Bilirubin From Heme Oxygenase-1 Attenuates Vascular Endothelial Activation and Dysfunction

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Objective—Heme oxygenase-1 (HO-1), the rate-limiting enzyme of heme degradation, has recently been considered to have protective roles against various pathophysiological conditions. Since we demonstrated that HO-1 overexpression inhibits atherosclerotic formation in animal models, we examined the effect of HO modulation on proinflammatory cytokine production, endothelial NO synthase (eNOS) expression, and endothelium-dependent vascular relaxation responses.

Methods and Results—After HO-1 induction by heme arginate (HA), vascular endothelial cell cultures were exposed to oxidized low-density lipoprotein (oxLDL) or tumor necrosis factor-α (TNF-α). HA pretreatment significantly attenuated the production of vascular cell adhesion molecule-1, monocyte chemotactic protein-1, and macrophage colony-stimulating factor, suggesting that HO-1 induction attenuates proinflammatory responses. In addition, HO-1 overexpression also alleviated endothelial dysfunction as judged by restoration of attenuated eNOS expression after exposure to oxLDL and TNF-α. Importantly, impaired endothelium-dependent vascular relaxation responses in thoracic aortic rings from high-fat–fed LDL receptor knockout mice were also improved. These effects were observed by treatment with bilirubin not by carbon monoxide.

Conclusions—These results suggest that the antiatherogenic properties of HO-1 may be mediated predominantly through the action of bilirubin by inhibition of vascular endothelial activation and dysfunction in response to proinflammatory stresses. (Arterioscler Thromb Vasc Biol. 2005;25:155-160.)

Key Words: heme oxygenase ■ oxidized LDL ■ endothelial nitric oxide synthase ■ bilirubin ■ carbon monoxide

Vascular endothelial cell activation by oxidized LDL (oxLDL) and cytokines such as tumor necrosis factor-α (TNF-α) is considered to play an essential role in the development of atherosclerotic lesions.1 Activated endothelial cells produce adhesion molecules, chemokines, and growth factors such as vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), and macrophage colony-stimulating factor (MCSF).2-4 Numerous studies have shown that these molecules promote multiple steps in the formation of atherosclerotic lesion.1,3,4 Endothelial dysfunction, which is associated with decreased bioavailability of NO from endothelial NO synthase (eNOS), is also considered to play an important role in atherogenesis.1,5 NO formed by eNOS has been shown to contribute to vascular smooth muscle cell relaxation and inhibition of platelet aggregation.1 Heme oxygenase (HO) catalyzes the rate-limiting step of heme degradation in mammals.6 The products of the reaction are biliverdin, carbon monoxide (CO), and free iron. It has been suggested that biliverdin and CO have cytoprotective effects against various cellular stresses.7-9 We demonstrated that the inducible form of HO (HO-1) is induced in cultured vascular endothelial cells, smooth muscle cells, and macrophages by oxidized low-density lipoprotein (oxLDL), and that high expression of HO-1 results in attenuation of monocyte chemotaxis by oxLDL.9 In fact, HO-1 is expressed in atherosclerotic lesions.10,11 We also demonstrated that overexpression of HO-1 inhibits the formation of atherosclerotic lesions by inhibiting lipid peroxidation and by affecting NO metabolism.11,12 These data suggest that HO-1 as an intrinsic antioxidant plays an important role against atherogenesis. However, it is still unknown1 which proinflammatory molecules are involved in the biological action of HO-1 and which HO-1 reaction product is predominantly responsible for vascular endothelial protection.

In this study, we screened for changes in genes after heme arginate (HA) treatment of vascular endothelium using cDNA microarray analyses to eliminate the possibility that use of heme derivatives to induce HO-1 may result in effects on genes other than the HO-1 gene. These analyses revealed that the HA treatment used in this study had the strongest effect on
the HO-1 gene and that effects on other genes were minimal. Endothelial HO-1 overexpression significantly attenuated production of inflammatory mediators and reversed the decrease in eNOS by oxLDL and TNF-α. In addition, HO-1 overexpression also improved the impaired vasodilatory responses of aortic segments treated with oxLDL. Importantly, these effects on vascular endothelium were predominantly observed by treatment with bilirubin not by CO. These results suggest the possibilities that HO-1 via the action of bilirubin is involved in suppression of endothelial dysfunction and activation in response to proinflammatory stresses.

**Methods**

**Reagents**

TNF-α was obtained from R&D systems and HA from Huhtamäki Oy Pharmaceuticals. Tricarbonyldichlororuthenium (II) dimer ([Ru(CO)3Cl2])2 was from Stream Chemicals. All other reagents were obtained from Sigma-Aldrich unless indicated otherwise. HA, Sn-protoporphyrin IX (SnPP IX), and bilirubin solutions were prepared in the dark.

**Cell Culture**

Human aortic endothelial cells (HAECs) were purchased from Clonetics. In all experiments, cells were used at passage 4-6. HO-1 activities were modulated by either 1 to 10 μmol/L HA for 16 hours or 10 μmol/L SnPP IX for 2 hours as described previously. To generate CO in cell cultures, tricarbonyldichlororuthenium (II) dimer ([Ru(CO)3Cl2]), a CO-releasing compound, was used. The amount of CO released was assessed spectophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin. The amount of CO released from 50 μmol/L [Ru(CO)3Cl2] in culture medium was ~38.7±2.5 μmol/L in medium (data not shown). Cells pretreated with bilirubin were not exposed to light to prevent degradation.

**Microarray Analysis of cRNA and Quantitative Analysis**

HAECs were cultured with 5 μmol/L HA and harvested after 16 hours. First-strand cDNA was generated with 10 μg of mRNA using T7-linked oligo(dT) primer (Takara). After second-strand synthesis, in vitro transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics). Resultant cRNA was purified on an affinity resin column (RNeasy; Qiagen). Biotinylated cRNA (40 μg) was fragmented and hybridized to oligonucleotide gene chip, human FL array (Affymetrix), and 6416 genes were analyzed as described previously. Quantitative analyses were performed with GeneChip Software (Affymetrix). The average differences and fold changes were defined and calculated as described previously.

**Lipoprotein Isolation and Modification**

LDL was isolated from the sera of normal blood donors by density-gradient ultracentrifugation. OxLDL was prepared by incubating freshly prepared LDL at 37°C for 24 hours in PBS (pH 7.4) containing 50 μmol/L CuSO4, and then dialyzing extensively against PBS.

**Western Blot Analysis**

Cell cultures and mouse aorta were washed, lysed, and homogenized in 10 mmol/L Tris-HCl (pH 7.4) containing 0.1% sodium dodecyl sulfate and a protease inhibitor cocktail (Boehringer Mannheim). A total of 20 μg of cell lysate and tissue homogenates was electrophoresed on 10% SDS-PAGE, blotted onto polyvinylidene difluoride membranes (ATTO), blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and incubated with polyclonal anti-HO-1 antibody (StressGen) or monoclonal anti-eNOS antibody (Transduction Laboratories). Bound antibody was detected using the enhanced chemiluminescence detection system (Amersham Pharmacia). The protein content was determined using a DC protein assay kit (BioRad).

**HO-1 Assay**

HO activity was measured in the microsomal fractions by determining the amount of bilirubin formed as described previously.

**ELISA for Inflammatory Mediators**

ELISA for VCAM-1, MCP-1, and M-CSF was performed with kits (R&D Systems) using conditioned media from HAEC cultures according to manufacturer protocol.

**RNA Extraction and Northern Blot Analysis**

Total RNA was isolated with TRizol reagent (GIBCO/BRL). A total of 20 μg of total RNA was electrophoresed, transferred to a nylon membrane (MSI), and hybridized with 32P-labeled rat HO-1 and VCAM-1 cDNAs as described previously.

**Plasma Lipids and Lipid Peroxidation Assay**

Blood was collected from mice fasted overnight. Total plasma cholesterol, triglyceride, and high-density lipoprotein (HDL) concentrations were determined enzymatically. Plasma lipid hydroperoxides were measured by the methylene blue hemoglobin method.

**Animal Handling and Vascular Relaxation Response**

All animal experiments were conducted in accordance with the guidelines of the Fukushima Medical University animal research committee. Two- to 3-month-old female LDL-receptor knockout mice with a C57BL/6J background (C57BL/6J-Ldlrtm1Her) were fed either standard rodent chow containing 5% fat and 0.075% cholesterol, and 0.5% cholic acid (Oriental Bio Service Kanto Inc.; HF group; n=6). The high-fat diet was started 1 week before the experiment for vascular endothelium response. To overexpress HO-1, HA (25 mg/kg) was injected intraperitoneally every other day during high-fat diet (HF+HA group; n=6). To examine the effects of CO, CO at 60 ppm was administered in a chamber for 2 hours per day during high-fat diet (HF+CO group; n=6). To examine the effects of bilirubin, bilirubin (25 mg/kg) was injected intraperitoneally every other day during high-fat diet (HF+BR group; n=6).

To examine endothelium-dependent vascular relaxation responses, isolated thoracic aortic strips were prepared. After euthanization, the descending thoracic aorta was excised, washed, and cut into aortic rings (2 mm). Aortic rings were then suspended in organ chambers in the Krebs’ buffer saturated with a 95% O2, 5% CO2 gas mixture. After equilibration for 90 minutes, the aorta was contracted with 1 μmol/L phenylephrine, and the endothelium-dependent and -independent responses were examined with acetycholine (10−9 to 10−5 mol/L) and sodium nitroprusside (10−10 to 10−7 mol/L).

**Data Analysis**

All values are expressed as means±SD. Significant difference was determined by 1-way ANOVA with the Fisher post hoc test. P<0.05 was considered significant.

**Results**

**HO-1 Induction in Vascular Endothelial Cells by HA**

To induce HO-1 in cultured vascular endothelial cells, we treated them with micromolar concentrations of HA. Figure 1A shows the dose-dependent induction of HO-1 protein and enzyme activity by HA. We did not observe any apparent toxic effects of HA as judged by trypan blue exclusion or lactate dehydrogenase release (data not shown).
HO-1 Induction Attenuates Proinflammatory Responses to Oxidized LDL and TNF-α in Vascular Endothelial Cells

To exclude pleiotropic effects of HA on genes other than HO-1, 6416 genes were screened with the GeneChip System before and after treatment with 5 μmol/L HA for 16 hours, the condition used to induce HO-1 in this study. The Table shows the genes, which were induced >3-fold or were downregulated <2-fold by HA. HO-1 was the gene that responded most and was induced up to 13-fold, although HA also upregulated several other genes such as NAD(P)H:menadione oxidoreductase and cytochrome P450 (CYP1A2), which have an antioxidant response element (ARE). In contrast, HA treatment reduced expression levels of 4 genes to <50%. The Table also includes the genes that are thought to be involved in endothelial activation or dysfunction, but HA treatment did not significantly change their expression. These results suggest that HA treatment induces HO-1 in a relatively specific manner.

HO-1 Induction Attenuates Proinflammatory Responses to Oxidized LDL and TNF-α in Vascular Endothelial Cells

To determine the effect of HO-1 on proinflammatory responses in vascular endothelium, we first examined the production of VCAM-1 in response to TNF-α (2 ng/mL) by ELISA (Figure 1B). HA pretreatment (5 μmol/L) itself did not induce the production and secretion of VCAM-1 into the conditioned media. Interestingly, VCAM-1 production after TNF-α exposure was significantly reduced by pretreatments with 1, 5, and 10 μmol/L HA. These effects were abolished by HO inhibitor SnPP IX (10 μmol/L). In addition, HA pretreatment also attenuated induction of VCAM-1 mRNA after TNF-α exposure (Figure 1C).

To examine the responsible HO-1 product that suppresses VCAM-1 production in response to TNF-α, we pretreated cells with [Ru(CO)3Cl2] (50 μmol/L), a CO-releasing compound, or bilirubin (5 μmol/L). Interestingly, this inhibitory effect on VCAM-1 production was predominantly observed by treatment with bilirubin not by CO. These results suggest a potential role for HO-1 and bilirubin in suppressing proinflammatory interactions between vascular endothelial cells and leukocytes.

To examine the effects of HO-1 on other proinflammatory molecules, production of MCP-1 and MCSF in vascular endothelium after exposure to oxLDL was also examined. After pretreatment with HA, SnPP IX, [Ru(CO)3Cl2], and bilirubin, cells were exposed to oxLDL (50 μg/mL) or TNF-α (2 ng/mL) for 3 hours, washed, incubated in fresh media for another 3 hours, and the levels of MCP-1 and MCSF were measured in the conditioned media by ELISA (Figure 1, available online at http://atvb.ahajournals.org). Production of MCP-1 and MCSF was also significantly suppressed by HA pretreatment. Similar effects of bilirubin on production of these molecules were also observed.
HO-1 Induction Preserves eNOS Expression After Exposure to OxLDL and TNF-α

To examine whether the antiatherogenic and vasculoprotective effects of HO-1 are via the effect of eNOS expression, we pretreated HAECs with HA and observed the resultant expression of eNOS after exposure to oxLDL and TNF-α (Figure 2). Untreated cells expressed a significant level of eNOS protein, whereas there was little expression of HO-1 mRNA and protein (Figure 2A, lane 1). Treatment with native LDL (50 μg/mL) had little effect on eNOS expression (Figure 2A, lane 3), whereas treatment with oxLDL (50 μg/mL) reduced eNOS expression accompanied by HO-1 induction (Figure 2A, lane 4). Interestingly, HO-1 overexpression by HA pretreatment significantly preserved eNOS expression (Figure 2A, lane 5). The effect of HO-1 induction on restoration of eNOS expression was abolished by cotreatment with SnPP IX (10 μmol/L), an HO inhibitor (Figure 2A, lane 6). Figure 2B shows the dose-dependent effect of HO-1 on attenuation of eNOS expression by TNF-α. HO-1 induction also recovered this attenuation.

To determine the HO-1 products responsible for preservation of eNOS expression, cells were pretreated with either 50 μmol/L [Ru(CO)3Cl2], a CO-releasing compound, or 5 μmol/L bilirubin for 2 hours (Figure IIA, available online at http://atvb.ahajournals.org), and then the cells were exposed to oxLDL (50 μg/mL) for 16 hours. Whereas [Ru(CO)3Cl2] treatment did not preserve eNOS expression, bilirubin treatment significantly preserved eNOS expression after exposure to oxLDL (Figure IIA). In addition, this effect was not inhibited by SB203580, an inhibitor of p38 mitogen-activated protein (MAP) kinase (Figure IIB).

HO-1 Induction Improves Impaired Endothelium-Dependent Vascular Relaxation

After we observed significant effects of HO-1 induction and bilirubin on the endothelial responses to oxLDL and TNF-α, we next examined whether these also affect endothelium-dependent vascular relaxation responses. To observe the changes of endothelium-dependent vascular relaxation response, isolated thoracic aortic rings from LDL-receptor knockout mice were prepared (Figure 3A). Aortic rings from mice fed high-fat diet for 1 week (HF group) showed impaired vasodilatory responses to acetylcholine compared with control (C group). Interestingly, aortic rings from mice fed high-fat diet and pretreated with HA (HF+HA group) showed improved vasodilatory responses on high-fat HF diet. Bilirubin pretreatment (HF+BR group) resulted in similar improvement, whereas CO exposure (HF+CO group) did not have apparent effects (Figure 3A). In contrast, there were no significant differences among the 4 groups in endothelium-independent relaxation responses (Figure 3B). To examine whether the improvement of vascular relaxation responses are mediated by restoration of eNOS by HA and bilirubin treatments, levels of HO-1 and eNOS expression in the aorta were examined (Figure 3C). After high-fat diet feeding, eNOS expression was significantly attenuated. In contrast, modest induction of HO-1 was observed. Importantly, HA pretreatment augmented this HO-1 induction and restored eNOS expression. Similar effects were confirmed after treat-
Recently, HO-1 induction by HA and bilirubin pretreatments improve endothelium-dependent vascular relaxation response (A and B), restore eNOS expression (C), and reduce plasma lipid hydroperoxides (D) in the LDL receptor knockout mice fed high-fat diet. Endothelium-dependent vascular relaxation responses to acetylcholine (Ach; 10^{-9}–10^{-5} mol/L; A) and endothelium-independent vascular relaxation responses sodium nitroprusside (SNP; 10^{-9}–10^{-5} mol/L; B) were examined with aortic rings. Aortic rings from mice fed chow diet (CH; ○), fed high-fat diet (HF; □), fed high-fat diet and pretreated with HA (HF+HA; ●), fed high-fat diet and pretreated with CO (HF+CO; ▽), and fed high-fat diet and pretreated with bilirubin (HF+BR; △). Samples from 6 mice in each group were analyzed. *P<0.01. C, Changes of HO-1 and eNOS protein expression in the aorta of LDL receptor knockout mice; Western blotting. D, Changes in plasma lipid hydroperoxides in the LDL receptor knockout mice. Duplicate samples from 6 mice in each group were analyzed. *P<0.01; **P<0.05.

Discussion

In this study, we examined whether vascular endothelial HO-1 expression is involved in the endothelial activation and insufficiency elicited by proinflammatory stresses, which are considered to be events essential for atherogenesis. Our results suggest that there are conditions under which HO-1 expression significantly suppresses production of proinflammatory mediators and reverses impaired eNOS expression in response to oxLDL and TNF-α.

To induce HO-1 in cultured endothelial cells, we used low concentrations of HA instead of hemin chloride because a recent study showed that HA is less toxic.18 Indeed, treatment with micromolar concentrations of HA did not have any injurious effects. We first examined the changes in gene expression in endothelial cells before and after treatment with 5 μmol/L HA for 16 hours, the conditions used to induce HO-1. A cDNA microarray analysis revealed that HO-1 was the gene most strongly upregulated, and that several other genes that have an ARE element also responded positively to this treatment (Table). Importantly, the microarray also revealed that HA treatment did not generate significant changes in other genes that may affect atherogenic processes in vascular endothelium. For instance, transcriptional changes of adhesion molecules, growth factors, and redox-sensitive transcription factors such as nuclear factor-κB were not significant (Table). Similarly, the eNOS gene considered to have antiatherosclerotic properties was not significantly altered by HA treatment.

Recent studies provide evidence that HO-1 functions as an intrinsic defense system in cardiovascular disorders.10–12,19,20 The protective effects of HO-1 may be attributed to its biological actions in vascular endothelium because endothelial HO-1 induction by HA significantly attenuated production of VCAM-1, MCP-1, and MCSF (Figures 1 and I) and preserved eNOS expression (Figures 2 and II) in response to TNF-α and oxLDL. The functional improvement of the endothelium-dependent vasodilatory response was also confirmed (Figure 3).

Cytoprotective effects of HO-1 have been observed recently in many kinds of cells, such as cardiomyocytes,20 hepatocytes,21 and fibroblasts,22 as well as in vascular endothelial cells.8 In this study, we examined the biological effects of the products of HO-1 in vascular endothelium. To observe the direct effects of CO, we treated cells with 50 μmol/L ([Ru(CO)3Cl2])2, a CO-releasing compound, and did not observe apparent biological effects, suggesting that CO is not an HO-1 product that protects endothelium (Figure II A), although recent studies have suggested there are multipotent actions of CO in endothelium.23,24 However, it is possible that CO produced from endothelium may regulate other cells in the artery wall. CO from endothelial cells may function to relax smooth muscle cells under conditions of insufficient NO bioavailability and suppress reactive oxygen species generation in macrophages infiltrated into the artery wall. Indeed, this is supported by a recent report that CO suppresses proinflammatory cytokine production via inhibition of the c-Jun N-terminal kinase pathway in macrophages.25

In this study, protective effects on vascular endothelium were observed with another HO-1 product, bilirubin. A physiological concentration of bilirubin suppressed activation of proinflammatory genes such as VCAM-1, MCP-1, and MCSF (Figures 1 and I), restored eNOS expression (Figures 2 and II), and improved endothelium-dependent vascular relaxation responses (Figure 3). Bilirubin seems to alleviate the effects of oxidative stresses on endothelium, which is considered to be a central factor in atherosclerosis. Because the anti-inflammatory action of CO in macrophages has been shown recently to be mediated via the p38MAP kinase pathway,26 we examined the effect of SB203580, an inhibitor of p38MAP kinase, on the action of bilirubin (Figure IIB). However, inhibition of the p38MAP kinase pathway did not abolish the effect of bilirubin. Thus, bilirubin seems to relieve endothelial dysfunction independent of this pathway. Previous studies have also indicated that bilirubin is a potent inhibitor of monocyte adhesion27 and chemotaxis9 to vascular
endothelium. Our study indicates that eNOS is one of the molecular targets of bilirubin in vascular endothelium, and that bilirubin functionally improves vasodilatory responses, although protective effects of bilirubin to other proinflammatory stimuli such as lipopolysaccharides and angiotensin II should also be explored (Figure 3). In conclusion, this study revealed that the antiatherosclerotic properties of HO-1 are mediated through effects on vascular endothelium and that these effects are predominantly caused by bilirubin.

Acknowledgments

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References

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**Fig. I.** HO-1 induction by HA and bilirubin pretreatment attenuates MCP-1 and MCSF production caused by oxLDL and TNF-α in HAEC. Cells were pretreated with 1, 5 and 10 µM HA for 16 hours or with SnPP IX (10 µM), [Ru(CO)₃Cl₂]₂ (50 µM), and bilirubin (5 µM) for 2 hours. Then, cells were exposed to oxLDL (50 µg/ml) (A, C) or TNF-α (2 ng/ml) (B, D) for 3 hours, washed, incubated in fresh media for another 3 hours. MCP-1 (A, B) and MCSF (C, D) production (pg/ml) were analyzed by ELISA with conditioned media in duplicate. Data shown are representative for three (A, C) and four (B, D) independent experiments. *; p<0.001, **; p<0.005.

**Fig. II.** Preservation of eNOS expression after exposure to oxLDL is not attributed to CO but to bilirubin. (A, B) Cells were pretreated with either 50 µM [Ru(CO)₃Cl₂]₂ or with 5 µM bilirubin for 2 hours. Then, cells were exposed to oxLDL (50 µg/ml) for 16 hours. Cells pretreated with [Ru(CO)₃Cl₂]₂ were exposed to a cold light to release CO. eNOS protein expression were examined by Western blotting. SB203580, an inhibitor of p38 MAP kinase, was added 2 hours before bilirubin pretreatment. Data shown are representative for three independent experiments.