Paradoxical Reduction of Fatty Streak Formation in Mice Lacking Endothelial Nitric Oxide Synthase

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Background—The endothelial isoform of nitric oxide synthase (eNOS) has been considered to exert an antiatherosclerotic role through synthesis of NO. However, eNOS has been shown to generate superoxide, which could oxidize LDL and promote atherosclerosis. We sought to determine the role of eNOS in diet-induced fatty streak formation through the use of eNOS-deficient mice.

Methods and Results—Mice were fed an atherogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate for 12 weeks, and atherosclerotic lesions at the aortic root were measured after oil-red O staining. Unexpectedly, eNOS-deficient mice developed much smaller aortic lesions than did wild-type control mice (2544±1107 versus 7023±1569 μm²/section; P=0.03). This reduction in lesion formation could not be explained by changes in plasma levels of lipids and susceptibility of lipoproteins to oxidation. To examine whether eNOS contributed to the oxidation of LDL within the arterial wall, endothelial cells were isolated from the aorta of mice and incubated with native LDL in the absence or presence of N-ω-nitro-L-arginine methyl ester (L-NAME), a specific NOS inhibitor. L-NAME significantly inhibited LDL oxidation by endothelial cells from wild-type animals (P<0.05), but it had no effect on LDL oxidation by endothelial cells from eNOS-deficient mice.

Conclusions—These data indicate that absence of eNOS-mediated LDL oxidation may contribute to the reduction of fatty-streak formation in eNOS-deficient mice. (Circulation. 2002;105:2078-2082.)

Key Words: nitric oxide synthase ■ atherosclerosis ■ endothelium ■ atherosclerosis ■ lipoproteins

Endothelium-derived NO, generated by the endothelial isoform of NO synthase (eNOS), has been considered an important endogenous antiatherosclerosis factor. In vitro, NO inhibits monocyte adhesion to the endothelium,1 suppresses smooth muscle cell proliferation and migration,2,3 inhibits platelet aggregation,4 induces apoptosis of macrophages and smooth muscle cells,5 and reduces the expression of inflammatory genes such as monocyte chemoattractant protein-1, vascular cellular adhesion molecule-1, and intracellular adhesion molecule-1.6 Consistent with the in vitro observations, oral administration of L-arginine, the substrate of NOS, inhibits atherosclerosis in rabbit and mouse models,7,8 and inhibition of NO production with NOS inhibitors promotes atherosclerosis.9 NO is synthesized from L-arginine by 3 NOS isoforms, including 2 constitutively transcribed forms, neuronal (nNOS) and endothelial (eNOS) enzymes, and 1 inducible form (iNOS) expressed in macrophages, vascular smooth muscle cells, and other types of cells. Because L-arginine and NOS inhibitors affect all 3 isoforms, it is difficult to dissect the individual role of NOS in the development of atherosclerosis. L-Arginine has been shown to have antioxidant properties and to influence levels of various hormones, including insulin10; thus, its antiatherogenic effects could be mediated through NO-independent pathways.

Recent studies have suggested that eNOS or NO may have proatherosclerotic properties. In addition to synthesizing NO, eNOS catalyzes superoxide formation.11 NO rapidly reacts with superoxide, leading to the formation of peroxynitrite.12,13 Both superoxide and peroxynitrite are potent oxidants, which can oxidize lipoproteins.14 Under the circumstance of hypercholesterolemia, eNOS may contribute significantly to the oxidation of LDL. Indeed, hypercholesterolemia or hypercholesterolemic levels of LDL have been shown to enhance production of NO and superoxide15,16 and impair the glutathione detoxification mechanism against peroxynitrite,17 thus leading to the accumulation of peroxynitrite.

Mice deficient in eNOS were examined for the development of atherosclerosis in an apolipoprotein E (apoE)–deficient mouse model.18,19 The results indicated a protective role of eNOS in atherosclerosis, and this antiatherosclerotic effect could not be fully explained by the influence on blood pressure. We have now examined the effects of eNOS...
deficiency on fatty-streak formation in a dietary model. Our results showed that eNOS-deficient mice developed smaller lesions, suggesting that the role of eNOS in atherogenesis is dependent on the stage of lesion development and on initiating factors that cause the disease.

Methods

**Mice and Diets**
Female eNOS-deficient mice and female C57BL/6J mice, 4 to 5 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, Maine). The eNOS-deficient mice, which were originally on a mixed B6.129 genetic background, had been sequentially backcrossed with C57BL/6J mice for 7 generations. Thus, the possibility that alleles associated with the NOS3 gene were derived from the 129 was minimal. Mice were fed a regular chow diet and maintained in a temperature-controlled room with a 12-hour light and dark cycle. At 8 weeks of age, mice were placed on an atherogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (TD 90221, Food-Tek, Inc) and maintained on this diet for 12 weeks. All procedures were in accordance with present National Institutes of Health guidelines and approved by the University of California Los Angeles Animal Research Committee.

**Tissue Preparation and Lesion Analysis**
Methods for the quantitation of atherosclerotic lesions in the aorta were as previously reported.20 Briefly, animals were sacrificed by cervical dislocation, and the heart and proximal aorta were excised and stored at

**Enzyme Immunoassay**
Measurement of 8-Isoprostane by Monocyt Chemotaxis Assay
Monocyte transmigration was determined using the protocol described previously.22 Cocultures of human aortic endothelial cells and smooth muscle cells were incubated with media containing 350 μg/mL of freshly isolated human LDL in the absence or presence of human or mouse HDL or with media containing 250 μg/mL of mouse LDL only for 6 hours. Monocyte chemotactic activity was measured using supernatants collected from the cocultures. The supernatants were added to a standard Neuroprobe chamber (Neuro Probe), with human monocytes added to the top. The chamber was incubated for 1 hour at 37°C. After wiping off nonmigrated monocytes, the membrane was air dried, fixed with 1% glutaraldehyde, and stained with 0.1% Crystal Violet dye. The number of migrated monocytes was determined microscopically and expressed as the mean±SEM of 9 fields counted in triplicate wells.

**Endothelial Cell Culture and Treatments**
Endothelial cells were isolated from the thoracic aorta of mice by an established explantation technique.23 Initially, the aortic segments were placed on Matrigel and incubated in DMEM supplemented with 15% FBS, 1% penicillin-streptomycin, 90 μg/mL heparin, 60 μg/mL endothelial cell growth supplements, and 100 U/mL fungizone. The vessel segments were removed once cell outgrowth was observed. The cells were passaged with Dispase (BD Biosciences) and then plated into gelatin-coated culture dishes. The subsequent passages were performed with 0.25% trypsin-EDTA. Immunostaining for the von Willebrand factor and the 1,1,3,3-tetramethylindo carbocyanine perchlorate-labeled acetylated LDL uptake experiment confirmed that this method generated pure endothelial cells. At passage 4, cells were plated into 6-well plates. Once cells grew to confluence, culture medium was removed and the cells were washed twice with sterile PBS. The cells were then incubated at 37°C with 2 mL Ham’s F-10 medium per well containing 250 μg/mL of LDL in the absence or presence of L-NAME (0.5 mmol) for up to 24 hours. After various lengths of incubation, the LDL-containing medium was removed and BHT was added to prevent additional oxidation. The amount of oxidized lipids in the LDL-containing medium was assessed by measuring thiobarbituric acid-reactive substances (TBARS).24 Briefly, 200 μL of TBARS agents (1.442 g thiobarbituric acid and 3.15 g trizma base in 100 mL H2O, pH 7.4) and 375 μL of 40% trichloroacetic acid in 1 N HCl were sequentially added to each sample (100 μL of 250 μg/mL LDL protein), and the resulting mixture was heated at 90°C for 45 minutes. After adding 900 μL of 0.9% NaCl solution, the samples were centrifuged at 2500 rpm for 15 minutes. The absorbance of the supernatants was read at 532 nm, and the values were expressed as nanomolar of malondialdehyde equivalents per mg of LDL protein.

**Measurement of 8-Isoprostane by Enzyme Immunoassay**
Plasma levels of 8-isoprostane, an index of lipid peroxidation, were determined by an enzyme immunooassay kit from Cayman Chemical. Briefly, 300 μL of plasma was first mixed with 200 μL of water, 10 μL of 0.5 mol/L EDTA, 30 μL of 10 mmol/L BHT, and 1 mL of ethanol, incubated at 4°C for 5 minutes, and spun at 1500g for 5 minutes to precipitate plasma proteins; 800 μL of the supernatant was then transferred to a clean test tube and treated with 800 μL of 15% KOH at 40°C for 1 hour to release 8-isoprostane and free fatty acids. The samples were then diluted to 10 mL by the addition of UltraPure water, and the pH of the samples was lowered to <4 by the addition of HCl. The samples were then loaded onto C18 solid-phase extraction cartridges (Waters, Millipore Inc) conditioned with 5 mL of methanol and 5 mL of UltraPure water. The cartridges were washed with 5 mL of UltraPure water and then with 5 mL of hexane. The lipids were eluted with 5 mL of ethyl acetate containing 1% methanol, and the samples were then dried using nitrogen gas resuspended in 250 μL of EIA buffer, and assayed in duplicate for 8-isoprostane using the enzyme immunoassay kit.

**Statistical Analysis**
Data are presented as mean±SEM, with n indicating the number of mice. The statistical significance of differences in aortic lesion areas and plasma lipid levels between the 2 groups was determined with Student’s t test. ANOVA was used to determine differences between
groups in LDL oxidation. Differences were considered statistically significant at *P* < 0.05.

**Results**

To examine the role of eNOS in fatty-streak formation, female eNOS-deficient mice, which were on the C57BL/6 genetic background, and C57BL/6 control mice were started on the atherogenic diet at the age of 8 weeks. After 12 weeks on the atherogenic diet, mice were killed and atherosclerotic lesions at the aortic root were measured after oil-red O staining. Quantitative analysis of atherosclerotic lesions at the aortic root showed that the mean lesion area in eNOS-deficient mice (n=12) was 2544 ± 1107 μm²/section, which was significantly smaller than the lesion area of 7023 ± 1569 μm²/section in the C57BL/6 control mice (n=11; *P* = 0.030; Figure 1). The lesions in both eNOS-deficient mice and control mice were primarily fatty-streak lesions. As shown in Figure 2, lesion areas were intensely stained with oil-red O, and immunostaining with the monoclonal antibody to mouse macrophages, MOMA-2, revealed that macrophage-derived foam cells were the primary cellular components of the lesions. Analysis of plasma levels of total cholesterol, HDL cholesterol, and triglyceride showed no significant differences between eNOS-deficient mice and C57BL/6 mice on chow and after 12 weeks on the atherogenic diet (Figure 3).

Plasma levels of 8-isoprostane, a biomarker of lipid peroxidation, were measured when mice were fed chow and atherogenic diets (Figure 4A). eNOS-deficient mice exhibited a higher plasma level of 8-isoprostane than the control mice on either chow (578 ± 89 versus 412 ± 40 pg/mL) or atherogenic (698.5 ± 68 versus 431 ± 80 pg/mL) diets. The influence of HDL and LDL isolated from the pooled plasma of mice on monocyte transmigration was assessed using a coculture model of the artery wall (Figure 4B). The coculture model, consisting of a monolayer of aortic endothelial cells overlying a layer of smooth muscle cells, provides a microenvironment in which exogenously added LDL becomes oxidatively modified. This oxidized LDL induces monocyte transmigration. As shown in Figure 4, monocyte transmigration was stimulated in cocultures preincubated with human LDL, and this stimulation was blocked by the addition of human HDL. HDL isolated from C57BL/6 mice also inhibited monocyte transmigration induced by human LDL. However, HDL isolated from eNOS-deficient mice failed to protect and actually increased monocyte transmigration induced by human LDL alone. We also examined the ability of LDL isolated from eNOS-deficient and control mice to induce monocyte transmigration after incubation in the coculture for 6 hours. LDL isolated from eNOS-deficient mice exhibited an increased ability to promote monocyte transmigration compared with LDL of the control mice.

To examine the involvement of eNOS in LDL oxidation, endothelial cells were isolated from the aorta of wild-type and eNOS-deficient mice and incubated with native LDL in the absence or presence of the eNOS inhibitor, L-NAME. Lipid peroxidation products in the LDL-containing medium were determined by measuring TBARS. Endothelial cells resulted in time-dependent oxidation of LDL (Figure 5). Treatment with L-NAME significantly reduced TBARS levels in the medium incubated with endothelial cells from the wild-type mice compared with the treatment without L-NAME (*P* = 0.002). In contrast, treatment with L-NAME had no significant influence on TBARS levels in the medium that
had been incubated with endothelial cells from eNOS-deficient mice. These results indicate that eNOS contributes to endothelial cell–induced LDL oxidation.

Discussion

eNOS is constitutively expressed by endothelial cells and produces NO under basal conditions and in response to shear stress and agonists such as acetylcholine and bradykinin.25 Using eNOS-deficient mice, we investigated the role of eNOS in the development of fatty streaks in a dietary model. The results demonstrated that the absence of eNOS decreased fatty-streak formation in the mice. Plasma lipid levels were unlikely to explain the reduction in lesion formation, because eNOS-deficient mice did not show significant differences from control mice in the lipid levels.

Plasma 8-isoprostane was somewhat higher in eNOS-deficient mice. Isoprostanes result from oxidative modification of arachidonic acid through a free radical–catalyzed mechanism.26 Formation of these compounds in vivo is considered to be a reliable marker for lipid peroxidation. The present finding suggests that eNOS-derived NO protects against lipid peroxidation, probably by reacting with free oxygen radicals. Obviously, this observation cannot explain the reduction of eNOS-deficient mice in lesion formation.

Oxidative modification of trapped lipoproteins in the subendothelial space is thought to be an important event in atherogenesis.27 In the present study, we observed direct evidence that eNOS contributes to the oxidation of LDL by endothelial cells. Indeed, the eNOS inhibitor L-NAME significantly reduced LDL oxidation induced by endothelial cells from wild-type mice but had no effect on the oxidation induced by endothelial cells from eNOS-deficient mice. Although the effect of eNOS on LDL oxidation was small, there was a time-dependent increase in the effect within 24 hours. Given that atherosclerosis is a chronic disease, the contribution of eNOS to LDL oxidation could be significant in vivo. Under the circumstance of hyperlipidemia, the contribution of eNOS to the oxidation of LDL could be more significant. Hypercholesterolemia or hypercholesterolemic levels of LDL have been shown to increase production of NO and superoxide.15 Thus, lack of the eNOS-driven oxidation of accumulated LDL in the vessel wall may contribute to the reduction of eNOS-deficient mice in atherosclerotic lesion formation. We also used the lucigenin chemiluminescence method to assess free oxygen radical release from the aorta of eNOS-deficient and C57BL/6 mice. Because of the small size of the aorta, we were unable to quantify free oxygen levels directly using the method.

On the apoE-deficient background, recent studies have shown that eNOS-deficient mice developed larger atherosclerotic lesions on chow or atherogenic diets. The reasons for the discrepant results between apoE-deficient and dietary mouse models are unclear. In the apoE-deficient model, increased atherosclerosis in eNOS-deficient mice was associated with elevation in blood pressure, because administration of enalapril reversed the effect of eNOS deficiency on atherosclerosis. However, the correlation efficient between blood pressure and the extent of atherosclerotic lesions was very low.
(r^2=0.2), and enalapril, an ACE inhibitor, has antiatherosclerotic effects independent of blood pressure. In a recent study, Chen et al.\(^\text{28}\) reported that hypertension does not account for the accelerated atherosclerosis in eNOS-knockout mice. In the present study, blood pressure likely was not a contributing factor, because eNOS-deficient mice showed a reduction in lesion formation. One possible explanation for the discrepancy is that the structural irregularity of aortic valves in eNOS-deficient mice may influence the extent of lesion formation differently in the 2 models. Lee et al.\(^\text{29}\) reported that some eNOS-deficient mice (\(~40\%\)) have a bicuspid aortic valve. In the present study, we also observed the aberrant aortic valve in the mice. In the dietary mouse model, atherosclerotic lesions are limited largely to the aortic sinus, whereas in the apoE-deficient model, atherosclerotic lesions occur not only at the aortic sinus but also at other regions of the aorta. Alterations in the morphology of the aortic sinus and flow characteristics through the region may affect lesion formation at the aortic sinus, but the influence may be less significant for the region distal from the valve. Similarly paradoxical results have also been observed in other disease models. For example, in trinitrobenzene-induced colitis, eNOS-deficient mice showed more severe colitis, whereas in dextran sodium sulfate–induced colitis, eNOS-deficient mice exhibited less severe inflammatory changes.\(^\text{30}\) Additional studies will be required to answer the unsettled questions.

The finding that absence of eNOS diminishes atherosclerosis in the dietary mouse model provides in vivo evidence for a dual role of eNOS in atherosclerosis. This study also provides the first direct evidence that eNOS contributes to the oxidation of LDL. The possibility that eNOS aggravates atherosclerosis by oxidatively modifying LDL is consistent with the concept that oxidation of LDL is the key step in the pathogenesis of atherosclerosis.

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**References**


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