Chronic Inhibition of Nitric Oxide Production Accelerates Neointima Formation and Impairs Endothelial Function in Hypercholesterolemic Rabbits

Antonio J. Cayatte, James J. Palacino, Kathleen Horten, Richard A. Cohen

Abstract To determine if endogenous local levels of nitric oxide (NO) modulate atherogenesis, we studied the effect of inhibiting NO with N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) on early neointima formation in cholesterol-fed rabbits. Male rabbits were fed for 5 weeks with a 0.5% cholesterol diet alone or treated in addition during the last 4 weeks with L-NAME (12 mg/kg per day SC) via osmotic minipump. Endothelial cell function was assessed in isolated aortic rings by vascular reactivity and levels of cyclic GMP. In L-NAME–treated rabbits there was inhibition of endothelium–dependent relaxations to acetylcholine and the calcium ionophore A23187 as well as impaired cyclic GMP accumulation in response to acetylcholine. Neointima formation in the ascending thoracic aorta was assessed by determining media and intima cross-sectional areas with computerized image analysis.

Nitric oxide (NO), a potent physiological vasodilator that accounts for the biological activities of endothelin-derived relaxing factor, is synthesized in endothelial cells from the terminal guanyldino nitrogen of L-arginine by the constitutive calcium-calmodulin-NADPH–dependent enzyme NO synthase (NOS). In humans and animals with hypercholesterolemia–induced atherosclerosis, endothelium–dependent vasodilation is impaired, suggesting reduced NO synthesis or action. NO has antiproliferative actions on vascular cells, supporting the hypothesis that the endothelial cell dysfunction observed in hypercholesterolemia could contribute to the initiation and progression of the atherosclerotic neointima. When administered in the diet of cholesterol–fed rabbits, the NO precursor L-arginine limits development of aortic atherosclerosis and improves endothelial cell function.

We recently reported a technique for the chronic administration to rabbits of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) and demonstrated its persistent systemic effects on endothelial cells and compared with rabbits that consumed the cholesterol diet alone, L-NAME–treated rabbits had significant increases in lesion area (0.29±0.04 versus 0.15±0.03 mm\textsuperscript{2}) and in lesion/media ratio (0.06±0.01 versus 0.03±0.01). Plasma levels of cholesterol and fluorescent lipid peroxide products were unchanged, suggesting no difference in cholesterol metabolism or oxidation. Because arterial blood pressure was not altered by L-NAME treatment, the increased atherogenesis could not be attributed to an increase in blood pressure. These results indicated that local inhibition of NO accelerates early neointima formation possibly because of modulating monocyte recruitment or foam cell lipid accumulation. (Arterioscler. Thromb. Vasc. Biol. 1994;14:753-759.)

Key Words • rabbits • nitric oxide • cholesterol • endothelium • atherogenesis

Microcirculation. We used this L-NAME rabbit model in this study to determine the effect of chronic inhibition of NO synthesis on atherosclerosis development during the initial weeks of cholesterol feeding. The observed acceleration of neointimal formation suggests that local NO production regulates neointimal growth in hypercholesterolemia.

Methods L-NAME Rabbit Model and Diets

Sixteen male New Zealand White rabbits (2.6±0.0 kg) were fed a diet of chow (Agway ProLab, Agway, Country Foods Division) supplemented with cholesterol (0.5% wt/wt) and peanut oil (4% vol/vol). Cholesterol was suspended in peanut oil and then mixed with normal rabbit chow. After 1 week of feeding, the rabbits were randomly assigned to control and L-NAME–treated groups. To study the effect of L-NAME treatment alone, 4 additional rabbits fed a standard diet were treated with L-NAME. Osmotic pumps (Alzet model 2ML4) were loaded with a sterile filtered solution of L-NAME (Sigma Chemical Co) that had been dissolved in deionized water and made up to a concentration based on the initial weight of each rabbit and the factory-determined mean pumping rate of 2.60±0.1 μL/h to deliver 12 mg/kg per day. The pumps were surgically implanted under sterile conditions in the scapular region. After the rabbits were anesthetized with xylazine (5 mg/kg IM) and ketamine (35 mg/kg IM), the area was shaved, and an incision (approximately 2 cm) was made laterally, exposing a subcutaneous pouch into which the pump was implanted with the flow moderator pointing away from the incision. The incised area was closed with a suture. After 4

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weeks the rabbits were anesthetized with pentobarbital sodium 50 mg/kg IV; after the administration of heparin 1000 U IV, blood was drawn via the heart, and vessels were removed for study.

**Tissue Preparation**

Immediately after exsanguination the aorta was dissected from the arch to the iliac bifurcation and rinsed with cold phosphate-buffered saline, pH 7.4, to remove traces of adherent blood. The descending thoracic aorta, free of periaortic tissue, was used for the vascular reactivity studies. The ascending thoracic portion from the heart to the left subclavian bifurcation was subdivided, immersion fixed overnight in 10% buffered formalin at room temperature, and used for morphometric studies after removal of adherent adventitia. This portion of the aorta was examined because it is most prone to lesion formation at the early stages examined in this study. Tissue segments were dehydrated in a graded series of ethanol and paraffin embedded for histological examination of neointima formation, which was done on sections prepared with van Gieson's elastic stain. One randomly chosen rabbit from each group was anesthetized, and the aorta was perfusion fixed at 80 mmHg pressure for en face examination. The aorta was mounted on a black background to enable visualization of early plaque localization.

**Morphometric Analysis**

After immersion fixation 5-μm sections were cut from three random sites at the proximal, mid, and distal ascending aortic arch. For morphometric analysis of neointimal size, measurements of aortic lesion area and media area were performed at random on two elastin-stained sections of each aorta by an examiner blinded to the study. An Olympus microscope equipped with an IBM personal computer, a digitizing pad, and OPTIMAS 3.1 image-analysis software (Biocscan, Inc) was used. Media area was determined with a ×10 objective. For accuracy, lesion area was determined with a ×40 objective, and areas in each field were added. After correcting for magnification, the lesion/media ratio was calculated, and a mean value for the two sections was calculated for each rabbit. For gross comparison of lesion areas in the two groups, ×10 magnified images of each entire elastin-stained aortic section were reflected on a digitizing pad using a microprojector, and contours of the lumen and the internal elastic lamina were traced to provide an image of the intima only. These images are presented in photographs of superimposed traces of the intima of all sections analyzed. This image-analysis technique was sensitive enough to quantify normal intimal area, because tissue preparation was sensitive enough to quantify normal intimal area, because

**Lipid Peroxide Assay**

Fluorescent lipid peroxidation products were assayed in the plasma of all rabbits. Briefly, rabbit blood was collected at the time of death and centrifuged to divide the plasma. Plasma was preserved with a final concentration of 1 mg/mL EDTA and frozen at –80°C until assay. When 1 mL plasma was treated with 6 mL methanol and 1 mL chloroform and vortexed. The mixture was allowed to incubate for 15 minutes at 4°C. After incubation, 4 mL 0.9% saline was added, and the mixture was centrifuged at 200g for 20 minutes at 4°C. One milliliter of the organic layer was added to 0.1 mL methanol, and fluorescent products were measured on a Perkin-Elmer model 650-10S spectrophotofluorimeter set at excitation 355 nm and emission 435 nm.

**Cholesterol Assay**

Plasma cholesterol levels were assayed spectrophotometrically by using a commercially available kit (Sigma Diagnostics 352-20, Sigma Chemical Co). Briefly, 5 μL plasma and 5 μL 0.9% saline were added to 1 mL cholesterol reagent and gently mixed, and the mixture was then incubated for 15 minutes at 37°C. Samples were analyzed 30 minutes after addition of the assay solution by reading in a Perkin-Elmer Coleman 44 spectrophotometer at 500 nm. Absolute cholesterol levels were calculated by comparison with lipid control-N (Sigma Diagnostics L-2008) using deionized water added to the reagent as a blank.
TABLE 1. Effects of 4-Week L-NAME Treatment on Cholesterol-Fed Rabbits

<table>
<thead>
<tr>
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<th>Cholesterol (n=8)</th>
<th>Cholesterol/L-NAME (n=8)</th>
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</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>3.3±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>100±2.4</td>
<td>107±3.1</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>81±5.8</td>
<td>86±5.3</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dL</td>
<td>1292±197</td>
<td>1510±208</td>
</tr>
<tr>
<td>Lipid peroxides, arbitrary units</td>
<td>18.1±3.7</td>
<td>15.5±1.8</td>
</tr>
</tbody>
</table>

L-NAME indicates N\(^{\text{G}}\)-nitro-L-arginine methyl ester. All values are mean±SEM. No significant differences between groups occurred.

Statistical Analysis

All data are presented as mean±SEM; n represents the number of different rabbits in each group. Relaxations are expressed as a percentage of contractions to phenylephrine and plotted against the negative log molar concentrations of acetylcholine, A23187, or sodium nitroprusside. Data for cGMP are expressed as femtomoles of cGMP per microgram protein, body weight is expressed in kilograms, blood pressure in millimeters of mercury, plasma cholesterol in milligrams per deciliter, and lipid peroxides as arbitrary units of fluorescence.

The statistical significance of the results comparing intimal area measurements between the two groups was analyzed by unpaired Student's t test.

Results

Characteristics of Cholesterol-Fed and L-NAME-Treated Rabbits

From an initial weight averaging 2.6 kg, both groups of rabbits gained weight equally over the 5-week feeding period and had similar body weights at the time of death (Table 1). There was no significant change in systolic or mean blood pressure over the 5-week cholesterol-feeding period, nor was there significant change caused by L-NAME treatment. Plasma cholesterol levels were greatly elevated in both groups of rabbits; there was no significant difference with L-NAME treatment. Plasma lipid peroxide levels showed no significant difference with L-NAME treatment (Table 1).

TABLE 2. Physiological Responses to Endothelium-Dependent and Endothelium-Independent Vasodilators in Cholesterol-Fed and Cholesterol-Fed, L-NAME-Treated Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol</th>
<th>Cholesterol/L-NAME</th>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>8.7±0.3</td>
<td>87.7±2.9</td>
</tr>
<tr>
<td>Acetylcholine After L-arginine</td>
<td>9.2±0.5</td>
<td>85.0±1.7</td>
</tr>
<tr>
<td>A23187</td>
<td>8.7±0.2</td>
<td>88.8±1.7</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>8.3±0.4</td>
<td>90.0±2.0</td>
</tr>
</tbody>
</table>

L-NAME indicates N\(^{\text{G}}\)-nitro-L-arginine methyl ester; NS, not significant. All relaxations of the proximal descending thoracic aortic rings were to a 1-μmol/L concentration of the agonists. The concentration of L-arginine was 1 mmol/L. Values are expressed as mean±SEM of the contractions caused by phenylephrine; relaxation is expressed as a percent of maximal relaxation achieved by addition of sodium nitroprusside (1 μmol/L). Probability values refer to comparisons between cholesterol-fed and cholesterol-fed, L-NAME-treated rabbits.
**FIG 2.** Bar graphs showing effect of N^6^-nitro-l-arginine methyl ester (L-NAME) on basal (open bars) and acetylcholine-stimulated (solid bars) levels of aortic cyclic GMP (cGMP). A, Without L-arginine (n=7); B, analysis of data after addition of L-arginine (1 mmol/L) (n=4 or n=5). Values are mean±SEM. L-NAME significantly inhibited acetylcholine-stimulated cGMP levels and L-arginine significantly reversed the effect of L-NAME. Asterisk indicates P<.05; chol, cholesterol; NAME, L-NAME; and f moles, femtomoles.

**Endothelium-Dependent Relaxations and cGMP Levels**

Without altering the contractions of aortic rings caused by phenylephrine, L-NAME treatment significantly inhibited relaxations caused by acetylcholine (10^-8 to 3×10^-6 mol/L; Fig 1 and Table 2). Relaxations caused by A23187 (10^-8 to 3×10^-6 mol/L) were also significantly inhibited in aortic rings from L-NAME-treated rabbits compared with untreated cholesterol-fed rabbits (Fig 1 and Table 2). Relaxations caused by sodium nitroprusside (10^-9 to 3×10^-6 mol/L) were not significantly different in the two groups (Fig 1). In vitro addition of arginine (10^-3 mol/L) significantly increased acetylcholine-induced relaxations only in L-NAME-treated rabbits (Table 2).

Basal aortic levels of cGMP were decreased 50% in L-NAME-treated cholesterol-fed rabbits, but this did not reach statistical significance (Fig 2A). However, after treatment with L-arginine, basal levels of cGMP were significantly less in the L-NAME-treated group (Fig 2B). Acetylcholine-stimulated cGMP levels were significantly reduced by the NOS inhibitor (Fig 2A). In vitro addition of L-arginine (10^-3 mol/L; Fig 2B) significantly increased acetylcholine-stimulated levels of cGMP in L-NAME-treated rabbits such that they were not significantly different from those found in untreated cholesterol-fed rabbit aorta in the presence or absence of L-arginine.

**Morphological Studies**

The aorta of one rabbit from each group was perfusion fixed (Fig 3). Macroscopically, surfaces of isolated
thoracic aortas were free of large plaques in the two groups. Small patches of white neointimal lesions could be seen only in the aorta of the L-NAME–treated cholesterol-fed rabbits.

Microscopic intimal lesions appeared as slightly raised focal areas typical of fatty streaks that separated the endothelium from the internal elastic lamina. The lesions were composed primarily of foam cells and were of similar cellular composition in both groups (Fig 4). Initial comparison of the superimposed contours of the neointima of one section from each rabbit in the two groups suggested that L-NAME–treated rabbit aortas had increased lesion cross-sectional area (Fig 5). Morphometric analysis substantiated this initial comparison. Calculated lesion areas (Fig 6) were significantly increased in cross sections of L-NAME–treated rabbit aortas (0.29±0.04 versus 0.15±0.03 mm², *P<.02, n=6 and n=7, respectively). Similarly, analysis of lesion/media area ratio revealed a significant increase in L-NAME–treated rabbit aortas (0.06±0.01 versus 0.03±0.01 mm², *P<.05). The cross-sectional area of the aortic media in both groups was similar (5.6±0.5 versus L-NAME, 4.7±0.4 mm²). No visible aortic intimal thickening or cell infiltration occurred in normocholesterolemic, L-NAME–treated rabbits. The intima that appeared to be normal measured 0.04±0.01 mm (n=4).

Discussion

In the present study chronic administration of L-NAME significantly increased neointimal size and impaired endothelial function. As the rabbits’ weight and plasma cholesterol levels were unchanged by administration of L-NAME, it is unlikely that the effect of the NOS inhibitor on atherogenesis resulted from any nutritional effect. Moreover, the L-NAME dose was chosen because it does not increase systolic blood pressure in rabbits, whereas higher doses of NOS inhibitors given to rats acutely or chronically can increase systolic blood pressure. In this study mean blood pressure was also estimated and was not altered by the dose of L-NAME used. Thus, the known ability of 30- to 40-mm Hg increases in blood pressure to accelerate atherosclerosis does not explain the greater neointimal formation observed in L-NAME–treated rabbits. In addition, because administration of L-NAME had no independent inflammatory or atherogenic effect in rabbits fed a standard diet, it is likely that L-NAME accelerates mechanisms associated with hypercholesterolemia–induced atherosclerosis.

Active local concentrations of L-NAME delivered systemically by the technique used are demonstrable in normal rabbits and in hypercholesterolemic rabbits in the present study by showing that the endothelium–dependent relaxations of isolated aorta caused by acetylcholine and A23187 are inhibited. Normalcy of relaxations to sodium nitroprusside, a source of exogenous NO, suggests that aortic tissue responsiveness to NO is unaffected by L-NAME treatment and supports the fact that L-NAME inhibits the synthesis of endogenous NO. NO mediates endothelium-dependent relaxations in blood vessels by increasing intracellular levels of cGMP in smooth muscle cells upon stimulation of soluble guanylate cyclase. cGMP levels in aortas from L-NAME–treated rabbits were decreased, indicating the effective inhibition of NO synthesis by endothelial cells in response to acetylcholine. Notably, acetylcholine relaxations persisted in the aorta of L-NAME–treated rabbits in the absence of a rise in cGMP. This has been explained by a cGMP-independent response to acetylcholine mediated by an endothelium–derived hyperpolarizing factor. The local effect of systemically administered L-NAME on acetylcholine–induced relaxations and increases in cGMP was reversible by exogenous L-arginine, further suggesting that the impaired endothelial cell function caused by L-NAME was directed specifically toward the L-arginine–NO pathway. In vitro administration of L-arginine failed to affect aortic relaxations in animals fed the cholesterol diet alone. Five weeks of the 0.5% cholesterol diet used in these studies caused no significant effect on endothelium–dependent relaxation compared with normal rabbits. In contrast, the effect of chronic or acute administration of L-arginine to reverse aortic endothelial dysfunction can possibly be explained because the
diet was higher in cholesterol and was maintained for a period of 10 weeks. Such diet significantly impairs endothelium-dependent relaxations, and aortas exhibit more extensive lesion involvement.\(^8\,9\,19\)

Because L-NAME was shown to act locally on the L-arginine–NO pathway, the increase in atherogenesis caused by the drug may have accelerated any one of a number of cellular events thought to be responsible for the initiation and progression of atherosclerosis. Such cellular events affected by L-NAME could include the recruitment and adherence of blood monocytes, the oxidation of low-density lipoprotein (LDL), or the formation of macrophage foam cells.

Focal adherence of circulating blood monocytes is one of the earliest pathological events observed during atherogenesis in hypercholesterolemia.\(^20\) After adherence, monocytes migrate into the intima, and they are believed to undergo transformation to lipid-laden macrophages. Accumulating evidence suggests that NO influences endothelial cell–leukocyte interactions.\(^22\,23\) Nitrovasodilators inhibit neutrophil adhesion,\(^21\) and L-NAME enhances leukocyte adhesion in the microcirculation in vivo.\(^22\) Exogenous NO inhibits monocyte chemotaxis and adhesion to cultured endothelium,\(^23\) suggesting that the decrease of NO in L-NAME–treated rabbits may have enhanced this initial step in atherogenesis.

The macrophage accumulates oxidized LDL via the scavenger receptor to become a lipid-laden foam cell, which is the hallmark of atherosclerosis.\(^24\) Oxygen-derived free radicals are believed responsible for the oxidation of LDL because antioxidants such as probucol inhibit atherogenesis\(^22\,25\) and improve endothelium-dependent relaxations.\(^19\) The plasma levels of lipid peroxides, which are elevated in hypercholesterolemic rabbits compared with their normocholesterolemic littermates,\(^19\) were unchanged by L-NAME, suggesting that L-NAME has no effect on lipid peroxidation. Nevertheless, blood levels of lipid peroxides may not accurately reflect tissue lipids, and aortic LDL peroxidation could be increased by L-NAME treatment. Moreover, because superoxide anion radicals, which can be implicated in local LDL oxidation, can be quenched by NO in the rabbit aorta,\(^26\) it is possible that free radical–mediated oxidation of LDL could be enhanced in the aorta of L-NAME–treated rabbits. The inhibitory effect of L-NAME on a possible protective role of NO in LDL oxidation could indirectly influence monocyte recruitment and adherence to the endothelium.\(^27\)

Oxidized LDL and cytokines such as interleukin-1 and tumor necrosis factor, which are produced by activated monocytes, are important chemotactic mediators for monocytes and induce the expression of vascular cell adhesion molecule–1 (VCAM-1), which selectively mediates adherence of monocytes to endothelium.\(^28\) The expression of VCAM-1 on aortic endothelium is a very early event in atherogenesis, occurring after only 1 week of cholesterol feeding.\(^29\) The intracellular signals that activate endothelial cell adhesion molecule expression are not yet fully understood, but the transcriptional regulatory mechanisms for VCAM-1 gene expression may be redox sensitive and inhibited by antioxidants.\(^30\)

During atherogenesis, localized vascular inflammatory reactions are believed to occur (see Reference 31 for review), and cytokines such as interleukin-1 and tumor necrosis factor are potential sources of NO and superoxide generation. Because scavengers of superoxide have been shown to increase NO half-life\(^29\) in vitro, NO may represent an important regulatory molecule in the redox-sensitive mechanisms that activate the expression of endothelial cell adhesion proteins in response to cytokine stimulation. Thus, by affecting the production of NO, L-NAME could indirectly affect the action of these important mediators in the early stages of atherosclerosis. Indeed, another NOS inhibitor, N\(^\text{\textsuperscript{G}}\) mono-methyl-L-arginine, increases adherence of monocytes to cytokine-activated, cultured endothelium,\(^23\) further suggesting that NO may regulate monocyte adhesion when the endothelium is activated, such as in atherosclerosis. The effect of L-NAME may be due to inhibition of the activity of a Ca\(^2+\)-independent NOS, which can be induced in atherosclerotic rabbit aorta as a result of cytokine activation.\(^34\,35\)

This study focused on the very early stages of atherosclerosis development to demonstrate an enhancement by L-NAME treatment. The fact that endothelium-dependent relaxations were normal in both the cholesterol-fed rabbits and the L-NAME–treated animals after arginine indicates that the impaired endothelial function was due to impaired NO synthesis rather than to the increased thickness of the aortic intima. This suggests that the abnormal endothelial function caused by L-NAME was key to the acceleration of atherosclerosis. This study thus supports the hypothesis that endothelial NO function is an important determinant of the progression of atherosclerosis.

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