

Hypertension Does Not Account for the Accelerated Atherosclerosis and Development of Aneurysms in Male Apolipoprotein E/Endothelial Nitric Oxide Synthase Double Knockout Mice

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Background—Apolipoprotein E (apoE)/endothelial nitric oxide synthase (eNOS) double knockout (DKO) mice demonstrate accelerated atherosclerosis and develop abdominal aortic aneurysms and aortic dissection, suggesting a role for eNOS in suppressing atherogenesis. To test whether accelerated atherosclerosis and aortic aneurysms were due to hypertension, we administered hydralazine to male apoE/eNOS DKO mice to reduce blood pressure.

Methods and Results—Male apoE/eNOS DKO mice were treated with hydralazine in their drinking water (250 mg/L) using a dose that lowers the blood pressure to levels seen in apoE KO mice. The mice were fed a Western-type diet for 16 weeks, and lesion formation was assessed by inspection of the vessel and staining with Sudan IV. Hydralazine-treated, normotensive male apoE/eNOS DKO mice developed increased aortic lesion areas ($30.0 \pm 2.8\%$, $n=11$) compared with male apoE KO mice ($14.6 \pm 0.8\%$, $n=7$). The extent of lesion formation was not significantly different from male apoE/eNOS DKO mice that were not given hydralazine ($28.3 \pm 3.1\%$, $n=9$). Four of 11 hydralazine-treated male apoE/eNOS DKO mice developed abdominal aortic aneurysms.

Conclusions—Hypertension is not required for the accelerated atherosclerosis seen in apoE/eNOS DKO animals, and control of hypertension during a 16-week period does not prevent aortic aneurysm formation. (*Circulation*. 2001;104:2391-2394.)

Key Words: arteriosclerosis ■ nitric oxide synthase ■ aneurysm ■ hypertension ■ hydralazine

Mouse models of atherogenesis, such as Western-type diet-fed apolipoprotein E (apoE) knockout (KO) mice,^{1,2} have been combined with mouse models of endothelial nitric oxide synthase (eNOS) deficiency^{3,4} to study the interactions between eNOS and atherogenesis.^{5,6} We previously found that apoE/eNOS double knockout (DKO) mice develop increased aortic lesions after 16 weeks of a Western-type diet; these lesions are also associated with distal coronary artery disease and left ventricular dysfunction. Furthermore, male apoE/eNOS DKO mice developed abdominal aortic aneurysm and aortic dissection.⁵ These complications have not been observed in apoE KO mice.^{1,2}

Because eNOS deficiency is associated with hypertension, both by itself³ and in the presence⁵ of apoE deficiency, it is possible that hypertension contributes to the increased extent of atherosclerotic lesions and the development of aortic aneurysms. To test this hypothesis, we treated male apoE/eNOS DKO mice with hydralazine at a dose that lowers the blood pressure down to the levels seen in apoE KO mice. Hydralazine-treated, normotensive, apoE/eNOS DKO mice

still developed increased atherosclerotic lesions compared with apoE KO mice. Hydralazine did not decrease the extent of lesion formation, because lesion areas were comparable between untreated and hydralazine-treated animals. Furthermore, 4 of 11 male mice treated with hydralazine developed abdominal aortic aneurysms, demonstrating that hypertension is not required for the development of aortic aneurysms in the apoE/eNOS DKO mouse model. These findings suggest that the effects of eNOS deficiency extend beyond hypertension and argue that eNOS plays important roles in suppressing atherogenesis separate from blood pressure regulation.

Methods

Mice

eNOS KO mice,³ apoE KO mice¹ (Jackson Laboratories, Bar Harbor, Maine), and apoE/eNOS DKO mice⁵ were all on a C57BL/6 genetic background. Mice were fed a Western-type diet (42% of total calories from fat; 0.15% cholesterol; Harlan-Teklad) for 16 weeks. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

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Blood Pressures and Lesion Areas of Animals Studied

	MAP, mm Hg	Lesion Area, %	No. of Aneurysms
ApoE KO	81.6±7.4 (11)	14.6±0.8 (7)	0/18
ApoE/eNOS DKO	101.1±3.0* (8)	28.3±3.1* (9)	5/12*
Hydralazine-treated apoE/eNOS DKO	85±6.6† (11)	30.0±2.8‡ (11)	4/11‡

Mean arterial pressure (MAP) and lesion area are mean±SEM (n). Number of Aneurysms is shown as number of animals with abdominal aortic aneurysm divided by the total number of animals examined in each group. Comparisons were made by ANOVA followed by Scheffe's F test for post-hoc comparisons.

* $P<0.05$ compared with apoE KO mice; † $P<0.05$ compared with untreated apoE/eNOS DKO mice (difference was not statistically significant when compared with apoE KO mice); ‡ $P<0.05$ compared with apoE KO mice (difference was not statistically significant when compared with untreated apoE/eNOS mice).

Blood Pressure Measurement and Hydralazine Treatment

Invasive blood pressure was measured by femoral artery catheterization using a Millar catheter.⁷ Hydralazine was added to the drinking water at 250 mg/L. Pilot experiments indicated that this dose of hydralazine reduces the blood pressure of eNOS KO mice and apoE/eNOS DKO mice to levels seen in apoE KO mice. The blood pressure of each treated apoE/eNOS DKO mouse was measured immediately before euthanization after 16 weeks of treatment on a Western-type diet.

Lesion Assessment

Animals were euthanized and perfused with PBS (pH 7.4). The aorta was opened longitudinally, and video images were captured using a dissecting microscope. To identify lipid-rich intraluminal lesions, the aortas were stained with Sudan IV. Image analysis was performed using 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. Aortic aneurysms were defined according to the criteria of the Society for Vascular Surgery, as an increase in vessel diameter of >50% over that of the proximal, adjacent, undilated segment.

Statistical Analysis

Statistical analysis was performed using StatView 4.51 (Abacus Concepts, Inc). ANOVA with Scheffe's F test for post hoc compar-

ison was used to compare the results from the different groups. $P<0.05$ was considered significant.

Results

Hydralazine was added to the drinking water at 250 mg/L throughout the entire study period. Male mice were fed a Western-type diet, which contains 42% of calories from fat, for 16 weeks. Immediately before euthanization, all of the hydralazine-treated apoE/eNOS DKO mice were subjected to invasive measurements of blood pressure. As shown in the Table, the mean arterial pressure of the hydralazine-treated mice was 85.0 ± 6.6 mm Hg, which is significantly lower than that of untreated apoE/eNOS DKO mice (101.1 ± 3.0 mm Hg). The hydralazine-treated mice have blood pressures comparable to apoE KO mice, which have a mean blood pressure of 81.6 ± 7.4 mm Hg.

Despite the lowering of blood pressure, the hydralazine-treated apoE/eNOS DKO mice still had increased lesion areas compared with apoE KO mice. In fact, hydralazine treatment did not significantly affect the extent of atherosclerosis in the apoE/eNOS DKO mice, as shown in the Table. The hydralazine-treated apoE/eNOS DKO mice developed lesions with

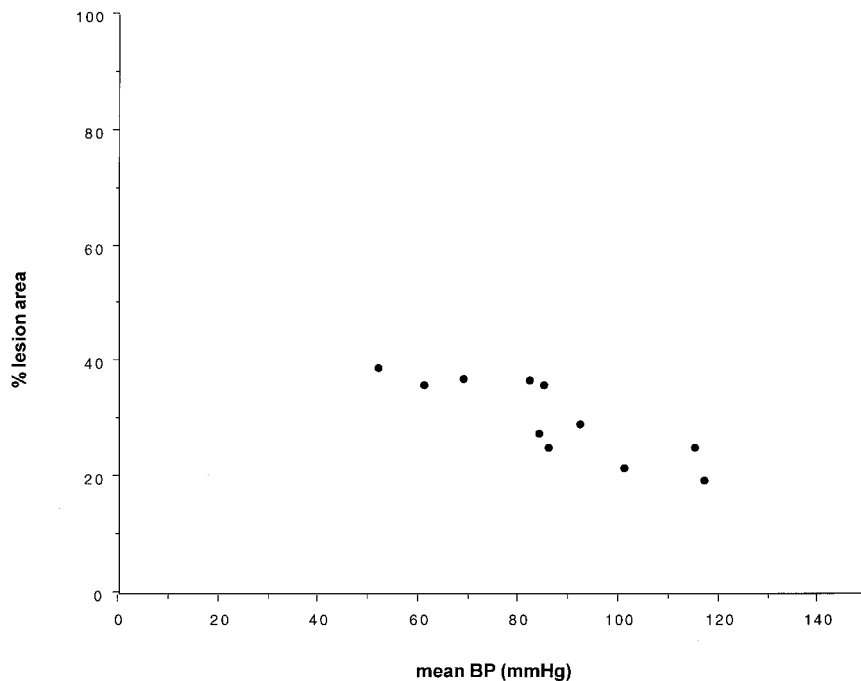


Figure 1. Scattergram showing percent lesion area and mean blood pressure (BP) of each hydralazine-treated apoE/eNOS DKO mouse.

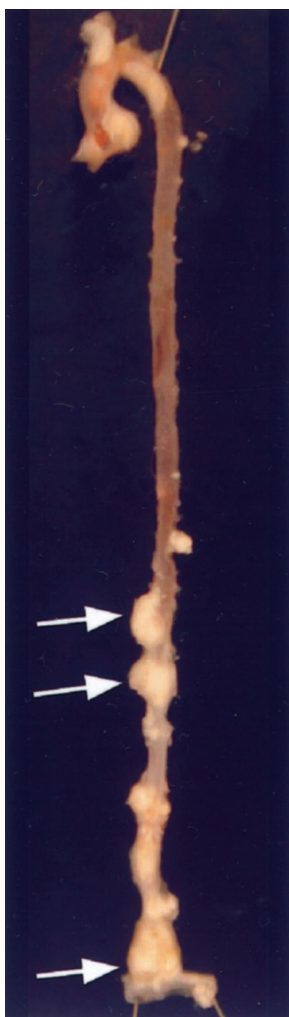


Figure 2. Unopened aorta from a hydalazine-treated apoE/eNOS DKO mice fed a Western-type diet for 16 weeks. Aortic aneurysms are indicated by white arrows. Similar aneurysms were found in apoE/eNOS DKO mice without hydalazine treatment, but not in apoE KO mice (data not shown).

areas of $30.0 \pm 2.8\%$ after 16 weeks, whereas the untreated apoE/eNOS DKO mice developed lesion areas of $28.3 \pm 3.1\%$ after 16 weeks. Furthermore, the percent lesion area did not increase with mean blood pressure, as shown in Figure 1.

Four of 11 male mice in this study developed abdominal aortic aneurysms, which were defined as an increase in aortic luminal diameter to $>50\%$ of the proximal segment, as seen in Figure 2. In untreated apoE/eNOS DKO mice after 16 weeks of a Western-type diet, 5 of 12 male mice developed aortic aneurysms; 2 additional mice had aortic dissection.⁵

Discussion

We and others have crossed eNOS KO mice with apoE KO mice to study the effect of eNOS gene deficiency on the development of atherosclerosis.^{5,6} ApoE/eNOS DKO mice develop atherosclerosis at an accelerated rate compared with apoE KO mice, confirming that eNOS protects against atherogenesis. However, apoE/eNOS DKO mice are hypertensive, with mean arterial blood pressures similar to eNOS KO mice.^{5,8} Because of this, one possibility is that the

accelerated atherosclerosis may be related to hypertension and not the other effects of eNOS deficiency. Furthermore, the development of aortic aneurysms in some of the male apoE/eNOS DKO mice may be due to hypertension. To test this hypothesis, we used hydalazine to lower the blood pressure of apoE/eNOS DKO mice to the level seen in control apoE KO mice.

Treatment with hydalazine lowered blood pressure in the apoE/eNOS DKO mice, but this did not affect the accelerated development of atherosclerosis in the animals. Lesion areas were the same in both hydalazine-treated and untreated groups. Furthermore, 4 of 11 male hydalazine-treated apoE/eNOS DKO mice developed aortic aneurysms compared with 5 of 12 untreated male apoE/eNOS DKO mice. These differences are not statistically significant.

Knowles et al⁶ also bred apoE/eNOS DKO mice to examine the role of eNOS deficiency in atherogenesis. Despite important differences between their study and our previous one,⁵ in both cases, apoE/eNOS DKO mice developed significantly greater atherosclerotic lesion areas than control apoE KO mice, confirming a role for eNOS in the suppression of atherogenesis. Knowles et al⁶ found that enalapril lowered the blood pressure of apoE/eNOS DKO mice and reduced the development of lesions.⁶ This indicates that the mechanism of enalapril action does not depend on eNOS. However, because of the intrinsic effects of ACE inhibitors on atherogenesis, enalapril treatment does not answer the question of whether the increase in lesion area seen in apoE/eNOS DKO animals is due to their increased blood pressure.

Aside from its potent neuroendocrine action, angiotensin II also has paracrine and autocrine vascular effects, including smooth muscle cell growth and migration, macrophage activation, platelet aggregation, and increased oxidative stress.^{9,10} Thus, the difference in outcome after treatment with hydalazine and enalapril may reflect the effects of enalapril itself on atherogenesis. Similar effects of ACE inhibitors that are independent of blood pressure lowering have also been noted in other studies.¹¹ Furthermore, a direct comparison of hydalazine and lisinopril at doses that lower blood pressure equally also shows additional effects of ACE inhibitors unrelated to blood pressure effects.¹²

In another study, Kauser et al¹³ reported that apoE KO mice treated with L-N-arginine methyl ester (L-NAME) at doses that do not raise blood pressure still have increased lesion areas. These results agree with ours in that the predominant effect of NOS inhibition on atherogenesis seems independent of blood pressure.

Aneurysm formation is thought to occur by a weakening of the media by tissue remodeling and local changes in the vessel wall. Hypertension may play an additional role in terms of mechanical forces and shear stress. However, the presence of aneurysms in hydalazine-treated male apoE/eNOS DKO mice suggest that the inherent changes in the vessel wall that predispose to aneurysm formation can occur independent of hypertension.

Our results indicate that the effects of eNOS gene deficiency on accelerating atherogenesis are not solely due to hypertension, in that lowering the blood pressure to normal in

the apoE/eNOS DKO mice did not reduce the lesion area. Furthermore, control of blood pressure in these animals does not prevent aneurysm formation. Overall, these results suggest the existence of blood pressure-independent mechanisms by which eNOS deficiency accelerates atherogenesis. These may include well-established effects of NO on vascular smooth muscle proliferation, leukocyte-endothelial interactions, and modulation of platelet aggregation.^{14,15}

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