essentially via preconditioning the tissue that limits the proinflammatory response. In each setting, CO prevents upregulation of cytokines/chemokines, adhesion molecules, and hyperproliferative signals such as growth factors that lead to cytoskeletal reorganization. The result is little to no activation of the cell in response to the ensuing stimuli. In contrast and perhaps more a rarity is that CO is also effective if started after a stress such as bacterial infection, PAH or myocardial infarction. In these settings, CO is proproliferative and induces tissue repair including stem cell function (see below), tissue remodeling (described above), and enhancing bacterial killing by augmenting the host immune response.12,164 Work by Lin et al125 showed that treatment with CO gas or CORM-2 promoted neovascularization and myocardial repair after coronary artery ligation. CO increased the activation of stromal cell-derived factor-1 via an Akt-dependent activating enhancer–binding protein 2α expression. Blockade of activating enhancer–binding protein 2α abrogated the beneficial effects of CO treatment. Collectively, whether CO is initiated before or after the insult, the result is salutary and befits the needs of the tissue. As such, perhaps the best definition for CO is homeodynamic in that its effects on the cell are dependent on the state of the cell at the time of exposure. HO-1 is similar in many ways. If HO-1 is absent, the ability to respond appropriately to any of the insults described above is exaggerated with increased morbidity and in most cases increased mortality.

**CO and Mitochondria**

One intriguing mechanism of action that has been proposed to explain the cytoprotective effects of CO in myocardial tissue is its ability to modulate mitochondrial activity and function. This may appear at first counterintuitive because CO gas is known to bind to hemoglobin with a 200× greater affinity than oxygen. In addition, CO inhibits tissue cytochrome c oxidase, which is fundamental for sustaining electron transport, oxygen consumption, and energy production in mitochondria.165 However, many of the studies showing the effects of CO on mitochondrial function were performed in isolated mitochondria, a model that is not fully representative of the cellular milieu. As observed for other signaling gases (such as NO and H2S166), modulation of mitochondrial function by CO is multifaceted and will depend on the concentration and status of mitochondrial targets. Controlled delivery of CO by CORM-3 to mice undergoing peritonitis-induced sepsis was shown to reduce inflammation by eliciting a mild oxidative stress response that leads to improved mitochondrial energetics and increased biogenesis in the heart.167 These data confirm previous results by Suliman et al98 showing that CO gas administered to mice in controlled amounts for limited periods of time act as a stimulus of retrograde signaling for cardiac mitochondrial biogenesis by triggering the production of mitochondrial hydrogen peroxide. The effect of CO was associated with a significant increase in mitochondrial DNA, and the coordinated expression of both mitochondrial and nuclear transcription factors that activate genes for mitochondrial proteins. These include mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). It is important to emphasize that the transcription of these cardiac genes by CO was stimulated by mechanisms that were independent of tissue hypoxia and did not involve the NO pathway. The response to CO was confirmed in cardiomyocytes demonstrating that in addition to an increased expression of transcription factors regulating mitochondrial biogenesis, CO stimulated cGMP production and activated the phosphatidylinositol-3 (PI3)-kinase/Akt pathway, which are both involved in prosurvival activities.98

In a second series of studies, Suliman et al92 reported that the HO-1/C0 pathway plays a crucial role in restoring the impairment in mitochondrial function induced by heart failure. Using a model of doxorubicin-induced cardiomyopathy in mice, it was observed that hearts damaged by doxorubicin lacked the ability to engage a nuclear program for mitochondrial biogenesis leading to severe depletion of mitochondrial DNA, sarcomere destruction, and cardiac fibrosis. Notably, periodic exposures to CO gas protected the heart from doxorubicin toxicity by mitigating the loss of mitochondrial DNA, reducing oxidative stress and maintaining a normal structure of the left ventricular wall. These results were confirmed in isolated cardiomyocytes demonstrating that overexpression of HO-1 prevented, whereas HO-1 gene-silencing exacerbated, doxorubicin-induced mitochondrial disruption and apoptosis. The possibility that an increase in ROS production by CO occurs via transient inhibition of cytochrome c oxidase is plausible156 and is in line with emerging evidence on the obligatory role of mitochondrial ROS in resistance to cardiomyopathy and cardiac failure, an adaptive response known as mitohormesis.168,169 Cardioprotective actions by HO-1 linked to modulation of mitochondrial function have been corroborated more recently in chronic heart failure induced by coronary ligation. In this model, transgenic mice overexpressing myocyte-specific HO-1 exhibited significantly improved cardiac ejection fraction and postinfarction survival in association with reduced hypertrophy, interstitial fibrosis, and oxidative stress.79 Moreover, mitochondria isolated from HO-1 transgenic hearts displayed markedly reduced oxygen consumption rates, an effect recapitulated in mitochondria of nontransgenic mice treated with CORM-3. Although the overall respiration was decreased, mitochondria treated with CORM-3 showed an increase in both state 3 (ATP linked) and state 4 (ADP independent) respirations, the latter being indicative of an uncoupling effect.39 Indeed, CORM-3 at low micromolar concentrations significantly uncouples mitochondrial respiration in heart isolated mitochondria, an effect accompanied by a mild decrease in membrane potential and reversed by the CO scavenger myoglobin.154,170 It is also interesting that the basal
production of ROS in heart mitochondria fed with pyruvate through complex I is increased by CORM-3, whereas excessive production of hydrogen peroxide in succinate-driven respiration through complex II is markedly attenuated by CO.\textsuperscript{154} Thus, on one side CO may temporarily inhibit cytochrome c oxidase to trigger production of ROS that in turn serves as signal for mitochondrial biogenesis, whereas, on the other hand, CO can transiently increase oxygen consumption and reduce ROS production by uncoupling respiration at the expense of oxidative phosphorylation (Figure 3).

Although the exact molecular target(s) and the sequence of events by which CO regulates uncoupling and energy metabolism remain to be characterized, the direct involvement of CO on cardiac energetics is supported by in vivo evidence. For instance, in pigs undergoing cardiopulmonary bypass, pretreatment for 2 hours with CO gas leading to 12% blood HbCO resulted in significantly higher cardiac ATP and phosphocreatine levels and reduced interstitial edema, heart fibrillation, and apoptosis.\textsuperscript{171} Similarly, pigs exposed to CO gas inhalation for 3 hours (5% HbCO) and subsequently subjected to coronary occlusion showed a much lower concentration of lactate in blood, less utilization of glucose, and increased pyruvate levels during ischemia compared with untreated animals, whereas cardiac ATP and energy charge were unchanged between the two groups.\textsuperscript{172} It is known that pyruvate dehydrogenase is inhibited in the heart by increased fatty acid β-oxidation,\textsuperscript{173} and it is tempting to suggest that reduced lactate in association with increased pyruvate are because of a CO-mediated switch in substrate utilization for energy. Altogether, these findings indicate that the HO-1/CO pathway exerts some of its cardiac protective effects by dynamically regulating aerobic and anabolic metabolisms, and thus counteracting the metabolic dysfunction occurring during stress conditions.

**Heme and HO-1–Derived Products: The Balance Between Cell Injury and Protection**

One outstanding issue that remains unsolved in the field of heme oxygenase is the source of heme to support its enzymatic activity and generate these essential cytoprotective products. It is known that the intracellular heme pool is tightly regulated by a precise balance between the rates of heme biosynthesis, catabolism, and export.\textsuperscript{174} However, no studies have been conducted to determine the source of heme used by heme oxygenase either during physiological conditions or when HO-1 is induced in disease states. What is plausible and is intrinsically accepted by all scientists working in this area is that under normal conditions, heme is reclaimed from the protein turnover that occurs during typical wear and tear of the cell. But is this an indiscriminate process where heme-containing proteins in the cytosol or in organelles such as the mitochondria or nucleus provide the heme substrate for heme oxygenase activity? Alternatively, might there be tighter regulation of heme catabolism? If under pathophysiologic conditions heme suddenly becomes available, as would occur after cell rupture in sickle cell anemia, trauma, or infection, is it the ECs and leukocytes that recognize and uptake the excess heme?
heme that would escape the prototypical heme scavengers haptoglobin and hemopexin? Can the internalized heme be used by the EC or leukocyte? Or heme is simply a DAMP arising from dying cells and much like other debris is internalized and removed from the damaged tissue? Given that heme has a cognate receptor in TLR4, is heme a signaling molecule that activates ECs and leukocytes via a specific MyD88 cascade? Could there be an increase in heme synthesis, which starts and ends in the mitochondria and is an energy-consuming process, simply to support increased enzymatic activity after HO-1 induction? It is clear that novel experimental approaches need to be developed to answer these fundamental questions. It has also become evident that the cytoprotective effects of HO-1-derived products may be stronger or better exploited where abundant heme sources are available. Thus, HO-1 and its products may have more influential roles in immune cells that are principally responsible for TLR4-mediated heme uptake. In such scenario, the products would then serve as additional signaling molecules, diffusing endogenously and exogenously to further respond, defend, and repair the environs. The fact that cardiac muscle contains high amounts of myoglobin and a high mitochondrial number suggests that the heart may benefit more than other tissues from the salutary effects of heme recognition and HO-1 induction.

Heme is essential to all living organisms. Its formation and degradation are highly regulated and depend on the needs of the cell and tissue. Heme, in and of itself, is characterized as a potent agonist of oxidative stress and highly detrimental to cells and tissues. Structurally, heme is an iron–protoporphyrin complex comprising four pyrrole rings. When the iron atom is in the ferrous state, the complex is called heme, versus when the iron atom is in the ferric state, where it is called hemin. The toxic effects of heme are extremely diverse, but a commonality is that heme increases the generation of iron-derived ROS. Hemin causes DNA damage and oxidation of lipids and proteins.175 Many of these conclusions are based, in large part, on data demonstrating that exogenous administration of heme in the presence of an additional cellular stressor such as cytokines, endotoxin, or hypoxia exacerbates tissue stress and damage.13 This is best demonstrated in sickle cell anemia or malaria where heme is released in large amounts (>20 µmol/L) because of hemolysis resulting in induction of a pro-inflammatory state with increased cell death.176 In the heart, heme production rises with injury caused by the activation of the heme synthesis enzyme δ-aminolevulinic acid synthase 2 in cardiomyocytes.177 Blockade of δ-aminolevulinic acid synthase 2 and heme synthesis abrogates cell death. It is clear that heme will be released in the presence of any tissue injury given the large heme pool inside cells and likely reflects why HO-1 induction occurs in the absence of extracellular heme administration such as in instances of UV irradiation, cytokines signaling, and agents that increase oxidative stress. Heme is a deadly molecule that needs to be eliminated when it is released from cells, it has recently been suggested to act as an alarmin and a DAMP13,178 and is recognized by its cognate receptor TLR4 in activation of inflammasome pathways.9 Heme is, however, actively secreted during erythropoiesis via the feline leukemia virus subgroup C receptor-related protein (FLVCR). The absence of this receptor results in midgestational lethality with severe deformities.179 Recent reports suggest that FLVCR is also critical in T-cell development and survival.180 Although these effects may validate that excess heme is toxic, it may also speak to the necessity of heme secretory pathways in cell and tissue development and ultimately innate immune functionality. Does heme serve a purpose when released from cells, much like HMGB1 and other mediators including cytokines/chemokines or even growth factors? We would propose that heme is actually a signaling molecule like other DAMPs such as ATP, mitochondrial DNA, and formyl peptides, which akin to other cellular mediators can be actively or passively released from cells and recognized by neighboring cells to elicit appropriate responses (Figure 4). Heme released as a result of tissue damage upregulates HO-1, which as presented throughout this review is absolutely critical in heart repair and survival. HO-1 in myocytes, vascular cells, stem cells, and immune cells each contribute to restoration of function.

There is no doubt that CO, at appropriate concentrations, binds to mitochondrial oxidases in mammals. There is a plethora of literature that concludes that by binding to these hemo-proteins CO increases ROS such as H2O2 and O2•− that function as potent signaling molecules and activators of transcription and modification of proteins such as IKK kinases or protein kinase A leading to expression of antioxidant genes (eg, MnSOD, thioredoxins) as well as HO-1 and Nrf2.95,98,100,181,182 Increased expression of HO-1 via ROS and the antioxidant responsive element has been well described. In this context, it is intriguing to speculate a feed-forward system where ROS indirectly increase HO-1 that results in generation of CO and CO in turns targets many of the ROS-generating systems like the mitochondria oxidases, catalase, NADPH oxidase, and xanthine oxidase. It is interesting to speculate that the powerful antioxidant bilirubin generated as the final product of heme catalysis serves to ultimately resolve the oxidative burden and return the cell to quiescence. These derivative or secondary effects of HO-1/CO must be considered in the context of the cell and tissue responses under both physiological and pathophysiologic circumstances.

Future Exploitation of HO-1/CO as Protective Therapies in the Heart

Implications of HO-1/CO in cell therapy

There is strong interest in the possibility of using stem cells for repairing and regenerating lost cardiac tissue during myocardial infarction because the postischemic heart has limited capacity for self-renewal and undergoes remodeling that inevitably impairs left ventricular function.183 Because the reparative potential of stem cells is severely compromised by their poor survival after transplantation into the infarcted heart, induction of cytoprotective genes such as HO-1 is a plausible stratagem to increase their viability and therapeutic efficacy. As a consequence, scientists have started to explore whether HO-1 has any active role in the repairing abilities of stem cells. Initial studies published in 2005 reported that mesenchymal stem cells (MSC) transfected with an HO-1 vector become more resistant to apoptosis induced by hypoxia-reoxygenation in vitro; in addition, HO-1 transfection increased the tolerance of MSC once engrafted in vivo, which promoted
significantly less ventricular remodeling and enhanced functional recovery of the infarcted heart. These protective effects were associated with a marked increase in VEGF expression, reduced expression of cardiac proinflammatory mediators (tumor necrosis factor-α, IL-1β, and IL-6) and augmented capillary density. Injection of human bone marrow MSC into the postischemic myocardium has also been shown to increase HO-1 expression both in MSC and in cardiomyocytes resulting in less ischemic damage and improved cardiac contractility. Interestingly, HO-1 gene transfer in cardiac tissue during myocardial infarction correlates with enhanced neovascularization via the production of angiogenic factors (VEGF and stromal cell–derived factor-1) and an increase in the number of c-kit stem cells recruited to the injured area several days after coronary artery ligation. These data indicate that increased HO-1 leads to a dual protective effect: (1) it promotes the paracrine activities of exogenously administered MSC, which helps in preventing myocardial cell injury and apoptosis and stimulates the formation of new vessels; (2) it triggers the recruitment of circulating progenitor stem cells, thus enhancing the endogenous regenerative capacities of the heart.

The ability of HO-1 to enhance cell therapy approaches has been confirmed in human late outgrowth endothelial progenitor cells which, after transduction ex vivo with HO-1 and the prosurvival gene Akt, improve their ability to adhere to extracellular matrix and migrate toward human cardiomyocytes showing a more robust paracrine profile under stress. Even in this instance, injection of human endothelial progenitor cells in the infarcted area of nude mice in vivo markedly attenuated inflammation, enhanced neovascularization, and reduced the negative remodeling ultimately ameliorating cardiac performance. A more recent study conducted in pigs showed, however, that although intracoronary delivery of allogeneic bone marrow–derived stem cells reduced infarct size after myocardial reperfusion injury, injection of stem cells overexpressing HO-1 did not further limit postischemic damage despite the hearts displaying an improved left ventricular ejection fraction. It is possible that the type of cells used and the mode and timing of intervention are crucial for a successful outcome, and more systematic procedures are required to fully exploit the potential of cell therapy as efficacious interventions for cardiac tissue repair. Nevertheless, manipulating stem cells to increase their therapeutic action by transferring HO-1 or other cytoprotective genes seems to be a promising approach. Consequently, increasing endogenous expression of these genes in stem cells could also be attainable by pharmacological means. Along this line, recent studies revealed that the HO-1 inducer cobalt protoporphyrin IX (CoPPIX) can render stem cells more resistant to oxidative injury and consequently increase their beneficial effects after transplantation into the infarcted myocardium. Treatment with C-kit+ human cardiac stem cells (hCSC), which are known to differentiate in vivo in cardiomyocytes, smooth muscle, and ECs, has been shown to improve heart function in different models of myocardial infarction and in patients with ischemic cardiomyopathy. Cai et al demonstrated the following: (1) induction of HO-1 by CoPPIX increases the resistance of hCSC to hydrogen peroxide–mediated oxidative stress and apoptosis, whereas silencing HO-1 abrogated the cytoprotective effects mediated by CoPPIX; (2) treatment of hCSC with CoPPIX stimulated the release of important growth factors into the media, and the same media applied to naïve cardiac stem cells conferred remarkable resistance against cellular damage and apoptosis; (3) CoPPIX in hCSC induces the expression of the transcription factor Nrf2 and increases the phosphorylation of
extracellular signal–regulated kinase 1/2, which are key players in the modulation of several detoxifying and prosurvival genes; (4) compared with untreated cells, hCSC preconditioned with CoPPIX survived much longer once injected in the infarcted myocardium of immunodeficient mice, at the same time the treatment markedly increased cardiac performance, promoted greater proliferation of cardiac cells, and reduced left ventricular remodeling.193 These data were then confirmed by Luo et al.196,197 showing that human embryonic stem cell–derived cardiomyocytes pretreated with CoPPIX exhibited a greater resistance to hypoxia-reoxygenation injury, increased VEGF production, and reduced ROS-mediated apoptosis. Moreover, when CoPPIX-treated stem cells were injected immediately after myocardial infarction in rats, human cardiomyocyte graft size increased ≤12% of the total ventricular area and was associated with increased human-derived capillaries and thus improved vascularization of the damaged tissue.197 Similar results were obtained more recently with adipose-derived stem cells pretreated with curcumin, another well-established and potent inducer of the Nrf2/ HO-1 axis.198,199 Specifically, adipose stem cells preconditioned with curcumin displayed increased resistance to oxidative stress and significantly ameliorated their efficacy in a model of rat myocardial IR injury by improving cardiac contractility, decreasing tissue damage and fibrosis and enhancing both capillary density and the formation of new vessels.200 Whether the products of HO-1 (biliverdin and CO) have an impact on the intrinsic capacities of stem cells to promote the repair of injured cardiac tissues remains to be fully established. A recent report supporting this possibility showed that rats treated with a compound that generates CO (methylene chloride) before coronary artery ligation displayed increased accumulation of c-kit+ stem progenitor cells in the infarcted cardiac tissue, and this effect was associated with formation of new coronary arteries and involvement of the expression of angiogenic factors such as HIF-1α, stromal cell–derived factor-1α, and VEGF-B.201 In addition, a new report by Suliman et al.202 revealed that HO-1 and CORM-2 can control the differentiation of embryonic stem cells into cardiomyocytes. This study demonstrated that CO-mediated mitochondria biogenesis is a crucial step in the maturation of embryonic stem cells into energetically efficient cardiomyocytes, providing additional evidence on the emerging and perhaps crucial role of the HO-1 pathway in the control of energetic metabolism not only in cardiac tissue but also during stem cell differentiation.203

Leveraging HO-1 and Its Products as Innovative Cardiotherapeutics

As demonstrated in the previous sections, substantial amounts of data have emerged indicating that HO-1 and its products can modulate the outcome of several cardiovascular diseases, and hence are obvious therapeutic targets for these conditions. There are now intense efforts underway by groups worldwide that are attempting to leverage and translate the benefits of these molecules for human use. There have been efforts toward inducing HO-1 by administering heme arginate, gene therapy strategies, and those targeting the regulation of HO-1 through transcriptional control of Nrf2. Although effective in inducing HO-1, this approach is still in the early phases but we note that HO-1 is induced by many approved agents such as statins, aspirin, NO, and even steroids. In many instances, mechanistic studies have shown that these agents act through and even require HO-1 to exert their effects.204 In addition, dimethylfumarate, which is approved by the Food and Drug Administration for the treatment of multiple sclerosis (Tecfidera), activates Nrf2 and induces HO-1, suggesting again the possibility that HO-1 may contribute to the pharmacological action of the drug.205 Targeting enzymatic activity, either endogenous or genetically, is fraught with uphill battles related to sufficient activity and potency of the induction. The goal is to generate sufficient and therapeutic amounts of the HO-1 products. Although a detailed discussion of the transcriptional regulation of HO-1 is beyond the scope of this review, it should be noted that Nrf2 is not the only factor that regulates the expression of HO-1, and indeed there are instances where the Hmox1 gene is induced despite a lack of Nrf2.214 The repressor BACH-1, HIF-1α, AP-1, and other factors can control HO-1 expression depending on the tissue and pathological condition considered.206 Thus, future investigations that will elucidate important mechanisms of HO-1 transcriptional regulation may identify novel pharmacological targets for drug design based on HO-1.

The most advanced of these efforts is in the use of inhaled CO, which has undergone substantial evaluation in numerous clinical trials (www.clinicaltrials.gov). To date, there are ongoing trials administering CO by mask in lung disease including pulmonary fibrosis, hypertension, and adult respiratory distress syndrome. Phase II studies in kidney transplant showed a trend toward improved renal function after transplant akin to what was observed in large animal studies in swine.207 The challenges with inhaled CO include precise delivery and dosing, nonpatient exposure to individuals administering the gas, cumbersome gas cylinder transport storage, and the necessity for hospital delivery because of safety concerns. In parallel, there has been the development of CO-RMs followed by extensive studies, showing their beneficial pharmacological activities in models of acute injury and inflammation. The first generation of CO-RMs was based on small metal-containing CO carriers that could release their CO over time in vitro and in vivo. These early molecules showed a spectrum of kinetics of CO release, and in most instances, recapitulated the salutary effects promoted by HO-1 induction. The challenge with the development of metal-based CO-RMs revolves around improving their solubility, stability, and potency while limiting toxicity related to the metal cores, even though to date only few studies have been published that objectively address in vivo the toxicity of CO-RMs arising from the metal.208 Likewise, no studies have characterized the effect of the inactive CO-RMs, that is, the chemical entities that remain in the circulation after CO has been liberated. Chemists have just begun to look into the design of nonmetal-based CO-RMs or compounds engineered to release CO in the presence of ROS, or light or even changes in pH as would occur in the stomach. However, the vast majority of these new compounds are still metal-based, and therefore the progress is somewhat limited if the elimination of the metal is the goal of this development. In addition, all transition metal carbonyls are intrinsically light.
sensitive, and therefore all metal CO-RMs are by definition photo-CO-RMs. In essence, the high affinity of CO for transition metals is what allows its physiological targeting and activity and is the property of CO that is exploited in CO-RMs. Importantly, one may see the formation of temporary endogenous CO-RMs in the binding of CO with different metal-containing proteins of the organism. The human body contains a most straightforward metal carbonyl CO-RM in the form of COHb. This has driven the creation of carbonmonoxy hemoglobin using both human (CO-MP4) and bovine pegylated hemoglobins that are being tested in human trials for a variety of disease indications. The major problem with the use of hemoglobin-based carriers and blood substitutes in general is related to their vasoconstrictive effects, alterations in fluid balances and, as mentioned above, an increase in heme-based toxicity. However, administration of MP4-CO did not show increases in mean arterial pressure and protected against myocardial infarction in rats and sickle cell disease to prevent vascular stasis. Additional approaches include organic-based click-and-release prodrugs that rely on chemical reactions for the generation of CO instead of simple release, and the efficacy of these molecules in ongoing models of inflammation is showing promising results (personal communication), but have not been tested in the setting of cardiac injury.

To circumvent toxicity of many of the above compounds, Steiger et al have developed an innovative approach defined as Therapeutic Gas Releasing Systems, which allows for controlled release of gases from sealed containers that permit generation and exposure to CO without the concern of toxic byproducts, solubility issues, and complex metabolites to identify, track, and characterize. Finally, and perhaps most intriguing is a simple concept that CO can be saturated within an oral formulation that can be well controlled and delivered with clear preclinical bioavailability, which would be amenable to both acute and chronic use, obviate concerns surrounding dosing, safety, and toxicity and take advantage of the ability of CO to rapidly diffuse into the bloodstream from the stomach. One commonality among each of the approaches described above is that CO enters the body as CO2, circulates, meets its targets, and is eventually eliminated (t1/2 in humans of 4 hours) from the body as CO exhaled through the lungs having undergone nearly no metabolism. Small amounts are converted to COHb. A recent report by Yuan et al delineate how CO influences carotid body signaling in response to changes in oxygen sensing as an elegant signaling cascade dependent on the delicate balance of hemoprotein sensor activation. This signaling also involves H2S and perhaps links peripheral and central neural activity related to breathing. Even though CO and CO-RMs exert pharmacological actions by interacting with different cellular targets, in some instances, they also induce Nrf2 and HO-1 as part of a preconditioning effect or as a stress response to supraphysiologic levels of CO. Thus, the initial protective activity obtained with exogenous CO can be amplified by the induction of intrinsic defensive mechanisms.

More recent strategies include the synthesis of hybrid molecules, termed HYCOs, designed to both deliver CO and induce HO-1 by Nrf2 activation. The premise is that HYCOs, containing an Nrf2 inducer bound to a CO-RM, will provide greater tissue protection by first limiting damage through CO delivery and subsequently promoting the endogenous upregulation of Nrf2-dependent defensive genes and proteins, a process that takes several hours because of transcription and translation processes. Thus, it is postulated that these molecules may offer a therapeutic advantage when compared with Nrf2/HO-1 inducers or CO-RMs alone. Although the characterization of several HYCOs is ongoing in models of inflammation with promising results, no data are yet available on their potential beneficial effects in cardiac pathologies.

The bile pigments have also been proposed to possess protective properties similar to HO-1 with preclinical data supporting their use to prevent IR injury after a heart transplant or intimal hyperplasia after balloon angioplasty driven, in large part, by the modulation of inflammation and smooth muscle cell proliferation, respectively. The limitations with this approach relate to obtaining a reliable and safe source of bilirubin, which can then be converted back to biliverdin. Of note, Asian cultures have been consuming bilirubin for centuries for medicinal purposes by eating the gall stones of various animal species. Biliverdin would, however, be the pigment of choice because of it being easier to solubilize and that it is converted to bilirubin by biliverdin reductase to also provide bilirubin. The relative impact of biliverdin versus bilirubin in terms of importance and efficacy has yet to be determined. Efforts are ongoing to use bacteria and yeast systems to generate biliverdin. Although proof of concept shows feasibility, generating large enough amounts for further preclinical and eventual clinical testing is still being explored. That a biliverdin/bilirubin-based therapy could be useful in the prevention of several human pathologies, and specifically heart disease is supported by epidemiological studies. The interesting findings of these reports highlight that subjects exhibiting modestly increased plasma bilirubin levels, such as those observed in Gilbert’s syndrome, display a reduced risk of developing cardiovascular disease and decreased mortality, and this is applicable also to diabetic patients and to patients with cancer.

Conclusion

Because the identification of heme oxygenases as metabolic enzymes designed to catabolize heme, there has been a remarkable increase in the number of laboratories evaluating and characterizing its ability beyond simple heme metabolism. The protective role of HO-1 and its products in the heart and cardiovascular system is unequivocal. The creation of the Hmox1−/− mice, the identification of the HO-1-deficient human, and the characterization of HO-1 polymorphisms showing that specific short GT repeats are associated with lower risk of heart disease, cardiac allograft vasculopathy, and susceptibility to restenosis after coronary stenting support this concept. In this review, we have provided a comprehensive perspective of the role of the HO-1 pathway in cardiovascular disease. Importantly, we have delineated how HO-1 has evolved from a straightforward catabolic protein into a critical enzyme necessary for normal heart and vascular function and finally to identifying its products as potential therapeutics for the treatment of a variety of heart, lung, and blood disorders. Heme is undeniably part of the foundation of cellular resources necessary for life of complex multicellular organisms. Without it, erythrocytes would be unable to deliver...
life-sustaining oxygen using hemoglobin, and tissues would be incapable of maintaining sufficient amounts of ATP without the heme-containing mitochondrial oxidases. But does this seemingly benign, iron-laden porphyrin ring carry the potential to wreak havoc if left unchecked, or does it serve a greater purpose? Is it a dangerous molecule and a signaling molecule, or is it perhaps a simple ring that holds great power within its bonds? Bonds that when broken by HO-1 set free a bioactive gas and bile pigments that provide the cell the defense and healing it demands.

Sources of Funding
Drs Foresti and Motterlini were supported by funding from Agence Nationale de la Recherche (MITO-CO and CO-HEAL), SATT Ile-de-France INNOV, AREMCAR Foundation, INSERM, and University Paris Est Creteil. Dr Otterbein was supported by the National Institutes of Health, the Julie Henry Foundation and the BIDMC Department of Surgery.

Disclosures
None.

References
Heme oxygenase-1 (HO-1) is a key mitochondrial enzyme that converts heme to biliverdin and carbon monoxide (CO), playing critical roles in various physiological processes. This review discusses the therapeutic potential of heme oxygenase-1 (HO-1) in the heart, with a focus on the HO-1-derived carbon monoxide (CO) and its cardioprotective effects. In recent years, HO-1 gene therapy has shown promising results in treating ischemia-reperfusion injury, with HO-1-derived CO molecules providing cardioprotection.

Carbon monoxide-releasing molecules (CO-RMs) have emerged as a novel class of drug candidates that can deliver CO to target tissues. These molecules can be administered systemically or locally, providing a versatile tool for cardioprotection.

CO-RMs have been shown to induce preconditioning in the heart, reducing the size of infarction and improving cardiac function after ischemia-reperfusion. The mechanism of action involves the activation of the peroxisome proliferator-activated receptor (PPAR)-γ, which enhances the expression of HO-1 and other antioxidant enzymes.

Furthermore, HO-1-derived CO has been implicated in the regulation of inflammation and vascular tone, contributing to anti-inflammatory and vasodilatory effects. This dualistic role of HO-1-derived CO in the heart highlights its potential as a therapeutic target for the treatment of cardiac diseases.

In conclusion, the therapeutic potential of HO-1-derived CO in the heart underscores the importance of ongoing research in this area. Further studies are needed to fully elucidate the mechanisms of action and to optimize the delivery of HO-1-derived CO for effective cardioprotection.

References:


The above references and additional literature highlight the promising role of HO-1-derived CO in cardioprotection, providing a promising platform for future therapeutic applications.


Heme Oxygenase-1 and Carbon Monoxide in the Heart


of myocardial function and viability by magnetic resonance. *Circulation*. 2012;126:S54–S64. doi: 10.1161/CIRCULATIONAHA.112.092627.


Heme Oxygenase-1 and Carbon Monoxide in the Heart: The Balancing Act Between Danger Signaling and Pro-Survival
Leo E. Otterbein, Roberta Foresti and Roberto Motterlini

Circ Res. 2016;118:1940-1959
doi: 10.1161/CIRCRESAHA.116.306588

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/118/12/1940

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/