Role of Nitric Oxide in Restenosis After Experimental Balloon Angioplasty in the Hypercholesterolemic Rabbit: Effects on Neointimal Hyperplasia and Vascular Remodeling

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OBJECTIVES

The purpose of this study was to assess the effects of L-arginine and NO-nitro-L-arginine methyl ester (L-NAME) on neointimal hyperplasia and vascular remodeling after balloon angioplasty in the hypercholesterolemic rabbit.

BACKGROUND

Restenosis after balloon angioplasty is a consequence of both neointimal hyperplasia and vessel remodeling. Nitric oxide inhibits neointimal hyperplasia, but its effect on vessel remodeling is unknown.

METHODS

Six weeks after induction of bilateral iliac atherosclerosis, 48 rabbits underwent successful angioplasty in 75 vessels. Eight rabbits (acute group) were sacrificed immediately after angioplasty. The remaining animals received either placebo (chronic control group), or a diet supplemented with either L-arginine (1.5 g/kg/day), or L-NAME (15 mg/kg/day) for 4 weeks after angioplasty.

RESULTS

The intimal area was significantly greater in the chronic control group compared to the acute group (2.60 ± 1.03 mm² vs. 1.35 ± 0.62 mm²). This increase in intimal area was lower in the L-arginine group (1.79 ± 0.61 mm²), and greater in the L-NAME group (3.23 ± 0.92 mm²). The area circumscribed by the internal elastic lamina (IEL) increased significantly in the control group compared to the acute group (from 2.52 ± 0.66 to 3.33 ± 0.85 mm²); a more marked increase occurred in the L-NAME group (3.90 ± 0.85 mm²). By contrast, IEL area was unchanged in the L-arginine group (2.41 ± 0.62 mm²). As a result, there was no significant difference in lumen area after 4 weeks in the chronic groups (control: 0.74 ± 0.38 mm²; L-arginine: 0.50 ± 0.43 mm²; L-NAME: 0.48 ± 0.42 mm²).

CONCLUSIONS

Our results demonstrate that L-arginine inhibits whereas L-NAME stimulates neointimal hyperplasia after experimental balloon angioplasty in the hypercholesterolemic rabbit. However, the lack of vessel enlargement in the L-arginine group resulted in a similar final lumen size in the L-NAME and L-arginine groups. (J Am Coll Cardiol 1999;33:876–82) © 1999 by the American College of Cardiology

There has been considerable interest in the potential role of nitric oxide (NO) in restenosis after experimental angioplasty. Studies performed in rats or in rabbits have shown that administration of L-arginine or of NO donors, or the inhalation of NO are associated with a decrease in neointimal hyperplasia after arterial injury (1–7). Moreover, administration of the NO synthase inhibitor NO-nitro-L-arginine methyl ester (L-NAME), has been associated with an increase in neointimal hyperplasia (2). Nitric oxide has thus been proposed as a potential “antirestenotic” molecule that may act by inhibition of smooth muscle cell proliferation and migration, by inhibition of the synthesis of extracellular matrix or by modulation of platelet and leukocyte function (8–15).

However, recent studies, performed in experimental models (16–20) and in humans (21,22), have shown that neointimal hyperplasia is not the sole factor implicated in restenosis and have demonstrated the major role of vascular remodeling. Studies performed in the atherosclerotic rabbit model have shown that vascular remodeling, not neointimal hyperplasia, was the main determinant of restenosis (17,18).

Although it has been speculated that NO may be implicated in vascular remodeling (23), its role in the adaptive remodeling process that occurs after angioplasty has never been specifically studied. We thus designed the present study...
study to analyze the role of NO in restenosis after experimental angioplasty in the hypercholesterolemic rabbit. The effects of L-arginine and L-NAME on both neointimal hyperplasia and vascular remodeling were studied.

METHODS

Male New Zealand White rabbits (3 to 3.5 kg) were used for this study. The investigation conformed to the guidelines of the American Physiological Society for the care and use of laboratory animals.

Induction of atherosclerosis. Bilateral iliac atherosclerosis was induced as previously described (17,24). The animals were anesthetized by intravenous injection of ethyl carbamate, 1 g/kg via a marginal ear vein. After exposure of the distal femoral arteries, a 3-F Fogarty balloon catheter was retrogradely inserted 20 cm and inflated until contact was made with the arterial wall. Deendothelialization of both iliac arteries was accomplished by advancing and withdrawing the catheter three times. The catheter was then removed, the distal femoral arteries were ligated, and the wound was closed. All rabbits received amoxycillin (50 mg/kg intramuscularly) and were fed a high cholesterol (2%) diet until sacrifice.

Angiography and balloon angioplasty. Six weeks after induction of atherosclerosis, the animals were anesthetized as described above. During the experimental procedure anesthesia was maintained by intermittent intravenous injection of ethyl carbamate 250 mg/kg. The right carotid artery was isolated by blunt dissection through a midline neck incision. A 5-F arterial sheath was then introduced via an arteriotomy and advanced to the abdominal aorta under fluoroscopic guidance. Angiograms were obtained with a Philips 17-cm image intensifier with a resolution of 4.4 line pairs per millimeter. Three minutes after administration of isosorbide dinitrate (ISDN; 0.2 mg), a baseline angiogram was recorded on videotape by injection of 3 ml of contrast medium (ioxaglate meglumine). A radio-opaque grid was placed over the pelvis to permit correction for magnification. Angioplasty was performed if a 50% to 99% iliac stenosis was present, as estimated visually from the angiogram. The angioplasty procedure was performed after administration of heparin (100 IU/kg). Under fluoroscopic guidance, a 0.014 in. (0.0355 cm) guide wire was inserted through the introducer sheath and advanced to the external iliac artery. A conventional balloon angioplasty catheter (2.5 mm in diameter, 20 mm long) was advanced to the site of the most severe iliac artery stenosis. Three 60-s inflations were performed at a pressure of 6 atmospheres, with a 1-min interval between each inflation. The result of the angioplasty procedure was documented by a second angiogram (3 min after administration of 0.2 mg of ISDN). The catheters were then removed, the arteries were ligated and amoxycillin (50 mg/kg intramuscularly) was administered. Successful angioplasty was defined as a >20% decrease in percent diameter stenosis.

Study groups. Fifty animals had unilateral or bilateral iliac stenoses and underwent angioplasty (Fig. 1). Successful iliac angioplasty was performed at 75 lesions in 48 rabbits. Eight rabbits (the acute group, 15 vessels) were sacrificed immediately after angioplasty. The iliac arteries were fixed by perfusion in situ with 4% paraformaldehyde (in a saline phosphate buffer solution) at 100 mm Hg for 30 min via a catheter introduced into the aorta. The aorta and iliac arteries were then removed and placed in 4% paraformaldehyde for 24 h. Forty rabbits (the chronic groups) were allowed to recover and were maintained on the 2% cholesterol diet for 4 weeks. Immediately after angioplasty, these animals were randomized into three groups: 14 rabbits received placebo (the chronic control group, 20 vessels); 12 rabbits received 1.5 g/kg/day of L-arginine hydrochloride (Sigma Chemical Co.) in the drinking water (the L-arginine group, 19 vessels), and 14 rabbits received 15 mg/kg/day of L-NAME (Sigma Chemical Co.) in the drinking water (the L-NAME group, 21 vessels). The dose of L-arginine was chosen because it has already been demonstrated to be effective in reducing neointimal hyperplasia in rabbit iliac arteries (3,4). The dose of L-NAME was chosen because it has been used in rabbits, by either oral or subcutaneous administration, without effect on arterial blood pressure (25,26). Six animals (one in the placebo group, three in the L-arginine group and two in the L-NAME group) died during the follow-up period. After 4 weeks, animals of the chronic groups (13 [19 vessels] in the

Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>EEL</td>
<td>external elastic lamina</td>
</tr>
<tr>
<td>IEL</td>
<td>internal elastic lamina</td>
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<tr>
<td>ISDN</td>
<td>isosorbide dinitrate</td>
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<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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Figure 1. Study design. L-NAME = N^G^-nitro-L-arginine methyl ester.
placebo group, 9 [14 vessels] in the L-arginine group and 12 [18 vessels] in the L-NAME group) underwent angiography as described above and were sacrificed. The arteries were perfusion-fixed, removed and placed in 4% paraformaldehyde in the same manner as the acute group.

Biochemical and physiologic measurements. Blood samples for determination of cholesterol levels were obtained at the time of angioplasty in all four study groups, and at the time of sacrifice in the chronic groups. Plasma cholesterol levels were measured with a Ciba-Corning 550 Express spectrophotometer using a commercially available enzymatic assay (Bio Mérieux).

In the chronic groups, blood samples were obtained at the time of sacrifice for measurement of plasma-free arginine levels. Plasma was deproteinized with 10% sulfosalicylic acid and analyzed for free arginine with an automated amino-acid analyzer (model LC 300, Biotronic Instruments).

Blood pressure was measured under general anesthesia via the catheter inserted into the carotid artery, in all groups immediately before angioplasty, and at the time of sacrifice in the three chronic groups.

Angiographic analysis. The reference diameters (a proximal angiographically normal segment of the external iliac artery and a segment of the common iliac artery) and the minimal lumen diameter were measured with calipers. Absolute diameters were calculated by correcting for magnification with the use of the radio-opaque grid. The measurements were performed in all groups immediately before and immediately after angioplasty, and in the three chronic groups at the time of the final angiogram.

Histological analysis. After review of the angiograms to guide sampling, a 10-mm long segment of the artery including the lesion was cut into three 3- to 4-mm long pieces, which were embedded in paraffin. Cross sections were taken at three locations (top, bottom, middle) within each block. Thus a total of nine sections were analyzed for each vessel. The sections were stained with Van Gieson elastin.

Morphometric analysis of the histologic cross sections was performed with a digital microscopic planimetry system (SMC 2002, Bioblock Scientific). For each lesion, the section that demonstrated the smallest lumen area was selected for further analysis. Lumen area, intimal area, medial area and the areas circumscribed by the internal elastic lamina (IEL) and the external elastic lamina (EEL) were measured by a pathologist who was unaware of the study design.

Statistical analysis. Data are expressed as mean ± SD. Differences between groups were assessed with use of analysis of variance and Scheffé test for multiple comparisons. For differences between two groups, Student’s t tests were used. A value of p < 0.05 was considered statistically significant. The sample size of the study groups was determined to give a power of 80% to detect a 40% increase in lumen area in the L-arginine group versus the chronic control group assuming a lumen area of 0.75 ± 0.32 mm² in control rabbits as seen in a preliminary study with this model in our laboratory.

RESULTS

Biochemical and physiologic measurements. As shown in Table 1, plasma cholesterol levels were similar among the four groups at the time of angioplasty and similar among the three chronic groups at the time of sacrifice. Plasma arginine levels were significantly higher in animals whose diet was supplemented with L-arginine compared with control animals. There were no significant differences in blood pressure measurements among the four groups at the time of angioplasty and among the three chronic groups at the time of sacrifice.

Angiography. Table 2 shows the angiographic results before angioplasty, immediately after angioplasty and at four-week follow-up in the four study groups. There was no significant difference in minimal lumen diameter before or immediately after angioplasty among the four groups. The reference vessel diameters were also similar among groups. At four-week follow-up, a significant decrease in minimal lumen diameter was observed in the three chronic groups,

<table>
<thead>
<tr>
<th>Table 1. Biochemical and Physiologic Parameters</th>
<th>Acute Group</th>
<th>Chronic Groups</th>
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<tr>
<td></td>
<td>Control (n = 8)</td>
<td>L-arginine (n = 9)</td>
</tr>
<tr>
<td>Plasma cholesterol (g/liter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preangioplasty</td>
<td>35 ± 11</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>Sacrifice</td>
<td>—</td>
<td>28 ± 9</td>
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<tr>
<td>Plasma L-arginine (mmol/liter)</td>
<td>—</td>
<td>114 ± 27</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td>—</td>
<td>113 ± 11</td>
</tr>
<tr>
<td>Preangioplasty</td>
<td>111 ± 8</td>
<td>103 ± 17</td>
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</table>

*p < 0.001 vs. control.

L-NAME = N^0-nitro-L-arginine methyl ester.
whereas no change in reference vessel diameters was observed. There was no significant difference in minimal lumen diameter at follow-up among the three chronic groups (control = 0.82 ± 0.37 mm, L-arginine = 0.63 ± 0.47 mm, L-NAME = 0.58 ± 0.46 mm).

**Morphometry.** Table 3 shows the morphometric results of the acute and chronic groups. In the control group, we observed a significant increase in intimal area compared to the acute group (from 1.35 ± 0.62 to 2.60 ± 1.03 mm²). This increase in intimal area was lower in the L-arginine group (1.79 ± 0.61 mm²), and greater in the L-NAME group (3.23 ± 0.92 mm²).

There was no significant difference in medial area among the four groups, although there was a trend for an increase in the L-NAME group.

In the control group, we observed a significant increase in the area circumscribed by the IEL or in the area circumscribed by the EEL compared with the acute group (from 2.52 ± 0.66 to 3.33 ± 0.85 mm² and from 3.23 ± 0.83 to 4.04 ± 0.92 mm², respectively). By contrast, in the L-arginine group, no increase in IEL or EEL area was observed (2.41 ± 0.62 mm² and 3.12 ± 0.78 mm², respectively). On the other hand, in the L-NAME group, a greater increase in IEL or EEL area was observed (3.90 ± 0.85 mm² and 4.81 ± 1.06 mm², respectively).

There was no significant difference in lumen area at 4-week follow-up among the three groups. The changes in lumen area during the follow-up period reflected both changes in intimal area and changes in vessel size. In the control group, the increase in IEL area accommodated 67% of the increase in intimal area (1.25 mm²) over the same time period; as a result, lumen area decreased only by 0.43 mm². In the L-arginine group, the potentially beneficial effect of the decrease in intimal area on lumen area was completely reversed by the lack of enlargement of the vessel. By contrast, in the L-NAME group, the increase in neointimal area was compensated by the greater degree of vessel enlargement.

Twelve vessels (two in the control group, four in the L-arginine group and six in the L-NAME group) were totally occluded at follow-up. In eight cases, a thrombus was clearly identifiable on the histologic cross sections. For these

### Table 3. Morphometric Analysis

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<tr>
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<th>Acute Group</th>
<th>Chronic Groups</th>
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<tr>
<td></td>
<td>Control (n = 15)</td>
<td>Control (n = 19)</td>
</tr>
<tr>
<td>Intimal area (mm²)</td>
<td>1.35 ± 0.62</td>
<td>2.60 ± 1.03*</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>0.70 ± 0.31</td>
<td>0.71 ± 0.18</td>
</tr>
<tr>
<td>IEL area (mm²)</td>
<td>2.52 ± 0.66</td>
<td>3.33 ± 0.85*</td>
</tr>
<tr>
<td>EEL area (mm²)</td>
<td>3.23 ± 0.83</td>
<td>4.04 ± 0.92*</td>
</tr>
<tr>
<td>Lumen area (mm²)</td>
<td>1.17 ± 0.31</td>
<td>0.74 ± 0.38*</td>
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*p < 0.05 vs. acute control group. †p < 0.05 vs. L-arginine. §p < 0.05 vs. chronic control group. Thrombus in three vessels in the L-arginine group and in five vessels in the L-NAME group accounts for the discrepancy between lumen + intimal area and IEL area.

IEL area = area circumscribed by external elastic lamina; IEL area = area circumscribed by internal elastic lamina; L-NAME = Nω-nitro-L-arginine methyl ester.
vessels, thrombus area was not included in intimal area; our results were, however, not altered if thrombus was measured as intimal area (data not shown). Similarly, a reanalysis of morphometric measurements after exclusion of the vessels with total occlusion at follow-up did not alter our results (data not shown).

**DISCUSSION**

The present study, performed in the atherosclerotic rabbit model, demonstrates that animals treated with L-arginine after balloon angioplasty have less marked neointimal hyperplasia than control animals, whereas animals treated with L-NAME have more marked neointimal hyperplasia. However, because vessel enlargement was observed in the L-NAME group but not in the L-arginine group, neither drug had a significant effect on the final lumen size.

**Mechanisms of restenosis.** Restenosis after balloon angioplasty has been traditionally associated with neointimal hyperplasia (27–29). Proliferation and migration of smooth muscle cells have been documented after experimental balloon angioplasty, in various species and in single-injury or double-injury models including the atherosclerotic rabbit model (20,27,30,31). Moreover, it has been shown that synthetic smooth muscle cells produce extracellular matrix proteins that constitute a large proportion of the neointimal volume. Neointimal hyperplasia has also been documented in humans; histologic studies have shown that intimal thickening in the restenotic lesion contains smooth muscle cells in an abundant extracellular matrix (28,29).

However, the lack of effect in humans of drugs that markedly inhibited neointimal hyperplasia in experimental models led to a reappraisal of the mechanisms of restenosis. Recently, concordant studies have been published showing that lumen renarrowing after angioplasty was also due to vascular remodeling (16–20). In the atherosclerotic rabbit model, Kakuta et al. and Lafont et al. demonstrated that vascular remodeling was the main determinant of lumen size at follow-up (i.e., restenosis) (17,18). In these studies, a linear correlation was observed between neointimal (or neointimal plus medial) area and vessel size (i.e., area circumscribed by the IEL or the EEL), suggesting that an increase in vessel size may compensate a large proportion of intimal growth. Our results are concordant with these previous studies; in the control groups, we found a significant enlargement in vessel size from immediately after angioplasty to sacrifice 4 weeks later; this increase in vessel size was able to accommodate 67% of the increase in intimal area over the same time period.

Importantly, vessel remodeling has also been confirmed as a major determinant of restenosis after angioplasty in humans (21,22). This may, at least in part, explain why drugs that effectively prevented neointimal hyperplasia in experimental models consistently failed to prevent restenosis in the clinical setting. In the light of these new findings on the mechanisms of restenosis, it is thus important to reevaluate drugs that have to date only been shown to affect neointimal hyperplasia. Indeed, depending upon their effect on vessel remodeling, these drugs may actually decrease restenosis, have no effect on restenosis or even increase restenosis.

**The NO pathway and neointimal hyperplasia.** Previous studies, performed in single-injury models, have shown that the NO pathway is an important determinant of neointimal hyperplasia after experimental angioplasty (1–3,7). Administration of L-arginine (the substrate for the enzyme NO synthase), administration of NO donors and inhalation of NO have been associated with an inhibition of neointimal hyperplasia in rats and rabbits (1–7). Conversely, administration of NO synthase inhibitors, such as L-NAME, has been associated with an increase in neointimal hyperplasia (2). To date, however, no data are available concerning the impact of the NO pathway in double-injury models such as the hypercholesterolemic rabbit. This model differs from single-injury models by the fact that angioplasty is performed on an already diseased vessel and by the number of foam cells in the neointimal thickening (24,30); this model may thus better reflect what could occur in human arteries. Our results clearly show that L-arginine has an inhibitory effect on neointimal hyperplasia, whereas the NO synthase inhibitor L-NAME has a stimulatory effect. Our results are thus concordant with those obtained in single-injury models. The mechanisms by which NO affects neointimal hyperplasia are not entirely clear but may relate to an inhibition of smooth muscle cell proliferation and migration (9–11), to an inhibition of extracellular matrix components production (12) or to a modulation of platelet and leukocyte functions (13–15).

**The NO pathway and vessel remodeling.** The production of endogenous vasodilators such as NO has been implicated in vessel remodeling (23). In an arteriovenous fistula model, chronic administration of L-NAME has been associated with decreased vascular enlargement, thereby suggesting that NO may participate in flow-induced remodeling of the rabbit common carotid artery (32). We thus speculated that NO might also participate in other forms of vessel remodeling such as remodeling after balloon angioplasty.

Vessel remodeling after balloon angioplasty may be described as a spectrum of changes in total vessel area ranging from constriction to enlargement. The model used in the present study does not allow us to analyze the exact mechanisms of vessel remodeling; our specific aim was to describe the effects of the NO pathway on vessel size at the lesion site and consequently, taking into account its effects on neointimal hyperplasia, to assess its effects on lumen size. L-arginine–treated animals had no change in vessel size, whereas L-NAME–treated animals had a major increase in vessel size. This observation conflicts with the hypothesis that NO could induce vascular enlargement after angioplasty. One possible explanation for these unexpected findings may be that the significant effects of L-arginine and
L-NAME on neointimal hyperplasia could have an impact on vessel remodeling. Previous studies have suggested that the extent of neointimal formation may be an important determinant of vessel enlargement after angioplasty (17,18), the inhibitory effect of L-arginine on neointimal formation may thus indirectly inhibit vessel enlargement; conversely, the stimulatory effect of L-NAME on neointimal formation may indirectly stimulate vessel enlargement. Whatever is the explanation for our findings, these results demonstrate that despite significant effects on neointimal growth, stimulation or inhibition of the NO pathway has no impact on final lumen size in this experimental model. These results underscore the importance of studying “antirestenosis” drugs in experimental models where the effects of both neointimal hyperplasia and vascular remodeling can be adequately assessed.

There are several limitations to this study. First, as stated above, this model is limited by the lack of a reference nondilated segment for calculation of relative values; this precludes a careful analysis of the mechanisms of the changes in vessel size (i.e., constrictive remodeling vs. lack of enlargement). Second, restenosis is a dynamic process, and both neointimal hyperplasia and vascular remodeling are time-related phenomena. This study used a 4-week time point, which has been the end point of most investigators studying neointimal hyperplasia and vascular remodeling in similar models (17,18); further time course experiments may provide additional information. Finally, our conclusion that the NO pathway had no impact on experimental restenosis may not apply to vascular stents. It has been shown that restenosis after stent implantation may be affected more by neointima, formation rather than vascular remodeling (33). In this instance, targeting the NO pathway to reduce neointimal formation would be important.

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REFERENCES


