Effects of Chronic Treatment With L-Arginine on Atherosclerosis in ApoE Knockout and ApoE/Inducible NO Synthase Double-Knockout Mice

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Objective—L-Arginine serves as a substrate for the formation of NO by the NO synthase (NOS) enzymes. In some studies, dietary supplementation of L-arginine reduces atherosclerosis through the restoration of NO release and improvement in endothelial function. In the present study, we investigate the effect of L-arginine supplementation on the development of atherosclerosis in a mouse model.

Methods and Results—Apolipoprotein E (apoE) knockout (ko) and apoE/inducible NOS (iNOS) double-ko mice were fed a western-type diet with or without L-arginine supplementation in the drinking water (25 g/L). L-Arginine did not affect the lesion area after 16 weeks or 24 weeks in apoE ko mice. However, L-arginine negates the protective effect of iNOS gene deficiency. In contrast to apoE/iNOS dko mice without arginine supplementation, lesion areas were increased in apoE/iNOS double-ko mice with arginine supplementation at 24 weeks. This was associated with an increase in thiobarbituric acid–reactive malondialdehyde adducts, nitrotyrosine staining within lesions, and a decrease in the ratio of reduced tetrahydrobiopterin to total biopterins.

Conclusions—Although L-arginine supplementation does not affect lesion formation in the western-type diet–fed apoE ko mice, it negates the protective effect of iNOS gene deficiency in this model. This raises the possibility that L-arginine supplementation may paradoxically contribute to, rather than reduce, lesion formation by mechanisms that involve lipid oxidation, peroxynitrite formation, and NOS uncoupling. (Arterioscler Thromb Vasc Biol. 2003;23:97-103.)

Key Words: atherosclerosis ■ arginine ■ apolipoprotein E ■ nitric oxide

Nitric oxide has been implicated in protection against atherosclerosis through the reduction of oxidative stress, inflammation, proliferation, and platelet aggregation. NO is formed from the terminal guanido nitrogen of L-arginine and molecular oxygen by NO synthase (NOS) enzymes. Dietary supplementation of L-arginine has been reported to improve endothelial reactivity in humans and to reduce atherosclerosis in hypercholesterolemic animal models by increasing endothelial NO production. However, in other studies, L-arginine does not reduce atherosclerosis. Although most studies support a protective role for endothelial production of NO by the endothelial NOS (eNOS) isoform, the inducible NOS (iNOS) isoform, present in activated macrophages and found within atherosclerotic lesions, may be proatherogenic. Genetic deficiency of iNOS reduces diet-induced atherosclerosis in the apoE knockout (ko) mouse model.13,14

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In the present study, we explore the chronic effects of L-arginine supplementation and the role of iNOS in the pathophysiology of diet-induced atherosclerosis in apoE ko and apoE/iNOS double-ko (dko) mice. We hypothesized that arginine would provide additional substrate for eNOS, resulting in reduction in the amount of atherosclerosis. We find that arginine supplementation does not affect the degree of atherosclerosis in apoE ko mice after 16 and 24 weeks of a western-type diet. Surprisingly, arginine negated the protective effect of iNOS deficiency in the apoE/iNOS dko mice. To explore the molecular mechanisms by which arginine worsens atherosclerosis, we measured plasma lipid peroxides as markers of lipid oxidation and performed immunohistochemistry for nitrotyrosine and NOS isoforms. We measured plasma total biopterin and tetrahydrobiopterin (BH4) levels, because oxidation of biopterins may cause NOS enzyme uncoupling.

Methods

Mice

ApoE ko and iNOS ko mice were obtained from Jackson Laboratories (Bar Harbor, Me). All mice were backcrossed for 10 generations to the C57BL/6J genetic background. ApoE ko and iNOS ko mice were crossed to generate double-heterozygous mice, which were
intercrossed, and the offspring were genotyped for iNOS and apoE using polymerase chain reaction.\textsuperscript{14} ApoE ko and apoE/iNOS dko mice were weaned at 21 days and fed a western-type diet (42% of total calories from fat and 0.15% from cholesterol, Harlan Teklad) for 16 or 24 weeks. L-Arginine was given to the animals by supplementing the drinking water with 25 g/L, a dose previously shown to reduce lesion formation in hypercholesterolemic rabbits\textsuperscript{7} and LDL-receptor ko mice.\textsuperscript{15} All procedures were approved by the institutional animal use and care committee and conform to National Institutes of Health guidelines.

**Lesion Assessment**

The aorta was dissected and analyzed as previously described.\textsuperscript{14,16} Animals were euthanized with pentobarbital (80 μg/kg IP), and the aorta was dissected from the aortic valve to the iliac bifurcation and fixed in 4% paraformaldehyde overnight. Adventitial tissue was removed, and the aorta was opened longitudinally and pinned onto a black wax surface by using microneedles (Fine Science Tools). Serial images of the submersed vessels were captured with a video camera and a still 35-mm camera. Lipid-rich intraluminal lesions were stained with Sudan IV. Image analysis was performed by using Image Pro Plus (version 3.0.1, Media Cybernetics). The amount of aortic lesion formation in each animal was measured as percent lesion area per total area of the aorta.

**Plasma Lipoperoxide Measurement**

Malondialdehyde–thiobarbituric acid adduct (MDA-TBA) was measured by high-performance liquid chromatography (HPLC) as previously described.\textsuperscript{16,17} Blood was drawn from the heart while the animal was under pentobarbital anesthesia, and it was collected in tubes containing 0.5 mol/L EDTA. Plasma was fractionated by using a C18 column (Micro Bondapak, Waters) with the use of an AKTA purifier HPLC system (Amersham Pharmacia Biotech). A standard curve was constructed by using tetraethoxypropane standards.

**Plasma BH\textsubscript{4} and Total Bioppterin Levels**

The amount of total bioppterin, which is made up of BH\textsubscript{4}, dihydrobiopterin (BH\textsubscript{2}), and oxidized bioppterin, was assayed according to the method of Fukushima and Nixon.\textsuperscript{18} Plasma (20 μL) was deproteinized by the addition of perchloric acid. After centrifugation, acidic iodine solution (1% iodine/2% KI in 1N HCl) was added to fully oxidize the reduced forms of bioppterin. The oxidation mixture was allowed to stand for 1 hour at room temperature, and the excess amount of iodine was reduced by the addition of ascorbic acid. Bioppterin was separated and quantified by HPLC with fluorescence detection (excitation at 350 nm and emission at 440 nm). For the measurement of alkaline-stable bioppterin (BH\textsubscript{4}+oxidized bioppterin), the deproteinized plasma was oxidized with alkaline iodine solution (1% iodine/2% KI in 0.1N NaOH). The amount of BH\textsubscript{4} was calculated by subtracting the amount of alkaline-stable bioppterin from total bioppterin.

**Plasma Total Cholesterol Assay**

Blood samples were obtained at the time of euthanasia for determination of total cholesterol by the enzymatic method of Sigma Diagnostics (Sigma kit 352) with the use of a microtiter plate reader at 500 nm (Spectra MAX 250, Molecular Devices).

**Immunohistochemistry and Confocal Imaging**

Immunohistochemistry was performed by using antibodies to iNOS, eNOS, neuronal NOS (nNOS), and nitrotyrosine and macrophage markers.\textsuperscript{14} The following antibodies were used: (1) goat anti-mouse iNOS antibody (1:100 dilution, N22920, Transduction Laboratories); (2) rabbit anti-eNOS antibody (1:50 dilution, N30030, Transduction Laboratories); (3) rabbit anti-nNOS antibody (1:50 dilution, N31030, Transduction Laboratories); (4) mouse anti-mouse nitrotyrosine antibody (1:100 dilution, 05-233, Upstate Biotechnology); and (5) macrophage marker (1:100 dilution, NCL-macro, Vector Laboratories). Sections from aortic arch, thoracic, and abdominal aorta were rehydrated, blocked with the use of normal serum, and incubated with primary antibody for 2 hours at room temperature. Visualization was performed by using a Vectastain ABC kit, with DAB used as the substrate in the case of iNOS, eNOS, nNOS, and nitrotyrosine and with AEC used in the case of the macrophage marker. Slides were counterstained with hematoxylin.

Multiple labeling and confocal imaging was performed for nitrotyrosine and macrophage markers. Sections were unmasked in 10 mmol citric acid at 95°C for 5 minutes and blocked with 5% host serum for the secondary antibody for 30 minutes, followed by incubation of primary antibody for 2 hours at room temperature. The secondary antibody was diluted 1:100. The sections were incubated with goat anti-mouse IgG FITC (1:100 dilution, 115-095-003, Jackson Immunology Research) for nitrotyrosine and with donkey anti-mouse IgG Texas Red (1:100 dilution, 715-075-156, Jackson Immunology Research) for the macrophage marker in the dark at room temperature for 30 minutes. Visualization was performed by using a confocal microscope with a BP530/30 filter for FITC and a LP590 filter for Texas Red with the use of a Leica TCS NT4D confocal imaging system (Leica). When the FITC (green) and Texas Red (red) signals colocalize, a yellow signal is seen.

**Western Blot Analysis**

Aortas were dissected, and samples were snap-frozen in liquid nitrogen. Western blots were performed with polyclonal anti-eNOS antibody (1:500 dilution, Transduction Laboratories) and anti-nNOS antibody (1:500 dilution, Transduction Laboratories). An anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:1000 dilution, Transduction Laboratories) was used.

**Statistical Analysis**

Statistical analysis was performed by using StatView 4.5.1 (Abacus Concepts, Inc). Two-way ANOVA was used for repeated measures, followed by the Scheffé F test. A probability value of \( P<0.05 \) was considered significant.

**Results**

**Lesion Surface Area**

At the time of euthanasia, there was no difference in body weight between the untreated and L-arginine–treated groups. L-Arginine did not change the lesion area in apoE ko mice after 16 weeks of treatment (16.4±0.7% [mean±SEM] without L-arginine and 17.5±0.8% with L-arginine, \( P>0.05 \) [not statistically significant]) or after 24 weeks of treatment (31.6±0.8% without and 29.0±1.1% with L-arginine, \( P>0.05 \)), as seen in Figure 1. Furthermore, L-arginine supplementation did not reduce atherosclerosis in apoE/iNOS dko mice. At the 16-week time point, lesion areas showed a trend toward an increase with L-arginine supplementation (12.9±0.6% without and 15.9±3.2% with L-arginine, \( P>0.05 \)). After 24 weeks, lesion areas were significantly higher in the L-arginine–treated apoE/iNOS dko mice (25.9±1.7%) than in the untreated apoE/iNOS dko group (19.4±1.0%, \( P<0.05 \)).

**Plasma MDA-TBA Levels and Cholesterol Levels**

 Plasma MDA-TBA adducts were determined as measures of lipid oxidation. As shown in Figure 2, the MDA-TBA level found in WT mice was 1.22±0.25 μmol/L. After 16 weeks, apoE ko mice had a level of 3.07±0.19 μmol/L. Although untreated apoE/iNOS dko mice had less MDA-TBA (2.01±0.2, 2.3 μmol/L), apoE/iNOS dko mice treated with L-arginine had a level of 2.92±0.27 μmol/L. After 24 weeks, apoE ko mice had an MDA-TBA level of 3.55±0.29 μmol/L. Untreated apoE/iNOS dko mice had a level of 2.70±0.33.
μmol/L, and apoE/iNOS dko mice treated with L-arginine had a level of 3.61 ± 0.59 μmol/L. These results indicate that the increase in atherosclerosis associated with L-arginine supplementation in the apoE/iNOS dko mice was associated with corresponding increases in markers of lipid oxidation. The total plasma cholesterol values with L-arginine treatment in apoE ko and apoE/iNOS dko mice at 16 or 24 weeks were not significantly different than those values in the untreated groups, nor did the values differ significantly between the 3 groups (data not shown).

**Bioperterin Levels**

To determine whether L-arginine supplementation affects bioperterin levels, we measured plasma bioperterin by HPLC under acidic and alkaline conditions. Under acid conditions, the levels measured reflect total bioperterin: BH$_4$, BH$_2$, and oxidized bioperterin. Under alkaline conditions, BH$_4$ is broken down, so the levels measured reflect BH$_2$ and oxidized bioperterin. The difference between the 2 conditions is the BH$_4$ level. As seen in Figure 3, the total bioperterin levels of apoE/iNOS dko mice treated with L-arginine at 6 months (138.7 ± 7.5 nmol/L) were significantly higher than those levels in untreated apoE/iNOS dko mice (92.4 ± 4.2 nmol/L, \( P < 0.05 \)) or apoE ko mice (95.8 ± 6.8 nmol/L, \( P < 0.05 \)). However, the absolute BH$_4$ levels were not statistically different between the 3 groups: 51.4 ± 5.9 nmol/L in apoE/iNOS dko mice treated with arginine, 38.1 ± 4.7 nmol/L in untreated apoE/iNOS dko mice, and 46.5 ± 5.9 nmol/L in apoE ko mice.

**Immunohistochemical Staining and Western Blot Analysis**

Immunohistochemical staining for iNOS after 4 months of L-arginine treatment was robust in apoE ko mice (Figure 4A) but was not detected in apoE/iNOS dko mice (Figure 4B). Staining for macrophage marker (Figure 4C) and for nitrotyrosine (Figure 4D) was observed in the apoE/iNOS dko mice at 4 months of L-arginine treatment. Multiple staining and confocal imaging showed that the distribution of nitrotyrosine in lesions matched that of the macrophage marker precisely (Figure 5). Nitrotyrosine staining in apoE/iNOS mice without L-arginine treatment (column 1) was weaker than the staining in apoE/iNOS dko mice with L-arginine treatment for 6 months (column 2) and in apoE ko mice without L-arginine treatment (column 3).

To determine whether eNOS or nNOS might be the source of NO in the apoE/iNOS dko mice, we performed immunohistochemistry for eNOS and nNOS in mice with and without
L-arginine treatment. As seen in Figure 6, eNOS and nNOS are present within lesions in both groups. eNOS is present predominantly in the endothelium, whereas nNOS is present in the intima. Quantification by Western blot analysis showed no difference in the intensity of the signals for either nNOS or eNOS between the untreated and L-arginine–treated groups.

Discussion
Endothelial production of NO serves important functions in blood vessels, including vasodilation, suppression of smooth muscle proliferation, and inhibition of leukocyte adhesion and platelet aggregation.1,2 Endothelial dysfunction, a common feature of hypertension, diabetes mellitus, and atherosclerosis, is associated with decreased bioavailability of endothelial NO. Supplementation with L-arginine has been considered as an approach to correct endothelial dysfunction by providing substrate for NO production by eNOS.3–5 However, nNOS and iNOS are expressed in atherosclerotic lesions and may play proatherogenic roles. In the apoE ko mouse model, genetic deficiency of eNOS increases atherosclerosis, consistent with a protective role of eNOS.16 In contrast, genetic deficiency of iNOS decreases atherosclerosis,13,14 suggesting that iNOS contributes to oxidative stress in the vessel wall.19

In the present study, we tested the hypothesis that L-arginine supplementation would reduce the atherosclerotic burden in the apoE ko mouse model. We postulated that this effect would be more pronounced in the apoE/iNOS dko mice, which lack the potentially proatherogenic iNOS isoform, by providing substrate for only the protective eNOS isoform. However, our results show that chronic oral administration with L-arginine does not reduce atherosclerosis in

Figure 3. Plasma biopterin levels at 6 months. Total biopterin levels are shown. Total biopterin levels are the sum of BH4 (solid bars) and BH2 and oxidized biopterin (open bars). Error bars are shown for BH4 and BH2 plus oxidized biopterin. *P<0.05 for differences between apoE/iNOS plus arginine group and the other 2 groups.

Figure 4. Immunohistochemistry for iNOS in apoE ko mouse showing staining with use of DAB (A), iNOS in apoE/iNOS dko mouse showing no staining with use of DAB (B), macrophage marker in apoE/iNOS dko mouse showing staining with use of AEC (C), and nitrotyrosine in apoE/iNOS dko mouse showing staining with use of DAB (D). All these animals were treated with L-arginine for 4 months.
apoE ko mice. Furthermore, it negates the protective effect of iNOS deficiency on atherosclerosis in apoE ko mice. To place our results in the context of other studies, L-arginine supplementation inhibits atherosclerosis in LDL receptor ko mice. However, in agreement with our results, it does not influence endothelial function or alter the lesion burden in apoE ko mice. In cholesterol-fed rabbits, L-arginine reduces lesion area and improves vasodilator

Figure 5. Multiple labeling and confocal imaging of nitrotyrosine and macrophage marker in aortic plaques. Columns are as follows: left column, apoE/iNOS dko without L-arginine; middle column, apoE/iNOS dko with L-arginine for 6 months; and right column, apoE ko mouse without L-arginine. Rows are as follows: top, immunofluorescence imaging of nitrotyrosine using FITC; middle, immunofluorescence imaging of macrophage marker using Texas Red; and bottom, confocal double imaging for nitrotyrosine and macrophage marker. Yellow signals represent the colocalization of signals from FITC and Texas Red.

Figure 6. Expression of eNOS and nNOS in apoE/iNOS dko mice at 6 months of western-type diet. A, Immunohistochemical staining for eNOS is seen in the endothelium, and staining for nNOS is seen in the intima of L-arginine-treated and untreated animals. B, Results of Western blot analysis of aortic lesions for eNOS and nNOS expression are shown. No significant difference was found in the levels of eNOS or nNOS between L-arginine–treated and untreated groups.
function in some studies, but not others. In humans, dietary supplementation and intravenous administration of L-arginine restore endothelial function. However, other studies fail to show a beneficial effect on coronary heart disease. Thus, there is no consensus on whether L-arginine supplementation definitively protects against atherosclerosis.

The molecular mechanisms by which L-arginine supplementation negates the protective effect of iNOS deficiency in the apoE/iNOS dko mice are not clear. Increased atherosclerosis after L-arginine supplementation was associated with an increase in MDA-TBA adducts, indicating more lipid oxidation. In addition, nitrosyltrosine immunoreactivity was more pronounced in L-arginine–treated than in untreated apoE/iNOS dko mice at 6 months. Nitrosyltrosine staining also colocalizes precisely with a macrophage marker in lesions. Nitrosyltrosine indicates the presence of peroxynitrite, which is formed by the reaction of NO with superoxide anion (O$_2^-$).

Indeed, these processes have been documented in apoE ko mice. Peroxynitrite is a powerful oxidant that can induce lipid peroxidation, oxidize LDL, promote nitrosyltrosine, and decompose to form toxic hydroxyl radicals. Peroxynitrite can also oxidize BH$_4$, which in turn causes pathological “uncoupling” of NOS enzymes, leading to further production of O$_2^-$. Because L-arginine supplementation increases nitrosyltrosine staining, it must either increase NO production in the setting of sufficient O$_2^-$ to form peroxynitrite, increase O$_2^-$ production, or both.

Although NAD[P]H oxidase is a major source of O$_2^-$ in the vasculature, another source is NOS enzymes themselves that have been uncoupled by cofactor deficiency. In the absence of sufficient BH$_4$, the oxidation of L-arginine is no longer coupled to NADPH consumption, and NOS isoforms catalyze the formation of O$_2^-$ at the oxygenase domain. In fact, BH$_4$ levels appear to regulate the ratio of O$_2^-$ and NO made by NOS enzymes; thus, abnormalities in biopterin metabolism are an important mechanism of endothelial dysfunction.

To determine whether L-arginine supplementation depletes BH$_4$ stores, leading to enzyme uncoupling, we measured plasma biopterin levels in apoE/iNOS dko mice with and without L-arginine supplementation. Although absolute BH$_4$ levels were not significantly different between groups, arginine supplementation was associated with a significant increase in total biopterin. These results suggest that L-arginine supplementation leads to oxidation of biopterin, with a compensatory increase in total biopterin levels to maintain BH$_4$. The ratio of BH$_4$ to BH$_2$ and the ratio of BH$_4$ to oxidized biopterin have been correlated with the uncoupling of NOS enzymes. Which NOS isof orm is responsible for peroxynitrite and superoxide formation? Because the iNOS gene has been disrupted in the apoE/iNOS dko mice, it cannot be iNOS. eNOS and nNOS isoforms are both present in the aortic atherosclerotic lesions in these animals (Figure 6), although there was no detectable upregulation in the amount of reactive protein by immunohistochemistry or Western blot. Thus, preexisting eNOS and nNOS may be the source of NO and, if uncoupled, superoxide.

In conclusion, we demonstrate that L-arginine supplementation does not reduce lesion formation in the western-type diet–fed apoE ko mouse model. Furthermore, it negates the protective effect of iNOS gene deficiency and is associated with increased serum markers of lipid peroxidation and nitrosyltrosine within lesions. These results raise the important possibility that L-arginine supplementation may paradoxically contribute to lesion formation by the generation of superoxide anion and peroxynitrite.

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