L-Arginine and L-NAME have no effects on the reendothelialization process after arterial balloon injury

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Abstract

Objective: Growth regulatory properties of nitric oxide (NO) in cultured endothelial cells is controversial. The aim of our study was to investigate the effect of L-arginine, the endogenous NO precursor, and L-NAME, an inhibitor of NO synthase on the reendothelialization process after angioplasty.

Methods: Fifty-five New Zealand White rabbits underwent denudation of the left iliac artery. After injury the rabbits were randomized in three groups: L-arginine 2.25% (n=19); N⁵-nitro-L-arginine methyl ester 15 mg/kg/day (L-NAME, n=19); and placebo (controls, n=17). Treatment was solubilized in drinking water. Reendothelialization was evaluated at 4 weeks by macroscopic evaluation of Evans blue staining and endothelial-specific immunostaining (CD-31) on cross sections. Intimal hyperplasia was evaluated by morphometric analysis.

Results: Despite a significant increase in plasma arginine (P<0.001) and a reduction in intimal hyperplasia (P<0.003) with L-arginine, neither agent had a significant effect on reendothelialization at 4 weeks (controls=36±4%, L-arginine=43±3%, L-NAME=33±4%; NS). Conclusion: These results suggest that, in spite of previously demonstrated effects on neointimal hyperplasia, the NO pathway does not influence the regrowth of macrovascular endothelial cells in vivo.

Keywords: Experimental; Vasculature; Circulatory physiology arteries; Endothelial factors; Nitric oxide; Angioplasty

1. Introduction

The response of the arterial wall to experimental endoluminal injury has been studied extensively during the past few years. Balloon denudation of blood vessels induces proliferation and migration of smooth muscle cells and accumulation of a large amount of extracellular matrix [1]. Endothelium plays a fundamental role in controlling vessel tone and, in addition, may regulate the growth of the underlying smooth muscle cells [1,2]. After experimental angioplasty, areas where the endothelium rapidly regenerates have less marked intimal thickening than areas where endothelial regeneration occurs later [2]. This observation may be related to inhibition of vascular smooth muscle cell (VSMC) proliferation by endothelium-derived substances.

Endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO) [3], has been shown to have growth-regulatory properties in cultured smooth muscle cells and cultured endothelial cells [4,5]. NO is derived from the metabolism of L-arginine to citrulline by nitric oxide synthase and L-NAME is an inhibitor of NO synthase. Long-term therapy with L-arginine, markedly reduced endothelial dysfunction and inhibited the development of atherosclerosis in hypercholesterolemic rabbits [6,7]. Moreover, long-term oral administration of L-arginine...
reduces intimal thickening and enhances neoendothelium-dependent acetylcholine-induced relaxation after arterial injury and endothelial denudation [8–10]. It was speculated that these effects might partly relate to an inhibitory effect of regeneration of the endothelium on smooth muscle hyperplasia.

The aim of our study was to investigate the effect of the NO precursor, l-arginine, and an inhibitor of NO synthase, l-NAME, on the reendothelialization process after balloon denudation of iliac arteries in normocholesterolemic rabbits.

2. Methods

2.1. Study protocol

Fifty-five male New Zealand White rabbits (3–3.5 kg), fed normal rabbit chow, underwent balloon denudation of one iliac artery (see below). Immediately after the procedure the rabbits were randomized into three groups: 19 rabbits received 2.25% l-arginine hydrochloride (Sigma Chemical Co, St. Louis, MO) solubilized in 200 ml drinking water (l-arginine group); 19 rabbits received 15 mg/kg/day of N⁶-nitro-l-arginine methyl ester (l-NAME; Sigma) solubilized in 200 ml drinking water (l-NAME group); and 17 rabbits received 200 ml normal drinking water alone as a placebo (control group). The dose of l-arginine was chosen because it has been shown by our group and by others [10–12] to result in a six-fold increase in daily l-arginine intake and in a reduction of intimal hyperplasia in rabbits. The dose of l-NAME was chosen because it has been used in rabbits, either by oral or subcutaneous administration, to inhibit NO production and achieve biological effects without significant changes in arterial pressure [12–15]. All animals were sacrificed 4 weeks later for analysis of the reendothelialization process. Forty-two animals were used for macroscopic evaluation of reendothelialization and 13 for histological analysis. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996).

2.2. Balloon denudation

All rabbits were anesthetized with ethyl carbamate (1 g/kg i.v.). After exposure of the left femoral artery, a 3F Fogarty balloon catheter was passed retrogradely to the junction of aorta with the iliac artery and inflated until contact was made with the endothelium. Left iliac artery deendothelialization was accomplished by advancing and withdrawing the catheter three times [10]. This technique has previously been shown to produce effective deendothelialization and a detectable loss of VSMCs in this model [10]. The catheter then was removed, the femoral artery was ligated, and 125 mg amoxicillin was applied locally.

2.3. Biochemical and physiological measurements

Blood samples were obtained at the time of the procedure, before first treatment administration, and at 3 weeks 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).

In biological fluids nitric oxide is rapidly deactivated by oxidation to nitrite (NO₂⁻) and nitrate (NO₃⁻) by physically dissolved oxygen and water. To ensure that treatment with l-arginine was associated with an increase in NO production, NOx measurements were performed in blood samples in l-arginine and control groups according to the method described by Giovannoni et al. [16]. Nitrate was stoichiometrically reduced to nitrite by incubation for 15 min. at 37°C with reduced nicotinamide adenine dinucleotide (Sigma) and nitrate reductase (Boehringer Mannheim). After incubation at room temperature for 15 min, the resulting solution was incubated with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye. Absorbance was read at 550 nm. Nitrite concentration was determined from a linear standard curve between 6.25 and 150 μM potassium nitrate. Measurements were performed in quadruplicate.

Blood pressure was measured under anesthesia via a catheter inserted into the carotid artery at the time of sacrifice (4 weeks) in the three groups.

2.4. Macroscopic evaluation of reendothelialization

Forty-two animals were sacrificed at 4 weeks to perform macroscopic evaluation of reendothelialization. This evaluation was performed by examining fresh iliac artery specimens following in vivo injection of 5 ml 1% Evans blue, as previously described [17,18]. Specimen were macrophotographed, the resulting pictures were digitized and planimetric analysis was performed using computerized sketching program (COLORIMAGE 1.32, NIH, Bethesda, MD) by a single observer, who was unaware of the treatment allocation. The initially denuded area was defined as the total surface of the iliac segment. The reendothelialized area was defined as the area that was not stained with Evans blue.

2.5. Histological morphometry

Thirteen animals were sacrificed at 4 weeks to perform histological analysis. A catheter was introduced into the abdominal aorta and the iliac arteries were fixed by perfusion in situ with 4% paraformaldehyde at a pressure of 110 mmHg over 30 min to maintain the vessels in their in vivo dimensions for subsequent histological analysis.
After further immersion fixation (in 4% paraformaldehyde for 24 h), each iliac artery was divided in three equal portions (proximal, middle and distal), cryoprotected by immersion in sucrose (30% for 4 h), embedded in OCT and frozen in isopentane. Three non-consecutive 6-μm thick sections stained with orcein and selected at random in each of the three predefined portions of the iliac artery were analyzed. Morphometric analysis of the sections was performed with a digital macroscopic planimetry system (Morphometry-System, Bioblock Scientific, Illkirch, France). Neointimal and medial areas were measured in each section and the neointima/media ratio was calculated.

2.6. Endothelial immunostaining

To confirm the endothelial versus vascular smooth muscle cells origin of the cells present at the luminal surface of the vessel wall, endothelial immunostaining was

![Fig. 1. Examples of complete (A) and partial (B) reendothelialization as demonstrated on cross sections by endothelial immunostaining (CD31).](image-url)
performed in the animals sacrificed for histological analysis. Cryosections were incubated with peroxidase blocking reagent, rinsed in PBS for 10 min and in 10% horse serum in PBS for 10 min. A mouse prepared monoclonal primary antibody to CD31 (Dakopatts A/S, Copenhagen, Denmark), diluted to 1:20 in PBS was incubated at 37°C overnight. After extensive washing, anti-mouse biotinylated secondary antibody was applied for 1 h. After washing in PBS, sections were incubated for 1 h in a solution of avidin–biotin–peroxidase preformed complex (ABC Vector-Vectastain kit, Vector laboratories Inc., Burlingame, CA). The peroxidase activity was revealed using hydrogen peroxide and diamino-benzidine, as a chromogen, and counterstaining was performed with hematoxylin. Three arterial cryosections selected at random in three of the predefined portions (proximal, middle and distal) were analyzed. The percent endothelialization in each section was measured with use of a computerized

Table 1
Effects of L-NAME and L-arginine on arterial blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>L-arginine</th>
<th>L-NAME</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Systolic arterial pressure (mmHg)</td>
<td>120±4</td>
<td>112±7</td>
<td>115±4</td>
<td>NS</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>106±3</td>
<td>93±8</td>
<td>100±5</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic arterial pressure (mmHg)</td>
<td>94±3</td>
<td>84±7</td>
<td>90±5</td>
<td>NS</td>
</tr>
</tbody>
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Fig. 2. Plasma arginine values at baseline and 3 weeks after the beginning of the diet. Measurements were performed in animals fed a normal diet (controls) or a diet supplemented with 2.25% L-arginine (L-arginine) or 15 mg/kg/day L-NAME (L-NAME). *P=0.001 versus L-arginine.

Fig. 3. Quantitative analysis of the reendothelialization process by macroscopic analysis of Evans blue staining. Results are expressed as absolute (A) and relative values (B). No differences were detected among groups.
sketching program (Morphometry-System, Bioblock Scientific). Examples of sections with various degree of endothelialization are shown in Fig. 1.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. Data were analyzed by ANOVA followed by Scheffé’s F-test. Statistical significance was assumed at P<0.05.

3. Results

3.1. Biochemical and physiological measurements

Plasma-free arginine levels were similar in the three groups at baseline. At 3 weeks, these plasma levels were significantly higher in animals whose diet was supplemented with L-arginine (L-arginine) compared with animals on normal diet (control; P=0.001) or on normal diet with L-NAME (P=0.001, Fig. 2). In the L-arginine group, the increase in plasma-free arginine was associated with an increase in NOx (124±11 μmol/l) compared to controls (86±11 μmol/l, P=0.04). At the time of sacrifice, there were no significant differences in hemodynamic measurements between the three groups (Table 1).

3.2. Effects of L-arginine and L-NAME on reendothelialization

Macroscopic analysis of reendothelialization was performed using Evans blue staining. The initially denuded area was similar among groups (controls=110±2, L-arginine=110±6, L-NAME=111±5 mm²; Fig. 3A). At 4 weeks, the reendothelialized area did not differ significantly among the groups (controls=39±4, L-arginine=49±4, L-NAME=37±4 mm²; ns, Fig. 3A). Analysis of percent reendothelialization defined as the ratio of the reendothelialized area divided by the initially denuded area (×100), confirmed these results (controls=36±4%, L-arginine=43±3%, L-NAME=33±4%; ns, Fig. 3B).

Representative examples of control, L-arginine, and L-NAME treated animals are shown in Fig. 4. Analysis of Evans blue pattern demonstrated that reendothelialization was most complete in the midportion, close to the origin of the internal iliac artery, and was less marked in the proximal and distal portions.

Microscopic assessment of reendothelialization was performed using endothelial immunostaining on cross sections. This immunostaining confirmed the endothelial origin of the cells at the luminal surface of the vessel wall and the lack of difference in reendothelialization among groups (controls=43±7%, L-arginine=51±8%, L-NAME=40±2%; ns, Fig. 5). Separate analysis in the three different segments (proximal, middle and distal) confirmed these results. Consistent with the macroscopic findings, a
trend for a greater degree of reendothelialization was seen in middle segments (Fig. 5).

3.3. Effects of L-arginine and L-NAME on intimal hyperplasia

Histomorphometric analysis revealed that the neointimal area was decreased in animals receiving L-arginine supplementation compared to controls ($P=0.003$) and L-NAME treated animals ($P=0.02$; Fig. 6). Intimal area was reduced from $0.33\pm0.01$ and $0.30\pm0.01 \text{ mm}^2$ in the control and L-NAME groups, respectively, to $0.19\pm0.01 \text{ mm}^2$ in the L-arginine group.

4. Discussion

Our results demonstrate that long-term administration of L-arginine, the precursor of EDRF-NO, or of L-NAME, an inhibitor of NO synthase, have no effects on the reendothelialization process after angioplasty in the rabbit iliac artery. These results have several implications in the understanding of the response of the vessel wall after injury, in particular the response of endothelial cells.

Even though NO is considered as a growth factor for microvascular endothelial cells, our results suggest that it is not the case for macrovascular endothelial cells in vivo. Ziche et al. have demonstrated that NO donors induce proliferation and migration of microvascular endothelial...
endothelial cells, but rather with a slight decrease in propagation of endothelial cells from adjacent areas re-induced proliferative response of cultured bovine aortic [31] and reendothelialization [32], our results suggest that application of exogenous NO was not associated with an endothelial progenitor cells are involved in angiogenesis the observations of Yang et al. [20]. In their study the In view of the recent demonstration that circulating a growth factor for these cells. Our results are supported by internal iliac artery were more prone to re-endothelialize. The absence of effect of L-arginine and L-NAME on endothelial regrowth in our model suggests that NO is not artery tract suggested that areas close to the origin of the ischemia [19]. Little data are available, however, on the effects on reendothelialization after balloon injury in rabbit iliac artery. These results suggest that, in spite of previous-ly demonstrated beneficial effects in various models of balloon injury, the NO pathway does not influence the regrowth of macrovascular endothelial cells in vivo.

Our study confirms and extends the results of earlier studies using Evans blue staining [30], suggesting a critical role of major side branches as a reservoir for endothelial cells in the regeneration of the endothelial monolayer. As in those earlier studies, the results of Evans blue staining in our model of denudation of the common iliac-external iliac artery tract suggested that areas close to the origin of the internal iliac artery were more prone to re-endothelialize. In view of the recent demonstration that circulating endothelial progenitor cells are involved in angiogenesis [31] and reendothelialization [32], our results suggest that propagation of endothelial cells from adjacent areas remains a major mechanism of the regrowth of the endothelial monolayer.

In conclusion, L-arginine levels, and L-NAME have no effects on reendothelialization after balloon injury in rabbit iliac artery. These results suggest that, in spite of previously-demonstrated beneficial effects in various models of balloon injury, the NO pathway does not influence the regrowth of macrovascular endothelial cells in vivo.

References


