Long-term Oral Administration of L-Arginine Reduces Intimal Thickening and Enhances Neoendothelium-Dependent Acetylcholine-Induced Relaxation After Arterial Injury

Martial Hamon, MD; Benoit Vallet, MD; Christophe Bauters, MD, FESC; Nicolas Wernert, MD; Eugène P. McFadden, MRCPI, FESC; Jean-Marc Lablanche, MD, FESC, FACC; Bernard Dupuis, MD, PhD; Michel E. Bertrand, MD, FESC, FACC

Background Nitrergic oxide (NO), in addition to its potent vasorelaxant properties, may participate in growth regulation of cultured smooth muscle cells. It was recently demonstrated that in vivo endothelial injury induces the production of NO from L-arginine in the arterial wall.

Methods and Results We studied the effects of long-term administration of L-argiine, the precursor of NO, on neointimal thickening and on neoendothelium-dependent vasorelaxation 4 weeks after balloon denudation of normocholesterolemic rabbit iliac arteries. Rabbits were fed with either a standard diet or a diet supplemented with L-arginine (2.25%) in their drinking water 3 days before and during 4 weeks after balloon denudation. The effectiveness of L-arginine supplementation was confirmed by measurement of plasma arginine levels. L-Arginine had no effect on hemodynamic parameters. All animals were killed 4 weeks after balloon denudation, and a digital histomorphometric analysis of three serial nonconsecutive histological cross sections per iliac artery was performed. Intimal thickening was reduced (P<.05) from 0.43±0.08 (SE) mm² in controls (n=8) to 0.24±0.02 mm² in treated animals (n=8). Ten animals (n=5 in each group) were used for in vitro vasoreactivity assessment 4 weeks after balloon denudation. Neoendothelium-dependent acetylcholine-induced relaxation (10⁻⁵ mol/L to 3.10⁻⁵ mol/L) in treated animals (Emax=-24.1±5.5%) was significantly greater than in controls (Emax=-8.9±2.2%). Endothelium-independent relaxation did not differ between groups (Emax=-58.1±6.5% in L-arginine-supplemented animals versus -52.9±6.8% in controls).

Conclusions Our results demonstrate that L-arginine, a precursor of NO, reduces neointimal thickening after balloon denudation and improves neoendothelial-dependent acetylcholine-induced relaxation. (Circulation. 1994;90:1357-1362.)

Key Words • endothelium-derived factors • balloon • acetylcholine • endothelium

The response of the arterial wall to experimental endoluminal injury has been studied extensively during the past few years.1-3 Blood vessel balloon denudation evokes a series of events including permeation of platelet factors into the vessel wall, smooth muscle cell (SMC) proliferation and migration to the intima of the injured vessel, and accumulation of a large amount of extracellular matrix. Endothelial cells play a fundamental role in controlling vessel tone and may additionally regulate the growth of the underlying SMCs.4,6 After experimental angioplasty, areas where the endothelium rapidly regenerates have less marked intimal thickening than areas where endothelial regeneration occurs later.6 This observation may be related to inhibition of SMC proliferation by endothelium-derived substances. Endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO)7 or a closely related compound, in addition to its potent vasorelaxant properties, has been shown to have growth-regulatory properties in cultured SMCs.8 In a recent study, long-term therapy with L-arginine, a precursor of EDRF-NO, markedly reduced endothelial dysfunction and inhibited the development of atherosclerosis in hypercholesterolemic rabbits.9 We studied the effects of long-term oral administration of L-arginine, the precursor of EDRF-NO, on the degree of neointimal thickening and the response to acetylcholine, an endothelium-dependent vasoactive agent, 4 weeks after balloon denudation of normocholesterolemic iliac rabbit arteries.

Methods

The experimental design is illustrated in Fig 1. The investigation conformed to the guiding principles of the American Physiological Society for the care and use of laboratory animals.

Administration of L-Arginine

Normal diet was supplemented with 2.25% L-arginine hydrochloride (Sigma Chemical Co) in the drinking water of animals randomly assigned to active treatment for 3 days before balloon denudation and for 28 days afterward until death. This amount of L-arginine supplementation in drinking water has been shown to result in a sixfold increase in daily L-arginine intake.5 Supplementation with this dose of L-arginine was begun 3 days before balloon denudation and resulted in a significant increase in plasma-free arginine levels after 3 days of treatment as ascertained by a pilot study.

Received January 27, 1994; revision accepted May 20, 1994.

From the Departments of Cardiology (M.H., C.B., E.P.M., J.-M.L., M.E.B.), Pharmacology (B.V., B.D.), and Pathology (N.W.), University of Lille (France).

Correspondence to M.E. Bertrand, MD, Service de Cardiologie B et Hémodynamique, Hôpital Cardiologique, Blvd du Professeur J Leclercq, 59037 Lille Cedex, France.

© 1994 American Heart Association, Inc.
In the study design, areas denudation, biocom (system, Biochem). In each group of animals, balloon denudation arterial blood was obtained from animals in each group 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). Four weeks after balloon denudation arterial blood pressure was measured under general anesthesia at time of death via a catheter inserted into the right carotid artery just before vessel fixation.

**Histological Morphometry**

Four weeks after balloon denudation, 16 animals were killed (n = 8 in each group). A catheter was introduced into the aorta through the right carotid artery and the iliac arteries were fixed by perfusion in situ with 4% paraformaldehyde (in a saline phosphate buffer solution) at a pressure of 110 mm Hg over 30 minutes to maintain the vessels in their in vivo dimensions for subsequent histological analysis. After further immersion fixation (in 4% paraformaldehyde for 24 hours), vessels were embedded in paraffin. Cross sections of vessels were cut and stained with hematoxylin-eosin-saffron-astra blue. Morphometric analysis of the histological cross sections was performed with a digital microscopic planimetry (morphometry-system, Biocom). Three serial nonconsecutive histological cross sections per iliac artery were analyzed. The mean areas of the neointima and of the media were calculated. All measurements were performed by a pathologist unaware of the study design.

**In Vitro Vasoreactivity**

Ten animals (n = 5 in each group) were used for assessment of vasoreactivity. Four weeks after balloon denudation, the iliac arteries were removed and placed in iced oxygenated Krebs-Henseleit (KH) solution consisting of the following (mmol/L): NaCl 118, KCl 4.6, NaHCO3 27.2, MgSO4 1.2, KH2PO4 1.2, CaCl2 1.75, Na2EDTA 0.03, d-glucose 11.1 (pH 7.35 to 7.45). Heparin (1000 U IV) was given before removal of the vessels to prevent coagulation. Vessels were cleaned of surrounding fat and connective tissue and cut into rings 4.5 to 5 mm long. Rings were then suspended in organ chambers (Radnoti Glass Technology) filled with 40 mL warmed (37°C) and oxygenated (95% O2/5% CO2) KH. Rings were connected to force transducers, and changes in isometric force were recorded continuously. During a 60-minute period, the vascular rings were stretched to 3.0 to 4.0 g, previously determined as the optimal point of their length-tension. The output from the transducers was amplified by signal conditioners and sent to an Intel 486-based computer (Kenitec) for analog/digital conversion. In each animal, the nondenuded contralateral iliac artery served as a control. For each iliac artery, two rings were studied. The contractile response to a depolarizing concentration of potassium chloride (KCl) (70 mmol/L) provided a measure of maximal contractile responsiveness in each ring. After duplicate 5-minute exposure to KCl, rings were washed and allowed to stabilize at a resting tension for a further period of 40 minutes. All of the rings then were constricted with phenylephrine [(PE) 10⁻⁷ to 10⁻³ mol/L]. Endothelium-dependent or -independent relaxant effects then were established when the constrictor response to PE was stable. First, the relaxant response to acetylcholine [ACH] 10⁻⁸ mol/L to 3.10⁻⁵ mol/L] was investigated. When the maximal responses produced by this agonist were stable, the rings were washed and allowed to stabilize at a resting tension. The smooth muscle relaxant sodium nitroprusside (10⁻² mol/L) then was given to vessels preconstricted with PE (10⁻⁴ mol/L).

**Drugs**

L-Arginine hydrochloride, ethyl carbamate, heparin sodium, PE hydrochloride, ACh, and sodium nitroprusside were purchased from Sigma Chemical Co. The drugs were dissolved in 0.9% NaCl solution. Gases were purchased from the Compagnie Francaise des Produits Oxygenes and were within a tolerance of 1% of the desired mixture.

---

**Fig 1.** Flow diagram illustrating study sequence of events. During the treatment period, 26 animals were fed normal rabbit chow supplemented with 2.25% l-arginine hydrochloride in the drinking water of animals assigned to active treatment (n = 13) for 3 days before balloon denudation and for 28 days afterward until death. Assessment of vasoreactivity or neointimal thickening was performed at death.

[534.4±67.2 mmol/L versus 173.8±16.8 mmol/L, P<.0001; l-arginine (n=7) versus control (n=7)].

**Balloon Denudation**

Male New Zealand rabbits (2.5 to 2.7 kg) fed with normal rabbit chow were anesthetized with ethyl carbamate (1 g/kg IV). After exposure of the right femoral artery, a 3F Fogarty balloon catheter was passed retrogradely to the junction of aorta with iliac artery and inflated until contact was made with the endothelium. Right iliac artery denudation was achieved by gently advancing and withdrawing the catheter three times. This technique previously has been shown to produce denudation and a detectable loss of SMCs in this model.² The catheter then was removed, the femoral artery was ligated, and 250 mg amoxicillin was injected locally.

**Biochemical and Physiological Measurements**

Before the initiation of treatment, 3 days later at time of balloon denudation, and 28 days after balloon denudation at time of death, blood samples were obtained from animals in each group 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). Four weeks after balloon denudation arterial blood pressure was measured under general anesthesia at time of death via a catheter inserted into the right carotid artery just before vessel fixation.

**Histological Morphometry**

Four weeks after balloon denudation, 16 animals were killed (n = 8 in each group). A catheter was introduced into the aorta through the right carotid artery and the iliac arteries were fixed by perfusion in situ with 4% paraformaldehyde (in a saline phosphate buffer solution) at a pressure of 110 mm Hg over 30 minutes to maintain the vessels in their in vivo dimensions for subsequent histological analysis. After further immersion fixation (in 4% paraformaldehyde for 24 hours), vessels were embedded in paraffin. Cross sections of vessels were cut and stained with hematoxylin-eosin-saffron-astra blue. Morphometric analysis of the histological cross sections was performed with a digital microscopic planimetry (Morphometry-System, Biocom). Three serial nonconsecutive histological cross sections per iliac artery were analyzed. The mean areas of the neointima and of the media were calculated. All measurements were performed by a pathologist unaware of the study design.

**In Vitro Vasoreactivity**

Ten animals (n = 5 in each group) were used for assessment of vasoreactivity. Four weeks after balloon denudation, the iliac arteries were removed and placed in iced oxygenated Krebs-Henseleit (KH) solution consisting of the following (mmol/L): NaCl 118, KCl 4.6, NaHCO3 27.2, MgSO4 1.2, KH2PO4 1.2, CaCl2 1.75, Na2EDTA 0.03, d-glucose 11.1 (pH 7.35 to 7.45). Heparin (1000 U IV) was given before removal of the vessels to prevent coagulation. Vessels were cleaned of surrounding fat and connective tissue and cut into rings 4.5 to 5 mm long. Rings were then suspended in organ chambers (Radnoti Glass Technology) filled with 40 mL warmed (37°C) and oxygenated (95% O2/5% CO2) KH. Rings were connected to force transducers, and changes in isometric force were recorded continuously. During a 60-minute period, the vascular rings were stretched to 3.0 to 4.0 g, previously determined as the optimal point of their length-tension. The output from the transducers was amplified by signal conditioners and sent to an Intel 486-based computer (Kenitec) for analog/digital conversion. In each animal, the nondenuded contralateral iliac artery served as a control. For each iliac artery, two rings were studied. The contractile response to a depolarizing concentration of potassium chloride (KCl) (70 mmol/L) provided a measure of maximal contractile responsiveness in each ring. After duplicate 5-minute exposure to KCl, rings were washed and allowed to stabilize at a resting tension for a further period of 40 minutes. All of the rings then were constricted with phenylephrine [(PE) 10⁻⁷ to 10⁻³ mol/L]. Endothelium-dependent or -independent relaxant effects then were established when the constrictor response to PE was stable. First, the relaxant response to acetylcholine [ACH] 10⁻⁸ mol/L to 3.10⁻⁵ mol/L] was investigated. When the maximal responses produced by this agonist were stable, the rings were washed and allowed to stabilize at a resting tension. The smooth muscle relaxant sodium nitroprusside (10⁻² mol/L) then was given to vessels preconstricted with PE (10⁻⁴ mol/L).

**Drugs**

L-Arginine hydrochloride, ethyl carbamate, heparin sodium, PE hydrochloride, ACh, and sodium nitroprusside were purchased from Sigma Chemical Co. The drugs were dissolved in 0.9% NaCl solution. Gases were purchased from the Compagnie Francaise des Produits Oxygenes and were within a tolerance of 1% of the desired mixture.
Hamon et al
L-Arginine and Arterial Injury 1359

Fig 3. Photomicrographs of representative cross sections of rabbit iliac arteries from a control animal (A) and from an L-arginine treated animal (B). There is much less marked intimal thickening in the L-arginine-treated animal. Arrow indicates the internal elastica lamina. Magnification ×250.

A

B

Statistical Analysis

Data are expressed as mean±SEM. Data were analyzed by ANOVA followed by Scheffé’s F tests to determine if differences existed between groups. A one-way ANOVA with a design for repeated measures followed by Student Newman-Keuls tests was used for comparison of changes in plasma arginine levels and for comparison of sequential changes in vasoreactivity in vessel rings after incremental concentrations of vasoactive agents. A P value <.05 was considered to indicate statistical significance. The n refers to the number of animals in a group.

Results

Biochemical and Physiological Measurements

Plasma arginine levels were significantly higher in animals whose diet was supplemented with L-arginine compared with animals on normal diet (control). This elevation in plasma-free arginine levels was maintained throughout the course of the study (Fig 2). There were no significant differences in hemodynamic measurements between the two groups of animals 4 weeks after balloon denudation. Systolic arterial pressure (136±1.8 mm Hg versus 138±2.3 mm Hg), diastolic arterial pressure (97±1.7 mm Hg versus 97.2±2.8 mm Hg), and mean arterial pressure (110±2.2 mm Hg versus 111±3.5 mm Hg) were similar in both control animals and animals supplemented with L-arginine, respectively.

Histological Studies

Histomorphometric analysis revealed that the medial cross-sectional areas were not different between the two groups. By contrast, a significant decrease in the neointimal cross-sectional area was noted in animals receiving L-arginine supplementation compared with control animals (Fig 3). Intimal cross-sectional area was reduced from 0.43±0.08 mm² to 0.24±0.02 mm² [control animals (n=8) versus animals supplemented with L-arginine (n=8), respectively, P<.05]; neointima/media ratio was reduced from 107.4±5.6% to 60.9±8.2% (P<.0004).

In Vitro Vasoreactivity

Previously Denuded Iliac Arteries

In previously denuded iliac arteries, endothelium-independent responses did not differ significantly between the
Nondenuded arteries, associated denuded arteries, relaxation ACh-induced between groups.

The maximal responses. Supplementation with L-arginine was not associated with a significant change in endothelium-independent responses. In denuded arteries, supplementation with L-arginine was not associated with a significant change in endothelium-independent responses. The maximal constriction induced by either potassium chloride (KCl 70 mmol/L) or phenylephrine (PE 10^-6 mol/L) (percent change from baseline tension) did not differ between groups. The maximal relaxation induced by sodium nitroprusside (SNP 10^-6 mol/L) (percent change from precontraction with PE) was also similar in the two groups.

two groups. The maximal tension induced by either KCl (70 mmol/L) or PE (10^-6 mol/L) was not different between groups (Fig 4). Similarly, there were no differences between groups in maximal vasodilation to sodium nitroprusside (10^-7 mol/L); Emax = -58.1±6.5% in L-arginine-supplemented animals versus -52.9±6.8% in controls (Fig 4). In contrast, the maximal endothelium-dependent ACh-induced relaxation of the L-arginine group was significantly greater than that of the control group; Emax = -24.1±5.5% in L-arginine-treated animals versus -8.9±2.2% in control animals (Fig 5).

Nondenuded Iliac Arteries

In nondenuded iliac arteries, endothelium-independent responses (Fig 4) and endothelium-dependent responses did not differ significantly in arginine-treated and control animals (Fig 5).

Discussion

Our results demonstrate that long-term administration of L-arginine, the precursor of EDRF-NO, reduces neointimal thickening after balloon denudation and concomitantly improves endothelial-dependent ACh-induced relaxation.

In this animal model of balloon denudation, the response to injury is well characterized and has some features in common with the restenotic process in humans after percutaneous transluminal coronary angioplasty. Proliferation and migration to the intima of medial SMCs are critical events in the arterial wall response to injury. Alterations in the release of EDRF-NO are known to persist after endothelial injury and may be a factor in the development of neointimal thickening. After balloon denudation, products of activated platelets such as thromboxane, serotonin, and platelet-derived growth factor may lead to vasoconstriction and the proliferation of vascular SMCs. In addition to its role in endothelial-dependent vasorelaxation, EDRF-NO inhibits in vitro platelet aggregation, leukocyte adhesion, and SMC growth, all key components in the development of neointimal thickening after arterial injury. Diminished NO production may facilitate atherosclerosis and restenosis following angioplasty by promoting interactions between platelets and the vessel wall through loss of NO-mediated platelet inhibition. In addition to limiting platelet-endothelial interactions, NO may play an important role in maintaining the normal mitogenic state of vascular smooth muscle by stimulating the production of cyclic guanosine monophosphate (cGMP) to inhibit the proliferation of vascular SMCs. DFRF-NO is synthesized from L-arginine by a constitutive enzyme NO synthase in endothelial cells, but also can be produced through activation of inducible NO synthase in some cells such as macrophages, endothelial cells, and SMCs. NO synthase activity is induced in arterial wall following in vivo injury by balloon catheterization even in the absence of endothel-
Kinesin released from blood cells at sites of vascular injury is the most prominent cell type in the arterial wall. The mediator that induces NO synthase activity in the arterial wall remains to be determined, but interleukin-1β or cytokines released from blood cells at sites of vascular injury are potential candidates. A recent study looked at the antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. The authors showed that supplementation of dietary L-arginine, the EDRF-NO precursor, improves endothelium-dependent vasorelaxation and is associated with a reduction in atherogenesis. Inhibition of catheter-induced intima hyperplasia by L-arginine was demonstrated recently, but no studies have examined the effect of L-arginine on neoendothelial ACh-induced relaxation. Our results confirm that L-arginine reduces neointimal hyperplasia and, in addition, show that L-arginine partially normalizes neoendothelium-dependent ACh-induced relaxation.

The improvement of the endothelium-dependent relaxation was associated with an inhibition of intimal thickening in animals that were supplemented with L-arginine; in the control animals, severe impairment of vasorelaxation was associated with an inhibition of intimal thickening, associated with an improvement of neoendothelium-dependent vasorelaxation and a significant inhibition of intimal thickening after arterial injury may have important clinical implications. Of course, there is marked discrepancy between the numerous positive results obtained in animal models and the results of pharmacological trials with the same molecules in humans. Additional studies in other species are needed before L-arginine can be considered a candidate for the prevention of human restenosis. Interestingly, a recent large clinical trial has demonstrated a beneficial effect of molsidomine, a direct NO donor, in the prevention of restenosis after coronary angioplasty.

In conclusion, modest supplementation of dietary L-arginine improves neoendothelium-dependent relaxation in rabbits 4 weeks after balloon denudation. This improvement in EDRF-NO activity is associated with a reduction in intimal thickening. Taken together, these results are consistent with the hypothesis that endothelium-derived NO may contribute to growth regulation of SMCs in the vessel wall.

Acknowledgments

We acknowledge the financial support of Groupe Lillois de Recherche en Pathologie Vasculaire and the CIVIS (Service Commun d’Imagerie Vasculaire Interventionnelle, CHRU Lille).

References


Long-term oral administration of L-arginine reduces intimal thickening and enhances neoendothelium-dependent acetylcholine-induced relaxation after arterial injury.

M Hamon, B Vallet, C Bauters, N Wernert, E P McFadden, J M Lablanche, B Dupuis and M E Bertrand

_Circulation_. 1994;90:1357-1362
doi: 10.1161/01.CIR.90.3.1357

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 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/90/3/1357

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/