Induction of Heme Oxygenase-1 In Vivo Suppresses NADPH Oxidase–Derived Oxidative Stress

Srinivasa R. Datla, Gregory J. Dusting, Trevor A. Mori, Caroline J. Taylor, Kevin D. Croft, Fan Jiang

Abstract—Our previous studies suggest that heme oxygenase (HO)-1 induction and/or subsequent bilirubin generation in endothelial cells may suppress superoxide generation of from reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase. In this study, we examined the consequence of HO-1 induction in vivo on NADPH oxidase activity. Three doses of hemin (25 mg · kg⁻¹, IP, every 48 hours), with or without cotreatment with the HO inhibitor tin protoporphyrin-IX (15 mg · kg⁻¹, IP), were given to apolipoprotein E–deficient mice, which display vascular oxidative stress. Hemin treatment increased HO-1 expression and activity in aorta (undetectable at baseline) and kidney (by 3-fold) and significantly reduced both NADPH oxidase activity (by ≈25% to 50%) and superoxide generation in situ. The increase in HO-1 activity and inhibition of NADPH oxidase activity by hemin were reversed by tin protoporphyrin-IX and not associated with changes in Nox2 or Nox4 protein levels. Hemin also reduced plasma F₂-isoprostane levels by 23%. The inhibition of NADPH oxidase activity by hemin in the aorta was mimicked by bilirubin in vitro (0.01 to 1 μmol/L). Bilirubin also concentration-dependently reduced NADPH oxidase–dependent superoxide production stimulated by angiotensin II in rat vascular smooth muscle cells and by phorbol 12-myristate 13-acetate in human neutrophil-like HL–60 cells. HO-1 overexpression by plasmid-mediated gene transfer in rat vascular smooth muscle cells decreased NADPH-stimulated superoxide production. Thus, systemic expression of HO-1 suppresses NADPH oxidase activity by mechanisms at least partly mediated by the bile pigment bilirubin, thereby reducing oxidative stress. (Hypertension. 2007;50:636-642.)

Key Words: bilirubin ■ heme oxygenase-1 ■ NADPH oxidase ■ oxidative stress ■ reactive oxygen species

Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase expressed in nonphagocytic cells has a critical role in influencing the redox balance in vasculature. This enzyme consists of the membrane-bound Nox and p22phox subunits, the cytosolic p47phox and p67phox subunits, and a small GTPase, Rac. Activation of this enzyme results in a multicomponent electron transfer machinery that carries single subunits, the cytosolic p47phox and p67phox subunits, and a small GTPase, Rac. Activation of this enzyme results in a multicomponent electron transfer machinery that carries single subunits, the cytosolic p47phox and p67phox subunits, and a small GTPase, Rac. Activation of this enzyme results in a multicomponent electron transfer machinery that carries single subunits, the cytosolic p47phox and p67phox subunits, and a small GTPase, Rac. Activation of this enzyme results in a multicomponent electron transfer machinery that carries single...
idemic apolipoprotein (E)-deficient (ApoE<sup>0</sup>) mice, which exhibit increased oxidative stress.

**Materials and Methods**

An expanded Methods section is available online at http://hyper.ahajournals.org.

**Cell Culture**

Rat aortic smooth muscle cells (RASMCs)<sup>15</sup> and HL-60 cells<sup>16</sup> were cultured as described previously.

**Animal Treatment and Sample Collection**

All of the animal studies were carried out in accordance with the guidelines of the institutional animal ethics committee and the National Health and Medical Research Council of Australia. Male ApoE<sup>0</sup> mice maintained on normal diet until 26 to 30 weeks of age were used. Animals were randomly divided into 3 groups: vehicle (control), hemin (3× IP injections at 25 mg·kg<sup>−1</sup> every 48 hours), and hemin plus SnPP (3× IP injections at 15 mg·kg<sup>−1</sup> every 48 hours). Twenty-four hours after the last injection, animals were euthanized, and blood, aorta, and kidney samples were collected. Plasma was quickly separated and stored at −80°C for bilirubin and isoprostane analysis.

**Lucigenin Enhanced Chemiluminescence**

NADPH oxidase activity in cells and tissues was assessed by lucigenin-enhanced chemiluminescence as described previously.<sup>15</sup>

**In Situ Superoxide Detection**

Dihydroethidium (DHE) fluorescence was used to detect in situ superoxide levels as described previously.<sup>17,18</sup>

**HO-1 Activity Assay**

HO-1 activity in microsomal preparations from the kidney and aorta was measured by bilirubin generation according to the method described by Nath et al.<sup>19</sup>

**Plasma F<sub>2</sub>-Isoprostane Measurement**

Plasma F<sub>2</sub>-isoprostane levels were measured by capillary gas chromatography and electron capture negative ionization mass spectrometry as described previously.<sup>20</sup>

**Plasma Bilirubin Measurement**

Plasma bilirubin levels were determined using a total bilirubin kit (Thermo Electron Corporation) following the protocol provided by the manufacturer.

**HO-1 Overexpression**

The full-length cDNA of human HO-1 was purchased from OriGene Technologies. A nonexpression vector containing human HO-1 cDNA was used as the control.<sup>21</sup>

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical significance (P<0.05) between the experimental groups was determined by unpaired Student’s t test or 1-way ANOVA, as appropriate.

**Results**

**Hemin Induces HO-1 Expression and Activity In Vivo**

In ApoE<sup>0</sup> mice maintained on normal diet, the NADPH oxidase–dependent superoxide production was increased in the aorta as compared with wild-type controls (199±51 count per second per mg in ApoE<sup>0</sup> versus 75±18 count per second per mg in C57BL/6; P<0.05; n=6). Although oxidative stress, per se, can induce HO-1 expression,<sup>22</sup> in Western blot experiments, we found that HO-1 expression in the aorta and kidney from these ApoE<sup>0</sup> mice was extremely low, suggesting that the increase in oxidative stress in these mice was not sufficient to induce HO-1 expression. In contrast, hemin administration caused a significant increase in HO-1 expression in both aorta (Figure 1A) and kidney (Figure 1B), whereas the levels of Cu/Zn superoxide dismutase were not changed. Cotreatment with the HO-1 inhibitor SnPP seemed to enhance the HO-1 expression, but this effect was not significant (Figure 1). Immunohistochemistry confirmed the enhanced HO-1 expression by hemin and demonstrated that HO-1 was mainly localized in the medial layers of aorta (Figure S1). In the aorta, HO-1 activity was undetectable under basal conditions; treatment with hemin markedly increased HO-1 activity, which was inhibited by SnPP (Figure 2A, pooled sample from 6 aortas in each group). Similarly, HO-1 induction in the kidney was accompanied by a significant increase in HO-1 activity, which was inhibited by SnPP (Figure 2B). The plasma bilirubin level was also significantly increased by hemin treatment (0.27±0.04 mg/dL in control versus 0.53±0.07 mg/dL in hemin-treated ones; P<0.05; n=12), and this was reversed
by SnPP (0.30±0.05 mg/dL; *P* > 0.05 versus control; *n* = 7). None of the treatments had any effect on body weight (30.7±0.6, 31.3±0.5, 31.3±0.8, and 31.0±0.4 g in control, hemin, hemin plus SnPP, and SnPP alone groups, respectively; *n* = 8 to 15).

**HO-1 Expression Suppresses NADPH Oxidase Activity and In Situ Superoxide Production**

NADPH oxidase activity in both aorta and kidney was reduced in hemin-treated mice (Figure 2), and this was reversed by SnPP (Figure 2). In both tissues, SnPP alone did not significantly alter the NADPH oxidase function (Figure 2). As found previously, the NADPH-stimulated superoxide release was blocked by the NADPH oxidase inhibitor diphenyleneiodonium (DPI) but not affected by the NO synthase inhibitor Nω-nitro-l-arginine methyl ester, the xanthine oxidase inhibitor allopurinol, the mitochondrial electron transport chain inhibitor rotenone, the cyclooxygenase inhibitor indomethacin, or the cytochrome P450 inhibitor 17-octadecynoic acid (data not shown). Consistent with the decreased NADPH oxidase activity, in situ superoxide production measured by DHE fluorescence was also significantly reduced in tissues from hemin-treated animals, and SnPP blocked this effect of hemin (Figure 3 and Figure S2).

To confirm the source of superoxide detected by DHE fluorescence, we incubated aortic segments ex vivo in solutions containing various inhibitors before sectioning. As shown in Figure S3, the DHE fluorescence was reduced by the NADPH oxidase inhibitor DPI but not affected by other enzyme inhibitors.

**Hemin Decreases Plasma F₂-Isoprostanes and Increases the Nitrate/Nitrite Level**

We examined whether HO-1 expression affected measures of systemic oxidative stress. As shown in the Table, the plasma F₂-isoprostane levels were significantly decreased in hemin-treated animals. Interestingly, SnPP alone also reduced plasma F₂-isoprostanes. On the other hand, hemin treatment did not further change the plasma F₂-isoprostanes in the presence of SnPP (Table). We also measured the plasma nitrate/nitrite level. Interestingly, we found that there was a slight increase in the nitrate/nitrite level in the hemin-treated group (Table).

**HO-1 Expression Does Not Affect Nox Expression**

We also investigated whether HO-1 induction had an impact on the protein abundance of the heme-containing Nox sub-
units of NADPH oxidase. In the aorta, the Nox2 isoform could be readily detected, whereas in the kidney, only Nox4 was detectable. Nox1 was not detected with Western blot in either of these tissues. Hemin treatment with or without SnPP had no significant effect on the levels of either Nox2 in aorta or Nox4 in the kidney (Figure 4).

Bilirubin Inhibits NADPH Oxidase Activity in Both Vascular and Phagocytic Cells

To explore whether the inhibitory effect of HO-1 on NADPH oxidase activity could be mediated by its end product, bilirubin, we carried out ex vivo experiments using isolated aortic rings from untreated ApoE0 mice. Preincubation of the tissues with bilirubin (10 nmol/L to 1 μmol/L) significantly inhibited the NADPH oxidase activity in a time- and concentration-dependent manner (Figure 5). To determine whether bilirubin had a similar effect on phagocytic NADPH oxidase, we used HL-60 cells. Stimulation of HL-60 cells with phorbol 12-myristate 13-acetate triggered a sustained superoxide release, which was blocked by the NADPH oxidase inhibitor DPI and significantly reduced by bilirubin (30 nmol/L to 10 μmol/L) in a concentration-dependent manner (Figure 5). The superoxide-scavenging effect of bilirubin is relatively weak (IC50 = 75 μmol/L) as compared with the concentrations used in this study,23 and to exclude that ROS are scavenged by bilirubin directly, we repeated the experiments in HL-60 cells that had been washed extensively after bilirubin pretreatment. Indeed, the inhibitory effect was largely preserved ≥20 minutes after removing bilirubin from the assay medium (online Figure S4).

Bilirubin Inhibits Angiotensin II–Induced NADPH Oxidase Activation in Vascular Smooth Muscle Cells

We also tested whether bilirubin could suppress NADPH oxidase activation under pathophysiological conditions using cultured RASMCs stimulated by angiotensin II (Ang II). Ang II significantly increased the NADPH-stimulated superoxide production (Figure 6). In all of the experiments, no chemiluminescence signal above the background could be detected in the presence of the NADPH oxidase inhibitor DPI. Preincubation of the cells with bilirubin (0.03 to 10 μmol/L) concentration-dependently reduced NADPH oxidase–dependent superoxide production in both resting (Figure 6A) and Ang II–treated cells (Figure 6B). Notably, the NADPH oxidase activity in Ang II–treated cells was decreased to a level comparable to that in resting cells in the presence of bilirubin from 0.3 to 10 μmol/L, which was also confirmed with DHE fluorescence microscopy (online Figure S5).

Table 1. Effects of Hemin±SnPP on Plasma F2-Isoprostanes and Nitrate/Nitrite Levels

<table>
<thead>
<tr>
<th>Plasma Concentration</th>
<th>Vehicle</th>
<th>Hemin</th>
<th>Hemin+SnPP</th>
<th>SnPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2-isoprostanes, nmol/L</td>
<td>3.8±0.2</td>
<td>2.9±0.1*</td>
<td>2.2±0.2*</td>
<td>2.6±2.2*</td>
</tr>
<tr>
<td>Nitrate/nitrite, μmol/L</td>
<td>33.6±2.3</td>
<td>48.3±5.6*</td>
<td>34.9±3.0</td>
<td>36.2±1.1</td>
</tr>
</tbody>
</table>

*P<0.05 vs vehicle control; n=5 to 10.

The plasma nitrate/nitrite was measured by the Griess reaction.

Figure 5. Effects of bilirubin on NADPH oxidase activity in isolated aortic rings of ApoE0 mice (A; n=5) and DMSO-differentiated (1.5% for 3 to 4 days), neutrophil-like HL-60 cells (B; n=3). Tissue or cells were incubated with bilirubin of increasing concentrations, which was blocked by the NADPH oxidase inhibitor DPI and significantly reduced by bilirubin (30 nmol/L to 10 μmol/L) in a concentration-dependent manner (Figure 5).

Figure 4. Effects of hemin or hemin plus SnPP administration in ApoE0 mice on Nox protein expression in the aorta (A) and kidney (B) measured with Western blot. The Nox2 isoform was readily detected in the aorta, whereas Nox4 was the predominant isoform in the kidney. C, Quantitated densitometry data normalized to β-actin (n=3 to 4).
dependent superoxide production. This may be an important showing that HO-1 expression suppresses NADPH oxidase–ment with our own studies in human endothelial cells ApoE0 mice, and these lesions show significant monocyte/macrophage infiltration, making it difficult to precisely define the cellular site of suppression of NADPH oxidase function. In measuring aortic NADPH oxidase activity, we deliberately chose segments of the thoracic aorta without visible atherosclerotic lesions. DHE fluorescence microscopy demon-
F₂-isoprostanes. The low level of F₂-isoprostanes in the hemin plus SnPP group, however, could indicate that the dose of SnPP used might not be sufficient to inhibit HO in vivo. However, this is unlikely, because the enzyme activity of HO in the kidney was significantly reduced by SnPP. Interestingly, we found that treatment with SnPP alone also reduced the F₂-isoprostane level, and this effect of SnPP prevented us from establishing a direct link between HO-1 expression and systemic F₂-isoprostane levels. The mechanism of this effect of SnPP remains unclear. Of note, F₂-isoprostanes are products of phospholipid peroxidation induced by free radicals, and we propose that SnPP might act as a chain-breaking antioxidant given its protoporphyrin structure. In addition, we found that the plasma nitrate/nitrite level was increased in the hemin-treated group. Although this effect may not be directly linked to improved endothelial function, given the recent findings that ROS may oxidize the cofactors of endothelial NO synthase, and reduced NO production, our results indicate that HO-1 expression and subsequent reduction in ROS release might improve the endothelial NO synthase function.

Perspectives
Induced expression of HO-1 has been shown to lower blood pressure in several animal models of hypertension. Recently, Wang et al reported that continuous administration of hemin plus SnPP group, however, could indicate that the dose of SnPP used might not be sufficient to inhibit HO in vivo. However, this is unlikely, because the enzyme activity of HO in the kidney was significantly reduced by SnPP. Interestingly, we found that treatment with SnPP alone also reduced the F₂-isoprostane level, and this effect of SnPP prevented us from establishing a direct link between HO-1 expression and systemic F₂-isoprostane levels. The mechanism of this effect of SnPP remains unclear. Of note, F₂-isoprostanes are products of phospholipid peroxidation induced by free radicals, and we propose that SnPP might act as a chain-breaking antioxidant given its protoporphyrin structure. In addition, we found that the plasma nitrate/nitrite level was increased in the hemin-treated group. Although this effect may not be directly linked to improved endothelial function, given the recent findings that ROS may oxidize the cofactors of endothelial NO synthase, and reduced NO production, our results indicate that HO-1 expression and subsequent reduction in ROS release might improve the endothelial NO synthase function.

PERSPECTIVES
Induced expression of HO-1 has been shown to lower blood pressure in several animal models of hypertension. Recently, Wang et al 10 reported that continuous administration of hemin to adult spontaneously hypertensive rats for 3 weeks produced a sustained normalization of the systolic blood pressure, and this effect was associated with increased HO-1 expression and activity in peripheral arteries. Also, it has been shown that HO-1 has remarkable protective effects against atherosclerosis, endothelial dysfunction, neointimal NO synthase, leading to uncoupled endothelial NO synthase expression and subsequent reduction in ROS release might improve the endothelial NO synthase function.

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Disclosures
None.

References


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