Heme Oxygenase-1 Deficiency Accelerates Formation of Arterial Thrombosis Through Oxidative Damage to the Endothelium, Which Is Rescued by Inhaled Carbon Monoxide

Andrea L. True, Michelle Olive, Manfred Boehm, Hong San, Randal J. Westrick, Nalini Raghavachari, Xiuli Xu, Edward G. Lynn, Michael N. Sack, Peter J. Munson, Mark T. Gladwin, Elizabeth G. Nabel

Abstract—Heme oxygenase (HO)-1 (encoded by Hmox1) catalyzes the oxidative degradation of heme to biliverdin and carbon monoxide. HO-1 is induced during inflammation and oxidative stress to protect tissues from oxidative damage. Because intravascular thrombosis forms at sites of tissue inflammation, we hypothesized that HO-1 protects against arterial thrombosis during oxidant stress. To investigate the direct function of HO-1 on thrombosis, we used photochemical-induced vascular injury in Hmox1−/− and Hmox1+/+ mice. Hmox1−/− mice developed accelerated, occlusive arterial thrombus compared with Hmox1+/+ mice, and we detected several mechanisms accounting for this antithrombotic effect. First, endothelial cells in Hmox1−/− arteries were more susceptible to apoptosis and denudation, leading to platelet-rich microthrombi in the subendothelium. Second, tissue factor, von Willebrand Factor, and reactive oxygen species were significantly elevated in Hmox1−/− mice, consistent with endothelial cell damage and loss. Third, following transplantation of Hmox1−/− donor bone marrow into Hmox1+/+ recipients and subsequent vascular injury, we observed rapid arterial thrombosis compared with Hmox1+/+ mice receiving Hmox1+/+ bone marrow. Fourth, inhaled carbon monoxide and biliverdin administration rescued the prothrombotic phenotype in Hmox1−/− mice. Fifth, using a transcriptional analysis of arterial tissue, we found that HO-1 determined a transcriptional response to injury, with specific effects on cell cycle regulation, coagulation, thrombosis, and redox homeostasis. These data provide direct genetic evidence for a protective role of HO-1 against thrombosis and reactive oxygen species during vascular damage. Induction of HO-1 may be beneficial in the prevention of thrombosis associated with vascular oxidant stress and inflammation. (Circ Res. 2007;101:893-901.)

Key Words: heme oxygenase ■ thrombosis ■ endothelium ■ apoptosis ■ vascular biology

H-1 is an inducible enzyme with broad tissue expression that is upregulated in response to oxidant stress and inflammatory stimuli and preserves vascular homeostasis.1-3 HO-1 protects tissues during inflammatory stress through degradation of prooxidative heme, production of bilirubin and carbon monoxide (CO), and regulation of cellular iron.9,10 In contrast, HO-1 inhibition is associated with tissue pathology in atherosclerosis11-13 and other inflammatory conditions associated with intra-vascular thrombosis such as septic shock,14 hypoxia,15 and graft rejection.16-18 HO-1 and its byproducts regulate inflammatory responses through repression of proinflammatory genes,19,20 suggesting a role for HO-1 in the regulation of thrombosis associated with inflammation.

At a cellular level, HO-1 prevents apoptosis by inhibiting reactive oxygen species (ROS) formation and attenuates platelet activation in vitro.22 Vascular smooth muscle cell-derived CO, generated by HO-1, attenuates platelet activation in vitro by increasing platelet cGMP,23 and platelets themselves possess functionally active HO-1.24 Deletion of Hmox1 in humans is characterized by severe and persistent endothelial cell (EC) damage with alterations in coagulation, fibrinolysis, including elevations in circulating von Willebrand Factor (vWF).25 Inhibition of HO-1 during pulmonary ischemia/reperfusion results in additive increases in plasminogen activator inhibitor-1 and fibrin deposition.26

These studies led us to hypothesize that HO-1 would prevent intravascular thrombosis associated with EC damage. We used a genetic model in vivo to directly investigate the role of HO-1 on arterial thrombosis in Hmox1−/− mice. We demonstrate that Hmox1 deletion leads to accelerated thrombosis by several mechanisms, including endothelial disrup-
tion and apoptosis, platelet activation, elevations in tissue factor (TF) and vWF, and a failure of HO-1–deficient bone marrow (BM)-derived progenitor cells to prevent thrombosis. This prothrombotic phenotype in Hmox1−/− mice is rescued by CO inhalation and biliverdin administration. Our data indicate that HO-1 directly preserves endothelial integrity and prevents arterial thrombosis during vascular injury.

Materials and Methods
An expanded Materials and Methods section is in the online data supplement at http://circres.ahajournals.org.

Carotid Artery Injury
Photochemical injury was induced in carotid arteries as described37 with approval from the NIH Animal Care and Use Committee.

Tissue Analysis
Transmission electron microscopy was performed on carotid segments using ultra-thin sections and examined with a Nikon E800 light microscope. An terminal deoxyribonucleotidyl transferase–mediated TUNEL assay was used to detect DNA fragmentation and apoptosis in situ. Protein carbonylation concentration in murine carotid arteries was determined using the Biocell PC test kit (BioCell Corp, Auckland, New Zealand).

Clinical Laboratory Measurements
Prothrombin time, complete blood count, and bilirubin measurements were made using citrated murine plasma or heparin. Bleeding times were evaluated as described previously.28 TF concentration in carotid arteries was determined using the Actichrom TF kit (American Diagnostica, Stamford, Conn). Murine plasma was subjected to ELISA using a polyclonal Asserachrom vWF kit (Diagnostica Stago Inc, Parsippany, NJ), a high-affinity means for evaluating circulating murine vWF.29 (Molecular Innovations Inc, Southfield, Mich). In vitro platelet aggregation assay was performed on citrated whole blood samples obtained from the inferior vena cava.

p38 Mitogen-Activated Protein Kinase Inhibitor and Biliverdin Treatment
The p38 mitogen-activated protein kinase (MAPK) inhibitor SKF-86002 was injected subcutaneously 30 minutes before photochemical injury.29 Biliverdin was injected intraperitoneally 3 hours before photochemical injury.30

BM Transplantation
BM, derived from Hmox1+/+ and Hmox1−/− mice, was injected intravenously into the tail veins of irradiated recipient mice. Twelve weeks later, successful engraftment was confirmed by quantitative PCR, and engrafted animals underwent photochemical injury.

CO Inhalation
CO inhalation studies were performed in chambers with a 0.4% CO/balanced air mixture blended with balanced air on site to obtain a stable chamber concentration of 500 ppm CO and O2 level of 20% to 21%. CO and O2 levels were continuously monitored inside the chamber for 24 hours or 15 minutes before or during photochemical injury.

Gene Expression Analysis
RNA was isolated and amplified as described.31 Microarray data processing and analysis were conducted on hybridized Affymetrix chips using Affymetrix GCOS version 1.4.32 Transformed data were subjected to a principal component analysis to detect outliers. Paired and unpaired t tests were performed to detect differentially expressed genes between Hmox1−/− and Hmox1+/+ injured and noninjured arteries. To address the multiple comparisons, fold-cut off filters and false-discovery rate analysis filters were applied.33 Two-way hierar-

chical clustering was used to bring together sets of samples and genes with similar expression patterns. Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems Inc, Redwood City, Calif). Gene expression was validated by quantitative real-time PCR.

Statistics
Statistical analyses were performed using ANOVA or t test as appropriate. Results are expressed as means±SEM and were considered significant at P<0.05.

Results
Deletion of Hmox1 Accelerates Thrombus Formation
We hypothesized that HO-1 would prevent intravascular thrombosis during vascular damage. To test this hypothesis, we used an established photochemical injury model and measured time to occlusive thrombosis in the carotid arteries of Hmox1−/−, Hmox1+/+, and Hmox1−/− mice. Hmox1−/− carotid arteries occluded at 54.7±3.3 minutes, whereas thrombus formed in Hmox1−/− arteries in a significantly shorter time, 20.9±3.4 minutes (P<0.001). Hmox1+/+ mice demonstrated an intermediate time to thrombosis, 39.9±3.4 minutes (P<0.05) (Figure 1A). Occlusive thrombosis was confirmed by hematoxylin/eosin staining of injured vessels (Figure 1B).

To further test the hypothesis that accelerated thrombosis is the direct result of Hmox1 deficiency, biliverdin, the byproduct of oxidative degradation of heme by HO-1, was administered to Hmox1−/− and Hmox1−/− mice 3 hours before photochemical injury. Biliverdin rescued the prothrombotic phenotype in Hmox1−/− mice: time to thrombosis for Hmox1−/−, 50.0±10.0 minutes; versus Hmox1−/−, 59.0±1.0 minutes (P=NS) (Figure 1C). These data suggest that HO-1 directly regulates thrombosis following vascular injury.

Endothelial Damage and Denudation Are Present in Hmox1−/− Mice
We hypothesized that oxidative injury in Hmox1−/− mice leads to EC damage, denudation, exposure of the subintima, and intravascular thrombosis. To test this hypothesis, carotid arteries were examined by electron microscopy 15 to 20 minutes after photochemical injury. Arteries from Hmox1−/− mice had extensive endothelial damage characterized by nuclear condensation and cytoplasmic vacuolization (Figure 2A and 2B). Endothelial denudation was evident with degranulated platelet-rich microthrombi (Figure 2C). In contrast, the endothelium in Hmox1+/+ arteries was intact (Figure 2D). These findings suggest that EC damage and denudation may contribute to rapid thrombosis in Hmox1−/− arteries.

To investigate the mechanism of EC damage and loss, we examined EC apoptosis in Hmox1−/− and Hmox1+/+ arteries, and we delivered a p38 MAPK inhibitor, SKF-86002, to Hmox1−/− mice before photochemical injury. A significant increase in apoptotic ECs was evident in Hmox1−/− arteries following vascular injury (Hmox1−/−, 8.3±0.4%; versus Hmox1+/+, 1.7±0.7%; P<0.0005) (Figure 3A through 3C). In addition, inhibition of p38 MAPK increased EC apoptosis in Hmox1−/− injured arteries (7.7±2.6%; versus non–inhibitor-treated Hmox1+/+ arteries, 1.7±0.7%; P<0.05) (Figure 3A) and accelerated thrombosis formation (38.3±6.4 versus
54.7±3.3 minutes; \( P<0.05 \). Thus, EC apoptosis contributes to accelerated thrombosis in injured \( \text{Hmox}^{1-/-} \) arteries and is likely mediated through p38 MAPK.

To further pursue the hypothesis that oxidative injury in \( \text{Hmox}^{1-/-} \) mice leads to EC damage and subsequent thrombosis, we measured levels of ROS in \( \text{Hmox}^{1-/-} \) and \( \text{Hmox}^{1+/-} \) arteries using a protein carbonylation assay. We found a significant elevation in ROS in \( \text{Hmox}^{1-/-} \) arteries before photochemical injury compared with \( \text{Hmox}^{1+/-} \) arteries (1.34±0.24 versus 0.90±0.06 nmol/mg, respectively; \( P<0.05 \)), consistent with \( \text{Hmox}^{1} \) deficiency. Following photochemical injury, ROS rose in \( \text{Hmox}^{1+/-} \) mice to 1.66±0.13 nmol/mg (\( P<0.01 \)) compared with \( \text{Hmox}^{1+/-} \) mice at baseline. ROS remained elevated in \( \text{Hmox}^{1-/-} \) mice following injury 1.54±0.18 nmol/mg (Figure 3D). Elevations in ROS in \( \text{Hmox}^{1-/-} \) mice were consistent with a deficiency of \( \text{Hmox}^{1} \) and superimposed oxidative injury leading to EC damage and thrombosis.

**Hemostatic Function in \( \text{Hmox}^{1-/-} \) Mice**

To investigate the mechanisms of thrombosis, we first asked whether abnormalities in hemostasis were present in \( \text{Hmox}^{1-/-} \) mice. No significant differences were present in bleeding time, platelet counts, or prothrombin times between...
Hmox1<sup>−/−</sup> and Hmox1<sup>+/+</sup> mice (P=NS), suggesting that abnormalities in primary and secondary hemostasis did not account for accelerated thrombosis (Table). Significant differences were observed, however, in hematocrit, hemoglobin concentration, and mean corpuscular volume of Hmox1<sup>−/−</sup> compared with Hmox1<sup>+/+</sup> and Hmox1<sup>+/+</sup> mice, indicative of an anemia associated with Hmox1 deficiency.25

Because heme catabolism by HO-1 leads to the formation of the antioxidants bilirubin and CO, which serve protective functions in the vasculature, we assayed for baseline arterial hemostasis, bilirubin, or COHb. Deficiency is not attributable to defects in primary or secondary hemostasis, bilirubin, or COHb.

### Hemostasis Measurements in Hmox1<sup>+/+</sup> and Hmox1<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Hmox1 Genotype</th>
<th>Bleeding Time, sec (n=5)</th>
<th>Prothrombin Time, sec (n=5)</th>
<th>Platelet Count, K/μL (n=5)</th>
</tr>
</thead>
<tbody>
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<td>Hmox1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>90.56±6.22</td>
<td>10.3±0.19</td>
<td>1177</td>
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<tr>
<td>Hmox1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>79.50±8.80</td>
<td>9.9±0.09</td>
<td>1356</td>
</tr>
</tbody>
</table>

Elevations in TF and vWF in Hmox1<sup>−/−</sup> Mice

To further investigate the mediators of thrombosis, we assayed arterial TF and circulating vWF, factors known to be associated with oxidant damage and pathological thrombosis.33 Differences in carotid TF levels were not present at baseline between Hmox1<sup>−/−</sup> and Hmox1<sup>+/+</sup> mice, but TF levels did significantly rise in Hmox1<sup>−/−</sup> arteries 15 minutes after the onset of photochemical injury and before the detection of occlusive thrombus (Hmox1<sup>−/−</sup>, 1.9-fold increase versus Hmox1<sup>+/+</sup>; P=0.02) (Figure 4A). Significant differences in circulating vWF levels were not present at baseline, but significant elevations in circulating vWF were observed in Hmox1<sup>−/−</sup> mice following photochemical injury (Hmox1<sup>−/−</sup>, 156.4±28.7; versus Hmox1<sup>+/+</sup>, 71.03±17.1; P<0.05) (Figure 4B). Elevations in TF and vWF following vascular injury may contribute in combination with EC damage to promote thrombus formation in Hmox1<sup>−/−</sup> mice.

### BM-Derived Cells Contribute to Accelerated Thrombosis in Hmox1<sup>−/−</sup> Mice

BM-derived progenitor cells are increasingly recognized as key components in vascular regeneration.34,35 We hypothesized that BM-derived cells lacking HO-1 may be defective in protection of damaged endothelium following injury. Accordingly, we transplanted Hmox1<sup>−/−</sup> BM into Hmox1<sup>+/+</sup> mice, Hmox1<sup>−/−</sup> BM into Hmox1<sup>+/+</sup> mice, and Hmox1<sup>+/+</sup> BM into...
**Figure 4.** Arterial TF and plasma vWF levels are elevated in Hmox1−/− mice. A, TF levels in Hmox1+/+ arteries (left) and Hmox1−/− arteries (right) following injury. Values are expressed as fold activation from Hmox1+/+ arteries. *P=0.02, Hmox1+/+ vs Hmox1−/− (n=6 mice in each group). B, Plasma vWF levels following injury in Hmox1+/+ mice (left) and Hmox1−/− mice (right). *P<0.05, Hmox1−/− vs Hmox1+/+ (n=6 mice in each group).

**Figure 5.** Transplantation of Hmox1+/− marrow into Hmox1+/+ mice leads to accelerated thrombosis. A, BM from Hmox1+/+ or Hmox1−/− mice (donor) were transplanted into Hmox1+/+ (left), Hmox1+/+ mice (middle), or Hmox1+/+ mice (right) (recipient), and injury was performed 14 weeks later. *P<0.05 compared with Hmox1+/+ BM transplanted into Hmox1+/+ recipients (n=7 to 9 mice in each group). B, TF levels in BM-transplanted arteries, as described in A. Values are expressed as fold activation from the Hmox1−/− arteries. *P<0.05, compared with Hmox1+/+ BM transplanted into Hmox1+/+ recipients. C, vWF levels in BM-transplanted mice, as described in A. *P<0.05, **P<0.01, compared with Hmox1+/+ BM transplanted into Hmox1+/+ recipients.

**Hmox1+/+ mice.** Engraftment was confirmed, photochemical injury was performed, and the time to thrombosis was measured. Transplantation of Hmox1−/− BM into Hmox1+/+ mice led to significantly accelerated thrombosis compared with transfer of Hmox1+/+ BM into Hmox1+/+ mice (42.8±3.4 versus 67.6±9.6 minutes; P<0.05) (Figure 5A), suggesting that Hmox1−/− BM-derived cells contribute to accelerated thrombosis. Transfer of Hmox1+/+ BM into Hmox1−/− mice led to a thrombosis time similar to transfer of Hmox1−/− BM into Hmox1−/− mice (40.3±5.2 minutes) (Figure 5A), suggesting that the partial absence of HO-1 in the arterial wall created a local milieu in which endothelial damage still led to accelerated thrombosis, even in the setting of BM-derived cells with full HO-1 complement.

To determine whether Hmox1+/+ progenitor cells could rescue the accelerated thrombosis phenotype in Hmox1−/− mice, we transplanted Hmox1+/+ BM into Hmox1−/− mice. Unexpectedly, engraftment did not occur, and injury experiments could not be performed.

We postulated that TF and vWF may also contribute to accelerated thrombosis when Hmox1−/− BM is transplanted into Hmox1+/+ mice. TF was increased 2-fold in this setting compared with TF levels following transplantation of Hmox1+/+ marrow into Hmox1−/− recipients (P<0.05), and vWF was similarly elevated (136.8±12.4 versus 52.3±8.6; P<0.005; Figure 5B and 5C). TF levels were also elevated 2-fold in Hmox1−/− mice receiving Hmox1+/+ BM, likely reflecting local EC damage in Hmox1−/− mice (Figure 5B and 5C).

To determine whether Hmox1−/− platelets aggregate abnormally independent of Hmox1−/− endothelium, in vitro platelet aggregations assays were performed in response to collagen or ADP. Equivalent numbers of Hmox1−/− and Hmox1+/+ platelets were exposed to agonists, and the extent and rate of aggregation was determined using an optical impedance method. No significant differences were observed in response to collagen (2.5 to 10 μg/mL) or ADP (5 to 20 μmol/L), indicating that HO-1 deficiency does not alter platelet aggregation (data not shown).

**CO Inhalation Rescues the Thrombosis Phenotype in HO-1 Deficiency**

Because CO attenuates platelet aggregation in vitro,36 we investigated whether inhalation of sublethal doses of CO...
would rescue the thrombotic phenotype in $Hmox1^{+/+}$ and $Hmox1^{+/−}$ mice. $Hmox1^{+/+}$ and $Hmox1^{+/−}$ mice were exposed to balanced room air containing CO at 500 ppm in a controlled chamber for ≈24 hours and underwent photochemical injury, and the time to arterial occlusion was measured. Inhaled CO did not modify thrombosis in $Hmox1^{+/+}$ mice (room air, 54.7 ± 2.3 minutes; versus inhaled CO, 52.0 ± 10.5 minutes; $P=NS$) (Figure 6A). In contrast, inhaled CO rescued the prothrombotic phenotype in $Hmox1^{+/−}$ mice by increasing the time to arterial occlusion (room air, 20.9 ± 3.4 minutes; versus inhaled CO, 36.6 ± 1.6 minutes; $P<0.05$) (Figure 6B). Delivery of CO just before or during injury had no effect on time to thrombosis.

**Hmox1 Deficiency Leads to Thrombosis Through a Failure of Arterial Repair Mechanisms**

The elevations in ROS in $Hmox1^{+/+}$ arteries led us to further hypothesize that HO-1 induction following arterial injury is critical to mediate the generation of ROS. Accordingly, we investigated HO levels at baseline and following photochemical injury, and the time to arterial occlusion was measured. Inhaled CO did not modify thrombosis in $Hmox1^{+/+}$ mice (room air, 54.7 ± 2.3 minutes; versus inhaled CO, 52.0 ± 10.5 minutes; $P=NS$) (Figure 6A). In contrast, inhaled CO rescued the prothrombotic phenotype in $Hmox1^{+/−}$ mice by increasing the time to arterial occlusion (room air, 20.9 ± 3.4 minutes; versus inhaled CO, 36.6 ± 1.6 minutes; $P<0.05$) (Figure 6B). Delivery of CO just before or during injury had no effect on time to thrombosis.

Intravascular thrombosis is a well-recognized complication of vascular inflammation. HO-1 has vascular protective properties, including inhibition of vascular smooth muscle cell growth,\textsuperscript{8,12,23} induction of vasodilatation,\textsuperscript{12} and protection against inflammation and oxidant damage.\textsuperscript{1–4,20,21} Although HO-1 guards against platelet-dependent thrombosis,\textsuperscript{37} the direct role of HO-1 in the prevention of arterial thrombosis, a known complication of vascular oxidant damage, has not been explored. Here, we used a genetic model of $Hmox1$ deletion to directly investigate the regulation of intravascular...
thrombosis by HO-1. We found that HO-1 protects against intravascular thrombosis associated with EC damage. *Hmox1* deletion leads to accelerated thrombosis by several mechanisms, including EC disruption and apoptosis, platelet activation, elevations in TF and vWF, and a failure of BM-derived progenitor cells to protect the artery. This prothrombotic phenotype in *Hmox1*⁻/⁻ mice is rescued by CO inhalation and biliverdin administration, known byproducts of HO-1 metabolism of heme. Our findings demonstrate that HO-1 directly preserves endothelial integrity, attenuates platelet activation, and protects against arterial thrombosis during acute vascular injury.

Previously, the role of HO-1 in thrombosis has been studied indirectly through animal models in which stimuli
such as hypoxia, lipopolysaccharide, or xenotransplantation provoke inflammation. In these studies, HO-1 mediates against the induction of proinflammatory cytokines such as tumor necrosis factor-α, IL-1, and monocyte chemoattractant protein-1; it attenuates lipopolysaccharide-induced and monocyte-derived endothelial activation and limits expression of TF and plasminogen activator inhibitor-1, which can result in intravascular thrombosis. In this study, we directly demonstrate, through genetic approaches, a critical role for HO-1 to mediate the induction of ROS following oxidant injury and prevent arterial thrombosis.

Extensive endothelial damage with elevations in circulating vWF and thrombomodulin has been reported previously in a human case of Hmox1 deficiency, which, when combined with factor VIIa to activate X and induce thrombosis, was also significantly elevated in Hmox1−/− mice through their effects on fibrin and the coagulation cascade. Our observations linking TF to accelerated thrombosis in Hmox1 deficiency is reinforced by the finding that CO inhibits MAPK-driven expression of Egr-1 and subsequent TF expression. Indeed, the concept that CO is a cytoprotective product of HO-1 is further supported by the reversal of rapid thrombosis in Hmox1−/− mice following CO inhalation.

In addition to vascular ECs, platelets and other circulating blood cells possess functional HO-1, and CO generated by HO-1 in cultured vascular smooth muscle cells attenuates platelet activation via elevations in platelet cGMP. Although platelet counts and bleeding times were not significantly different in Hmox1−/− and Hmox1+/* mice, we hypothesized that the loss of HO-1 in platelets might also contribute to accelerated thrombosis in concert with EC damage. Our findings that BM from Hmox1−/− mice transplanted into Hmox1−/− recipients leads to accelerated thrombosis compared with Hmox1+/* mice transplanted with Hmox1+/* BM suggest that BM-derived cells, which lack HO-1, directly contribute to thrombosis formation as well.

Hmox1−/− platelets did not demonstrate increased aggregation in vitro, and acute inhalation of CO just before and during photochemical injury did not alter the thrombotic phenotype, suggesting that Hmox1−/− platelets are not solely responsible for thrombosis in the setting of Hmox1−/− deficiency. Other studies using indirect in vitro approaches have suggested a possible role for HO-1 in platelet-dependent thrombosis; these differences may be attributable to technical factors, such as use of Hmox1−/− platelets.

Endothelial progenitor cells, originating from the BM, have been shown to be recruited to sites of vascular injury, where they differentiate into ECs and contribute to adaptive vascular remodeling. Interestingly, thrombosis was significantly reduced following balloon angioplasty of rabbit carotid arteries transplanted with endothelial progenitor cells overexpressing HO-1. These findings are consistent with the observations from our BM transplantation findings; that is, BM-derived progenitor cells from Hmox1−/− mice are more susceptible to apoptosis and are associated with rapid thrombus formation. These Hmox1−/− progenitor cells lack the antithrombotic and antiinflammatory properties of HO-1, likely because of a lack of protection against ROS.

CO prevents oxidant-induced endothelial damage in the lung, directly protects ECs from apoptosis, and attenuates platelet aggregation following elevations in cGMP. Chronic CO inhalation reversed the prothrombotic phenotype in Hmox1−/− mice but had no effect on Hmox1+/+ mice, suggesting that the endogenous generation of CO by HO-1 is a potent mechanism by which HO-1 protects the vasculature from thrombosis under conditions of oxidative stress. Similarly, biliverdin, another byproduct of heme metabolism by HO-1, rescued the prothrombotic phenotype of Hmox1 deficiency in a p38 kinase-dependent manner, providing an additional mechanism by which HO-1 protects vascular integrity.

We conclude that HO-1 directly preserves vascular integrity and prevents thrombosis during oxidant stress by multiple mechanisms, including preservation of the endothelium, inactivation of platelets, and adaptive remodeling by BM-derived progenitor cells. Administration of byproducts of HO-1, CO, and biliverdin rescues the prothrombotic phenotype of Hmox1 deficiency during oxidative stress. Thus, therapies targeted toward the induction of HO-1 or its cytoprotective byproducts may be beneficial in preventing thrombosis observed in vascular disorders.

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Disclosures
None.

References


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Materials and Methods

Carotid artery injury

Photochemical injury was produced as described with minor modification\textsuperscript{27}. Hmox1\textsuperscript{+/+}, Hmox\textsuperscript{+/−}, and Hmox1\textsuperscript{−/−} mice (generous gift of Shaw-Fang Yet\textsuperscript{1}), were back crossed 6 generations against a C57Bl/6 background and were studied at an average weight of 23 grams. The left common carotid artery was isolated and gently positioned in a Doppler flow probe (T206 VB series, 0.5 mm, Transonic Systems, Inc., Ithaca, NY) connected to a flow meter (Transonic model T206). Rose Bengal (Fisher Scientific, Fair Lawn, NJ) was diluted to 10 mg/ml in sterile phosphate buffered saline (pH 7.4, Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free, Invitrogen, Carlsbad, CA) and injected into the cannulated right jugular vein (50-60 mg/kg) over a 5 minute period using a 1 ml latex free syringe (Becton Dickinson and Co., Franklin Lakes, NJ). Just prior to injection, a 1.5 mW hene-green light laser (540 nm, Melles Griot, Carlsbad, CA) was positioned over the carotid artery from a distance of 50 mm until the flow meter indicated stable cessation of blood flow, at which time vessel injury was stopped. Confirmation of occlusive thrombosis was determined by hematoxylin and eosin (H&E) staining of injured, flushed, and paraffin embedded carotid sections. Age matched littermates were used in all experiments. All experimental protocols were approved by the NIH Animal Care and Use Committee in compliance with the NIH Guidelines for the Use and Care of Laboratory Animals.

Tissue analysis

Transmission electron microscopy. Carotid arteries were injured for 15-20 minutes and processed for routine transmission electron microscopy (TEM) Sections were cut with a Leica Ultracut UCT ultra-microtome. Semi-thin sections were generated in order to orient a section plane across all layers of the vessel wall. Ultra-thin sections were produced from at least one plane of an artery block, selected from a total of 56 tissue blocks. Semi-thin sections were
stained with Epoxy Tissue Stain (Electron Microscopy Sciences, Fort Washington, PA), and examined with a Nikon Eclipse E800 light microscope. Images were acquired with a Sony DKC-ST5 digital camera. Ultra-thin sections were cut with a Diatome diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEOL 1000CX transmission electron microscope at an accelerating voltage of 60 kV using a 40 µm objective aperture.

**In situ nick-end labeling for detection of apoptosis.** The terminal deoxyribonucleotidyl transferase–mediated TUNEL assay was used to detect DNA fragmentation in situ. Briefly, the samples were preincubated with equilibration buffer for 10 minutes and subsequently incubated with deoxyribonucleotidyl transferase (Supertechs, Inc. Rockville, MD) in the presence of digoxigenin-conjugated dUTP (Roche, Nutley, NJ) for 2 hours at 37°C. The reaction was terminated by incubating the samples in 2xSSC at RT for 15 minutes. After three rinses with TBS, the sections were incubated with alkaline phosphatase labeled Streptavidin (Roche, Nutley, NJ) for 30 minutes.

**Protein carbonylation measurements.** Protein carbonylation concentration in murine carotid arteries was determined using the Biocell PC test kit (BioCell Corp, Auckland, New Zealand). All samples were evaluated in triplicate. Approximately 30 minutes after photochemical injury, arteries were flushed with saline and lysed in homogenization buffer: 0.2 mM Na citrate in 50 mM Tris-HCl, pH 7.4 + Complete inhibitors (Roche, Nutley, NJ). Carotids were homogenized using a Dounce homogenizer, and samples were centrifuged at 800 x g for 10 minutes at 4°C, and 10 µg of protein were used for the assay. The low protein method, which involves concentration of the protein by precipitation with trichloroacetic acid (TCA), was used. The results are expressed as carbonylation content of the carotid extract (nmol/mg protein).
Clinical laboratory measurements

PT, CBC and bilirubin measurements were conducted based on standard clinical laboratory procedures using citrated murine plasma (final 0.38% sodium citrate) or heparin (<20 U/ml), where appropriate. Bleeding times were evaluated as previously described.

Tissue factor (TF) concentration in murine carotid arteries was determined using the Actichrome TF kit (American Diagnostica, Standford, CT). All samples were evaluated in duplicate. Arteries were flushed with saline and lysed according to manufacturer’s recommendations. Two carotid arteries were pooled, and TF concentration was expressed relative to 100 ng total protein. Absorbance was read using a Spectramax plus microplate reader controlled by and interpreted with SOFTmax Pro software (Molecular Devices, Sunnyvale, CA).

Murine plasma was subjected to ELISA using a polyclonal Asserachrom vWF kit (Diagnostica Stago Inc., Parsippany, NJ), a high-affinity means for evaluating circulating murine vWF. vWF was measured in duplicate at 3 dilutions (1:25, 1:51 and 1:102) and analyzed by ELISA. All values were normalized to 1:51 dilution. 100% corresponds to the amount of vWF present in C57Bl/6 pooled murine plasma at a 1:51 dilution. Regression analysis was performed on all samples to standard curves of pooled murine and human plasma for vWF. Absorbance was read using a Versamax tunable microplate reader controlled by and interpreted with SOFTmax Pro software (Molecular Devices, Sunnyvale, CA).

In vitro platelet aggregation assay was performed using citrated whole blood samples obtained from the inferior vena cava and spun for 15 minutes at 100 g to obtain platelet rich plasma (PRP) and for an additional 15 minutes at 2400 g to obtain platelet poor plasma (PPP). Platelets were automatically counted (Coulter Counter Z2, Beckman Coulter, Inc., Fullerton, CA) using a 50 µM aperture and equivalent counts (200-250,000 platelets/µl) were exposed to the
indicated agonist. Aggregation was measured by the optical impedance method using a Chrono-log aggregometer (Chrono-log, Havertown, PA).

**p38 MAP-kinase inhibitor and biliverdin treatment**

The p38 MAP-kinase inhibitor, SKF-86002, was dissolved in DMSO-ethanol-saline (30:30:40 by volume) and injected subcutaneously at a dose of 20 mg/kg body weight 30 minutes prior to the beginning of photochemical injury. Biliverdin (Frontier Scientific, Logan, UT) was dissolved in 0.2 N NaOH and adjusted to a final pH of 7.4 with HCl. After diluting in saline, biliverdin was injected intraperitoneally at a dose of 50 mg/kg in 1 ml. Mice were pretreated with biliverdin 3 hours before laser injury.

**Bone marrow transplantation**

BM was derived from *Hmox1−/−* and *Hmox1+/+* CD45.2 age matched donors following CO₂ euthanasia. Marrow cell suspensions were flushed from femurs and tibias, filtered, and stored on ice until use. Recipient mice were lethally irradiated with 900 rads of whole body irradiation. Five million unfractionated cells were injected intravenously into the tail veins of recipient mice. Twelve weeks later, successful engraftment was confirmed by quantitative PCR to determine the presence or absence of *Hmox1* or *Sry* to distinguish female and male BM cells and by flow cytometry (FACS) using antibodies directed against CD45.1 and 45.2 after gating on CD3+ cells (BD PharMingen, San Diego, CA). Engrafted animals were injured at least 14 weeks post-transplantation. Six to nine females were studied per group.

**CO inhalation**

For CO inhalation studies, animals in their original cages were placed in a chamber with food and water ad libitum. A 0.4% CO/balanced air mixture was further blended with balanced air on site to obtain a stable chamber concentration of 500 ppm CO and O₂ level of 20-21%.
Both CO and O₂ levels were continuously monitored inside the chamber for 24 hours prior to photochemical injury, 15 minutes prior to injury, or during injury (Dräger Safety, Inc., Pittsburgh, PA). Following photochemical injury to mice carotid arteries, the time to arterial occlusion was measured.

**Gene expression analysis**

**RNA isolation and amplification.** Total RNA from carotid arteries was extracted using a RNAeasy mini kit (Qiagen, MD) following the manufacturer’s directions. Arteries were homogenized and lysed in lysis buffer containing guanidinium thiocyanate, and the lysate was mixed with ethanol and applied to a silica based filter that selectively binds RNA. Genomic DNA was removed by DNase treatment. The concentration of the isolated RNA was determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Quality and integrity of the total RNA isolated was assessed on the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

To allow for an analysis of gene expression profiles on individual mice, T7 based RNA amplification was carried out on 10 ng of the isolated total RNA from a single mouse carotid artery using a Riboamp OA two-round amplification kit as suggested by the manufacturer (Arcturus, Mountain View, CA). Briefly, total RNA was incubated with oligo dT/T7 primers and reverse transcribed into double stranded cDNA. *In vitro* transcription of the purified cDNA was performed using T7 RNA polymerase at 42ºC for 6 hours. The amplified RNA was purified and subjected to a second round of amplification and biotin labeling using Affymetrix’s IVT labeling kit following the manufacturer’s directions. The yield and integrity of the biotin labeled cRNA were determined using the Nanodrop ND-1000 spectrophotometer and the Agilent 2100 bioanalyzer. Twenty µg of biotin-labeled RNA was fragmented to ~200 bp size by incubating in fragmentation buffer containing 200 mM Tris-acetate pH 8.2, 500 mM potassium acetate and 500 mM magnesium acetate for 35 minutes at 94ºC prior to hybridization. Fragmented RNA was
assessed for relative length on an Agilent 2100 bioanalyzer and hybridized to mouse genome 430A chips for 16 hours, washed, stained on an Affymetrix fluidics station and scanned using an Affymetrix genechip scanner.

**Microarray data processing and analysis.** Affymetrix GCOS version 1.4 was used to calculate the signal intensity and the percent present calls on the hybridized Affymetrix chip. The signal intensity values obtained for probe sets in the microarrays were transformed using an adaptive variance-stabilizing, quantile-normalizing transformation. Transformed data from all the chips were subjected to a principal component analysis (PCA) to detect outliers. Paired and unpaired t-tests were performed to detect differentially expressed genes between \( Hmox1^{-/-} \) and \( Hmox1^{+/+} \) injured and noninjured arteries. To address the multiple comparisons, fold-cut off filters and false discovery rate (FDR) analysis filters were applied. Two-way hierarchical clustering was used to bring together sets of samples and genes with similar expression patterns. The hierarchical cluster was run from the JMP5.1 statistical software package (SAS Institute, Cary, NC) using the ward method. Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA).

**Validation of gene expression data by Q-PCR.** First-strand cDNA was synthesized using 1 µg of amplified RNA and random primers in a 20 µl reverse transcriptase reaction mixture using Invitrogen’s Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA) following the manufacturer’s directions. Quantitative real-time PCR assays were carried out with the use of gene-specific double fluorescently labeled probes in a 7900 Sequence Detector (PE Applied Biosystems, Norwalk, CT). Probes and primers were obtained from Applied Biosystems. In brief, PCR amplification was performed in a 384 well plate with a 20-µl reaction mixture containing 300 nm of each primer, 200 nm probe, 200 nm dNTP in 1x real time PCR buffer and passive reference (ROX) fluorochrome. The thermal cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 second denaturation at 95°C and 1 minute annealing and extension at 60°C. Samples were analyzed in duplicate and the Ct values
obtained were normalized to the housekeeping gene β actin. The comparative $C_T$ ($\Delta\Delta C_T$) method which compares the differences in $C_T$ values between groups was used to achieve the relative fold change in gene expression between $Hmox1^{-/-}$ and $Hmox1^{+/+}$ mice.

**Statistics**

Gene expression analysis is described above. Standard statistical analyses (ANOVA or t-test, where appropriate) were performed using the GraphPad Prism program (San Diego, CA). Results are expressed as the mean ± the standard error of the mean (S.E.M.) and were considered significant at a $P<0.05$ level.

**References**


**Supplemental Table 1: List of genes with 10-fold or greater upregulation by injury and Hmox deficiency.** The gene list is selected by comparing (t-test) injured arteries with uninjured arteries, using a maximum 10% false discovery rate, a minimum 10-fold change cutoff, and a 2.5-fold or greater change in Hmox1\(^{−/−}\) vs. Hmox1\(^{+/+}\).

<table>
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<tr>
<th>Genes induced more by injury in Hmox1(^{−/−}) arteries</th>
<th>Genes induced more by injury in Hmox1(^{+/+}) arteries</th>
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<tr>
<td>actin, alpha 2, smooth muscle, aorta</td>
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<td>proteoglycan 1, secretory granule</td>
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<td>GATA binding protein 1</td>
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<td>ras-related associated with diabetes</td>
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