Effects of Induction/Inhibition of Endogenous Heme Oxygenase-1 on Lipid Metabolism, Endothelial Function, and Atherosclerosis in Rabbits on a High Fat Diet

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Abstract. The heme oxygenase-1 (HO-1) / carbon monoxide (CO) system has been presumed as a therapeutic target for preventing atherosclerosis. However, the exact mechanism(s) underlying this system remains largely undefined. This study aims to examine the influence of induction/inhibition of HO-1 on atherosclerotic plaque using pharmacological approaches and to elucidate potential mechanisms. Rabbits were randomly assigned to receive a standard diet (control group), high fat diet (HFD), HFD plus HO inducer hemin (HFD + H group), and HFD plus an HO inhibitor, zinc protoporphyrin-9 (ZnPP9, HFD + Z group). Atherosclerotic plaque was evaluated using oil red O staining and histological analyses. Immunohistochemistry, western blotting, and RT-PCR were employed to study the expression of HO-1 and endothelin-1 (ET-1). Levels of CO, nitric oxide (NO), eNOS/iNOS activities, NF-κB activity, and TNF-α level were determined. No significant differences of serum lipid levels were observed among the HFD, HFD + Z, and HFD + H groups. In rabbits, HFD induced typical atherosclerotic plaque and increased intima/media thickness ratio, which was markedly reduced in the HFD + H group and further aggravated in the HFD + Z group. Furthermore, hemin increased HO-1 expression, CO levels, and eNOS activity, while decreasing iNOS levels, ET-1 expression, NF-κB activity, and TNF-α level. ZnPP9 caused opposite effects. Induction of the endogenous HO-1/CO system by hemin can prevent atherosclerosis though increasing CO levels, regulating eNOS activity, NF-κB activity, TNF-α levels, and ET-1 levels in rabbits. Our results add new evidence for the importance of HO-1 in the genesis and development of atherosclerosis and provide several possible mechanisms underlying the anti-atherosclerosis effects of HO-1.

Keywords: heme oxygenase-1, endothelium-derived factor, inflammatory factor, endothelial dysfunction

Introduction

Atherosclerosis is a disease of the vasculature characterized by endothelial dysfunction and arterial plaque formation that result from complicated underlying mechanisms (1, 2). Generally, endothelial dysfunction is thought to contribute to the initial stages of atherosclerosis. Stable atherosclerotic plaques, which may be asymptomatic, are rich in extracellular matrix and vascular smooth muscle cells (VSMCs) (1, 2). Unlike stable plaques, most unstable plaques are rich in macrophages and foam cells, with the extracellular matrix separating the lesion from the arterial lumen. Furthermore, the fibrous cap is usually weak and prone to rupture (1, 2). It is well accepted that inflammatory mechanisms and oxidative stress play critical roles during the genesis and development of atherosclerosis. Thus, atherosclerosis is often viewed as an inflammatory disease (1, 3 – 5).

Heme oxygenase (HO) is the first and rate-limiting enzyme that catalyzes the degradation of heme to bili-
verdin, iron, and carbon monoxide (CO). Heme oxygenase-1 (HO-1) is the inducing form of HO and has been demonstrated to exert marked anti-oxidative and anti-inflammatory properties (6). Considerable evidence has demonstrated that HO-1 plays important roles in the stress response to various pathological stimuli to serve a cytoprotective function (6, 7). HO-1 has been reported to be involved in the atherosclerosis process (8). In low density lipoprotein (LDL) receptor–knockout mice, HO-1 is abundantly expressed in atherosclerotic lesions after dietary cholesterol feeding. In this mouse model, HO overexpression resulted in a reduction of plaque formation, whereas HO inhibition promoted lesion development (9). A study using the rabbit model showed that HO-1 has antiatherogenic properties in vivo and that the antiatherogenic properties of HO-1 are conducted through the prevention of lipid peroxidation (10). Hemin, the most prominent and frequently used HO inducer, has been proposed to increase not only the production of CO from HO-1, but also that of NO. Since arginate is a derivative of l-arginine, it raises concerns about potential adverse effects, particularly when used for the chronic upregulation of HO-1 (8). However, the exact effects of induction/inhibition of endogenous HO-1 on atherosclerosis plaque formation in vivo remain largely unknown. Moreover, the effects of inhibition/induction of HO-1 on endothelial/inducible nitric oxide synthase (e/iNOS) in atherosclerosis are currently being elucidated. Therefore, in this study, we investigated the role of endogenous HO-1 in atherosclerotic lesions using hemin (HO-1 agonist) and zinc protoporphyrin-9 (ZnPp9, HO-1 inhibitor).

Material and Methods

Animals and treatments

Forty New Zealand white rabbits, weighing 1.8 – 2.2 kg, were provided by the Animal Research Centre of the Guiyang Medical College (Certificate No. SCXXK [Qian2002-0001]) (Guiyang, Guizhou, China). Animals were acclimatized to the laboratory conditions for one week prior to implementation of experimental protocols. All experimental procedures were approved and carried out in accordance with the guidelines of the ethical committees of Guiyang Medical College and The Third Military Medical University. Rabbits were randomly divided into four groups: Control group (CTRL), high-fat diet group (HFD, 1.5% cholesterol and 21% lard), HFD plus heme group (HFD + H), and HFD plus zinc group (HFD + Z) (n = 10 in each group). The rabbits in the CTRL group were fed with standard laboratory chow and the rabbits in HFD group was fed with a high fat diet and given saline (i.p.) once daily. The rabbits in the HFD + H group and HFD + Z group were fed with HFD and injected daily (i.p.) with hemin (15 mg/kg) or ZnPp9 (45 μmol/kg), respectively. The interventions were conducted for 12 weeks. Hemin and ZnPp9 were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Determination of blood biochemical parameters

Blood biochemical parameters were measured at baseline and at 4, 8, and 12 weeks of dietary intervention. Concentrations of serum lipids including total cholesterol (TC), triglyceride (TG), high density lipoprotein–cholesterol (HDLC), and LDL-cholesterol (LDLC) were determined by routine enzymatic methods using kits provided by the Shanghai Kehua Bio-engineering (Shanghai, China). Oxidized LDL (ox-LDL) was determined by the double antibody sandwich method using kits obtained from Shanghai Rongsheng Biotech (Shanghai, China).

Tissue collection

Twelve weeks later, the weight of rabbits is 2.3 – 3.1 kg. Following collection of blood samples, animals were sacrificed by injecting an overdose of 20% urethane (5 mL/kg) via the ear vein. Aortic arch, thoracic aorta, abdominal aorta, and arteria iliaca were then removed from each animal. A part of the aortic arch was fixed in 4% paraformaldehyde for pathological and immunohistochemical assays and the other part of aortic arch was cut into 1 x 1 mm² pieces and placed in cold fixative solution (2.5% glutaraldehyde) for ultrastructural examination. The upper section of thoracic aorta was homogenated and used for eNOS/iNOS activities, NO/CO generation, nuclear protein extraction, RT-PCR, and western blot determination. The lower section of thoracic aorta, abdominal aorta, and arteria iliaca were excised longitudinally and preserved for gross morphological observation (oil red O staining).

Aortic oil red O staining

Longitudinally dissected vessels were fixed in 10% neutral buffered formalin–calcium chloride solution, dehydrated, and stained with oil red O. Fatty streaks and plaques were stained red and photographed using JCV CCD camera and thereby analyzed by the Kontron IBAS 2.5 automatic image analyzing system (Kontron, Munich, Germany). The total area and plaque area were obtained to calculate the ratio of plaque area.

Hematoxylin and eosin (HE) staining

Fixed aortic samples were dehydrated, embedded in paraffin, sectioned (4-μm thickness), and stained with HE as previously reported (11). Sections (n = 8 per rabbit) were examined using a Zeiss microscope and analyzed by a Kontron IBAS 2.5 automatic image ana-
lyzing system. The thickness of intimal and media was calculated by two independent observers.

Transmission electron microscope (TEM) examination

The samples were fixed in 2.5% glutaraldehyde at 4°C for 24 h and a semi-thin sectioning approach was used to locate the area of interest before the samples were prepared as ultrathin sections as described previously (12). Ultrastructure was observed using a Hitachi H600 TEM (Hitachi, Tokyo).

Determination of aortic NO/CO generation and NOS activity

NO2 content in the aortic tissue homogenate, used as a marker of NO generation, was determined using a commercially available NO assay kit (Institute of Military Medical Sciences, Beijing, China) according to the manufacturer’s instructions. Aortic CO generation was determined as previously described (13). Briefly, sections of the thoracic aorta were cut into 3-mm pieces and placed in 2 mL DMEM medium. After incubation in 95% O2 for 24 h and a semi-thin sectioning approach was used to locate the area of interest before the samples were prepared as ultrathin sections as described previously (12). Ultrastructure was observed using a Hitachi H600 TEM (Hitachi, Tokyo).

Determination of nuclear factor-kappaB (NF-κB) activity and TNF-α level in the aorta

Aortic tissues were homogenized and lysed in ice-cold NP-40 lysis buffer (0°C) for 30 min. After homogenization, the lysate was centrifuged at 4°C at 12,000 rpm for 15 min. The supernatant was collected and protein concentrations were determined using the Lowry protein assay method. NF-κB activity was determined by using an ELISA assay kit (Active Motif Ltd., Carlsbad, CA, USA) according to the instructions of the manufacturer (16). Aortic TNF-α activity was determined by using an ELISA kit as reported previously (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (17).

Immunohistochemical detection of HO-1 and endothelin-1 (ET-1) protein expression in the aorta

Immunohistochemical analysis was performed as described previously (18). Paraaffin-embedded sections were dewaxed in xylene, dehydrated with a graded ethanol series, and treated with 0.3% hydrogen peroxide solution. Antigen retrieval was carried out using microwave heating (80°C, 30 min × 3). The sections were then blocked with goat serum, incubated with appropriate primary antibodies (goat anti-mouse polyclonal antibodies against HO-1 and ET-1, dilution 1:1000; both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with horseradish peroxidase-labeled rabbit anti-goat IgG monoclonal antibody (1:500; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The sections were counterstained by hematoxylin and examined by optical microscopy (Zeiss microscope; Zeiss, Oberkochen, Germany).

RT-PCR detection of HO-1 and ET-1 mRNA expression in the aorta

Total RNA in the aorta was extracted using Trizol reagent (Roche, Mannheim, Germany) (19). After determination of RNA purity and concentration, RNA was reverse-transcribed at 50°C for 30 min, 99°C for 5 min, and 5°C for 5 min. The PCR reaction was then performed using a commercially available RT-PCR kit (Takara Bio, Inc., Otsu). Rabbit HO-1, ET-1, and β-actin (internal standard) gene sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Primers were designed using DNASTAR software (Hitachi Software Engineering Co., Ltd., Tokyo) and synthesized by Shanghai BioAsia Biotechnology Co., Ltd. (Shanghai, China). The primer set for HO-1 was 5′-CACGTACTGCAGGAGGT TT TA-3′ (forward) and 5′-GGAAATGACGCGGCGC G TAG-3′ (reverse), with a product of 118 bp. The primer set for ET-1 was 5′-AGATCCACGGCAGATGGA GAGCG-3′ (forward) and 5′-CGGTGAGCTGCGTCC TCCTTGATGG-3′ (reverse), with a product of 543 bp. The primer set for β-actin was 5′-CCATCTAGCGG GCTACGC-3′ (forward) and 5′-CAGGAGGAGG GCTGAAAC-3′ (reverse), with a product of 312 bp. For HO-1 amplification, the PCR reaction was performed according to the following conditions: pre-denaturation at 94°C for 2 min followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. For ET-1 amplification, the PCR reaction was performed according to the following conditions: pre-denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 3 min, and a final extension at 72°C for 7 min. A 10-μL aliquot of each PCR product was subjected to 2% agarose gel electrophoresis and DNA bands were scanned with a gel imaging system ChemiImager™ 5500 (Alpha Innotech, San Leandro, CA, USA). The results are expressed as the ratios of optical density values of HO-1 or ET-1 relative to that of β-actin (20).
Western blot analysis of HO-1 and ET-1 protein expression in the aorta

Western blot analysis was performed as previously reported (21). The aorta samples were cut into pieces and lysed in RIPA lysis buffer (1 mL/100 g tissue; Shanghai Biocolor BioScience & Technology, Shanghai, China) containing PMSF on ice for 30 min. The lysed cells were then centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was transferred to a clean tube and the protein concentration determined using the Coomassie brilliant blue method (Bradford method). Proteins (40 μg/lane) were loaded onto a SDS-polyacrylamide gel (10% separating gel, 5% stacking gel). After electrophoresis was completed, proteins in the gel were blotted to a polyvinylidene fluoride (PVDF) membrane using semi-dry blotting conditions. The membrane was then blocked at room temperature for 1 – 2 h, incubated with appropriate primary antibody (goat anti-mouse polyclonal antibodies against HO-1 and ET-1 and goat anti-mouse monoclonal antibody against GADPH, 1:1000 dilution; all from Santa Cruz Biotechnology) at 4°C overnight. After rinsing, the PVDF membrane was incubated with secondary antibodies (horseradish peroxidase–labeled rabbit anti-goat IgG monoclonal antibody, 1:500 dilution) at 37°C for 1 h. Finally, the signal was developed using chemiluminescence, and the results were expressed as the ratios of the optical density values of HO-1 or ET-1 relative to those of GADPH.

Statistical analysis

Data are expressed as the mean ± S.D. Comparisons of values among groups were made using one-way analysis of variance ANOVA methods (SPSS, Chicago, IL, USA) followed with the SNK-test (q-test). Statistical signifi-
cance was set at $P < 0.05$.

**Results**

*Hemin induces expression of HO-1 in atherosclerotic lesions*

As shown in Fig. 1A, HO-1 was expressed in endothelial cells in the control group. HFD increased the expression of HO-1 in the media of arteries. This increase of HO-1 expression was suppressed by ZnPP9 (HFD + Z group) and further enhanced by hemin (HFD + H group). Western blotting and RT-PCR analyses were performed to confirm this result. As shown in Fig. 1B, HFD increased HO-1 protein expression (approximately 2.7-fold). Treatment with ZnPP9 markedly inhibited the HO-1 protein expression, while exposure to hemin further increased HO-1 protein expression. Similar phenomena were observed in HO-1 mRNA expression (Fig. 1C).

*Effects of induction/inhibition of HO-1 on atherosclerosis lesions*

Oil red O staining was used to evaluate atherosclerotic plaque area. As shown in Fig. 2, A and B, there was no visible atherosclerotic plaques in aortae of control rabbits. HFD induced readily observed atherosclerotic plaques (red, Fig. 2A). Inhibition of HO-1 by ZnPP9 increased the HO-1 atherosclerotic plaque area ($62.32 \pm 3.85\%$ vs. $52.16 \pm 4.19\%, P < 0.01$), whereas hemin significantly decreased atherosclerotic plaque area ($16.95 \pm 2.83\%$ vs. 

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**Fig. 2.** Effects of induction/inhibition of HO-1 on atherosclerosis lesions. A and B: representative oil red O staining (A) and quantitative analysis (B) showing the influence of induction/inhibition of HO-1 on atherosclerotic plaque in the rabbit aortae. The red area is atherosclerotic plaque. C and D: Representative images of hematoxylin and eosin (HE) staining (C) and transmission electron microscopy (TEM, D) of the rabbit aortae. Black arrow: normal endothelial cells, blue arrow: atherosclerotic material, yellow arrow: lipid vacuoles, pink arrow: collagen, red arrow: macrophage. E and F: effects of induction/inhibition of HO-1 on intima thickness (E) and intima–media thickness ratio (F). CTRL: control, HFD: high fat diet, Z: ZnPP9, H: hemin. *$P < 0.05$ vs. control, **$P < 0.05$ vs. HFD group; $n = 10$ per group.
52.16 ± 4.19%, *P < 0.01).

Aortic HE staining in the control group showed that the intimal structure was well-defined and intact. Furthermore, the medial structure composed of elastic fibers and smooth muscle was well-organized (Fig. 2C). In the HFD group, the intima was markedly thickened, and endothelial cells were detached and a significant proliferation of foam cells was noted in the subendothelial layer. ZnPP9 aggravated the HFD-induced damage, whereas hemin attenuated this damage.

Similar results were observed under electron microscopy (Fig. 2D). In the control group, TEM showed the structure of the endothelium and the internal elastic lamina to be well-defined with a few elastic fibers evident between the layers. There were numerous pinosomes in normal endothelial cells (black arrow). In the HFD group, the endothelium disappeared and was replaced by atherosclerotic material (blue arrow). The structure of the elastic and smooth muscle layers was disordered, lipid vacuoles (yellow arrow) were evident in the cytoplasm of smooth muscle cells, and collagen was apparent (pink arrow) in the matrix. ZnPP9 aggravated the HFD-induced damage, evidenced by the disappearance of endothelium and internal elastic layer and the disruption/degeneration of smooth muscle cells. Moreover, macrophages (red arrow) can be observed, whereas hemin attenuated this damage.

In addition, we examined intimal thickness (Fig. 2E) and intimal-media thickness ratio (Fig. 2F). HFD increased the intimal thickness and intimal-media thickness ratio significantly compared with the control group. ZnPP9 further augmented the HFD-induced intimal thickening. In contrast, hemin partially attenuated the intimal thickening.

Effects of induction/inhibition of HO-1 on aortic CO level, NO level, eNOS activity, and iNOS activity

As shown in Fig. 3A, the CO level was enhanced by HFD (60.21 ± 5.78 vs. 33.24 ± 4.23 nmol/mg tissue, *P < 0.01) compared with that in the control group. The CO level was further increased in the HFD + H group (76.32 ± 6.37 vs. 60.21 ± 5.78 nmol/mg tissue, #P < 0.01) compared with that in the HFD group. Conversely, ZnPP9 significantly suppressed the increase of CO level by HFD (*P < 0.05).

As shown in Fig. 3B, the NO levels in the HFD, HFD + Z, and HFD + H groups were lower than that in the control group. ZnPP9 further increased the NO level (103.47 ± 15.34 vs. 79.21 ± 14.32 nmol/mg tissue, *P < 0.01), whereas hemin slightly but not significantly increased the NO level (89.09 ± 13.27 vs. 79.21 ± 14.32 nmol/mg tissue, §P > 0.05).

Fig. 3. Effects of induction/inhibition of HO-1 on aortic CO level (A), NO level (B), eNOS activity (C), and iNOS activity (D). CTRL: control, HFD: high fat diet, Z: ZnPP9, H: hemin. *P < 0.05 vs. control, #P < 0.05 vs. HFD group, §P < 0.05; n = 10 per group.
Figure 3, C and D, showed that the eNOS activity in the HFD group was decreased by 48% \((P < 0.01)\), whereas the iNOS activity in the HFD group was increased by 59% \((P < 0.01)\) compared to that in the control group. The ZnPP9 further decreased the eNOS activity \((\text{Fig. } 3\text{C}, P < 0.05)\) and further increased the iNOS activity \((\text{Fig. } 3\text{D}, P < 0.05)\). In contrast, hemin slightly but significantly raised the eNOS activity \((\text{Fig. } 3\text{C}, P < 0.01)\) and lowered the iNOS activity \((\text{Fig. } 3\text{C}, P < 0.01)\).

**Effects of induction/inhibition of HO-1 on aortic ET-1 expression**

ET-1 expression was first examined using immunohistochemistry (Fig. 4A). There were no visible ET-1–positive cells in aortae from control rabbits. In contrast, HFD markedly increased ET-1 expression. Treatment with ZnPP9 plus HFD induced ET-1 expression in both endothelium and media, while hemin attenuated the HFD-induced ET-1 expression. Western blotting (protein expression, Fig. 4B) and RT-PCR (mRNA expression, Fig. 4C) analyses confirmed these results.

**Fig. 4.** Effects of induction/inhibition of HO-1 on aortic ET-1 expression. A: immunohistochemistry analysis of ET-1 expression in rabbit aortae. The brown area is thought to be ET-1–positive cells. B and C: representative image and quantitative analysis of ET-1 protein (B) and mRNA (C) expression by western blotting and RT-PCR, respectively. CTRL: control, HFD: high fat diet, Z: ZnPP9, H: hemin. *\(P < 0.05\) vs. control, #\(P < 0.05\) vs. HFD group; \(n = 10\) per group.
Effects of induction/inhibition of HO-1 on inflammatory factors

We assessed the effects of induction/inhibition of HO-1 on two important inflammatory factors: NF-κB (Fig. 5A) and TNF-α (Fig. 5B). As expected, HFD induced activation of aortic NF-κB and TNF-α. ZnPP9 further increased the levels of aortic NF-κB and TNF-α, whereas hemin attenuated the increase of aortic NF-κB and TNF-α induced by HFD.

Effects of induction/inhibition of HO-1 on serum lipid levels

HFD induced significant increases in serum TC, TG, LDL-C, and ox-LDL levels. Treatment with either ZnPP9 or hemin did not impact these observations (Fig. 6).

Discussion

Although the impact of modulation of endogenous HO-1 or gene transfer of HO-1 on atherosclerosis has frequently been described in experimental research (10, 22 – 24), much less is known about the molecular mechanisms underlying the influences of induction/inhibition of HO-1 on NO level, eNOS/iNOS activities, ET-1 expression, NF-κB activity, and TNF-α levels. In the present study, we show that induction of HO-1 by hemin attenuated HFD-induced atherosclerosis plaque formation in rabbits. The influence of hemin on CO levels, NO/ eNOS/iNOS activity, ET-1 expression, NF-κB activity, and TNF-α levels may contribute to its beneficial effects. Conversely, inhibition of HO-1 by ZnPP9 caused adverse effects with a greater degree of atherosclerosis being evident.

Previous studies have showed that ox-LDL and lipometabolic products can induce HO-1 mRNA and protein upregulation (9, 10). Some previous data suggested that the induction of HO-1 by high fat diet may be a compensational anti-oxidative mechanism that forms the second line of defense against oxidative stress in addition to the glutathione system (25). The beneficial effects of HO-1 on atherosclerosis have been well-established by the use of either an HO-1 inhibitor or agonist. Ishikawa et al. have reported that HO-1 functions as an intrinsic protective factor against atherosclerotic lesion formation, possibly by inhibiting lipid peroxidation in rats (9) and in rabbits (10). In these two studies, the authors used Sn-protoporphyrin 9, another HO-1 inhibitor, to show the anti-atherosclerosis effects of HO-1. Recently, Lin et al. demonstrated that hemin-induced upregulation of HO-1 could stabilize vulnerable plaques (26). Our data are in agreement with these results. Treatment with hemin reduced atherosclerotic plaque from 63% (HFD group) to 18% (HFD + H group). Moreover, the morphology of aortae was significantly improved by hemin, evidenced by both HE staining and electron microscopy. Thus, we believe that hemin, a HO-1 agonist, is an effective anti-atherosclerosis agent in the HFD-fed rabbit model.

It is well-known that endothelial dysfunction is viewed as the initial step in the pathogenesis of atherosclerotic lesions (1, 2, 27). Recent evidence suggests that the HO-1/CO system also plays a beneficial role in atherosclerosis via activating soluble guanylate cyclase, modulating the activity of mitogenic signaling pathways and protecting against thrombosis (28). As the main enzymatic product of HO-1, CO is an important endothelial-derived relaxation factor as well as NO. CO and NO play complementary or equivalent roles in maintaining the normal physiological functions of blood vessels (27, 29). Earlier studies have suggested that CO might bind to and activate guanylate cyclase, thereby increasing intracellular levels of cyclic guanine monophosphate (cGMP), a target of NO (29, 30). Under physiological conditions, CO production is rather low, and the ability of CO to increase cGMP is much weaker compared with NO. In contrast, CO production from VSMCs is increased under
pathological conditions, particularly when NO production from the endothelium is impaired. Therefore, CO exerts an important regulatory and compensatory role in pathological conditions (29).

The most important finding of our study is that we demonstrate for the first time the effects of inhibition/inhibition of HO-1 on CO, NO levels, and eNOS/iNOS activities in an animal model of atherosclerosis. In our study, as expected, HFD markedly impaired eNOS activity and NO generation and increased the iNOS activity and CO generation. Hemin treatment successfully reduced iNOS activity, whereas hemin also increased eNOS activity. Although the NO free radical production increased in the HFD group, the endogenous NO synthesis was reduced due to the HFD-induced impairment. Thus, the NO level was still significantly lower than that in the control group. In HFD + H Group, although the atherosclerotic lesion was attenuated, synthesis of endogenous NO was still less than that in the control group. Because the atherosclerotic lesion in the HFD + Z group was most severe, the endogenous NO synthesis was naturally lower than that in the control group. However, since the free NO radical generated from iNOS was also increased, the total NO level in the HFD + Z group was higher than that in the HFD group.

Considering the unchanged total NO level in the
HFD + H group compared with the HFD group, we proposed that the beneficial effects of hemin on atherosclerosis may be mainly due to the marked increase of CO levels induced by HO-1. In other words, the HO-1/CO system may exert a major effect in inhibiting the development of atherosclerosis. The marked increase of CO induced by hemin can lead to increased cGMP levels and relaxation of blood vessels and compensate for insufficient NO production by endothelium-derived eNOS and promote eNOS activity by improving endothelial function (29, 31). On the other hand, induced HO-1 expression and increased CO production can inhibit iNOS activity and generation through the following potential mechanisms, thus alleviating arterial wall injury: 1) iNOS is a cytochrome P450-like hemoprotein and cytochrome P450 and is a substrate of HO-1. Therefore, an increase in HO-1 activity can accelerate the degradation of iNOS; 2) since the active site of iNOS requires two heme molecules, increases in HO-1 activity accelerates the degradation of heme and reduces the activity of iNOS (32); 3) CO can bind and inactivate iNOS; 4) free iron generated by heme decomposition can inhibit iNOS production by suppressing nuclear transcription. Given that the interaction between the iNOS/NO system and the HO-1/CO system underlies the regulatory mechanisms involved in injury/protection under various stresses, we propose that the inhibitory effects of HO-1/CO on iNOS is particularly important under pathological conditions such as atherogenesis.

In addition to the effect of hemin on HO-1/CO and the NO/eNOS/iNOS system, we also showed that the increased aortic ET-1 expression, NF-κB activity, and TNF-α level were all decreased by hemin. These inflammatory factors are all considered to be pro-atherogenic (1, 33). NF-κB activation is a critical process for accelerating atherosclerosis and neo-intima hyperplasia (34). Pae et al. found that the HO-1 inducer cobalt protoporphyrin can inhibit TNF-α–induced NF-κB activation in human umbilical vein endothelial cells and that the effect could be reversed by the HO-1 inhibitor tin protoporphyrin (35). Our present results also show that NF-κB activity and TNF-α levels were highest in the HFD + Z group and lowest in the HFD + H group, indicating the anti-inflammatory effect of the HO-1 system. Thus, the actions of hemin in suppressing NF-κB activity and TNF-α level contribute to its anti-atherosclerosis effect. Another study showed that hypoxia induced HO-1 expression and CO production in VSMCs in a co-culture system of endothelial cells and VSMCs, which in turn increased cGMP levels and inhibited ET-1 expression in endothelial cells, thus inhibiting hypoxia-induced neointimal proliferation (36). As ET-1 expression is associated with endothelial dysfunction and neointimal proliferation, which are the key processes in the development of atherosclerosis, our data therefore establish ET-1 as an anti-atherosclerosis target of the HO-1/CO system.

In conclusion, our results demonstrate the anti-atherosclerosis actions of the HO-1/CO system in the HFD-fed rabbit model. The mechanisms underlying this protection appear to involve the regulation of HO-1 on the NO/eNOS/iNOS system, NF-κB activity, TNF-α level, and ET-1 expression. These observations highlight the importance of HO-1 in the genesis and development of atherosclerosis and may motivate future efforts to explore the possibility of developing new chemical inducers of HO-1 as a promising anti-atherosclerosis drug in the future.

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