Rapid and Efficient Vascular Transport of Arginine Polymers Inhibits Myointimal Hyperplasia

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Background—We recently discovered that short polymers of arginine efficiently translocate across the cytoplasmic membrane independent of the basic amino acid transporter. We evaluated the kinetics and biological effects of heptamers of L-arginine and D-arginine (L-R7 and D-R7, respectively) in vascular cells. We assessed the effects of these peptides on the NO synthesis pathway and vascular cell proliferation.

Methods and Results—Human umbilical vein endothelial cell and rabbit vascular segments were incubated in medium containing biotin-labeled L-R7 or D-R7. Both polymers rapidly translocated through the vessel wall and into the vascular cells in a dose- and time-dependent fashion. At a dose of 10 μmol/L for 30 minutes, 100% of the endothelial cells showed evidence of cytoplasmic and nuclear localization of the peptides. To evaluate the biological effects of the polymer translocation on myointimal formation, rabbit jugular vein segments were incubated with polymers (10 μmol/L, 30 minutes) or vehicle before arterial interposition grafting. Planimetric measurement 28 days after surgery revealed that L-R7 and D-R7 substantially reduced myointimal formation compared with the control condition (intima/media ratio: control 1.50.5, L-R7 0.40.2, and D-R7 0.80.2; P<0.05). Furthermore, basal nitrate and nitrite production from L-R7-treated grafts was significantly higher than that from both control and D-R7-treated veins. Studies in vitro of cultured vascular smooth muscle cells revealed that both polymers also exhibit an NO-independent inhibition of vascular smooth muscle cell proliferation.

Conclusions—Short polymers of arginine have the unique ability of vascular cell translocation, and they also have direct biological effects. These attributes are potentially useful in treating myointimal hyperplasia. (Circulation. 2000;102:2629-2635.)

Key Words: amino acids ■ nitric oxide ■ muscle, smooth

Myointimal hyperplasia is a vascular response to injury that contributes to the development of vein graft disease, restenosis after angioplasty, and atherosclerosis.1,2 Myointimal hyperplasia involves the migration and proliferation of vascular smooth muscle cells (VSMCs) as well as the elaboration of extracellular matrix in the intima.3,4 Vascular NO inhibits myointimal formation by suppressing monocyte adherence and infiltration and VSMC proliferation and by inducing VSMC apoptosis.5–8 Systemic or local supplementation of L-arginine, an NO synthase substrate, enhances vascular NO production and inhibits vascular myointimal formation.9–13 Ordinarily, NO synthesis is not substrate-limited. However, under certain circumstances, L-arginine can become rate-limiting, as with elevated plasma levels of asymmetric dimethylarginine, a circulating NO synthase antagonist.14,15 or with the expression of the inducible NO synthase (iNOS).16–18 Both of these abnormalities are operative in the setting of vascular injury.19–21 Furthermore, in some pathological conditions, impairment of L-arginine uptake occurs through its membrane transporters.22,23 Under these circumstances, the efficient intracellular delivery of L-arginine may attenuate myointimal hyperplasia.

We recently have observed that short polymers of between 6 and 15 residues of arginine, but not lysine or histidine, efficiently translocate across the cytoplasmic membrane of cultured Jurkat cells independently of the membrane basic amino acid transporter.24 In addition to their role as intracellular transporters, polymers of L-arginine might be useful as an intracellular source of L-arginine, which in vascular tissue might result in therapeutically useful NO release. To test this hypothesis, we evaluated the kinetics of heptamers of L-arginine and D-arginine (L-R7 and D-R7, respectively) in vascular cells and tissue and also the biological effects of these polymers on myointimal hyperplasia in a vein graft model.

Methods

Peptide Synthesis

Peptides were synthesized by using solid-phase techniques and Fmoc amino acids, resins, and reagents (PE Biosystems and Bachem) on an Applied Biosystems 433 peptide synthesizer.25 The reagents used in the present study were (1) L-R7 (NH2-RRRRRR-COOH, R=L-arginine), (2) D-R7 (NH2-rmmm-COOH, r=D-arginine), (3) L-R5 (NH2-RRRRR-COOH), (4) D-R5 (NH2-rmmm-COOH), (5) biotin-labeled L-R7 (bL-R7, 2000;102:2629-2635.)
biotin-Aca-RRRRRRR-CONH₂), (6) biotin-labeled D-R7 (bD-R7, biotin-Aca-RRrrrrr-CONH₂), and (7) biotin-labeled L-K7 (bL-K7; biotin-Aca-KKKKKKK-CONH₂, K=L-lysine).

**In Vitro Translocation Study**
Spontaneously transformed human umbilical vein endothelial cells (ECV304, American Type Culture Collection) were cultured in medium M199 (Irvine Scientific) containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (GIBCO-BRL). Confluent cells were washed and placed in serum-free medium. After 2 hours, the cells were incubated in the presence of biotin-labeled peptides, such as bL-R7, bD-R7, or bL-K7 (0.1, 1.0, and 10.0 μmol/L). To assess the role of endocytosis in the cellular uptake of the peptides, experiments were performed at 4°C. To determine whether the uptake of R7 was an energy-requiring process, some experiments were carried out in the presence of sodium azide (1.0%) to deplete the cells of ATP and GTP. The cells were exposed to sodium azide for 30 minutes before addition of the peptides. After 30 minutes of incubation, cells were stained with trypan blue to assess cell viability or were washed 3 times with PBS, fixed in ethanol/acetone, washed in PBS, incubated for 30 minutes with peroxidase suppressor (ImmunoPure, Pierce) to block endogenous peroxidase activity, and then incubated with 5 μg/mL horseradish peroxidase–conjugated streptavidin (Pierce) for 30 minutes. A substrate of HRP, diamino-biotin (DAB, Sigma Chemical Co.), was added to the cells. The reaction was terminated by washing in distilled water after a 60-second incubation.

**Ex Vivo Translocation Study**
Carotid artery and jugular vein segments of male New Zealand White rabbits were used. To test the dose dependency of R7 translocation, vascular segments were incubated for 30 minutes with either bL-R7 or bD-R7 solution (0.1, 1.0, and 10.0 μmol/L) in serum-free DMEM (GIBCO-BRL). To test the incubation time dependence, vascular segments were incubated with 10.0 μmol/L biotin-labeled R7 (bR7) solution for 10 seconds, 60 seconds, 5 minutes, 10 minutes, and 30 minutes. To determine the ability of R7 to penetrate through the vessel wall, R7-containing medium was instilled into the lumen, and the vessel was ligated proximally and distally to expose only the luminal surface to R7 (10.0 μmol/L) for 30 minutes. To test the temperature dependence of translocation, vascular segments were incubated with 10.0 μmol/L bR7 solutions at 37°C or 4°C. To determine the disappearance time course of translocated R7, vascular segments were incubated with bR7 solutions (10.0 μmol/L) at 37°C for 30 minutes and then reincubated in DMEM with 10% FBS up to 5 days. Vascular segments were harvested 1, 2, and 5 days later.

**Histological Detection of Internalized bR7**
After incubation with bR7, vascular segments were frozen in OCT compound (Miles Scientific). Frozen sections, 5 μm thick, were fixed with acetone for 10 minutes. Internalized biotin was detected
by using the staining procedure described above. Methyl green was
used for nuclear counterstaining.

To quantify the efficiency of R7 nuclear translocation, the num-
bers of both DAB-positive nuclei and total nuclei were counted in the
intima and media separately at 3400 magnification with a video
image analysis system (Automatrix). The frequency of R7 nuclear
translocation was expressed as the percent staining of nuclei, defined
as the ratio of the number of DAB-positive nuclei to that of all
nuclei, in the intima and media.

Surgical Procedure
Male New Zealand White rabbits (3.0 to 3.5 kg) were anesthetized
with a mixture of ketamine (40 mg/kg) and xylazine (5 mg/kg)
intramuscularly. The left external jugular vein was excised and
immersed in PBS (control) or PBS containing either L-R7
(10.0 μmol/L) or D-R7 (10.0 μmol/L) for 30 minutes. As controls,
L-R5 and D-R5 (10.0 μmol/L) were also used, which in Jurkat cells
do not translocate across the cell membrane.24 The right common
carotid artery was exposed and clamped at the proximal and distal
ends. The treated vein segment was anastomosed in a reverse
end-to-side fashion into the carotid artery with use of continuous 8-0
polypropylene sutures. The common carotid artery was ligated and
dissected between the 2 anastomoses, and the wound was closed with
3-0 nylon suture. The experimental protocols were approved by the
Administrative Panel on Laboratory Animal Care of Stanford
University.

Vessel Morphometry
Vein graft segments were harvested on the 28th surgical day. Graft
segments were fixed in 10% buffered formalin with gentle intralu-
minal pressure. The middle portions of the paraffin samples were
sectioned (5 μm) and stained with hematoxylin-eosin. Three sections
of each graft, taken at 0.5-mm intervals, were analyzed by planim-
etry by a observer blinded to the treatment group. The cross-sectional
areas of the lumen, intima, and media were digitized by use of the
Image Analyst program (Automatrix). The intima-to-media (I/M)
area ratio was calculated.

Measurement of Ex Vivo NOx Production From
Vein Grafts
Vein grafts were harvested 3 days after surgery. Vein graft segments
were incubated in 1 mL Hanks' buffered saline solution (HBSS,
Irvine Scientific) containing calcium (1.0 mmol/L) and L-arginine
(100 μmol/L, Sigma) at 37°C for 2 hours. Nitrate and nitrite (NOx)
production was measured either in the absence (basal) or presence
(stimulated) of calcium ionophore (A23187, 10.0 μmol/L, Sigma).
Samples of the medium (80 μL) were collected, and NOx measure-
ment was performed by using the Griess reaction and a commercial
colorimetric assay (Cayman Chemical).

VSMC Proliferation Assay
Rat aortic VSMCs were grown to 50% confluence in 96-well cell
culture plates. VSMCs were incubated with serum-free DMEM for
48 hours to obtain quiescent cells. Western analysis confirmed the
absence of iNOS in these cells. Thereafter, VSMCs were treated with
vehicle, L-R7 (10.0 μmol/L), or D-R7 (10.0 μmol/L) for 30 minutes.
After the treatment, cells were washed and incubated for 48 hours
with serum-containing DMEM (0.5%, FBS). Cell count was per-
formed by use of a proliferation assay kit with spectrophotometry
(XTT, Boehringer-Mannheim). As a negative control, cells treated
with vehicle and incubated with serum-free medium were used. As an index of cell proliferation, the optical density ratio of each treatment group to the negative control group was calculated as an index of cell proliferation.

Statistical Analysis
All values in text are expressed as mean ± SEM. Means were compared by ANOVA, and a value of $P < 0.05$ was accepted as statistically significant.

Results

In Vitro Translocation of R7
No internalized biotin was detected by light microscopy in control cultures exposed to either vehicle or bL-K7 (Figure 1A and 1B). On the other hand, even at the lowest concentration of b-R7 (0.1 μmol/L), internalized biotin was observed in the cytoplasm of all endothelial cells (Figure 1C). At 10.0 μmol/L b-R7 for 30 minutes, internalized biotin was also detected in the nucleus of virtually all exposed cells, with accumulation in the nucleoli (Figure 1D). There were no observable differences in the distribution or intensity of internalized biotin between L-R7 and D-R7. This transport was an energy-requiring process, because when rat vascular smooth muscle cells were exposed to 1% sodium azide for 30 minutes before incubation with bR7, neither cytoplasmic nor nuclear staining was observed. Trypan blue staining indicated that these concentrations of the arginine polymers did not induce cytotoxicity. These findings indicate that both L-R7 and D-R7 are very efficient at translocating across both cytoplasmic and nuclear membranes of endothelial cells in culture and act as carriers for a second molecule, biotin.

Ex Vivo Translocation of R7
When the carotid artery and jugular vein were incubated with bL-K7 (10 μmol/L), no uptake of biotin was apparent (Figure 2A and 2B). On the other hand, after incubation for 30 minutes at a dose of 10.0 μmol/L bL-R7, a distinct biotin signal was observed in virtually all intimal cells, medial cells, and adventitial cells (Figure 2C and 2D and Figure 3A). Jugular vein segments, incubated with bL-R7, exhibited a similar staining pattern (Figure 2E).

The extent and the intensity of staining increased in a time-dependent manner, so that within 30 minutes, virtually all vascular cells exhibited a distinct biotin signal in both the cytoplasm and nucleus (Figure 3B and 3C). Furthermore, when b-R7 was instilled intraluminally, biotin signals were detected even in the adventitial cells, which stained intensely after intraluminal exposure for 30 minutes (Figure 2F). There were no differences between D-R7 and L-R7 in their ability to penetrate the vascular wall and translocate into cells. Intriguingly, translocation of bL-R7 into vascular tissue occurred when the experiments were performed at 4°C, indicating that R7 translocation was not dependent on classic endocytosis (Figure 3B and 3C).

To estimate the relative stability of D-R7 and L-R7 in vivo, the disappearance of the biotin signal over time from vascular segments ex vivo was studied. Residual nuclear biotin in both endothelial and medial cells was greater in vascular segments treated with bD-R7 at days 1 and 2 after exposure (Figure 2G and 2H). No significant positive staining was observed with either form of R7 by day 5 (Figure 4A and 4B).

Biological Effects of R7 on Myointimal Formation of Vein Grafts
All vein grafts treated with vehicle developed significant myointimal hyperplasia 28 days after surgery (Figure 5). By contrast, vessel segments treated with L-R7 or D-R7 had substantially less myointimal formation (intimal area: control 1.70.8 mm², L-R7 0.50.2 mm², and D-R7 1.10.4 mm²;
Treatment was more potent with L-R7 than with D-R7, and L-R7 treatment reduced the intimal area by >70%. The I/M ratio of L-R7–treated vein grafts was also significantly less than that of both control and D-R7–treated grafts (I/M ratio: control 1.50.5, L-R7 0.40.2, and D-R7 0.80.2; P<0.05) (Figure 6). Treatment using the smaller oligopeptide (eg, R5) did not inhibit myointimal formation.

Mechanisms by Which R7 Inhibits Myointimal Hyperplasia

Three days after surgery, the basal NOx production from L-R7–treated vein grafts was significantly higher than that from both control and D-R7–treated vein grafts (NOx production: control 356 nmol/L per milligram tissue per hour, L-R7 8014 nmol/L per milligram tissue per hour, and D-R7 488 nmol/L per milligram tissue per hour; P<0.05). There was no significant difference in basal NOx production between D-R7 and vehicle-treated vein grafts. Calcium ionophore stimulation of eNOS did not affect NOx production by the vein grafts (Figure 7A).

Because D-R7 significantly reduced myointimal formation (although to a lesser degree than L-R7) without enhancing NO production, we evaluated whether arginine polymers had direct cytostatic effects. Indeed, cell proliferation assays revealed that (in the absence of NOS enzyme) VSMC proliferation was significantly inhibited by pretreatment with both L-R7 and D-R7 compared with vehicle incubation. There were no significant differences between isomer treatment groups in this NO-independent cytostatic effect (Figure 7B).

Discussion

The salient observations of the present study are as follows: (1) Arginine heptamers (but not pentamers) are extraordinarily efficient at translocating through the vessel wall and into the cytoplasm and nucleus of vascular cells. (2) Arginine heptamers suppress vascular smooth muscle cell proliferation and reduce myointimal hyperplasia in vein grafts by NO-dependent and -independent pathways. These observations are significant because they may herald a new and effective approach for local delivery of vascular therapeutics. The unique characteristics of R7 to rapidly translocate across the cytoplasmic and nuclear membrane of vascular cells may
make it an excellent candidate as a carrier for vascular therapeutics.

Recently, we observed that polymers of arginine, but not lysine or histidine, efficiently enter cultured cells in vitro. In the present study, we found that heptamers of arginine (R7) translocated into cultured vascular cells and vascular tissue in a dose- and time-dependent manner. Moreover, when applied intraluminally, R7 was capable of penetrating deeply into the vessel wall. The ability to cross biological membranes is not due to the polycationic character of the peptide but rather to the guanidinium groups of arginine, because K7 did not enter the cells effectively. Furthermore, when biotin was linked to the peptides, it was delivered into the vessel wall. Peptides composed of D-arginine are more resistant to hydrolysis than are those composed of L-arginine, and this property may be advantageous for certain forms of drug delivery.

The cellular uptake of R7 is an energy-dependent process, because sodium azide blocked the uptake and nuclear localization of the peptide. However, R7 translocated into the cells even at 4°C, which suggests that conventional endocytic pathways do not play an important role in R7 translocation. Once in the cell, R7 also crossed the nuclear membrane. In the lymphocyte cell line, the Tat protein of HIV-1 efficiency translocates across cytoplasmic and nuclear membranes. It has been suggested that the short basic region of Tat, which is composed of an arginine-rich sequence (residues 49 to 57, RKKRRQRRR), is responsible for this unique property.

We found that R7 peptides are extraordinarily effective at translocating across the cytoplasmic membrane of vascular cells. We hypothesized that R7 itself, as a source of NO synthase substrate, might have an effect on myointimal hyperplasia. To test this hypothesis, an established rabbit vein graft model of myointimal hyperplasia was used. Interposition of a vein graft in an arterial circulation causes hemodynamic injury to the endothelium and vascular smooth muscle of the graft. Within 24 hours of vascular injury, VSMCs express iNOS, Under these conditions, vascular NO synthesis can be enhanced by exogenous L-arginine mainly via the action of iNOS. We found that brief (5-minute) treatment with L-R7 significantly increased local NO production and markedly reduced intimal expansion. We used both vehicle and L-R5 treatment as controls. Treatment for the same brief duration with the pentamers of L-R5 (which does not translocate across the cell membrane in the rapid fashion of L-R7) was no different from treatment with vehicle. This finding suggests that the observed inhibitory effects were due to translocation of the heptamers of arginine and not simply to the availability of polyarginine, perhaps complexed to the cell membrane.

The increase in NO synthesis induced by L-R7 was predictably associated with an inhibition of myointimal hyperplasia. The L-R7 polymer induced a 73% reduction in I/M thickness of the vein grafts, which was significantly greater than the 47% reduction by the D-arginine polymer. Nevertheless, because D-arginine is not a substrate for NO synthase, its modest inhibitory effect on myointimal hyperplasia was surprising. The effect of D-R7 also might have been due to NO production after epimerization of D- to L-arginine. Alternatively, D-arginine may be nonenzymatically oxidized to D-citrulline and NO by a nonenzymatic reaction involving hydrogen peroxide. It is also possible that some D-arginine was converted to the L form before its metabolism by iNOS.

Alternatively, there may be an NO-independent mechanism(s) of R7 action. One possible NO-independent effect of the arginine polymers might be mediated by a cationic interaction with nucleic acid. It is possible that after nuclear translocation, highly positively charged arginine polymers interact with RNA in the nucleus and may interfere with the translation required for VSMC proliferation. Many proteins that interact with ribosomal RNA have arginine-rich sequences. These proteins are largely found in the nucleus and appear to be involved in RNA processing and transcriptional control. Typically, these residues are methylated by specific
protein methyl arginine transferases. It is possible that R7 competes for these protein methyl arginine transferases or otherwise interferes with protein-RNA interaction. The aggregate data indicate that arginine polymers may inhibit VSMC proliferation by NO-dependent and -independent mechanisms.

In summary, short polymers of arginine (R7) are efficient at penetrating the vessel wall and translocating across the cytoplasmic membrane of vascular cells even while coupled to another molecule. These peptides may be efficient research tools for intracellular delivery of therapeutic agents. Furthermore, these compounds may have intrinsic biological effects on the vessel wall that may be useful in preventing or treating vascular disease.

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References

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