Accelerated Atherosclerosis, Aortic Aneurysm Formation, and Ischemic Heart Disease in Apolipoprotein E/Endothelial Nitric Oxide Synthase Double-Knockout Mice

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Background—To test whether deficiency in endothelial nitric oxide synthase (eNOS) affects atherosclerosis development, we compared lesion formation in apolipoprotein E (apoE)/eNOS-double knockout (DKO) and apoE-knockout (KO) control animals.

Methods and Results—After 16 weeks of “Western-type” diet, apoE/eNOS-DKO males and females showed significant increases in lesion area of 93.6% and 59.2% compared with apoE-KO mice. All apoE/eNOS-DKO animals studied developed peripheral coronary arteriosclerosis, associated with perivascular and myocardial fibrosis, whereas none of the apoE-KO mice did. Transthoracic echocardiography showed a significantly increased left ventricular wall thickness and decreased fractional shortening in DKO animals. Mean arterial pressure was increased in DKO mice and was comparable in degree to eNOS-KO animals. Male DKO animals developed atherosclerotic abdominal aneurysms and aortic dissection.

Conclusions—eNOS deficiency increases atherosclerosis in Western-type diet–fed apoE-KO animals and introduces coronary disease and an array of cardiovascular complications, including spontaneous aortic aneurysm and dissection. This phenotype constitutes the first murine model to demonstrate distal coronary arteriosclerosis associated with evidence of myocardial ischemia, infarction, and heart failure. Hypertrophy and reduced left ventricular function cannot be explained by increased blood pressure alone, because eNOS-KO animals do not develop these complications. (Circulation. 2001;104:448-454.)

Key Words: arteriosclerosis ■ nitric oxide synthase ■ coronary disease ■ aneurysm ■ hypertension

Nitric oxide (NO) plays important roles in endothelium-dependent vasodilation. It is released by the vascular endothelium in response to various stimuli, including acetylcholine and blood flow. Diseased segments of coronary arteries constrict, rather than dilate, in response to the endothelium-dependent vasodilator acetylcholine, a phenomenon called endothelial dysfunction. The molecular mechanisms responsible may lie in substrate (L-arginine) or cofactor (tetrahydrobiopterin) deficiency for nitric oxide synthases (NOS), alterations in membrane signaling, or enhanced degradation of NO. Aside from effects on vascular tone, NO has physiological properties that may be antiatherogenic, including inhibition of smooth muscle cell proliferation, platelet aggregation and adhesion, and leukocyte activation and adhesion. NO may serve as an oxidant, however, as well as an antioxidant. Under certain conditions of substrate and cofactor deficiency, NOS enzymes can produce superoxide rather than NO. In human atherosclerotic plaques, all 3 isoforms, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), are present. Although most experimental data support a protective role of eNOS, the precise roles of nNOS, iNOS, and eNOS in lesion formation have not been proved.

Atherosclerotic lesions do not develop at random locations throughout the vasculature but rather occur in areas of predilection, such as areas of curvature or branch vessels with nonlaminar flow. The eNOS gene is regulated acutely by shear force through changes in enzyme activity and chronically by changes in gene expression. Local changes in eNOS expression or activity have been proposed to target lesion development to areas of predilection.

To study the contribution of eNOS to lesion formation, we combined a genetic model of eNOS deficiency with a mouse model of atherosclerosis, the hyperlipidemic apolipoprotein E–knockout (apoE-KO) mouse. Unlike most mouse strains, which are highly resistant to atherosclerosis, apoE-KO mice develop atherosclerotic lesions in a distribution closely resembling human disease. Disease progression can be sub-
stantially accelerated by a “Western-type” atherogenic diet. By breeding eNOS-knockout (eNOS-KO) mice with apoE-KO mice, we bypassed problems due to lack of specificity of NOS inhibitors. To avoid genetic background effects, we used highly inbred animals, all backcrossed for 10 generations to the C57BL/6J strain.

In the present study, we show that chronic deficiency of eNOS increases atherosclerosis in the Western-type diet–fed apoE-KO mouse model. Furthermore, in the absence of eNOS, peripheral coronary disease, chronic myocardial ischemia, heart failure, and an array of vascular complications develop that have not been observed in apoE-KO animals.

Methods
Mice
All mice were backcrossed for 10 generations to the C57BL/6J genetic background. eNOS-KO mice and apoE-KO mice (Jackson Laboratories, Bar Harbor, Me) were crossed to generate double heterozygous mice. These mice were then crossed and the offspring genotyped for eNOS by Southern blotting and for apoE by polymerase chain reaction. ApoE-KO animals that were wild-type, heterozygous, or knockout for eNOS were weaned at 21 days and fed a Western-type diet (42% of total calories from fat; 0.15% cholesterol; Harlan-Teklad) for 16 or 24 weeks.

Lesion Assessment
Animals were anesthetized (80 g/mL pentobarbital IP) and perfused with PBS, pH 7.4, and the aorta was dissected from the aortic valve to the iliac bifurcation and fixed in 4% paraformaldehyde overnight. The vessel was opened longitudinally and pinned onto a black wax surface. Serial images were captured with a video camera mounted on a stereo microscope. To identify lipid-rich intraluminal lesions, the aortas were stained with Sudan IV. Color photographs were taken with a Leitz 35-mm camera and used as a reference to identify lesions on the video images. Image analysis was performed with Image Pro Plus (Version 3.0.1; Media Cybernetics). The amount of lesion formation in each animal was expressed as percent lesion area per total area of the aorta.

Tissue Preparation and Histology
The left ventricle (LV) was cannulated, and the animals were perfused with 0.9% NaCl followed by 10% phosphate-buffered formalin. The heart was embedded in paraffin, and serial 5-μm sections were taken in the midventricular short axis and through the aortic valve. Sections were stained with hematoxylin/eosin or Masson’s trichrome. Aortic aneurysms were evaluated according to the Society for Vascular Surgery, International Society for Cardiovascular Surgery. The size of the abdominal aortic aneurysm was calculated from the circumference of the luminal surface (diameter=luminal circumference/r), compared with the circumference of the adjacent, undilated, proximal aortic segment. Aneurysms were defined as an increase in vessel diameter of >50%.

Lipids and Lipoprotein Characterization
Animals were fasted for 4 hours, and plasma total cholesterol was measured with reagents from Sigma (kit 352). Lipoprotein cholesterol distribution was evaluated after fractionation by fast protein liquid chromatography (FPLC) gel filtration with a Superose 6 column.

Blood Pressure Measurements
Invasive blood pressure was measured as previously reported from our laboratory.

Echocardiography
Mice were lightly sedated (20 mg/kg pentobarbital IP) and secured to an imaging platform. M-mode and 2D echocardiographic images were obtained. Measurements were taken from the 2D pictures, and M-mode images served as a reference. Heart rate was measured from the cycle length on the M-mode echocardiograms. Fractional shortening <30% was taken as evidence for impaired LV systolic function.

Statistical Analysis
Statistical analysis was performed with StatView 4.51 (Abacus Concepts, Inc). Two-way ANOVA was used for repeated measures, followed by Scheffé’s F test. A probability value of P<0.05 was considered significant.

Results
The total area of the aorta, total cholesterol, and lipoprotein profile by FPLC did not differ between apoE-KO mice and apoE/eNOS-DKO mice fed the Western-type diet for 16 weeks. Body weight did not differ between genotypes among animals of the same sex. Table 1 shows some hemodynamic characteristics of the animals studied. The mean arterial pressure of apoE/eNOS-DKO animals was elevated by 25 mm Hg compared with apoE-KO control animals and was similar to the blood pressure of eNOS-KO animals.

apoE-KO animals also had a significantly elevated mean arterial pressure compared with age-matched C57BL6 mice, but the elevation was less marked than in apoE/eNOS-DKO mice. Mean heart rate did not differ between apoE/eNOS-DKO, apoE-KO, and C57BL6 animals.

TABLE 1. Characteristics of Animals Studied

<table>
<thead>
<tr>
<th></th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
<th>n</th>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>72.0±5.3</td>
<td>463±13</td>
<td>9</td>
</tr>
<tr>
<td>ApoE-KO</td>
<td>81.6±7.4†</td>
<td>498±14</td>
<td>11</td>
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<tr>
<td>ApoE/eNOS-DKO</td>
<td>101.1±3.0†</td>
<td>449±19</td>
<td>8</td>
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</table>

Mean arterial pressure (MAP) and heart rate (HR) are shown ± SEM. n is the number of mice.

*Significant difference between apoE-KO mice and C57BL6 mice (P<0.01).
†Significant difference between apoE/eNOS-DKO mice and apoE-KO mice (P<0.0007).

Inspection of the longitudinally opened vessels revealed that apoE/eNOS-DKO and apoE-KO control animals develop aortic atherosclerotic lesions in the same areas of predilection, namely, at sites of branch vessels or curvature (Figure 1). After 16 weeks on a Western-type diet, apoE/eNOS-DKO males showed an increase in lesion area of 93.6% compared with apoE-KO males, and females showed an increase of 59.2% compared with apoE-KO females (Figure 2). Consistent with the reports of Caligiuri et al, female apoE-KO control animals had significantly greater lesion areas at the 4-month time point than male apoE-KO animals. This difference at 4 months was not present between female and male apoE/eNOS-DKO animals. ApoE-KO males and females heterozygous for eNOS showed lesion areas that were not significantly different from those of apoE-KO animals (data not shown).

Of 12 male apoE/eNOS-DKO animals that were on the Western-type diet for 16 weeks, 3 developed abdominal aortic aneurysms, with a maximal increase in vessel diameter...
of 180% (Figure 3a). The aneurysms were classified as atherosclerotic suprarenal abdominal aneurysms. Two additional male apoE/eNOS-DKO mice developed acute Stanford type B aortic dissections (Figure 3b). Figure 4 shows the histology of an aortic dissection, with a clearly defined true lumen and false lumen, and atherosclerotic lesions within the true lumen. Neither we nor others have observed macroscopic aortic aneurysms or aortic dissections in apoE-KO mice, regardless of diet.

In a second set of experiments, apoE-KO animals were fed the Western-type diet for 24 weeks to determine whether vascular complications seen in DKO animals at 16 weeks would develop in apoE-KO animals, only later. None of the analyzed apoE-KO animals (n=17) developed macroscopic aortic aneurysms or dissected in apoE-KO mice, regardless of diet.

Aortic lesion area increased in apoE-KO animals fed a Western-type diet for 6 months (males: mean area $31.3 \pm 1.6\%$, n=11; females: mean area $32.1 \pm 3.3\%$, n=6). These values are comparable to those in apoE/eNOS-DKO animals at 16 weeks. Furthermore, like the apoE/eNOS-DKO animals, the lesion areas did not differ between male and female animals.

Histological evaluation of cross sections through the midventricular short axis of the hearts of DKO animals revealed distal coronary arteriosclerosis in all apoE/eNOS-DKO animals studied (n=10), regardless of sex. Coronary arteriosclerosis was associated with perivascular (Figure 5F and 5G) and endomyocardial (Figure 5E) fibrosis and endomyocardial scars consistent with nontransmural infarction (Figure 5H and 5I). Neither distal coronary disease nor fibrosis was seen in apoE-KO control animals (n=7).

To evaluate whether these histological findings coincide with impaired cardiac function, we studied a separate set of apoE/eNOS-DKO and apoE-KO control animals fed a Western-type diet for 16 weeks by transthoracic echocardiography (Table 2). ApoE/eNOS-DKO animals showed a significant increase in the thickness of the interventricular septum and the posterior wall compared with apoE-KO, eNOS-KO, or C57BL6 control animals. Moreover, DKO animals displayed a decrease in fractional shortening. Two of 10 DKO animals displayed massive LV dilation (Figure 6C), with a reduction in fractional shortening to <30%. LV wall thickness and fractional shortening did not differ between age-matched apoE-KO, C57BL6, and eNOS-KO animals.

**Discussion**

Atherosclerotic lesions develop as a result of chronic inflammation of the vessel wall, orchestrated by the interaction of endothelium, monocytes, T lymphocytes, smooth muscle cells, and fibroblasts.\(^{26}\) eNOS, expressed in the endothelial layer, is strategically positioned to affect luminal and abluminal processes. Ample evidence suggests that it plays a protective role. To test the hypothesis that eNOS deficiency, a hallmark of endothelial dysfunction, affects the development of atherosclerosis, we compared lesion formation in apoE-KO mice and apoE/eNOS-DKO mice after 16 weeks of Western-type diet.

We found that genetic deficiency of eNOS significantly increases atherosclerosis in the Western-type diet–fed apoE-KO mouse model. The location of the lesions was similar in apoE/eNOS-DKO animals to that in apoE-KO mice. Aortic lesions still developed in areas of predilection with disturbed flow characteristics. Thus, absence of eNOS does...
not initiate or determine the site of lesion formation in the aorta but appears to accelerate its development.

The apoE/eNOS-DKO mice showed evidence of peripheral coronary disease, chronic myocardial ischemia, and impaired LV function. Nakashima et al.\textsuperscript{19} reported that apoE-KO mice fed a Western-type diet for \textgreater20 weeks develop coronary lesions, but these progress from aortic root lesions and are limited to the proximal coronary arteries. To the best of our knowledge, no studies have found distal coronary lesions in mouse models of atherosclerosis. We found both proximal and distal coronary arteriosclerosis in apoE/eNOS-DKO animals. The lesions were associated with perivascular and endomyocardial fibrosis, suggestive of chronic hypoxic stress, and myocardial scars, consistent with myocardial infarction. We did not detect coronary arteriosclerosis in any of the apoE-KO animals. Transthoracic echocardiography

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Left, Intact normal male apoE-KO aorta and apoE/eNOS-DKO aorta with a suprarenal abdominal aortic aneurysm. Atherosclerotic lesions appear white. Inset to right shows higher-magnification views of abdominal aortas. White arrows indicate left renal artery. Right, apoE/eNOS-DKO aorta from male mouse with Stanford type B dissection extending past renal arteries. Red arrows indicate start and end of dissection.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Histochemistry of aortic dissection displayed in Figure 3 (right), stained with hematoxylin-eosin (left) and Masson’s trichrome (right). Top, Proximal thoracic aorta with atherosclerotic lesion with fibrous cap and necrotic core (red arrows). White arrows indicate displaced outermost layer of aortic wall. Middle, Midthoracic aorta showing atherosclerotic lesion (red arrows) in true lumen (TL) and false lumen (FL). Bottom, Abdominal aorta showing large, red cell-filled false lumen.}
\end{figure}
revealed that apoE/eNOS-DKO animals had globally impaired systolic function and significant reduction in fractional shortening. This was associated with an increase in LV end systolic diameter. Furthermore, the DKO animals developed concentric LV hypertrophy. Neither hypertrophy nor dilation was found either in age-matched apoE-KO animals or in eNOS-KO animals.

Five of 12 male apoE/eNOS-DKO animals developed peripheral vascular complications of aortic dissection and aortic aneurysm. Aneurysm formation is thought to occur by weakening of the elastic laminae in the media by matrix metalloproteinases and/or other proteases. Microscopic aneurysm formation and medial elastin destruction have been observed after a diet containing the toxic component cholic acid in apoE-KO mice and LDL receptor–KO mice. Aneurysms have also been induced in wild-type mice by perfusing the aorta with elastase. Spontaneous aortic dissection and macroscopic aneurysm formation, however, have not been reported in mouse models of atherosclerosis. The addition of eNOS deficiency to the Western-type diet–fed

<p>| TABLE 2. Transthoracic Echocardiographic Measurements |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>ApoE-KO (n=10)</th>
<th>ApoE/eNOS-DKO (n=10)</th>
<th>C57BL/6 (n=10)</th>
<th>eNOS-KO (n=9)</th>
</tr>
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<tbody>
<tr>
<td>FS, %</td>
<td>52±3.7</td>
<td>40±3.3††</td>
<td>55±2.4</td>
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<tr>
<td>PWT, mm</td>
<td>0.62±0.04</td>
<td>1.00±0.04††</td>
<td>0.71±0.03</td>
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<tr>
<td>IVS, mm</td>
<td>0.87±0.02</td>
<td>1.06±0.02††</td>
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<td>LVIDs, mm</td>
<td>1.60±0.01</td>
<td>2.02±0.03††</td>
<td>1.51±0.01</td>
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<tr>
<td>LVIDd, mm</td>
<td>3.27±0.01</td>
<td>3.26±0.02</td>
<td>3.43±0.01</td>
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<tr>
<td>HR, bpm</td>
<td>456±36</td>
<td>491±40</td>
<td>469±21</td>
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Fractional shortening (FS), posterior wall thickness (PWT), interventricular septum thickness (IVS), LV inner diameter at end systole (LVIDs) and end diastole (LVIDd), and heart rate (HR) are shown. Values are mean±SEM; n=number of mice. ApoE-KO and apoE/eNOS-DKO mice were fed the Western-type diet for 16 weeks. C57BL/6 and eNOS-KO mice were fed normal chow diet.

*Significant difference vs apoE-KO mice.
†Significant difference vs C57BL/6 mice.
‡Significant difference vs eNOS-KO mice.
apoE-KO mouse model results in the development of aneurysm and aortic dissection without the additional injury of cholic acid or elastase treatment.

The sex difference between males and females at 16 weeks of Western-type diet in apoE-KO mice is no longer seen at 24 weeks, suggesting that the rate, not the ultimate extent, of lesion formation is greater in female apoE-KO mice. Lesion areas in the apoE/eNOS-DKO mice fed for 16 weeks were comparable to those of apoE-KO mice fed for 24 weeks, and no differences were noted between male and female DKO mice. Despite equivalent lesion burdens, apoE-KO mice fed for 24 weeks did not show coronary arteriosclerosis, aortic aneurysm, or dissection. Although coronary disease was present in all the apoE/eNOS-DKO mice studied, female DKO mice did not develop aneurysms or aortic dissection. The reasons for this are unclear, because female DKO mice have comparable lesion burdens and blood pressure elevations.

Our results differ from those of Knowles et al,30 who also found increased atherosclerosis in apoE/eNOS-DKO mice, using a different eNOS-KO mouse for the generation of the DKO animals. These authors did not find distal coronary lesions, myocardial ischemia, heart failure, or vascular complications of aortic aneurysm and aortic dissection. Although coronary disease was present in all the apoE/eNOS-DKO mice studied, female DKO mice did not develop aneurysms or aortic dissection. The reasons for this are unclear, because female DKO mice have comparable lesion burdens and blood pressure elevations.

Hypertension may certainly play a role in the development of atherosclerosis and its cardiac and vascular complications. ApoE-KO animals progressively develop endothelial dysfunction and hypertension.31,32 We found that apoE-KO animals had higher blood pressure than C57BL6 control animals. ApoE/eNOS-DKO animals, however, showed a more marked increase in blood pressure, comparable to that of eNOS-KO mice.23 This indicates that the blood pressure effects of apoE gene deficiency and eNOS gene deficiency are not additive but rather reflect different degrees of endothelial dysfunction. Furthermore, hypertension alone cannot account for the cardiac hypertrophy, impaired contractility, or vascular complications seen in the apoE/eNOS-DKO mice, because these complications do not occur in eNOS-KO mice. ApoE-KO animals heterozygous for eNOS did not show an increase in lesion formation or an increase in blood pressure compared with apoE-KO control animals, suggesting a threshold, rather than a gene-dose, effect of eNOS deficiency on both parameters.

The mechanisms by which hypertension contributes to atherosclerosis have not been fully elucidated. Inasmuch as eNOS plays important roles in recruitment of inflammatory cells, oxidative stress, and vascular response to injury, eNOS deficiency/endothelial dysfunction could be one of the molecular mechanisms linking hypertension to atherosclerosis. Knowles et al30 attempted to separate the effects of hypertension from those of NO deficiency by administering the ACE inhibitor enalapril to their apoE/eNOS-DKO mice. Enalapril corrects both the hypertension and the enhanced atherosclerosis, but this may be because ACE itself and its products, angiotensin II and bradykinin, play important roles in atherogenesis.33,34

In summary, eNOS deficiency changes the disease pattern of atherosclerosis in Western-type diet–fed apoE-KO animals, introducing peripheral coronary disease, signs of chronic myocardial ischemia, and vascular complications, such as aortic dissection and abdominal aortic aneurysm formation. The phenotype of apoE/eNOS-DKO mice more closely resembles the spectrum of cardiovascular complications seen in human atherosclerosis and constitutes the first murine model to demonstrate spontaneous distal coronary arteriosclerosis associated with LV dysfunction. These findings support the concept that restoration of eNOS function in patients with atherosclerosis is an important therapeutic goal.
Acknowledgments
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References
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