Heme Oxygenase-1 Inhibits Atherogenesis in Watanabe Heritable Hyperlipidemic Rabbits

Kazunobu Ishikawa, MD; Daisuke Sugawara, MD; Jun Goto, MD; Yasuyuki Watanabe, MD; Keiichi Kawamura, MD; Masashi Shiomi, PhD; Hiroyuki Itabe, PhD; Yukio Maruyama, MD

Background—Heme oxygenase-1 (HO-1) is proposed to have a variety of adaptive responses against oxidative stress. To examine the function of HO-1 against atherogenesis in vivo, we observed the effects of HO-1 inhibition on atherosclerotic lesion formation in Watanabe heritable hyperlipidemic rabbits (WHHL).

Methods and Results—During 4 weeks of a 1% cholesterol diet, intravenous injections of Sn-protoporphyrin IX to inhibit HO-1 (S group, n=10) and saline as a control (C group, n=10) were given to 3-month-old WHHL rabbits. The percentages of en face atherosclerotic lesion areas in total descending aorta by Sudan IV staining (EFA) and the ratio of intima to media in microscopic atherosclerotic lesions in the ascending aortas (I/M) were calculated. Two different quantitative methods revealed significantly greater atherosclerotic lesions in the S group than the C group (EFA, P<0.001; I/M, P<0.005). HO-1 expression in atherosclerotic lesions was confirmed by Northern blot and immunohistochemical analyses. The dominant cell types expressing HO-1 were macrophages and foam cells, in which oxidized phospholipids were also accumulated. HO inhibition increased plasma and tissue lipid peroxide levels without affecting plasma lipid composition.

Conclusions—These results suggest the possibilities that HO-1 has antiatherogenic properties in vivo and that the antiatherogenic properties of HO-1 are conducted through the prevention of lipid peroxidation. (Circulation. 2001;104: 1831-1836.)

Key Words: heme oxygenase ■ hypercholesterolemia ■ atherosclerosis

An inducible form of heme oxygenase (HO), HO-1, is transcriptionally induced by a variety of pathophysiological conditions or substances in the cardiovascular system, such as hypoxia, ischemia/reperfusion, hypertension, proinflammatory cytokines, or oxidized LDL. The HO reaction, which is a rate-limiting step of heme degradation in mammals, mediates the conversion of heme into biliverdin, carbon monoxide, and free iron. The elimination of prooxidant heme and the production of these molecules are proposed to provide cytoprotective roles against different pathophysiological stresses in the vascular wall.

Atherosclerosis, which is characterized by a progressive accumulation of lipids and fibrous elements in artery wall, is a primary cause of heart attack or stroke in westernized societies. Oxidative modification of lipoproteins in vascular wall is believed to be essential for the initiation and development of atherosclerosis. Various preparations of oxidatively modified LDL have been shown to stimulate vascular endothelial cells to produce a number of proinflammatory molecules or to inhibit the production of nitric oxide, which has various antiatherogenic properties.

Although accumulating studies have shown that HO-1 provides cytoprotection against a variety of pathological conditions, particularly if HO-1 was induced before the stress, the function of HO-1 in atherogenesis has not been elucidated. Ishikawa et al showed that minimally oxidized LDL markedly induced HO-1 in cultured arterial wall cells and that the augmented induction of HO-1 attenuated oxidized LDL (oxLDL)–dependent monocyte chemotaxis, which is an essential event in early atherosclerotic lesion formation. This observation suggests the possibility that HO-1 functions as an intrinsic antiatherogenic molecule. Indeed, HO-1 is expressed in atherosclerotic lesions in humans and several experimental animals. To examine the possible effect of HO-1 on atherogenesis in vivo, we used both murine and rabbit models, because these 2 species sometimes exhibit different responses in atherosclerotic lesion development to certain reagents, such as probucol. We used LDL-receptor–knockout mice and Watanabe heritable hyperlipidemic (WHHL) rabbits, which develop atherosclerotic lesions in a short
period when they are challenged by a high-fat diet.\textsuperscript{17,18} We recently reported that HO modulation in LDL-receptor–knockout mice resulted in antiatherogenic and antioxidative effects of HO-1.\textsuperscript{19}

WHHL rabbits develop marked hyperlipidemia and atherosclerotic lesions similar to those observed in human familial hypercholesterolemia.\textsuperscript{18,20} This experimental animal model has been used extensively to analyze the cause of atherogenesis or to examine the effects of drugs and other agents on atherosclerosis.\textsuperscript{12,21,22} Using this atherosclerotic animal model, we inhibited HO activity by the administration of Sn-protoporphyrin IX (SnPP IX) and examined the resultant atherosclerotic lesions. Significant progression of atherosclerotic lesion formation was observed in the HO-inhibited animals. The antiatherogenic mechanism of HO-1 was accompanied by elevated plasma lipid hydroperoxides and tissue lipid peroxides. These in vivo results suggest the possibility that the HO pathway functions as an intrinsic antioxidant system through inhibiting lipid peroxidation.

Methods

Animal Handling and Procedures
Three-month-old male WHHL rabbits were bred in a room kept at 22°C and equipped with laminar-flow filters. All the experimental protocols were approved by the Committee for Animal Experiments of Fukushima Medical University. Animals were fed a standard rabbit chow (Oriental Bio Service) until they were given a high-fat diet. A high-fat diet containing 1% cholesterol (Oriental Bio Service) was fed for 4 weeks to promote atherosclerotic lesion formation. HO activities were inhibited with SnPP IX, a competitive inhibitor of HO.\textsuperscript{1,2,4,14–16} SnPP IX (7.5 mg/kg) was injected 5 times per week into the car vein for 4 weeks (5 group, n=10). An equal volume of saline was administered to a control group (C group, n=10). The SnPP IX solution was prepared in the dark because of its light sensitivity.

RNA Extraction and Northern Blot Analysis
Total RNA was isolated with TRIzol reagent (Gibco BRL) from aorta. Twenty micrograms of total RNA was electrophoresed in a formaldehyde/1% agarose gel, then transferred to a nylon membrane (Millipore) and cross-linked to the membrane. The blots were hybridized with \(^3^P\)-labeled HO-1 and HO-2 cDNA as described previously.\textsuperscript{5}

Quantitative Analyses of Aortic Atherosclerotic Lesions
After 4 weeks of HO modulation, animals were euthanized by intravenous injection of sodium pentobarbital (50 mg/kg). En face Sudan IV staining of the excised aortas from the arch to the common iliac levels (descending aortas) was performed after fixation in phosphate-buffered 10% formaldehyde. Microscopic examination showed no evidence of atherosclerotic lesions at this age (data not shown). In contrast, HO-1 mRNA was hardly present in the aortas from 2-month-old WHHL rabbits fed a Chow diet. HO-1 mRNA was expressed in the atherosclerotic aortas from 4-month-old WHHL rabbits. The expression of HO-1 mRNA was examined by Northern blot analysis (Figure 1). HO-1 mRNA was visualized with ethidium bromide staining.

Plasma Lipoprotein, Lipid Hydroperoxides, and Tissue Lipid Peroxidation

Blood was collected from rabbits fasted overnight. Total plasma cholesterol, triglyceride, and HDL concentrations were determined enzymatically. Plasma lipid hydroperoxides were measured by the method of Lowry et al.\textsuperscript{24} Estimates of lipid peroxidation in aortas and livers were obtained by measuring malondialdehyde (MDA) (Oxis, LPO-586 kit) in homogenates and conjugated dienes in extracted lipoproteins.\textsuperscript{26} Oxidative stress was determined by the method of Lowry et al.\textsuperscript{24} Oxidative stress was determined by the method of Lowry et al.\textsuperscript{24}

Immunohistochemistry

An avidin-biotinylated peroxidase system was used for immunohistochemical analyses of the sections of ascending aortas with a Vectastain Elite ABC kit (Vector Laboratories) and counterstaining with hematoxylin. The following primary antibodies were used: goat polyclonal antibody against HO-1 (Santa Cruz Biotechnology), mouse monoclonal antibody against oxidized phospholipids,\textsuperscript{23} mouse monoclonal antibody against rabbit macrophages (Dako), or mouse monoclonal antibody against human smooth muscle actin (Dako).

Figure 1. A, HO-1 and B, HO-2 mRNA expression in arteries of 2 WHHL rabbits from each group. Total RNA (20 \(\mug\)) was electrophoresed in formaldehyde/1% agarose gel, blotted to nylon membrane, and hybridized with HO probe as described in Methods. Lanes 1 and 2, 2-month-old WHHL rabbits fed Chow diet. Lanes 3 and 4, 4-month-old WHHL rabbits fed high-fat diet. Lanes 5 and 6, 4-month-old WHHL rabbits fed high-fat diet and injected with SnPP IX as described in Methods. 28S ribosomal RNA was visualized with ethidium bromide staining.

Data Analysis

All values are expressed as mean±SD. Differences were evaluated for significance by ANOVA followed by a Fisher post hoc test.

Results

HO Expression in the Atherosclerotic Lesions of WHHL Rabbits

To examine HO-1 induction in the atherosclerotic lesions of WHHL rabbits, the expression of HO mRNA was examined by Northern blot analysis (Figure 1). HO-1 mRNA was hardly present in the aortas from 2-month-old WHHL rabbits fed a high-fat diet (Figure 1A, lanes 1 and 2). Histological examination showed no evidence of atherosclerotic lesions at this age (data not shown). In contrast, HO-1 mRNA was induced in the atherosclerotic aortas from 4-month-old HO-2 mRNA expression in arteries of 2 WHHL rabbits from each group. Total RNA (20 \(\mug\)) was electrophoresed in formaldehyde/1% agarose gel, blotted to nylon membrane, and hybridized with HO probe as described in Methods. Lanes 1 and 2, 2-month-old WHHL rabbits fed Chow diet. Lanes 3 and 4, 4-month-old WHHL rabbits fed high-fat diet. Lanes 5 and 6, 4-month-old WHHL rabbits fed high-fat diet and injected with SnPP IX as described in Methods. 28S ribosomal RNA was visualized with ethidium bromide staining.

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To examine HO-1 induction in the atherosclerotic lesions of WHHL rabbits, the expression of HO mRNA was examined by Northern blot analysis (Figure 1). HO-1 mRNA was hardly present in the aortas from 2-month-old WHHL rabbits fed a high-fat diet (Figure 1A, lanes 1 and 2). Histological examination showed no evidence of atherosclerotic lesions at this age (data not shown). In contrast, HO-1 mRNA was induced in the atherosclerotic aortas from 4-month-old HO-1 mRNA was expressed in the atherosclerotic aortas from 4-month-old WHHL rabbits. The expression of HO-1 mRNA was examined by Northern blot analysis (Figure 1). HO-1 mRNA was visualized with ethidium bromide staining.
WHHL rabbits fed a high-cholesterol diet (Figure 1A, lanes 3 to 6), whereas mRNA of HO-2, a constitutive form of HO, was similarly expressed in both normal and atherosclerotic arteries (Figure 1B). Representative aortic atherosclerotic lesions are shown in Figures 3 and 4 and will be discussed later.

Immunohistochemical analyses also demonstrated that HO-1 was expressed in the atherosclerotic arteries of 4-month-old WHHL rabbits fed a high-cholesterol diet (Figure 2A). The major cells expressing HO-1 seem to be macrophages, as judged by immunostaining with anti-macrophage antibody (Figure 2, A and B). Immunostaining with a monoclonal antibody against oxLDL revealed that oxLDL was localized in relatively deep intimal lesions, where there was predominant immunostaining of macrophages and HO-1 (Figure 2C).

**Figure 2.** Representative photomicrographs of immunohistochemical staining of aortic atherosclerotic lesions in 4-month-old WHHL rabbits fed high-fat diet. Serial 5-μm sections were stained with anti–HO-1 (A), anti–macrophage (B), anti–oxidized phospholipids (C), and anti–α-smooth muscle actin (D). E. Control staining with nonimmune sera. All sections were immunostained with immunoperoxidase technique, then counterstained with hematoxylin.

### Effects of HO Inhibition on Atherosclerotic Lesion Formation in WHHL Rabbits

To examine the effect of HO modulation on atherosclerotic lesion development in WHHL rabbits, we inhibited HO activity in rabbits treated with a competitive inhibitor of HO, SnPP IX (S group, n = 10), and compared the extent of resultant atherosclerotic lesions to that in control rabbits (C group, n = 10). To shorten the period of SnPP IX treatment, which may evoke skin and liver toxicity in the animals, we put animals on a hypercholesteremic diet and accelerated atherosclerotic lesion development. The en face Sudan IV–positive lesion areas of the descending aorta in the SnPP IX treatment group (37.1 ± 9.5%) were significantly greater than in the C group (14.7 ± 5.3%) (P < 0.001) (Figure 3). Microscopic analyses of the ascending aorta also revealed a significantly greater mean atherosclerotic intimal area in the

**Figure 3.** Effect of HO inhibition on development of aortic atherosclerotic lesions after high-fat diet (A) in WHHL rabbits. Rabbits were treated by intravenous injections of SnPP IX (right) or saline (left, control) during 4 weeks of high-fat diet (A); 2 representative cases in each group are presented. Atherosclerotic lesion areas were determined by en face Sudan IV staining of descending aorta. Data are mean ± SD (B, n = 10 each). *P < 0.001.
S group (4.96 ± 0.99 mm²) compared with the C group (1.73 ± 1.25 mm²) (P < 0.0001) (Figure 4, A to C). The mean medial smooth muscle area was significantly decreased in the S group (Figure 4D). The ratio of intima to media in microscopic atherosclerotic lesions of the ascending aorta was significantly greater in the S group (67.7 ± 19.5%) than the C group (16.9 ± 9.4%) (Figure 4F, P < 0.005). Thus, SnPP IX treatment resulted in marked promotion of atherosclerotic lesion formation. HO inhibition by SnPP IX was confirmed by assay of HO activity in the aorta (Table).

**Elevation of Lipid Peroxides by HO Inhibition**

To examine the mechanism of the promotion of atherosclerotic lesions by HO inhibition, we measured plasma lipid levels before and after HO inhibition (Figure 5). There were no significant influences on total cholesterol, triglyceride, or HDL. Plasma lipid hydroperoxide levels were elevated after the high-fat diet in both groups. Interestingly, this elevation was significantly higher in the S group (85.4 ± 30.5 nmol/mL) than in the C group (37.7 ± 6.1 nmol/mL) (P < 0.05) (Figure 6A). Figure 6, B through E, shows the effects of SnPP IX treatment on tissue lipid peroxides in aorta and liver. Lipid peroxides in aortas and livers, estimated by measurement of MDA in homogenates (Figure 6, B and C) and conjugated dienes in extracted lipoproteins (Figure 6, D and E) were significantly elevated in the S group compared with the C group (P < 0.01, P < 0.001, respectively).

**Discussion**

In this study, we found that HO-1, an inducible form of HO, was expressed in the atherosclerotic arteries of WHHL rabbits. HO-1 expression seems to be a common response in different species during atherosclerotic lesion formation because HO-1 expression in human and experimental murine models has recently been reported.11,19 In contrast, HO-1 expression was not evident in nonatherosclerotic arteries of WHHL rabbits, as in murine models.19

Immunohistochemical analyses demonstrated that HO-1 is localized mainly in macrophages in the lesions of WHHL rabbits. Interestingly, these lesions were rich in oxidized LDL, as judged by immunostaining with specific antibody against oxidized phospholipid.23 Because biologically active oxidized phospholipids in oxLDL induced HO-1 in our previous cell culture study,5 these results suggest the possibility that oxidized phospholipids also induced HO-1 in this animal model.

The synthetic heme analogue SnPP IX has been widely used as a competitive inhibitor of HO, 1,2,4,14 –16 although a recent report shows its effects on other enzymes.28 Because prolonged treatment with metalloporphyrins may provoke such complications as light sensitivity or liver toxicity, 15,16

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**Figure 4.** A and B, Representative photomicrographs of atherosclerotic lesions of WHHL rabbits after high-fat diet for 4 weeks. Serial sections were stained by Azan-Mallory method. C through F, Quantitative analyses of microscopic atherosclerotic lesions in ascending aortas of WHHL rabbits. Lesion areas were calculated from cross sections of thickest 5-mm atherosclerotic lesions from ascending aortas. Fifty 5-μm sections at 100-μm intervals were stained by Azan-Mallory method and analyzed in each animal, and data were averaged in each group. Mean intimal (C), medial (D), and total (E) areas were quantified by computer-assisted planimetry and analyzed with NIH Image software. F, Ratio of intimal and medial area. Data are mean ± SD. *P < 0.005.
we put WHHL rabbits on a high-fat diet to further promote atherosclerotic lesion formation during a short period. Importantly, HO inhibition resulted in a significant progression of atherosclerotic lesion formation as judged by both en face Sudan IV staining of the descending aorta and quantitative microscopic analyses of the ascending aorta.

It is important to elucidate the mechanism of how HO-1 exhibited the antiatherogenic effects in WHHL rabbits. Cytoprotective effects of HO-1 have been attributed to the 3 reaction products in a variety of pathophysiological conditions. Antiatherogenic properties of HO-1 may also be conducted through these products, as well as the elimination of pro-oxidant heme. Carbon monoxide has been suggested to exhibit properties similar to those of nitric oxide through enhancing intracellular cGMP levels and direct action on Ca-dependent potassium channels, although the potency of vasodilatory action in vivo seems to be controversial. Biliverdin and bilirubin inhibit lipid peroxidation to a degree similar to that of vitamin E and protect albumin-bound linoleic acid from peroxyl radical–induced oxidation in vitro. Biliverdin and bilirubin inhibit monocyte transmigration elicited by minimally oxidized LDL and oxidant-induced microvascular leukocyte adhesion to vascular endothelium. Free iron induces ferritin, which serves as an antioxidant by sequestering iron. Indeed, HO inhibition resulted in a marked increase of atherosclerotic lesion development in WHHL rabbits, suggesting the possibility that these HO-1 reaction products function as antiatherogenic molecules in vivo.

Systemic administration of SnPP IX exhibited plasma lipid peroxidation without affecting plasma lipid profiles in our experiments. Furthermore, the promotion of lipid peroxidation was also observed in tissue lipid peroxide levels in aorta and liver. These results are consistent with our recent observations in LDL-receptor knockout mice. This is the first observation showing antiatherogenic and antioxidative properties of HO-1 in experimental hyperlipidemic rabbit models. In rabbits, antioxidative effects of HO-1 on lipoproteins may be attributed to biliverdin, because relatively lower activity of biliverdin reductase has been reported previously. Further experiments to determine which products of HO-1 are predominantly responsible for antiatherogenesis are also needed.

Recent studies have shown that HO-1 is induced by several atherogenic risk factors, such as oxidized lipoproteins.
hypertension,\textsuperscript{3} and diabetes mellitus.\textsuperscript{35} Antiatherogenic properties of HO-1 may also be increased through relieving the deleterious effects of these risk factors. HO-1 may widely affect the homeostasis inhibiting inflammatory processes in vascular wall and should be considered one of the new target molecules for future therapeutics of atherosclerotic disease.

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\section*{References}
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