Effect of Chronic Treatment With the Inducible Nitric Oxide Synthase Inhibitor N-Iminoethyl-L-Lysine or With L-Arginine on Progression of Coronary and Aortic Atherosclerosis in Hypercholesterolemic Rabbits

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Background—We examined the implications of iNOS in atherosclerosis progression using the selective inducible NO synthase (iNOS) inhibitor N-iminoethyl-L-lysine (L-NIL) in hypercholesterolemic rabbits.

Methods and Results—Nine rabbits were fed a 0.3% cholesterol diet for 24 weeks (Baseline group); 25 animals were maintained on the diet and treated for 12 extra weeks with L-NIL (5 mg kg⁻¹·d⁻¹, L-NIL group, n=8), vehicle (Saline group, n=9), or L-arginine (2.25%, L-Arg group, n=8). In abdominal aortas of Saline rabbits, the lesions (53.7±5.7%, Baseline) increased to 75.0±5.0% (P<0.05) but remained unaltered in the L-NIL group (63.4±6.6%). Similar results were obtained for the intima/media ratio in thoracic aortas. In coronary arteries, the intima/media ratio was comparable in Baseline (0.68±0.18) and Saline (0.96±0.19) rabbits but decreased to 0.34±0.19 (P<0.05) in L-NIL rabbits. L-Arginine had beneficial effects only in abdominal aortas. An increased thoracic aorta collagen content was found in Saline and L-Arg but not in L-NIL rabbits. In thoracic aortas of the Saline group, acetylcholine caused modest relaxations that slightly increased by L-arginine but not by L-NIL. Relaxations to nitroglycerin were ameliorated by L-NIL.

Conclusions—This is the first study showing that chronic treatment with an iNOS inhibitor, L-NIL, limits progression of preexisting atherosclerosis in hypercholesterolemic rabbits. Increased intimal collagen accumulation may participate in iNOS-induced atherosclerosis progression. (Circulation. 2000;102:1033-1038.)

Key Words: nitric oxide synthase ■ L-NIL ■ arginine ■ atherosclerosis ■ inhibitors

Nonendothelial NO synthase (NOS) activity in atherosclerosis was first suggested in 1993 in hypercholesterolemic rabbits.¹⁻³ Later, mRNA and protein of inducible NOS (iNOS) was described in experimental⁴,⁵ and human atherosclerosis.⁶,⁷ High aortic cGMP and decreased vasoconstrictions illustrate that iNOS in atherosclerosis is functional.¹²,⁹ Can NO formed from iNOS interfere with plaque development? Because matrix metalloprotease (MMP) activity is enhanced by NO,¹⁰ iNOS could cause plaque progression by promoting its vulnerability. Angiogenic actions of NO could accelerate plaque formation through neovascularization.¹¹ NO could contribute to necrotic core development of plaques through its proapoptotic properties.¹² However, NO could inhibit plaque development by its antiadhesive, antioxidant, and antiproliferative properties¹³,¹⁴; eg, gene transfer of iNOS reduces transplant atherosclerosis,¹⁵ and endothelium-leukocyte interactions augment it, in iNOS knockout mice.¹⁶ Selective iNOS inhibitors, such as N-iminoethyl-L-lysine dihydrochloride (L-NIL), are now available¹⁷; their impact on iNOS activity in atherosclerosis is not known. Nonselective NOS inhibitors enhance lesion formation, probably through eNOS inhibition¹⁸; they lose activity when administered after 8 months of hypercholesterolemia.¹⁹ We evaluated the impact of iNOS inhibition on atherosclerotic lesion progression. Because iNOS is present only in well-developed lesions, rabbits were given a 0.3% cholesterol–enriched diet for 24 weeks and were then treated with L-NIL for 12 weeks.

iNOS induction in atherosclerosis might decrease substrate availability, causing an imbalance between NO and superoxide generation from NOS. L-Arginine given with the hypercholesterolemic diet reduces atherosclerotic lesions and restores endothelial-dependent relaxations²⁰⁻²²; when it was administered after the onset of the pathological lesions, some authors found a reduced lesion progression and amelioration.
of endothelium-dependent relaxations,\textsuperscript{12,23} whereas others suggested short-lasting effects of L-arginine.\textsuperscript{24,25} In humans, the beneficial effects of L-arginine may depend on the stage of coronary artery disease\textsuperscript{26–29}; thus, L-arginine improved endothelium-dependent vasodilatation in young hypercholesterolemic humans\textsuperscript{26–28} but failed to improve cardiovascular performance in severely hypercholesterolemic patients.\textsuperscript{29} We therefore studied L-arginine treatment on atherosclerosis progression by a protocol similar to that for L-NIL.

**Methods**

**Study Design**

Male New Zealand White rabbits (8 weeks) started a 0.3% cholesterol diet as described previously.\textsuperscript{1,2,9,30} After 24 weeks, atherosclerosis was evaluated in 9 rabbits (Baseline group). The remaining rabbits were separated into 3 groups kept on the diet for 12 more weeks. Eight rabbits received treatment with L-NIL 5 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} (L-NIL group), administered by 2 subcutaneously implanted ML4 miniosmotic pumps (Charles River) that delivered the drug in the jugular vein. Nine rabbits were treated with saline (Saline group), and 8 rabbits received L-arginine HCl (2.25%) in drinking water (L-Arg group), using conditions previously described.\textsuperscript{22} Experiments were conducted in accordance with institutional guidelines for animal studies.

At monthly intervals, total cholesterol, triglycerides, and HDL cholesterol plasma levels were determined. Blood pressure and heart rate were measured before and at the end of the treatment.

**Drugs**

The following drugs were used: acetylcholine, ADP, phenylephrine, and L-arginine HCl (Sigma); L-NIL (A. Cordis); asymmetrical di- methyl arginine (ADMA) and symmetrical dimethyl arginine (SDMA) (Calbiochem); nitroglycerin (Besins-Iscovesco); and collagen and ATP (Coultronic).

**Plasma L-Arginine, ADMA, SDMA, and L-NIL Concentrations**

Plasma concentrations of L-arginine, ADMA, SDMA, and L-NIL were monitored as previously described.\textsuperscript{31} After acetonitrile extraction, samples were incubated with OPA reagent (5.4 mg/mL Na\textsubscript{2}CO\textsubscript{3}, 2.64 g/L) containing 0.4% 2-mercaptoethanol and analyzed with high-performance liquid chromatography. Separation was obtained with a C\textsubscript{18} Hypersil BDS-CPS 3-μm column, followed by analytical separation on a Kromasil 100-3.5 C\textsubscript{18} column with a fluorescence detector. Samples were eluted with carbonate buffer. Drug concentrations were determined against internal standards.

**Tissue Preparation**

Animals were anesthetized with sodium pentobarbital (30 mg/kg IV), and carotid arteries were cannulated for blood sampling (10 mL). Thoracic aortas and hearts were harvested. Hearts were immersed in Bouin’s fixative before paraffin embedding; sections (4 μm) were cut to examine left common coronary arteries by histomorphometry after hemalun-eosin staining.

Aortic rings 3 mm wide were cut: the first ring, just below the aortic arch, was embedded in OCT compound (Ames, snap-frozen in liquid nitrogen, sectioned (7 μm), and stained by hemalun-eosin or orcein or stored after acetone fixation at −80°C. The next ring was used for cGMP determination and the following rings for reactivity studies. Abdominal aortas were fixed in 10% buffered formalin, immersed in oil red O solution (Bio-optica) diluted in ethanol (1:1), and rinsed in water before being processed.

**Tissue cGMP**

Thoracic aortas were homogenized in a glass potter homogenizer (50 mmol/L, phosphate buffer, 1 mmol/L theophylline, pH 7.4) and sonicated. An aliquot was used for DC protein assay (Bio-Rad). The remaining homogenate was centrifuged at 3000g for 15 minutes, and supernatants were frozen at −30°C. cGMP was quantified with a competitive enzyme immunoassay (Cayman) as previously described.\textsuperscript{8}

**Histomorphometry**

Sections of thoracic aortas and coronary arteries were analyzed with an automated computerized image analyzer (Visiologic 1000, Bio-com). The intimal cross-sectional area (mm\textsuperscript{2}) was determined by subtracting the lumen area from the area enclosed by the internal elastic lamina. The medial area was determined by subtracting the area enclosed by the internal elastic lamina from that enclosed by the external elastic lamina. Mean areas were calculated to deduce intima/media (I/M) ratios.

**Planimetry**

Fixed abdominal aortas were incised longitudinally, pinned open, and placed flat on a Sygland glass with transillumination for photography. The plaque surface was expressed as percentage of total surface quantified with the image analyzer.

**Sirius Red Polarization Method for Collagen**

Thoracic aortas were cut into sections, rinsed with distilled water, and incubated with 0.1% Sirius red F3BA (BDH) in saturated picric acid for 90 minutes. Sections were mounted in permanent nonaqueous media (Kindler) and visualized by polarization microscopy.\textsuperscript{52} Intimal Sirius red-stained areas were measured with the image analyzer and calculated as percentage of total intimal area.

**Immunohistochemistry**

The following antibodies were used: anti-iNOS monoclonal antibody (1/50) (Transduction) and isotype-matched mouse IgG (Dako) as control antibodies and the specific cell markers (Dako) anti-α-actin (HHF-35) for smooth muscle cells (SMCs) (1/200), anti-rabbit macrophages (RAM-11) (1/250), and anti-CD45 RO (clone UCHL1) (1/100) for activated T cells.

Thoracic aorta sections were rehydrated in Tris-buffered saline (TBS: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.6; Dako), then in TBS containing 0.5% BSA (Sigma); nonspecific immunoglobulin-binding sites were blocked by TBS containing 4% BSA. Sections were incubated with a monoclonal primary or a negative control antibody for 2 hours at 20°C. The presence of antigen was revealed with biotinylated goat anti-mouse antibodies followed by incubation with streptavidin–alkaline phosphatase complex (Vector). Alkaline phosphatase was visualized with Vector red alkaline phosphatase substrate incubation. Slides were rinsed with TBS, counterstained with hematoxylin (Gill’s formula; Vector), and mounted in permanent nonaqueous medium. SMCs, macrophages, T lymphocytes, and iNOS were quantified on the intima with the automated image analyzer. Positive areas (% of total intima) were determined with a predefined threshold.

**Hematology and Platelet Aggregation**

Platelets, red blood cells, leukocytes, hemoglobin, and hematocrit levels were measured on whole blood with a T-540 counter (Coultronics). Platelet aggregation to ADP or collagen and ATP release were measured in whole blood aggregometers (Chrono-log). For technical reasons, blood samples from 2 Baseline and 1 Saline rabbit could not be tested.

**Vascular Reactivity**

Segments (3 mm) of thoracic aortas were mounted in organ chambers as previously described.\textsuperscript{9,30} Segments were placed at the optimal resting tension (8 g) for atherosclerotic rabbit aortas.\textsuperscript{2,30} During contractions with phenylephrine (1 μmol/L), concentration-response curves to acetylcholine (0.01 to 3 μmol/L) or nitroglycerin (0.001 to 3 μmol/L) were performed.

**Statistical Analysis**

Results are expressed as mean ± SEM. Statistical analysis was performed by 1-way ANOVA with Newman-Keuls complementary anal-
ysis or 2-way ANOVA (treatment versus time of treatment) with repeated measures. For vascular reactivity studies, IC₅₀ values were determined by linear interpolation. Treatment-effect relationships were compared by 2-way ANOVA with repeated measures on factor concentration. A value of P<0.05 was considered statistically significant.

Results

Physiological and Biological Parameters

No differences in body weight, mean arterial pressure, or heart rate (Saline group: 4.2±0.1 kg, 86±2 mm Hg, and 213±10 bpm) or levels of total cholesterol, HDL cholesterol, or triglycerides (Saline group: 18.7±2.4, 0.39±0.09, and 1.05±0.20 mmol/L) were detected.

Plasma L-arginine concentrations in Baseline rabbits averaged 72.1±11.5 μmol/L and were similar in the 4 groups at 24 weeks; they increased 2-fold after 4 weeks of L-arginine supplementation and remained elevated throughout treatment (176±2.6 μmol/L; P<0.05, 2-way ANOVA); they were unaffected in Saline and L-NIL rabbits. ADMA and SDMA concentrations (0.64±0.07 and 0.27±0.06 μmol/L) were comparable in all groups at 24 weeks and remained stable throughout. L-NIL plasma concentrations in the L-NIL group averaged 11.4±2.3 μmol/L after 4 weeks, 7.8±0.8 μmol/L after 8 weeks, and 10.6±0.9 μmol/L after 12 weeks.

iNOS and Inflammatory Cell Staining

Immunohistochemistry of thoracic aorta sections with the anti-iNOS showed a strong positive signal in all groups (Figure 1); no quantitative differences existed between the groups (Table 1). Lesion infiltration by inflammatory components such as macrophages (RAM-11 antibody) and T lymphocytes (CD45RO antibody) averaged 85% and 6% of the total intimal area, respectively, and was similar in all groups (Table 1).

Planimetry

In the Baseline group, the surface area occupied by atherosclerosis averaged 53.7±5.7%. This was increased in the Saline group (P<0.05, 1-way ANOVA) but not in the L-Arg and the L-NIL groups (Figure 2).

Histomorphometry

The I/M ratio of thoracic aortas of the Baseline group averaged 0.98±0.04. This ratio was increased in the Saline and L-Arg groups (P<0.05, 1-way ANOVA) but not in the L-NIL group (Table 2; Figure 3). Cross-sectional areas of medias were not different between the groups (Table 2).

The I/M ratio of coronary arteries of the Baseline group averaged 0.68±0.18. This ratio was not altered in the Saline and L-Arg groups but decreased in the L-NIL group (P<0.05, 1-way ANOVA) (Table 2; Figure 3).

![Figure 1. Immunostaining of iNOS on cross sections of thoracic aortas of rabbits from Baseline (a), Saline (b), L-NIL (c), and L-Arg (d) groups. L indicates lumen. Magnification x10.](http://circ.ahajournals.org/)

![Figure 2. Surface (expressed as % of total intimal area) occupied by atherosclerotic plaques in abdominal aortas of 4 groups of rabbits. In Saline but not in L-Arg or L-NIL groups, lesion area was significantly increased (P<0.05 Baseline vs Saline, 1-way ANOVA).](http://circ.ahajournals.org/)
**SMC and Collagen Staining**

In the Baseline group, the percentage of total intimal surface of thoracic aortas stained by SMCs averaged 48.1 ± 7.4%. This area was not altered in the Saline, L-Arg, or L-NIL group (Table 1).

In the Baseline group, the percentage of collagen-stained areas in thoracic aortas averaged 21.3 ± 4.5%; this was increased in the Saline and L-Arg groups (*P < 0.05, 1-way ANOVA) but not in the L-NIL group (Figure 4; Table 1).

**Hematological Analysis and Platelet Aggregation**

Levels of platelets (418 ± 32 × 10³/µL), white (6.9 ± 0.5 × 10³/µL) and red (4.8 ± 0.1 × 10³/µL) blood cells, hemoglobin (10.9 ± 2 g/dL), and hematocrit (31.9 ± 0.9%) were comparable in all rabbits (values from Saline group). Collagen (10 µg/mL) induced aggregation (26.5 ± 1.9%) and ADP (3 µmol/L) induced aggregation (6.6 ± 1.3%) of platelets in whole blood. These responses were comparable in all groups.

**Discussion**

The principal findings of our study show that the selective iNOS inhibitor L-NIL limits progression of preexisting ath-
Atherosclerosis in cholesterol-fed rabbits. L-Arginine influences only less severe lesion development in abdominal aortas. The data thus suggest that iNOS expression may aggravate development of atherosclerosis.

After 24 weeks of 0.3% cholesterol diet, rabbits developed atherosclerosis in thoracic and abdominal aortas and in coronary arteries; iNOS is present in the neointima, sometimes in the media or endothelium, of these plaques, confirming earlier studies.1-2,9,30 Between 24 and 36 weeks of diet, the I/M ratio of thoracic aortas and the plaque surface in abdominal aortas increased; also, collagen accumulation became augmented, suggesting matrix accumulation. The observation that cGMP accumulation in thoracic aortas was higher at 36 than at 24 weeks probably reflects iNOS activity, and the desensitization of guanylate cyclase that may result from it may explain the decreased relaxations to nitroglycerin noted at 36 weeks. Only modest endothelium-dependent relaxations to acetylcholine were noted at 24 weeks, and they were further decreased at 36 weeks.

The first goal of our study was to evaluate the effect of treatment with the iNOS inhibitor L-NIL on progression of atherosclerosis. The plasma concentration of 10 μmol/L L-NIL obtained with the treatment inhibits iNOS in vivo without influencing endothelium-dependent relaxations. L-NIL treatment prevented the increased aortic cGMP levels at 36 weeks of hypercholesterolemia, demonstrating its inhibitory action on plaque iNOS activity. In abdominal aortas, L-NIL treatment abolished the increased lesion area, whereas in thoracic aortas and coronary arteries, it prevented the increased I/M ratio noted in untreated animals. No changes in SMC infiltration occurred between 24 and 36 weeks of hypercholesterolemia or in L-NIL–treated rabbits. However, increases in collagen accumulation were noted in saline-treated but not in L-NIL–treated rabbits. Collagen is an essential factor in plaque evolution, and L-NIL may decrease its synthesis; indeed, increased expression of iNOS in wounds precedes collagen synthesis.31 Activation of MMPs by iNOS seems unlikely, because NO activates MMPs.10 A decreased collagen accumulation by L-NIL without modification of SMC infiltration suggests changes in SMC phenotype that can be influenced by collagen through adhesion receptors.34

L-NIL treatment had only modest effects on relaxations in hypercholesterolemic rabbit aortas. It did not influence endothelium-dependent relaxations, which contradicts evidence that iNOS induction in injured arteries inhibits platelet adhesion and restores blood flow.35 In the latter study, iNOS may influence the acute response to endothelial denudation, or else platelets see less NO than do the SMCs in which iNOS is induced. L-NIL treatment prevents reduced nitroglycerin relaxations; the receptor for NO, soluble guanylate cyclase, becomes desensitized by excessive activation occurring after iNOS induction, especially when this occurs in SMCs, and this may explain the reduced activity for NO donors.

Thus, the selective iNOS inhibitor L-NIL possesses beneficial antiatherogenic effects, limiting progression of atherosclerosis in both advanced (thoracic aorta) and less developed (abdominal aorta) lesions of hypercholesterolemic rabbits.

Several studies in atherosclerosis showed beneficial effects of l-arginine treatment on lesion development or endothelial dysfunction.20,24,26-28 When the effects of L-arginine treatment on preexisting lesions were studied,12,23-25 positive effects depended on the protocol used: Böger et al23 and Wang et al12 reported decreased intimal arterial growth, augmented apoptosis, and restoration of endothelium-dependent relaxations, whereas Candipan et al,25 who fed rabbits a cholesterol diet for 23 weeks with l-arginine supplementation for the last 13 weeks, reported nonsustained effects of l-arginine, as also suggested by Jeremy et al.24

In our study, dietary l-arginine, which increased plasma l-arginine levels 2-fold,22,31 failed to influence lesion progression and composition in thoracic aortas and coronary arteries but decreased it in abdominal aortas. Lesion progression is more important in thoracic than in abdominal aortas,9,30 and studies in cholesterol-fed rabbits illustrated a greater benefit of l-arginine on plaque formation in distal parts of the aorta21,24; regional variation of eNOS activity was described in rat aortas,36 and iNOS expression is more pronounced in more developed lesions of thoracic aortas and coronary arteries.4 These findings may explain the regional difference in l-arginine activity. The lack of antiatherogenic effect of l-arginine on severe lesions may be related to the duration of hypercholesterolemia or to unexpected effects, such as blockade of the increased cGMP production. The latter may be due to transformation of l-arginine by iNOS into oxygen-derived free radicals.37 Yet l-arginine treatment had a modest, beneficial effect on endothelium-dependent relaxations in thoracic aortas.

Our data with l-arginine contribute to the debate on proatherogenic or atherogenic functions for NO. l-Arginine ameliorates...
manifestations of cardiovascular disease, but our data suggest that this effect may be limited, at least in the case of hypercholesterolemia, as previously shown in patients. Further investigations should clarify this issue as well as that of the hypercholesterolemic rabbit model to study NO in atherosclerosis.

In conclusion, although iNOS activity within developed atherosclerotic plaques does not influence infiltration and proliferation of cellular components, it accelerates progression of plaque development, which may be related to intimal collagen accumulation. Thus, contrary to eNOS activity, which exerts antiatherogenic effects on early lesions, iNOS activity may aggravate atherosclerotic lesion progression. Selective inhibitors of iNOS, eg, L-NIL, can interrupt this proatherogenic action. Our study is the first to illustrate negative effects of iNOS induction in rabbit atherosclerosis. If predictive for human atherosclerosis, the data suggest iNOS inhibition as a target for disease treatment. The results stress the role of inflammatory mechanisms on atherosclerosis progression.

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