Increased Expression of Heme Oxygenase-1 and Bilirubin Accumulation in Foam Cells of Rabbit Atherosclerotic Lesions

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Abstract—Heme oxygenase-1 (HO-1) catalyzes the regiospecific oxidative degradation of heme to biliverdin IX\(\alpha\), iron, and carbon monoxide. Biliverdin IX\(\alpha\) is subsequently reduced to bilirubin IX\(\alpha\) by biliverdin reductase. HO-1 expression is induced under various disease conditions, including atherosclerosis, but it is unknown whether HO-1 catalyzes heme breakdown in the regions at risk. Using hypercholesterolemic rabbits fed a cholesterol-enriched diet, we attempted to demonstrate the involvement of HO-1 induction and bilirubin IX\(\alpha\) production in atherosclerotic regions. Expression levels of HO-1 mRNA were elevated in the aortas of hypercholesterolemic rabbits. In situ hybridization and immunohistochemistry revealed that mRNA and protein of HO-1 are induced in endothelial cells and foam cells (lipid-filled macrophages) in atherosclerotic lesions. Furthermore, immunohistochemistry with the use of an anti-bilirubin-IX\(\alpha\) monoclonal antibody, 24G7, demonstrated accumulation of bilirubin IX\(\alpha\) in foam cells, indicating that heme is actually degraded in atherosclerotic lesions. Remarkably, bilirubin IX\(\alpha\), like HO-1 protein, is predominantly accumulated in the perinuclear regions of foam cells. These results provide the first in vivo evidence of the colocalization of HO-1 and bilirubin IX\(\alpha\) in foam cells, suggesting a role of HO-1 induction in the modulation of macrophage activation in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2001;21:1373-1377.)

Key Words: cholesterol ■ atherosclerosis ■ foam cells ■ heme oxygenase 1 ■ bilirubin IX\(\alpha\)

Oxidative stress has been implicated in the pathogenesis of atherosclerosis. In the early stage of atherosclerosis, LDL is subjected to oxidative modification in the vessel wall.1,2 Oxidized LDL may injure the vascular wall, leading to endothelial dysfunction and altered gene expression in vascular cells.3,4 Recent studies have shown that antioxidants suppress the progression of atherosclerosis and endothelial dysfunction.5–7 Oral administration of the antioxidant probucol decreased the incidence and severity of restenosis after coronary angioplasty.5 Treatment with vitamin C or vitamin E prevented endothelial dysfunction in patients with coronary heart disease.6,7

Heme oxygenase (HO) is an enzyme that catalyzes the region-specific oxidative degradation of iron protoporphyrin IX (heme) to biliverdin IX\(\alpha\), iron, and carbon monoxide (CO).8 Biliverdin IX\(\alpha\) is subsequently reduced to bilirubin IX\(\alpha\) by biliverdin reductase. These bile pigments are regarded as endogenous antioxidants that scavenge a variety of active oxygen species, including superoxide anions and lipid peroxides.9–11 Several lines of evidence support the role of bilirubin in the prevention of oxidative damages associated with cardiovascular diseases. For example, bilirubin has been shown to inhibit the oxidation of LDL,10 monocyte chemotaxis induced by oxidized LDL,12 and the adhesion of neutrophils elicited by ischemia/reperfusion or exposure to hydrogen peroxide.13 On the other hand, CO has been recognized as an endogenous gas mediator that regulates vascular function.14–16 Morita et al17 have reported that CO upregulates cGMP and/or modulates the expression of platelet-derived growth factor-B, resulting in the inhibition of proliferation of vascular smooth muscle cells.17

A number of reports have shown that the expression of HO-1, an inducible isozyme of HO, is increased by a variety of stimulants, including heavy metals, NO donors, hyperoxia, UV light, and heme, a substrate of the enzyme.18–23 Thus, HO-1 has been considered a stress-responsive protein that protects cells from oxidative stress. This notion is supported by the phenotypic consequences in HO-1–targeted mice24,25 and in a patient with HO-1 deficiency.26 Recently, Wang et al27 have shown that HO-1 is expressed in atherosclerotic vessels from humans and apoE-deficient mice. However, little information is available regarding whether HO-1 catalyzes the heme breakdown in atherosclerotic lesions in situ. Furthermore, cellular components in the lesions responsible for the HO-1–mediated generation of the products are largely unknown.
In the present study, we examined the microtopographic distribution of HO-1 expression and its end product, bilirubin IXα, in atherosclerotic lesions from hypercholesterolemic rabbits fed a cholesterol-enriched diet. The present study provides evidence that foam cells (lipid-filled macrophages) constitute a major cellular component responsible for HO-1 induction in atherosclerotic lesions. Furthermore, the enzyme induction is accompanied with local accumulation of bilirubin IXα in the foam cells, suggesting that these cells generate the heme-degrading products through HO-1 induction.

**Methods**

**Animal Protocol**

Twelve New Zealand White male rabbits (aged 11 weeks) were individually housed in stainless-steel cages. Rabbits were maintained for 8 weeks on a 2% cholesterol–containing diet or a normal diet. All experiments were performed in accordance with a protocol approved by the Ethics Committee of Tohoku University School of Medicine.

**Tissue Preparation**

Rabbits were anesthetized with an intravenous injection of pentobarbital at a concentration of 50 mg/kg body wt. Aortic tissues (descending aortas) were fixed in 4% paraformaldehyde and 6% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce Chemicals) for 6 hours at 4°C and snap-frozen with OCT compound (Sakura Fine Technical Co, Ltd). Cryostat sections were cut at 4 μm.

**Northern Blot Analysis**

Total RNA was extracted from the descending aortas of control rabbits or cholesterol-fed rabbits. The descending aortas from cholesterol-fed rabbits contained morphologically normal portions and atherosclerotic lesions. The probe for human HO-1 mRNA was the Xho1-Xhol fragment of a human HO-1 cDNA, pHHO1.23 The probe for β-actin mRNA was the Smal-Scal fragment (nucleotide positions 124/1050) derived from the full-length human β-actin cDNA. These probes were labeled with [α-32P]dCTP by the random-priming method. The RNA blot membrane was prehybridized in a solution consisting of 5× standard saline citrate (SSC, containing 0.75 mol/L sodium chloride and 0.075 mol/L sodium citrate), 1% SDS, 50% formamide, 5× Denhardt’s solution, and 0.2 g/L salmon testis DNA for at least 3 hours and then hybridized overnight at 42°C. The hybridized filter was washed at 50°C with 1× SSC and 0.1% SDS. Radioactive signals were detected with a Bioimage analyzer (BAS 1500, Fuji Film). The intensity of hybridization signals was quantified with the Bioimage analyzer (BAS 1500, Fuji Film). The intensity representing HO-1 mRNA was normalized with the intensity of the β-actin band, and the normalized values in various samples were compared.

**In Situ Hybridization**

The BamHI-Xho1 fragment of pHHO1 was subcloned into pBlue-Script vector (Stratagene). The antisense and sense HO-1 RNAs were synthesized by SP6 and T7 RNA polymerase, respectively, and labeled with digoxigenin (DIG)-UTP (Dig RNA Labeling Kit, Boehringer-Mannheim). Prehybridization was carried out in 50% formaldehyde and 1× SSC at 50°C for 60 minutes. Hybridization was carried out in hybridization buffer (50% formamide, 1× SSC, 10% dextran, 1× Denhardt’s solution, and 1 μg/mL tRNA) at 42°C overnight. After a washing with 2× SSC and 1× SSC in each buffer containing 50% formaldehyde at 50°C for 30 minutes, the sections were incubated with anti-DIG alkaline phosphatase–conjugated antibody. Excess antibody was washed away with PBS, and the color substrate (nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate) was added, according to the manufacturer’s protocol.

**Immunohistochemistry**

Immunostaining was performed by using the Vectastain ABC kit (Vector Laboratories), as previously reported.29 Monoclonal antibodies against α-smooth muscle actin and CD31 antigen were purchased from NeoMarkers, and RAM11 monoclonal antibody was purchased from Dako. Sections were pretreated with 2% hydrogen peroxide to exhaust endogenous peroxidase activities and preincubated with 10% normal goat serum. The anti-rat HO-1 monoclonal antibody, GTS-1,30 was used at a dilution of 1:50. Immunopositive cells were characterized by immunostaining the serial sections for α-smooth muscle actin (smooth muscle cell marker), RAM11 (rabbit macrophage marker), and CD31 (endothelial marker). Monoclonal antibodies against α-smooth muscle actin, RAM11, and CD31 were used at dilutions of 1:300, 1:200, and 1:300, respectively.

The alkaline phosphatase–conjugated bilirubin IXα monoclonal antibody, 24G7,31 was a gift from Shino-Test Co, Tokyo, Japan. This monoclonal antibody specifically recognizes the conjugated and unconjugated forms of bilirubin IXα but not other isomers of bilirubin.32 Bilirubin IXα is produced by the reduction of biliverdin IXα, a product of the HO-1 reaction. Thus, immunohistochemistry with the use of 24G7 allowed us to assess the HO-1–specific heme degradation in vivo. Atherosclerotic lesions with 3 different levels of severity were fixed with 6% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce Chemicals) at 4°C for 6 hours. Sections were washed with PBS incubated with 0.1% Triton X-100 for 15 minutes at room temperature and washed twice with PBS. Cells were preincubated for 1 hour with 4% normal goat serum in PBS and incubated with anti-bilirubin antibody in 2% BSA overnight at 4°C. Most of the steps were performed in a dark room to prevent isomerization and photo-oxidation of bilirubin. Sections were then washed and dyed with a Fuchsin kit (Dako) according to the manufacturer’s protocol. The number of stained foam cells was counted in at least 3 different fields of the tissue section.

**Statistical Analysis**

Statistical analysis was performed by the Student t test. Data are shown as mean±SEM.

**Results**

**Increased Expression of HO-1 mRNA in Aortas of Hypercholesterolemic Rabbits**

The serum levels of total cholesterol were significantly increased in rabbits maintained on a cholesterol-enriched diet for 8 weeks (2148.3±139.2 mg/dL, n=4); the levels were ~30 times those found in normal diet–fed rabbits (63.7±8.6 mg/dL, n=4). Atherosclerotic lesions were found in the descending aortas of cholesterol-fed rabbits, expanding the lumenal surface of the aorta >50%, but these lesions not found in the arterial walls of control rabbits. We compared the expression of HO-1 mRNA in the aortas from both groups of animals. HO-1 mRNA expression was not detected in the aortas of control rabbits (Figure 1, lanes 1 to 4) but was remarkably increased in the aortas of hypercholesterolemic rabbits (lanes 5 to 8). In contrast, the expression of β-actin mRNA remained unchanged after 8 weeks of cholesterol feeding.

**In Situ Hybridization of HO-1 mRNA in Aortas of Hypercholesterolemic Rabbits**

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**In Situ Hybridization of HO-1 mRNA in Atherosclerotic Lesions**

To localize the regions expressing HO-1 mRNA in the aortas of cholesterol-fed rabbits, we performed in situ hybridization analysis (Figure 2). Positive signals were detected with the

![Figure 1. Northern blot analysis of HO-1 mRNA expression in the rabbit aorta. Each lane contains 15 μg of total RNA extracted from aortic tissue of 4 rabbits fed a normal diet (lanes 1 to 4) and 4 cholesterol-fed rabbits (lanes 5 to 8). Bottom panel shows β-actin mRNA as an internal control.](http://atvb.ahajournals.org/DownloadedFrom)
antisense HO-1 RNA probe in the morphologically normal endothelial cells (A) and fatty streaks of hypercholesterolemic rabbits (Figure 2A and 2B) but not with the sense RNA probe (Figure 2C). HO-1 mRNA was also detectable in the endothelial cells of the aortic walls of control rabbits (Figure 2D and 2E). These results indicate that HO-1 mRNA is normally expressed in endothelial cells of the aorta and that its expression is remarkably increased in the fatty streaks of atherosclerotic lesions.

Immunohistochemistry of HO-1 Protein in the Aortic Wall

We initially analyzed whether GTS-1 anti-rat HO-1 monoclonal antibody reacts with rabbit HO-1. Positive immunostaining was detected in Kupffer cells of the normal rabbit liver (data not shown), as reported for the rat liver.30 Thus, the monoclonal antibody against rat HO-1 used in the present study is able to cross-react with rabbit HO-1. We then performed immunohistochemical analysis of the vascular tissues with the anti–HO-1 monoclonal antibody. Positive immunoreactivity for HO-1 was not detected in sections of the normal vessel wall (Figure 3A), whereas positive immunoreactivity for HO-1 occurred in the fatty streaks and intima of the atherosclerotic lesions from a cholesterol-fed rabbit (Figure 3B).

The cellular sources of positive HO-1 immunostaining in fatty streaks were then explored by immunostaining the serial sections with antibodies to RAM11 (rabbit macrophage marker, Figure 3E), CD31 (endothelial cell marker, Figure 3F), and α-actin (smooth muscle cell marker, Figure 3G). Positive HO-1 immunostaining was detected in endothelial cells and medial smooth muscle cells of atherosclerotic lesions (Figure 3C). Figure 3D shows a negative control of a serial section that was not treated with the HO-1 antibody. HO-1 protein was prominently expressed in foam cells (Figure 3C), which also express RAM11 (Figure 3E). It is noteworthy that HO-1 is expressed in the perinuclear regions and cytoplasm of foam cells (Figure 3C), whereas RAM11 is mainly expressed in the cytoplasm. Figure 3H shows a control section stained with hematoxylin-eosin.

Bilirubin IXα Accumulation in Atherosclerotic Lesions

To demonstrate actual degradation of heme through the HO-1 reaction in atherosclerotic lesions, we analyzed the accumulation of bilirubin IXα in 3 lesions of different severity by using the 24G7 monoclonal antibody. In the early lesion, positive foam cells are detectable in the thickened intima, constituting 9±0.7% of the foam cells (Figure 4A and 4B). The population of positive foam cells appears to increase to 15±0.7% in the more advanced lesion (Figure 4C and 4D). No positive immunostaining was detectable in endothelial...
cells and medial smooth muscle cells. Weak staining was also detected in the cytoplasm of foam cells. In the most severe atherosclerotic lesion, positive foam cells are distributed in the entire lesion (22±0.5% positive) and are more intensely stained (Figure 4E and 4F). Bilirubin IXα was mainly detected in the perinuclear regions of foam cells, which is consistent with the expression pattern of HO-1 (see Figure 3C). It is noteworthy that only a portion of foam cells (≈20%) was positive for bilirubin IXα, despite the fact that almost all foam cells appear to express HO-1 protein. This difference may be due to the sensitivity of the detection method used, which allowed us to detect the foam cells that accumulate sufficient amounts of bilirubin IXα. Alternatively, individual foam cells may produce and/or excrete different levels of bilirubin IXα or accumulate different levels of the substrate (heme).

**Discussion**

We have provided evidence that foam cells constitute a major cellular component responsible for the HO-1-mediated heme degradation in the rabbit model of atherosclerosis. The foam cells exhibited upregulation of HO-1 at mRNA and protein levels, which is consistent with a previous report by other investigators on atherosclerotic lesions in apoE knockout mice and humans. However, these findings did not provide evidence of the HO-1-mediated heme degradation in situ. The present study, which used the anti--bilirubin IXα monoclonal antibody, showed the accumulation of bilirubin IXα, an HO-1–specific end product, in foam cells of atherosclerotic lesions, suggesting that the foam cells actually degrade heme in situ and generate bilirubin IXα and CO in atherosclerotic lesions.

Oxidative LDL caused the induction of HO-1 in cultured macrophages, smooth muscle cells, and endothelial cells. Therefore, it is conceivable that high levels of LDL, containing oxidized LDL, may induce HO-1 expression in the aortic walls of hypercholesterolemic rabbits. It is noteworthy that HO-1 protein is prominently expressed in the perinuclear region of foam cells. Likewise, the perinuclear location of HO-1 protein was reported in epithelial cells, fibroblasts, and smooth muscle cells of the human prostate. In this context, it has been reported that HO-1 mRNA is translated on free polysomes from alveolar macrophages, and it has been suggested that the newly synthesized HO-1 protein is incorporated into microsomal membranes through a hydrophobic domain at its C-terminus. Therefore, we suggest that HO-1 may be preferentially present and function in the perinuclear region under certain circumstances.

Bilirubin IXα is the most abundant endogenous antioxidant in adult mammalian tissues, accounting for the majority of antioxidant activity in serum. The induction of HO-1 in atherosclerotic lesions may ameliorate oxidative stress, which contributes to atherogenesis. The anti--bilirubin IXα monoclonal antibody, 24G7, used in the present study has been established to recognize specifically bilirubin IXα and its oxidative metabolites. Accordingly, positive immunostaining of foam cells with the 24G7 antibody indicates that the α-methylene bridge of heme is actually cleaved by HO-1 to release CO, iron, and biliverdin IXα. In this context, it remains to be investigated whether a constitutively expressed isozyme of HO, HO-2, may also contribute to the accumulation of bilirubin IXα in foam cells, although there are no reports involving the induction of HO-2 in macrophages or foam cells.

Unexpectedly, the perinuclear region of foam cells was strongly immunostained with the 24G7 antibody, which appears to be correlated with the perinuclear expression of immunoreactive HO-1. In foam cells, bilirubin IXα possibly exists as an unconjugated nonpolar form and is likely to be associated with membrane components, including the nuclear membrane. The weak immunostaining in cytoplasm of foam cells may represent the bilirubin IXα bound to cytosolic proteins, such as glutathione-S-transferase, or the oxidative metabolites of bilirubin IXα. The accumulation of the oxidative metabolites of bilirubin IXα may be related to the fact that foam cells are exposed to various species of endogenously generated radicals. On the other hand, we could not detect the immunoreactive bilirubin IXα and its oxidative metabolites in endothelial cells and medial smooth muscle cells. This may be partially due to the active release of bilirubin IXα and its metabolites from endothelial cells into the blood stream or the rapid degradation of these compounds in endothelial cells and smooth muscle cells. Moreover, there are in vivo data showing that high serum bilirubin reduces the risk of ischemic heart disease. We suggest that HO-1 expression in foam cells represents an adaptive response to hypercholesterolemia and that the production of bilirubin IXα...
and CO may reduce the progression of atherosclerosis and ameliorate endothelial dysfunction.

In conclusion, we show an increased expression of HO-1 mRNA and protein in the foam cells from the atherosclerotic lesions of rabbits fed a cholesterol-enriched diet. The colocalization of the induced HO-1 and bilirubin IXα in foam cells suggests that HO-1 catalyzes heme breakdown in atherosclerotic lesions.

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