Local L-Arginine Delivery After Balloon Angioplasty Reduces Monocyte Binding and Induces Apoptosis

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Background—Local administration of L-arginine after balloon angioplasty has been shown to enhance NO generation and inhibit lesion formation. In this study, we assessed the mechanisms by which local delivery of L-arginine inhibits lesion formation.

Methods and Results—New Zealand White rabbits (n=56) were fed a 1% cholesterol diet. After 1 week, both iliac arteries were balloon-denuded, and a local drug delivery catheter was introduced into both iliac arteries to deliver either L-arginine (800 mg/5 mL with and without 100 μCi L-[2,3-3H]-arginine) or saline. Monocyte-endothelial interaction was assessed by functional binding assay; NO activity was measured by chemiluminescence. Intramural administration of radioactively labeled L-arginine led to significantly higher counts in comparison to the contralateral segment for up to 1 week after delivery (676±223 versus 453±93 cpm/mg; P<0.02); this was associated with significantly higher NO levels in the L-arginine–treated segments (394.4±141.6 versus 86.3±34.3 nmol/mg; P<0.01). Even after 2 to 3 weeks, monocyte binding was significantly decreased by treatment with L-arginine as compared with saline infusion (P<0.01). After 4 weeks, there was a 9-fold greater number of apoptotic cells in the vessel wall of L-arginine as compared with the saline-treated segments (P<0.05).

Conclusions—Intramural delivery of L-arginine immediately after angioplasty causes a sustained increase in tissue L-arginine levels associated with enhancement of local NO synthesis. The local increase in NO synthesis is associated with an attenuation of monocyte binding and increased apoptosis of resident macrophages. This treatment strategy could be valuable for the prevention and management of restenosis. (Circulation. 1999;100:1830-1835.)

Key Words: atherosclerosis ■ catheterization ■ hypercholesterolemia ■ nitric oxide ■ restenosis

Although balloon angioplasty initially leads to an increase in luminal diameter of previously stenosed segments, its long-term benefits are limited by restenosis rates of 40% to 50%. Recent advances in coronary stenting have reduced the restenosis rate. However, neointimal hyperplasia continues to be a problem and causes in-stent restenosis in 15% to 25% of patients. Migrating and proliferating smooth muscle cells accompanied by deposition of extracellular matrix and monocyte adhesion and infiltration contribute to the neointimal formation. A substance delivered to the site of injury immediately after angioplasty could have tremendous impact on the restenosis if it could interfere with these processes. Endothelium-derived NO has been shown to antagonize key processes involved in atherogenesis and restenosis. NO inhibits monocyte adherence and chemotaxis, platelet adherence and aggregation, and vascular smooth muscle proliferation. Enhancement of NO activity in hypercholesterolemic rabbits reduces monocyte adherence and accumulation, whereas inhibition of NO activity enhances endothelial adhesiveness for monocytes and increases the extent of lesion formation. In a previous study, we have demonstrated the feasibility and effectiveness of a single intramural delivery of L-arginine (the precursor of NO) following balloon angioplasty and reported long-term improvement in vasomotion, attenuation of macrophage infiltration and neointimal lesion formation. In this study, we show that a single intramural delivery of L-arginine causes a sustained increase in levels of vascular L-arginine, associated with a persistent increase in NO elaboration, as well as long-term inhibition of monocyte adherence. Most recently, NO has been suggested to induce apoptosis in cell culture studies as well. We have extended this finding by demonstrating that intramural L-arginine induces apoptosis of vascular cells in vivo.

Methods

Fifty-six male New Zealand White rabbits weighing 3.8±1.5 kg were entered into the study after 1 week of acclimation in the housing facilities of the Stanford University Department of Comparative Medicine. All animals were inspected before the study by a veterinarian and monitored daily by technicians and investigators. The experimental protocols were approved by the Administrative
Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Animals were then fed a high cholesterol diet until euthanized (1%, Dyets).

**Anesthesia and Surgical Preparation**

One week after initiating the high cholesterol diet, rabbits were anesthetized using a mixture of ketamine (5 mg/kg) and xylazine (35 mg/kg). The right common carotid artery was exposed, carefully incised, and a 5F sheath was inserted under fluoroscopic control to the descending aorta. An angioplasty balloon (3 mm; Advanced Cardiovascular Systems) was advanced into either iliac artery and inflated distal to the deep femoral artery 6 times for 30 seconds at 8 atm with 30-second intervals between each inflation. Subsequently, the same procedure was repeated in the contralateral iliac artery.

**Local Drug Delivery**

Immediately following angioplasty, a local drug delivery balloon (3 mm; Dispatch, SciMed) was advanced to the left or right iliac artery and placed at the same position as the previous balloon injury site. The proximal end of the delivery catheter was placed at the internal iliac branch under fluoroscopic control for landmark reference. The balloon was inflated to 6 atm and L-arginine (800 mg/5 mL) or saline was infused for 15 minutes at a rate of 0.2 mL/min (total volume 3 mL). This procedure was subsequently repeated in the contralateral iliac artery. The iliac artery to receive L-arginine treatment was determined randomly. An intravenous bolus injection of cefazolin was given for prevention of infections.

**3H-L-arginine Delivery**

Saline and L-arginine infusions were prepared as described above, with the exception that 3H-L-arginine was added to the L-arginine infusion (800 mg/5 mL plus 100 μCi 1-[2,3-3H]-arginine [ie, 100 μL]).

**Harvesting of Tissue**

Animals were euthanized 1 hour, 1 day, 1 week, 2 to 3 weeks, and 4 weeks after the local delivery of L-arginine; the iliac arteries were carefully freed from adjacent tissue. To determine the amount of cell damage induced by the local drug delivery balloon, electron microscopy of the segment exposed to the local delivery balloon was performed in 3 rabbits.

**High Performance Liquid Chromatography**

In a series of experiments, we set out to measure the local concentration of L-arginine one day after delivery into the vessel wall. High performance liquid chromatography (HPLC) analysis of tissue arginine was based on a technique described in full elsewhere. Briefly, samples and internal standard (1-Homoarginine [10 μmol/L]) was added to 0.5 mL of dissolved tissue) were extracted on solid-phase extraction cartridges (CBA Bond Eluate, Varian). Eluates were dried over nitrogen and resuspended in double-distilled water for HPLC analysis. HPLC was performed on a computer-controlled Varian Star chromatography system consisting of a ternary gradient HPLC pump (Varian 9010), an automatic injector with automated sample-reagent mixing capabilities (Varian 9050), and a fluorescence detector (Varian Fluorichrom III). A 250x4.5-mm-ID 7-μm Nucleosil phenyl column (Supelco) was used.

**3H-L-arginine Measurement**

Iliac artery segments were blotted dry and weighed immediately after being excised. They were then immersed in a tissue solubilizing agent (100 mg tissue/1 mL Soluene-350, Packard Instruments) until complete digestion, and mixed with a scintillation cocktail (Hionic-Flour, Packard Instruments; 1:10 v/v). Emission was measured with the use of a β-scintillation counter (Packard liquid scintillation counter model 1500).

**NO Measurements**

Iliac artery rings were opened longitudinally and incubated in 2 mL of Hanks buffered saline (HBSS) solution (Irvine Scientific) containing calcium ionophore (1 μmol/L, A23187, Sigma) and L-arginine (1 mmol/L, Sigma, St. Louis) at 37°C. At 30, 60, and 120 minutes, samples of the medium were collected for measurement of nitrogen oxides, as previously described. Samples (100 μL) were injected into a reduction chamber containing boiling acidic vanadium-chloride III. In the reduction chamber, NO₂⁻ and NO₃⁻ are reduced to nitrogen oxide, which is then detected by chemiluminescence after reaction with ozone (Model 2108, Dasibi Environmental Corp). Signals from the detector were analyzed by a computerized integrator.

**Functional Monocyte Binding Assay**

Iliac arteries were harvested, cut into 10-mm segments, opened longitudinally, and fixed to the culture dishes with the endothelial surface to the medium containing isolated, TRITC labeled (3 μg/mL; Molecular Gibco/BRL Probes, Gaithersburg, Md) murine monocyteoid cells (WEHI 78/24, ATCC). Culture dishes were then placed on a rocking platform (Research Products International Corp) for 30 minutes. After washing, iliac artery segments were placed on glass slides for counting of adherent cells under epifluorescent microscopy. Data from L-arginine-treated vessels are expressed as a percentage of the number of adherent cell per high power field on iliac arteries treated with saline (control=100%).

**Apoptotic Cell Counting**

Quantitation of apoptotic cells was performed in histologic sections of iliac arteries 4 weeks after local drug delivery. The vessel rings were fixed in 10% buffered formalin, embedded in paraffin, and sectioned into 5-μm thick slices. Sections were deparaffinized and hydrated through xylene and graded alcohol series. Hoechst 33342, a membrane permeable dye (Molecular Probes), was added to a final concentration of 5 μg/mL and the sections were incubated for 60 minutes at room temperature in the dark. Immediately thereafter, the nuclear morphology was observed by fluorescence microscopy under ultraviolet light (Leica Laboratory-S, 100-W mercury bulb, excitation and emission filters to pass all wavelengths >400 nm). Individual nuclei were visualized at x400 to distinguish the normal uniform nuclear pattern from the characteristic condensed and fragmented chromatin pattern of apoptotic cells. Like TUNEL, this technique does not differentiate cell type because both apoptotic vascular smooth muscle cells and macrophages are being stained. A brightly staining, condensed nucleus with fragmentation into apoptotic bodies is required criteria for a cell to be considered apoptotic. Although chromatin undergoes condensation during mitosis, these cells can be readily distinguished from apoptotic cells by the fragmentation typical of apoptotic cells. To quantify apoptosis, nuclei from random microscopic fields were analyzed by an observer blinded to group assignment. The apoptotic nuclei were counted in 5 high-power fields for each of 3 cross-sections per vascular segment. These values were averaged and expressed as the number of apoptotic nuclei/10 mm² lesion area. This technique for quantification of apoptosis has been validated in vitro with time lapse videomicroscopy. The results using this technique are consistent with TUNEL staining and DNA fragmentation assessed by gel electrophoresis.

**Statistical Analyses**

Differences were considered significant if the 2-sided P≤0.05. Results are expressed as mean±SE. All calculations were performed using SPSS statistical software. A paired t test was used when values between 2 groups were compared. ANOVA was performed to identify a significant difference among the mean values of a variable measured in >2 groups. When ANOVA was significant, comparisons of the mean values were made by paired Student’s t test with Fisher’s exact test correction.

**Results**

**Determination of Arginine**

We performed HPLC measurements for L-arginine in tissue harvested 1 day after L-arginine delivery. There were no differences detected between sites of saline versus L-arginine delivery. This could mean that because of high levels of...
arginine already present in the vessel, the signal to noise ratio was too low and HPLC was thus not sensitive enough to detect increased levels. It could, however, also mean that L-arginine had already partially been metabolized. Thus, we set out to measure 3H-L-arginine after a single intramural delivery.

3H-L-arginine Measurement

Highest counts of β-emission from radioactively labeled L-arginine were detected 1 hour after delivery with a 2-fold increase in the 3H-L-arginine–treated segments (Figure 1). The difference between 3H-L-arginine and saline and uninjured segments remained significant for at least 1 day after delivery. Although the difference between vehicle- and L-arginine–treated segments of injured vessels was lost after 1 week, counts in the treated segments still remained significantly above those of uninjured proximal control segments.

NO Measurements

Vessels were harvested at 1 hour, 1 day, 1 week, and 2 to 3 weeks after local drug delivery in order to measure NO levels (Figure 2). Preparation and incubation of the tissue take 3 hours, so that the first measurements could be made 4 hours after L-arginine delivery. Although NO levels remained unchanged in the saline-treated segments, there was a significant increase in the L-arginine–treated vessel observed as early as 4 hours after delivery. NO levels further increased at day 1 to levels twice as high as in the saline-treated segments and reached a peak after 1 week, when NO activity was nearly 4 times that of saline-treated vessels. Differences between groups were not detectable 2 to 3 weeks after L-arginine delivery.

Monocyte Binding Assay

Binding assays were performed in the angioplastied segments that received either L-arginine or saline, as well as in uninjured control sites proximal to the injured areas. In comparison to these uninjured control segments, monocyte binding was significantly increased in all injured segments 1 hour and 1 day after intervention (irrespective of local delivery of L-arginine or saline) (Figure 3). Results were nearly identical in the injured and uninjured sites after 1 week. After 2 to 3 weeks, the relative number of adherent cells of the saline-treated segments was significantly more than that observed in the L-arginine–treated segments.

Determination of Apoptosis

Apoptotic cells were counted microscopically after visualization by Hoechst stain 4 weeks after drug delivery. We have previously shown that at this time there was a significant reduction in macrophage infiltration and neointima formation in L-arginine–treated segments. In this study, there were nearly 9 times more apoptotic cells observed in the L-arginine compared with the saline-treated sides (L-arginine: 2.6±0.9, saline: 0.3±0.3 apoptotic cells/10 mm² intimal surface, \( P<0.05 \)) (Figure 4). Furthermore, there was a significant inverse correlation between the extent of neointimal lesion formation and the number of apoptotic cells 4 weeks after L-arginine delivery (\( r=-0.73, P<0.003 \)).

Discussion

The main findings of this study are (1) after local administration, L-arginine levels remain significantly elevated in the
injured vessel wall for 1 week; (2) the local increase in tissue L-arginine levels is associated with a prolonged enhancement of local NO synthesis; (3) monocyte binding to the site of L-arginine delivery is attenuated; and (4) intramural administration of L-arginine augments apoptosis of vascular cells in vivo.

Restenosis rates after successful coronary balloon angioplasty remain high despite recent advances in stenting and are caused in part by smooth muscle cell proliferation; this has not yet been successfully inhibited by pharmacological means. Experimental studies have shown that vascular injury induces local expression of mitogens and chemotactic factors, which mediate neointimal lesion formation. This process is characterized in part by the abnormal migration and proliferation of vascular smooth muscle cells in the intima. In addition, the denudation of the endothelium after balloon injury results in the loss of endothelium-derived inhibitory factors such as endothelium-derived NO, which has been shown to be an important endogenous inhibitor of vascular lesion formation because of its multifactorial function in maintaining vascular homeostasis.

Enhancement of Tissue L-arginine
L-arginine serves as the substrate for the enzyme NO synthase. In cultured endothelial cells, NO synthase is not rate-limiting in the conversion from arginine to citrulline and NO, because the $K_m$ for NO synthase is in the micromolar range, whereas intracellular levels of arginine are in the millimolar range. However, there is substantial evidence that supplementation of L-arginine enhances NO production in certain disease states. In the balloon injury model, vessels are denuded of endothelium and NO is produced by other cells such as proliferating vascular smooth muscle cells and infiltrating monocytes; here, inducible NO synthase is responsible for NO production and L-arginine is rate-limiting. This induction of iNOS in vascular smooth muscle cells is transient because of its downregulation by mitogens activated after vascular injury. Long-term oral L-arginine supplementation and single intramural administration of L-arginine chronically enhance vascular NO generation, resulting in improved vasomotion and inhibition of lesion formation in hypercholesterolemic rabbits. In the present study, we dem-
onstrate that radioactively labeled L-arginine remains elevated until 1 week after intramural delivery, which is associated with persistent increase in NO release, inhibition of monocyte binding, and increased rates of apoptosis. Counts detected must stem from L-arginine or one of its breakdown products still resident in the vessel wall, thus documenting that pools were created in the vessel wall. Possible breakdown products that could carry the radioactive label include ornithine, citrulline, glutamate, agmatine, and urate. However, none of these could explain the observed effects, thus lending further support to our hypothesis that local delivery of L-arginine reduces intimal hyperplasia, as previously shown, and increases apoptosis, as reported in this study.

**NO Inhibits Monocyte Binding**

NO inhibits the interaction of circulating blood elements with the vessel wall and its activity is reduced in hypercholesterolemia and after vascular injury. Both oral and local administration of the NO precursor L-arginine have been shown to restore vascular NO activity in animals and in humans, and this is associated with reduced endothelial adhesiveness for monocytes and inhibition of intimal monocyte accumulation in the vessel wall. By contrast, long-term administration of NO synthase antagonists augments endothelial adhesiveness for monocytes and accelerates atherogenesis. In the present study, we show that local enhancement of NO activity reduces monocyte binding to the vessel wall as long as 2 to 3 weeks after L-arginine delivery. As a consequence of balloon angioplasty, the endothelium is removed at the time of the intervention and is not fully regenerated at the site of injury for several weeks in this animal model. Thus, inhibition of monocyte adhesion is most likely a result of the activity of NO synthase expressed by vascular smooth muscle cells. Indeed, it has been found that smooth muscle cells in the neointima express inducible NO synthase as early as 1 day after balloon catheter injury and this expression persists for up to 14 days. Also, transfection of vascular smooth muscle cells with a plasmid construct containing NO synthase-enhanced NO generation locally and inhibited myointimal hyperplasia. However, there is also evidence that infiltrating cells are generating iNOS. Under these circumstances, peroxynitrite as well as NO is being produced. Either of the nitrogen oxides could contribute to the apoptosis observed.

The mechanism by which NO inhibits monocyte adhesion is probably multifactorial. NO can inhibit monocyte adhesion to the endothelium, mediated by cGMP modulation of adhesion signaling. However, NO also downregulates the endothelial expression of monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1), which play critical roles in monocyte-vessel wall interaction. By contrast, inhibition of NO synthase increases the expression of endothelial proteins required for monocyte adhesion. Recent studies from our laboratory and others implicate the existence of an oxidant-sensitive transcriptional pathway that activates the expression of VCAM-1 and MCP-1. Cultured human aortic endothelial cells exposed to oxidized lipoprotein or cytokines, endogenous NO or exogenous NO donors, inhibit endothelial elaboration of superoxide anion, reduce the activity of NFκB, suppress the stimulated expression of VCAM-1 and MCP-1, and reduce endothelial adhesiveness for monocytes. NO may exert these effects in part by inhibiting the generation of superoxide anion by oxidative enzymes.

**L-arginine Induces Apoptosis In Vivo**

In response to a variety of stimuli and circumstances, cells have an intrinsic capacity to activate a gene-directed program that commits the cell to a suicidal death, described as apoptosis. It has become increasingly clear that the process of cell death by apoptosis is a relatively ubiquitous phenomenon observed in a variety of cell types; it occurs within the context of atherosclerosis and restenosis after angioplasty, as recently shown in studies of human vascular lesions. Also, in vitro studies have documented that both endothelial and VSMCs undergo apoptosis in response to the removal of mitogens such as PDGF-BB present within serum. Furthermore, there is immunohistochemical evidence for proteins like p53 and interleukin-1β-converting enzyme to be involved in apoptosis of atherosclerotic lesions and for interferon-γ, tumor necrosis factor-α, and interleukin-1β in vascular smooth muscle cells.

Whereas this and other studies show that NO induces apoptosis in vascular cells, NO donors have been demonstrated to have anti-apoptotic effects in cultured endothelial cells, suggesting that the effect of NO as a modulator of apoptosis is cell-specific and dependent on the presence of certain cytokines, growth factors, or oxidative stress. A rapidly emerging body of evidence suggests that vascular remodeling and lesion formation are determined in large part by the balance between cell growth and cell death by apoptosis. NO beneficially modulates both. Our laboratory and others have previously shown that vasodilators such as NO inhibit cell growth. Moreover, we have recently used an in vivo gene transfer experimental approach to demonstrate that the endogenous generation of NO reduces vascular lesion formation by inhibiting smooth muscle cell proliferation and migration after balloon injury. Moreover, Pollman et al have now elegantly demonstrated in an in vitro model that NO not only modulates the vascular structure by regulating cell growth but is also a potent inducer of vascular smooth muscle cell apoptosis via a second messenger signaling pathway involving cGMP. The present study is consistent with these observations but extends them by demonstrating in vivo that local enhancement of NO activity induces apoptosis, which, together with reduced monocyte binding, inhibits myointimal hyperplasia after balloon angioplasty.

It is most likely that iNOS expressed by intimal macrophages and vascular smooth muscle cells are responsible for the effect of L-arginine. This is in keeping with previous immunohistochemical studies that could demonstrate activation of iNOS in the intimal macrophages and vascular smooth muscle cells of human atherosclerotic plaque. In the presence of superoxide anion, which is produced under these conditions, the product of iNOS is quickly transformed into peroxynitrite anion, a highly reactive free radical which itself is cytotoxic and may also induce apoptosis by causing...
DNA strand fragmentation. Both NO or peroxynitrite anion could induce apoptosis of vascular smooth muscle cells.

Conclusion
Intramural delivery of L-arginine immediately after balloon injury leads to prolonged enhancement of local NO synthesis, attenuated monocyte binding, and augmented apoptosis of vascular cells. Furthermore, we show for the first time that intramural delivery of L-arginine induces apoptosis of resident macrophages in vivo, which thus provides further insight into the underlying mechanisms of the observed reduced rate of restenosis in the L-arginine–treated vessels. In view of this and other studies, this treatment strategy could be valuable for the prevention and management of restenosis.

References
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_Circulation_. 1999;100:1830-1835
doi: 10.1161/01.CIR.100.17.1830

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/17/1830

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