L-Arginine Improves Endothelium-Dependent Vasorelaxation and Reduces Intimal Hyperplasia After Balloon Angioplasty

Wallace C. Tarry, Raymond G. Makhoul

Abstract Reductions in nitric oxide (NO) activity persist after arterial intimal injury and may be a factor in the development of intimal hyperplasia. NO inhibits in vitro platelet aggregation, leukocyte adhesion, and smooth muscle cell growth, all of which are key components in the process of intimal hyperplasia. We hypothesized that long-term supplementation with L-arginine, the precursor of NO, would increase NO production and thereby improve endothelium-dependent vasorelaxation and simultaneously reduce intimal hyperplasia. Twenty-six New Zealand White male rabbits were fed standard rabbit chow either with or without 2.25% L-arginine in their drinking water for 3 weeks. Then the animals underwent unilateral iliac artery angioplasty and were continued on their respective diets. Four weeks after angioplasty, the iliac arteries were harvested for functional and morphometric studies. The iliac arteries from several animals from each group were processed for study by electron microscopy. Maximal endothelium-dependent vasorelaxation in injured arteries was significantly greater in L-arginine-supplemented animals (mean ± SEM, 71.8 ± 4.1%; n = 6) than controls (51.4 ± 4.0%, n = 7; P < .05). Furthermore, the intimal area in injured arteries was significantly reduced in L-arginine-supplemented animals (0.22 ± 0.03 mm², n = 5) compared with controls (0.34 ± 0.03 mm², n = 6; P < .05). These data suggest that L-arginine supplementation enhances NO production at sites of vascular healing and may reduce intimal hyperplasia. (Arterioscler Thromb 1994;14:938-943.)

Key Words • nitric oxide • endothelium-dependent relaxation • L-arginine • intimal hyperplasia • rabbits

Mechanically induced vascular intimal injury is characterized by prolonged reduction of endothelial cell nitric oxide (NO) release and concurrent development of intimal hyperplasia. Current evidence suggests that these phenomena may be causally related. Alterations in NO-mediated vasodilation persist in injured arterial segments even after regrowth of an endothelial cell monolayer. Recent in vitro experiments have shown that NO inhibits platelet aggregation, leukocyte adhesion, and smooth muscle cell growth, all of which are key components in the development of intimal hyperplasia. Furthermore, administration of L-arginine, the NO precursor, to hypercholesterolemic rabbits has been shown to reverse defects in NO activity and reduce the development of proliferative atherosclerotic lesions. We hypothesized that long-term administration of L-arginine would increase endothelial cell NO release at the site of balloon angioplasty injury, therefore increasing endothelium-dependent vasorelaxation while simultaneously reducing the intimal hyperplastic response to this injury.

Methods

Animals

Twenty-six New Zealand White male rabbits (3.5 to 5.0 kg body weight) were studied. All animals were housed individually in a controlled-temperature, standard light/dark environment and allowed to acclimate before any intervention or change in diet. Control animals received standard rabbit chow and water ad libitum. Experimental animals received standard chow and drinking water supplemented with 2.25% L-arginine (L-arginine hydrochloride, 9 g/d; Sigma grade, Sigma Chemical Co) for 3 weeks before unilateral iliac artery angioplasty. Animals were continued on their respective diets after angioplasty until they were killed, at which time the iliac arteries were excised and studied for vasomotor function and histomorphometric analysis. The L-arginine regimen was well tolerated by the animals, had no systemic side effects, and produced consistent elevations in systemic arginine levels over a 2- to 3-week period. All protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee in accordance with recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Angioplasty

After 3 weeks of dietary intervention, the rabbits were anesthetized with intramuscular injections of ketamine (35 mg/kg), acepromazine (1 mg/kg), and xylazine (5 mg/kg); supplemental oxygen was delivered by face mask; and prophylactic procaine penicillin (30 000 U/kg) was administered by intramuscular injection. The right carotid artery was exposed, and under fluoroscopic guidance, a coronary angioplasty catheter (2.5 x 20 mm) was passed over a stenotic guide wire into the iliac artery. Beginning at the distal portion of the external iliac artery, we inflated the contrast-filled balloon three times to 10 atm for 30 seconds separated by 30-second intervals. The partially deflated balloon (2 to 3 atm) was then withdrawn 1 cm and inflated three more times. This procedure was repeated to the level of the aortic bifurcation. Previous studies have demonstrated that this technique results in complete removal of the endothelium from a segment approximately 2 to 3 cm long. At the end of the procedure the angioplasty catheter was removed, the carotid artery ligated, and the wound closed.
Measurement of Arginine Levels
Blood was obtained at the time of angioplasty; plasma was isolated and stored at −20°C. Arginine levels were measured by reverse-phase high-performance liquid chromatography as described.7

Measurement of Systemic Blood Pressure
Four weeks after angioplasty, but before sacrifice, control and experimental animals were anesthetized as described above and mean arterial blood pressure was recorded via a 26G cannula inserted in the left middle ear artery and connected to a digital pressure transducer.

Measurement of Vasomotor Function
Four weeks after angioplasty the animals were given 500 U heparin IV and killed with an overdose of pentobarbital (20 mg/kg IV). Iliac arteries were excised and immersed in oxygenated Krebs-Henseleit physiological saline solution of the following composition (mmol/L): NaCl 118.1, KCl 4.7, MgSO4 0.56, KH2PO4 1.2, CaCl2 5.0, NaHCO3 25.0, and glucose 11.1, pH 7.4. The vessels were cleaned of adherent connective tissue and cut into rings (4 mm long, two rings each from the injured and contralateral uninjured iliac arteries). The rings were suspended on stainless steel stirrups connected to a force-displacement transducer (model FT03E, Grass Instrument Co) in an organ chamber containing 10 mL Krebs' solution at 37°C bubbled with 95% O2/5% CO2. The rings were individually stretched to the optimum point of their length-tension relation as determined by contraction to potassium chloride. To study endothelium-dependent vasorelaxation, rings were contracted with norepinephrine (NE) to a tension approximately one half of the maximal contraction (EC50) and then exposed to increasing concentrations of acetylcholine (ACh) in half-log-concentration increments (10⁻⁸ to 10⁻⁴ mol). The rings were rinsed until their tension returned to baseline and allowed to equilibrate for 30 minutes. To study endothelium-independent vasorelaxation, rings were again contracted with NE (EC50) and subsequently exposed to sodium nitroprusside (SNP) in half-log-concentration increments (10⁻⁴ to 10⁻⁴ mol). Responses to ACh and SNP were calculated and expressed as percent relaxation from precontracted tension induced by exposure to NE. To study vasoconstrictor responses, rings were exposed to NE in half-log-concentration increments (10⁻⁴ to 10⁻³ mol) with responses expressed as a percentage of maximal contraction.

Drugs
ACh chloride, SNP, and NE bitartrate (Sigma) were prepared daily in distilled water and administered in a volume of 50 μL. Krebs' solution was prepared daily.

Histology and Morphometry
Four weeks after angioplasty each animal was anesthetized and the abdominal aorta and vena cava were cannulated. After the animals were killed with an overdose of pentobarbital, they were exsanguinated and Krebs' solution was infused into the aortic cannula at a pressure of 90 mm Hg until the venous effluent was clear. The vessels were then perfusion fixed with NE (EQo) and subsequently exposed to sodium nitroprusside (SNP) and Krebs' solution was infused into the effluent was clear. The vessels were then perfusion fixed with 2% phosphate-buffered glutaraldehyde. Arterial segments were then dehydrated in alcohol, postfixed in 1% OsO4, and critical-point dried. The tissue blocks were affixed to aluminum stubs with silver paint and then sputter coated with gold. The stubs were examined and photographed in a JEOL JSM-820 scanning electron microscope (Edward Weck and Co).

Data Analysis
All data are expressed as mean±SEM. Mean vasomotor responses were calculated for individual groups at specific concentrations of ACh, SNP, and NE. Significant differences in vasomotor responses were determined by multivariate ANOVA. Significant differences in serum arginine levels; mean arterial blood pressures; body weights; and intimal, medial, luminal, and total vessel areas were determined by Student's t test for unpaired observations. Values of P<.05 were considered significant.

Results
Systemic Measurements
At the time of angioplasty, plasma arginine levels were consistently elevated in l-arginine-supplemented animals compared with controls (0.46±0.07 vs 0.18±0.02 μmol/mL, P<.05). Animal body weights at the time of angioplasty were not different between groups (4.21±0.11 vs 4.07±0.14 kg, P>.05). Animal body weights did not change significantly by the time the animals were killed and were not different between l-arginine-supplemented and control animals (4.24±0.14 vs 4.10±0.08 kg, P>.05). Mean arterial blood pressures at the time the animals were killed were not significantly different between l-arginine-supplemented and control animals (61.5±2.6 vs 63.2±2.5 mm Hg, P>.05).

Vasomotor Function
Endothelium-Dependent Vasorelaxation
Endothelium-dependent relaxation was significantly impaired in injured iliac arteries compared with the contralateral (uninjured) iliac arteries in control animals. However, endothelium-dependent relaxation was normalized in injured arteries from l-arginine-supplemented animals. In l-arginine-fed animals the relaxation responses in injured and uninjured vessels were not different, nor were there significant differences in the relaxation of uninjured arteries from l-arginine–fed and l-arginine–free animals (Fig 1).

Endothelium-Independent Vasorelaxation
Vasorelaxation in injured arteries in response to SNP was not significantly different between l-arginine–supplemented animals and controls. Responses to SNP in uninjured segments were also not different between groups (Fig 2).

Scanning Electron Microscopy
Scanning electron microscopy was used to determine the status of the endothelial surface of injured arterial segments. Separate control and l-arginine-supplemented animals were killed, exsanguinated, and perfused with Krebs' solution as described above. Vessels were perfusion fixed with 2% phosphate-buffered glutaraldehyde. Arterial segments were then dehydrated in alcohol, postfixed in 1% OsO4, and critical-point dried. The tissue blocks were affixed to aluminum stubs with silver paint and then sputter coated with gold. The stubs were examined and photographed in a JEOL JSM-820 scanning electron microscope (Edward Weck and Co).
Fig 1. Line plot of endothelium-dependent relaxations in rabbit iliac arteries 4 weeks after angioplasty. Responses are expressed as percent relaxation from preconstricted baseline values. Endothelium-dependent vasorelaxation in injured iliac arteries (○) was significantly less than in contralateral uninjured iliac arteries in control animals (●; n=7 animals, two rings per animal). However, endothelium-dependent vasorelaxation was normalized in injured iliac arteries of L-arginine-supplemented animals (▲). Responses to acetylcholine were not different in uninjured segments between control and L-arginine-supplemented (●) animals (n=6 animals, 2 rings per animal; P<.05: uninjured control vs injured control and injured L-arginine-treated vs injured control by ANOVA).

Vasoconstriction

Vasoconstriction in response to NE in injured arteries was not significantly different between L-arginine-supplemented and control animals. Likewise, responses to NE in uninjured segments were not different between groups (Fig 3).

Histology and Morphometry

Vessels demonstrated consistent circumferential intimal hyperplasia with largely intact internal and external elastic laminae. The neointima was characterized by a matrix of smooth muscle cells and surrounding connective tissue. The gross morphological appearance of the lesions was not different between groups. However, intimal area was significantly reduced in injured arteries from L-arginine-supplemented animals compared with controls (Fig 4 and the Table).

Scanning Electron Microscopy

Four weeks after angioplasty, scanning electron microscopy of the endothelial surface of the iliac arteries demonstrated coverage by endothelial cells. These cells appeared morphologically distinct from normal endothelial cells because of the irregular size and shape of the former. There were no differences in appearance of regenerated endothelial cells between control and L-arginine-supplemented animals.

Discussion

This report confirms that chronic dysfunction of the L-arginine/NO pathway at the site of arterial injury may play a causative role in the development of intimal hyperplasia. Our study is the first to demonstrate that long-term administration of L-arginine, the NO precursor, normalizes endothelium-dependent relaxation after mechanically induced arterial injury. This restoration of endothelial function was accompanied by a decrease in vascular intimal hyperplasia. These effects were seen in the absence of significant alterations in systemic hemodynamics or endothelium-independent vasorelaxation. The endothelium-dependent vasorelaxation and histological appearance of normal arterial segments were not altered by L-arginine supplementation.

The critical role of NO in the modulation of normal arterial tone has been well characterized. In 1980 Furchgott and Zawadski demonstrated the presence of an endothelium-derived relaxing factor (EDRF). EDRF was subsequently identified as NO, which is synthesized by endothelial cells from the precursor L-arginine by NO synthase. Systemic defects in endothelial NO production and/or release have been implicated in the pathogen-


### Vessel Areas in Control and Experimental Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-Arginine Treated</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total vessel area*</td>
<td>1.252±0.088</td>
<td>0.993±0.114</td>
<td>.78</td>
</tr>
<tr>
<td>Luminal area*</td>
<td>0.595±0.071</td>
<td>0.484±0.089</td>
<td>.33</td>
</tr>
<tr>
<td>Medial area*</td>
<td>0.299±0.018</td>
<td>0.264±0.023</td>
<td>.24</td>
</tr>
<tr>
<td>Intimal area*</td>
<td>0.336±0.034</td>
<td>0.216±0.033</td>
<td>.02</td>
</tr>
<tr>
<td>Intimal/medial area ratio</td>
<td>1.257±0.130</td>
<td>0.967±0.150</td>
<td>.16</td>
</tr>
</tbody>
</table>

Morphometric measurements are shown for iliac arteries after angioplasty in control (n=6) and L-arginine-supplemented (n=5) animals. There was a significant reduction in intimal area in the L-arginine group.

*In square millimeters.

The critical relationship between endothelial cells and the development of intimal hyperplasia is well recognized. Endothelial cells are known to release growth factors and heparin-like compounds, which likely have a major influence on surrounding cells and in maintaining normal vessel wall homeostasis. Similarly, abnormal NO-mediated vasorelaxation is observed after endothelial injury associated with ischemia and reperfusion in both coronary and peripheral vascular beds.

Previous work has documented that experimental balloon angioplasty results in prolonged dysfunction of the endothelial L-arginine/NO pathway. Electron microscopic studies demonstrate complete endothelium-denuding injury immediately after angioplasty, which corresponds to a complete loss of endothelium-dependent vasorelaxation and a tendency toward vasospasm and thrombosis. Endothelial regrowth generally occurs within 2 to 3 weeks; however, these regenerated endothelial cells display distinct morphological and functional alterations, with diminished production and release of NO and therefore, incomplete endothelium-dependent vasorelaxation. This was confirmed by our data as well. This long-standing deficiency in endothelial cell release of NO persists for as long as 1 to 2 months after injury and may contribute to the excessive growth of vascular smooth muscle cells characteristic of intimal hyperplasia.

The pathogenesis of the cardiovascular abnormalities that accompany hypercholesterolemia, diabetes, and hypertension is well recognized. Similarly, abnormal NO-mediated vasorelaxation is observed after endothelial injury associated with ischemia and reperfusion in both coronary and peripheral vascular beds. Endothelial regrowth generally occurs within 2 to 3 weeks; however, these regenerated endothelial cells display distinct morphological and functional alterations, with diminished production and release of NO and therefore, incomplete endothelium-dependent vasorelaxation. This was confirmed by our data as well. This long-standing deficiency in endothelial cell release of NO persists for as long as 1 to 2 months after injury and may contribute to the excessive growth of vascular smooth muscle cells characteristic of intimal hyperplasia. The critical relationship between endothelial cells and the development of intimal hyperplasia is well recognized. Endothelial cells are known to release growth factors and heparin-like compounds, which likely have a major influence on surrounding cells and in maintaining normal vessel wall homeostasis. Similarly, abnormal NO-mediated vasorelaxation is observed after endothelial injury associated with ischemia and reperfusion in both coronary and peripheral vascular beds.

Morphometric measurements are shown for iliac arteries after angioplasty in control (n=6) and L-arginine-supplemented (n=5) animals. There was a significant reduction in intimal area in the L-arginine group.

*In square millimeters.

### Figure 4

**A.** Histological cross section of an iliac artery 28 days after balloon angioplasty from a rabbit that did not receive L-arginine supplementation; note the large amount of intimal hyperplasia (IH). **B.** Cross section of an iliac artery 28 days after balloon angioplasty from a rabbit that did receive L-arginine supplementation. Note the decrease in the degree of intimal hyperplasia (hematoxylin-eosin stain; original magnification ×100). M indicates media; AD, adventitia.
proliferation. Prolonged dysfunction of the L-arginine/NO axis at the site of an injury may well result in loss of a critical inhibitor of smooth muscle cell proliferation.

We hypothesized that increased endothelial cell NO production at the site of injury would reduce intimal hyperplasia. The effectiveness of the NO precursor L-arginine to increase NO production and release has been confirmed by experimental studies. For example, with short-term administration of L-arginine, Weyrich et al²⁶ and Nakanishi et al²⁷ were able to reverse defects in coronary artery endothelium-dependent vasorelaxation after ischemia/reperfusion injury of myocardium. Likewise, short-term infusion of L-arginine corrects the deficit of endothelium-dependent vasorelaxation in hypercholesterolemic animals and humans.²⁸⁻³³ These effects are seen in the absence of changes in vascular responses to SNP and are stereospecific, thus supporting the theory that these phenomena are mediated by increased production of NO rather than a local or systemic effect of the amino acid arginine. More recently, Cooke et al³⁴ have demonstrated that long-term L-arginine supplementation to cholesterol-fed rabbits decreases atherosclerotic lesions and improves endothelium-dependent vasorelaxation, presumably by increasing NO production. The results of our study are similar, in that L-arginine supplementation normalized endothelium-dependent responses to ACh after injury but had no effect on endothelium-independent vasorelaxation, implying the existence of a common route by which the L-arginine/NO pathway suppresses pathological cellular proliferation in the arterial wall. Our study differs from that of Cooke et al, in that endothelial injury was mechanically induced by a balloon angioplasty catheter and our animals were not hypercholesterolemic. Because balloon angioplasty is a commonly performed clinical procedure, our results, coupled with those of Cooke et al, have important clinical implications. McNamara et al³⁵ have also recently demonstrated that L-arginine inhibits intimal hyperplasia in the rabbit aorta after injury induced by an embolectomy catheter. Our results confirm and significantly extend these findings, in that we have shown that reduced hyperplasia is accompanied by a normalization of NO-dependent relaxation.

This study provides important new evidence that augmentation of NO production at sites of arterial intimal injury might reduce the intimal hyperplastic response. Nonetheless, the specific mechanisms of this effect are likely multifactorial, and this study does not exclude a pivotal role of other key cellular elements or hemodynamic changes in mediating these effects. Because many early events associated with intimal hyperplasia occur before the endothelium is regenerated, the effect of L-arginine on intimal hyperplasia may be partially due to a direct effect of increased NO production by adjacent intact endothelium, platelets, leukocytes, and/or smooth muscle cells. Further studies to elucidate the mechanism of this action are needed.

Acknowledgments

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References


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