Role of endogenous nitric oxide in progression of atherosclerosis in apolipoprotein E-deficient mice

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EDNO is synthesized by NOS-3 during the oxidation of the substrate L-arginine to L-citrulline. L-Arginine treatment has been shown to be beneficial in conditions when EDNO synthesis is impaired (5, 6). The exact mechanism of the beneficial effect of L-arginine is not completely understood, but may be related to increased circulating levels of the endogenous inhibitor N\textsuperscript{G}\textsuperscript{-}dimethylarginine (asymmetric dimethylarginine, ADMA) (20). Opposite to L-arginine, the substrate analog N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) is an inhibitor of NOS and it has been shown to evoke acceleration of atherosclerosis in rabbits (4, 22, 23).

Atherosclerosis develops and progresses spontaneously in the apolipoprotein E-deficient [apoE-knockout (KO)] mouse, which makes this model ideal to investigate the impact of certain manipulations on the progression of the disease (21, 28, 29). However, a link between EDNO and atherosclerosis progression has not been studied yet in this model. In the present study we examined the pathogenic link between progression of atherosclerosis and changes in endogenous NO synthesis by investigating the effect of L-NAME as well as L-arginine administration on aortic lesion development.

METHODS

Animals and experimental design. Five-week-old male apoE-KO and age-matched male C57Bl/6J mice were obtained from Jackson Laboratories. At 4 mo of age apoE-KO mice were randomly allotted into three treatment groups (n = 10 in each group): 1) control group receiving tap water (control), 2) NOS inhibitor-treated group provided with 100 mg/l L-NAME in the drinking water (L-NAME), and 3) NOS substrate-treated group provided with 100 mg/l L-arginine in the drinking water (L-Arg). To determine endothelial function at the beginning of the study, age-matched 4-mo-old apoE-KO and C57Bl/6J mice were used. All mice were housed in groups of four per cage and provided with standard mouse chow and drinking water with or without L-NAME or L-arginine ad libitum. Water intake and weight gain were monitored weekly during the 8-wk duration of the study. The dose of L-NAME was selected on the basis of our previous experience using L-NAME treatment in rabbits (23) and a published report by Elhage et al. (7) using L-NAME treatment in apoE-KO mice. The efficacy of NOS inhibition was determined by measuring the inhibition of endothelial NO-mediated relaxation in isolated aortic rings. The experiments were conducted according to protocols approved by the Animal Care Committee at Berlex Biosciences, in agreement with the recommendation of the American Association for the Accreditation of Laboratory Animal Care.
Organ chamber studies. Two rings (~3 mm wide) of the distal end of the thoracic aortas were isolated from each mouse. The distal region of the thoracic aorta was purposely selected to avoid lesions in the rings used for the contractility studies. The isolated aortic segments were mounted in organ chambers (Schüler, Hugo Sachs Electronics) filled with physiological saline solution of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.17 KH₂PO₄, 2.0 NaHCO₃, 0.026 EDTA, 11 glucose, and 5 HEPES. Changes of isometric tension in response to drug treatment were measured with force transducers (model F30, Hugo Sachs Electronics) and recorded by a data acquisition and analysis system (MP100WSW, BIOPAC Systems). EDNO production was determined either by measuring endothelium-dependent increases in tension to N²-nitro-L-arginine (L-NNA, 100 μM) in rings half-maximally contracted by the thromboxane analog U-46619 (30 nM) or by measuring endothelium-mediated relaxation to ACh (10 nM-10 μM) in rings contracted with U-46619 (30 nM). These studies were performed in the presence of indomethacin (10 μM) to block the production of vasoactive prostanoids. Relaxation to sodium nitroprusside (1 nM-1 μM) was also investigated at the end of the study to test the responsiveness of the vascular smooth muscle to exogenous NO.

Atherosclerotic lesion size determination. The proximal part of the thoracic aorta up to the aortic origin was isolated and cleaned from adherent connective tissue. Atherosclerotic plaque area was quantified by analyzing the open luminal surface image of the formalin-fixed aortic arch and thoracic aorta. The atherosclerotic lesions were visible and clearly distinguishable from the not plaque covered areas on the luminal surface of the vessels without staining. The colored images were taken by a digital camera (Sony) mounted on a microscope (Nikon SMZ-2T) and attached to a computer (Dell). The images were recorded in 24-bit true image format. The data analysis was performed by image-analysis software (Simple, Compix Mars, PA) with the investigator setting the threshold color for plaque inclusion at the beginning of the evaluation of all groups and keeping the same setting between the analyses of each group. The plaque area was expressed as a percentage of the total luminal surface.

Serum cholesterol and triglyceride measurement. Serum samples were collected by cardiac puncture into serum collection tubes (Microtainer serum separator tubes, Becton Dickinson, NJ) at the time when the animals were killed by CO₂ inhalation and centrifuged at 1,000 g for 10 min. Serum total cholesterol and triglycerides were measured at Consolidated Veterinary Diagnostics (Sacramento, CA).

Blood pressure measurement in conscious mice. Mice were anesthetized with 100 mg/kg ketamine (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and 6 mg/kg xylazine (Rompun, Bayer, Shawnee Mission, KS) injected intramuscularly. A PE-50 tube, stretched to reduce the tip diameter to the size of the artery and filled with 20 U/ml heparin in saline was inserted ~1 cm into the right carotid artery. The tube was then exteriorized through the skin at the back of the neck. The incisions were closed and the catheter secured with a suture to the skin. The distal end of the catheter was attached to a blood pressure transducer (fluid-filled DTX Pressure Transducers, Viggo-Spectramed, Oxnard, CA). When the animals recovered from anesthesia, baseline mean arterial pressure was monitored until it became stable followed by recording for ~60 min.

Calculation and statistical analysis. The concentration of U-46619 (30 nM) causing half-maximal contraction (EC₅₀) was calculated from full dose-response curves. Increase in tension by L-NNA is expressed as a percentage of the initial contraction by U-46619. Relaxation evoked by ACh and sodium nitroprusside is expressed as percent inhibition of the contraction by U-46619. The concentration of drug (expressed as ~log[M]) required to produce EC₅₀ was determined by computer-assisted interactive nonlinear regression analysis (Graphpad Prism). Lesion area is expressed as a percentage of the total luminal surface of the thoracic aorta. Results are presented as means ± SE for the number of experiments indicated (n) using different animals. Multiple comparisons of the mean values were performed by ANOVA followed by Newman-Keuls test. Data were considered to be significantly different at P < 0.05.

RESULTS

Endothelial dysfunction in 4-mo-old apoE-KO mice. Endothelium-dependent increase in isometric tension of aortic rings contracted with U-46619 (30 nM) in response to L-NNA (100 μM) is shown in Fig. 1A. Rings from the 4-mo-old apoE-KO mice responded to L-NNA with significantly less tension development (38.4 ± 4.3%), indicating significantly attenuated basal EDNO production in these vessels compared with rings from age-matched C57Bl/6J mice (65.7 ± 11.5%) (P < 0.05). Responses to U-46619 were not different in apoE-KO (360 ± 60 mg) and in C57Bl/6J (370 ± 40 mg) mice, respectively. Endothelium-dependent relaxation of thoracic aortic rings to ACh are shown in Fig. 1B. ACh-induced EDNO-mediated relaxations were not statistically different between apoE-KO (EC₅₀ 6.9 ± 0.2 ~log[M]) and maximum relaxation 70.7 ± 3.8% and age-matched C57Bl/6J (EC₅₀ 6.8 ± 0.1 ~log[M]) and maximum relaxation 66.9 ± 4.8% mice at 4 mo of age. Similarly, endothelium-independent relaxation to sodium nitroprusside was not different between aortic rings isolated from apoE-KO (EC₅₀ 8.3 ± 0.1 ~log[M]) and maximum relaxation 93.8 ± 2.4% and C57Bl/6J (EC₅₀ 8.2 ± 0.1 ~log[M]) and maximum relaxation 86 ± 3.1% mice (Fig. 1C).

Effect of L-NAME and l-arginine treatment on endothelium-dependent vascular responses in the apoE-KO mouse. L-NNA-induced contraction was 36.1 ± 2.4% in control apoE-KOs as shown in Fig. 2A. L-NNAME-treated group (EC₅₀ 6.8 ± 0.1 ~log[M]) and maximum relaxation 35.4 ± 12.9% compared with the untreated age-matched apoE-KO controls (EC₅₀ 7.2 ± 0.1 ~log[M]) and maximum relaxation 82.9 ± 4.9% as shown in Fig. 2B. Thus 8-wk treatment with L-NNAME caused a more severe endothelial dysfunction than was present in apoE-KO mice at that age. ACh-induced relaxation was not significantly different in the L-arginine-treated group (EC₅₀ 7.3 ± 0.1 ~log[M]) and maximum relaxation 75.4 ± 4.5% compared with control apoE-KO mice. This indicates that delivery of the NOS substrate at 4–6 mo of age did not affect EDNO-mediated endothelium-dependent vasorelaxation in apoE-KO mice. Endothelium-independent relaxation to sodium nitroprusside was not altered either by the L-NNAME or by the
L-arginine treatments indicating no change in smooth muscle responsiveness to NO (Fig. 2C).

Effect of L-NAME and L-arginine treatment on atherosclerotic plaque development. L-NAME treatment of apoE-KO mice for 8 wk resulted in significant acceleration of plaque formation in the aortic arch and thoracic aortas. Figure 3 shows images of the isolated aortae prepared for luminal surface analysis taken from representative animals of the control (Fig. 3A), L-NAME-treated (Fig. 3B) and L-arginine-treated (Fig. 3C) groups at the end of the 8-wk treatment. In the control group, 22.6 ± 4.8% of the total aortic surface was covered with atherosclerotic plaques as determined by image analysis. In the L-NAME-treated group the calculated plaque-to-surface ratio was 64.3 ± 2.5%, significantly (P < 0.0001) greater compared with control (Fig. 4). Plaque-to-surface ratio in the L-arginine group (26.6 ± 1.9%) was not different from that of control (Fig. 4).

Effect of L-NAME treatment on mean arterial blood pressure and serum lipids. Mean arterial blood pressure of conscious mice was not significantly different.
between the groups on different treatments at the end of the 8-wk treatment period (Table 1). Serum total cholesterol and triglyceride levels were also not different between the treatment groups (Table 1).

**DISCUSSION**

The results of this study indicate for the first time that decreased production of endogenous NO is a significant progression factor in the development of atherosclerosis in the apoE-KO mouse. Novel findings include the demonstration of 1) endothelial dysfunction in 4-mo-old apoE-KO mouse, 2) significant acceleration of atherosclerotic plaque development in apoE-KO mice after NOS inhibition, and 3) lack of beneficial effect of L-arginine treatment on EDNO production and atherosclerosis in the apoE-KO mouse.

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Endothelial dysfunction in the apoE-KO mouse. We found that L-NNA-induced endothelium-dependent contraction is significantly reduced in 4-mo-old apoE-KO mice, whereas the ACh-induced EDNO-mediated vasorelaxation was not different compared with age-matched C57Bl/6J controls. The L-NNA-induced endothelium-mediated contraction is often called basal EDNO-mediated response (14, 26). It reflects the amount of constitutively available EDNO, which modulates contractile responses of the underlying smooth muscle of the vascular wall. ACh-induced relaxation has been referred to as stimulated EDNO release resulting in larger amounts of EDNO in response to receptor-activated Ca\(^{2+}\) influx into endothelial cells. Maintained responses to ACh were reported earlier in apoE-KO mouse aorta compared with apoE/LDL receptor (LDLR)-double knockout mice (2, 15).

Selective suppression of basal, but not ACh-induced EDNO release has been reported earlier in the aorta of rabbits as a result of ovariectomy (14), in the aorta of

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**Table 1.** Body weight, water intake, serum total cholesterol, serum triglycerides, and mean arterial blood pressure in three treatment groups of apoE-KO mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-NNAME</th>
<th>L-Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>32.6±0.8</td>
<td>31.8±0.7</td>
<td>33.8±1.2</td>
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<tr>
<td>Daily water intake, ml</td>
<td>6.3±1.9</td>
<td>4.0±0.6</td>
<td>4.3±0.7</td>
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<tr>
<td>Serum total cholesterol, mg/dl</td>
<td>852.5±76.8</td>
<td>970.2±83.2</td>
<td>1085.3±111.5</td>
</tr>
<tr>
<td>Serum triglycerides, mg/dl</td>
<td>113.7±10.9</td>
<td>239.8±76.1</td>
<td>234.7±75.8</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mmHg</td>
<td>84±6.1</td>
<td>97±8.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6–10 experiments in each group of 6-mo-old mice. apoE-KO, apolipoprotein E-deficient-knockout (KO) mice; ND, not determined; L-NNAME, N\textsuperscript{-}nitro-L-arginine methyl ester.
rats in response to superoxide anion inactivation (19), and in the aorta of estrogen receptor-deficient (ERKO) mice compared with wild-type controls (26). Differential impairment of basal and stimulated EDNO release suggests that separate pathways may regulate these processes. The finding that in the hypercholesterolemic apoE-KO mouse the basal EDNO-mediated vascular response is attenuated suggests that the mechanism responsible for basal EDNO production is sensitive to atherosclerotic milieu. Acylation of NOS-3 preferentially targets the enzyme to the plasma membrane of endothelial cells (9, 16) to caveolae where it localizes in close proximity to the structure protein caveolin (9, 16). Membrane localization of NOS-3 is important for optimal NO production of the enzyme in response to stimuli (10). Caveolin-1 exerts an inhibitory effect on NO production (11), which seems to be further augmented under hypercholesterolemic condition (8). It is possible that hypercholesterolemia in the apoE-KO mice contributes to suppression of basal EDNO production by a similar mechanism. Flow/shear stress-induced endothelium-dependent brachial artery vasodilation has been shown to be impaired in hypercholesterolemic patients (17) and is regarded as an early sign of endothelial dysfunction and atherosclerotic vessel disease (33).

Inhibition of NOS by L-NAME facilitates lesion progression in the apoE-KO mouse. To investigate the role of endogenous NO in the progression of atherosclerosis in apoE-KO mouse, we administered L-NAME in the drinking water of the animals similar to earlier studies in hypercholesterolemic rabbits (4, 22, 23). Treatment of the animals started at 4 mo of age and was carried out for 8 wk. This time period was chosen on the basis of our earlier observation of linear plaque progression in apoE-KO mice between 2 and 8 mo of age (28).

Eight-week treatment of apoE-KO mice with L-NAME resulted in a significant inhibition of NO-mediated, endothelium-dependent responses compared with untreated, age-matched control apoE-KO mice (Fig. 2). Aortic plaque measurement of these animals revealed significant acceleration of atherosclerotic lesion development in the thoracic aorta (Figs. 3 and 4). The 30% inhibition of EDNO-mediated responses was accompanied by a nearly 70% increase in plaque-covered aortic surface, supporting the anti-atherosclerotic effect of EDNO observed in other species (5, 23). Accelerated progression of atherosclerosis in apoE-KO mice has been reported earlier in response to high-cholesterol diet (21), hyperglycemia (29) and most recently to hypertension (31). However, it is unlikely that additional risk factors could contribute to the results of this study, because neither blood pressure nor serum cholesterol levels were significantly different in the treated animals compared with controls (Table 1).

NO is a pleiotropic molecule exerting several potentially anti-atherosclerotic effects, such as inhibition of platelet aggregation, leukocyte adhesion, chemokine expression, nuclear factor-kB induction, and smooth muscle proliferation (for review see 3, 12, 13, 30). It is also a potent scavenger of the oxygen-derived free radical, superoxide anion (27). Diminished production of EDNO may lead to an insufficient control of these events resulting in increases in plaque size because of the additive effects of these different pathophysiological processes. It has been shown that L-NAME treatment of ovariectomized female apoE-KO mice resulted in significant increase in oxidation of LDL (7). Because the oxidized form of LDL is highly atherogenic, it could be an important contributor to the acceleration of vascular lesions.

However, despite increased levels of modified lipoproteins in the L-NAME-treated ovariectomized female apoE-KO mice, no acceleration of plaque formation was observed (7). The difference between the present finding and that reported by Elhage et al. (7) is most likely the result of the differences in the assessment of vascular lesions. In the present study we measured total plaque surface area instead of the microscopic evaluation of sections taken from the aortic origin used by Elhage et al. (7).

NOS-3 may not be the only source of endogenous NO in the atherosclerotic lesion. Expression of inducible NOS (NOS-2) has been shown in advanced human atherosclerotic lesions (32). Whereas NOS-3-generated NO is implicated in vasoproliferation, the role of excessive NO generated by NOS-2 is implicated in plaque rupture in association with apoptosis (13). Because L-NAME is not a selective inhibitor of NOS-3, our study does not allow differentiating between the effects of inhibition of these two different NOS isoforms on the atherosclerotic process. However, the results of this study demonstrate that inhibition of endogenous NO synthesis, irrespective of the source of NO, facilitates progression of early atherosclerotic lesion development in apoE-KO mice. The effect of L-NNA, causing an increase in the tension of preconstricted vessels, was abolished after endothelium removal (data not shown) (26). This suggests that the source of NO in these vessels was restricted to the endothelium, most likely to the NOS-3 isofrom. This question can be more specifically addressed when a selective NOS isofrom inhibitor will be available or by crossing selective NOS isofrom-deficient mice with the apoE-KO mouse.

Lack of beneficial effect of L-arginine treatment in the apoE-KO mouse. Another approach to investigate the role of endogenous NO in the progression of atherosclerosis is to increase its production. Cooke and coworkers (5) reported that treating hypercholesterolemic rabbits with L-arginine, the substrate of NOS enzymes, resulted in partial restoration of endothelial function, which was associated with a significant reduction in the extent of atherosclerosis. This and similar experimental findings lead to clinical studies, demonstrating that L-arginine treatment of hypercholesterolemic patients can improve endothelial function (for review see Ref. 17).

L-Arginine treatment also reduced atherosclerotic plaque area in LDLR-deficient mice (LDLR-KO) (1). On the basis of these results, we also attempted to investigate the effect of L-arginine treatment on the development of atherosclerosis in apoE-KO mice. However, we could not find any changes in plaque develop-
ment in the thoracic aorta after 8 wk of treatment with the NOS substrate (Figs. 3 and 4).

The exact mechanism of the beneficial effect of L-arginine in atherosclerosis is not yet understood. Measurement of L-arginine concentration (both in cells and in plasma) showed that this substrate is in a sufficient amount in most situations studied. However, relative substrate deficiency may still occur under pathological situations like atherosclerosis. Changes in intracellular compartmentalization in response to LDL treatment of cultured endothelial cells have been suggested to cause substrate uncoupling of NOS enzyme activity leading to oxygen free radical generation (24). Increasing L-arginine concentration of the culture media reversed this phenomenon (24). Induction of other L-arginine-consuming enzymes, such as NOS-2 or arginase in activated macrophages under atherosclerotic conditions may also result in substrate limitation for the NOS-3 enzyme (25). Most recently circulating levels of the endogenous NOS inhibitor, the arginine analog ADMA, have been suggested to correlate with the extent of atherosclerosis and contribute to the endothelial dysfunction associated with the disease (20). Indeed, studies in isolated vessels and cultured endothelial cells demonstrated that ADMA is able to suppress endogenous NO production (18). In all of these studies decreased EDNO production or diminished endothelium-dependent relaxation was reported as a result of limited L-arginine availability.

The beneficial effect of L-arginine reported earlier, has been associated with the restoration of impaired ACh-induced EDNO-mediated relaxation. In our study the ACh-induced NO-mediated relaxation was not impaired in the 4- and 6-mo-old apoE-KO mice. The lack of dysfuction of the stimulated NO release may provide a potential explanation for the missing benefit on plaque development by L-arginine treatment. The fact that L-arginine treatment had no effect on the impaired basal EDNO response may support the hypothesis that basal and agonist-stimulated EDNO production is regulated by different mechanisms.

In conclusion, our study demonstrates that inhibition of endogenous NO synthesis accelerates atherosclerosis in the apoE-KO mouse. This finding supports the vasoprotective function of NO in cardiovascular diseases and provides further evidence that lack of endogenous NO, manifested in impaired NO-mediated vasorelaxation, is an important factor in the progression of atherosclerosis.

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