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Short Polymers of Arginine Rapidly Translocate Into Vascular Cells

— Effects on Nitric Oxide Synthesis —

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The present study was designed to determine the efficiency of translocation of short polymers of arginine into vascular smooth muscle cells (VSMC) and to determine their effect on nitric oxide (NO) synthesis. Immunostaining revealed that heptamers of L-arginine (R7) rapidly translocated into the VSMC. This rapid transport was not observed with shorter polymers of L-arginine (R5) nor heptamers of lysine (K7). Translocation of R7 was not inhibited by the addition of free L-arginine into the media. When cells were transiently pretreated with R7 or a nonamer of arginine (R9), NO_2 production from cytokine stimulated VSMC was significantly increased, whereas incubation with R5 and K7 had no effect. Short polymers of arginine not only have a unique ability of rapid VSMC translocation but once internalized enhance NO production. Heptamers (or larger polypeptides) of arginine may be useful in therapy to enhance NO production in the vascular system. (*Circ J* 2002; **66:** 1155 – 1160)

Key Words: Arginine; Atherosclerosis; Nitric oxide; Vascular smooth muscle cells

Vascular nitric oxide (NO) is synthesized from its precursor L-arginine by endothelial cells and contributes to vascular relaxation as well as maintenance of normal vascular structure![1,2] Vascular NO inhibits monocyte adherence and chemotaxis;[3] platelet adherence and aggregation[4,5] and proliferation of vascular smooth muscle cells (VSMC)[6] The vascular endothelium normally express endothelial NO synthase (eNOS), but in certain disease states, vascular cells also express inducible NO synthase (iNOS). Recent evidence suggests that preservation or enhancement of NO synthesis can prevent or reverse some of the pathophysiological processes that contribute to vascular proliferative diseases.

Because the intracellular levels of L-arginine far exceed the Km of the NOS enzyme, NO synthesis is ordinarily not dependent on extracellular supplementation;[7] but under certain circumstances, local L-arginine concentration can become rate-limiting, as with elevated plasma or tissue concentrations of the endogenous NO synthase antagonist ADMA (asymmetric dimethylarginine)[8,9] or in the context of inflammation and expression of the inducible NO synthase (iNOS)![10,11] Both of these abnormalities operate in the setting of vascular injury![12,13] in which we and others have demonstrated that oral, intravenous or local supplementation of L-arginine enhances NO synthesis and beneficially modulates vascular function and structure, including the atherosclerosis and myointimal formation after vascular injury![14–17]

Recently, we observed that short polymers of arginine (heptamers or greater) rapidly translocate through the cytoplasmic and nuclear membranes of vascular cells in a very efficient manner![18] Furthermore, we have shown that pretreatment of a vascular segment with short polymers of arginine inhibits myointimal formation after vein grafting![19] Although the mechanisms are still unclear, these polymers seem to translocate through the cytoplasmic membrane independently of the classical basic amino acid transport system. The present study was performed to extend these findings to vascular cells and in particular, to characterize the transport of L-arginine polymers and their effect on NO biosynthesis in cytokine-stimulated VSMC in culture. We chose those cells to study because after angioplasty, they rather than endothelial cells become the major source of NO production in the vessel wall.

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Methods

Reagents

Peptides were synthesized using solid-phase techniques and commercially available Fmoc amino acids, resins, and reagents (PE Biosystems, Foster City, CA, and Bachem, Torrence, CA, USA) on an Applied Biosystems 433 peptide synthesizer as previously described![20] The reagents used were: (a) penta-L-arginine (R5: NH_2-RRRRR-$CONH_2$, R = L-arginine), (b) hepta-L-arginine (R7: NH_2-RRRRRRR-COOH), (c) nona-L-arginine (R9: NH_2-RRRRRRRRR-COOH), (d) hepta-D-arginine (D-r7: NH_2-rrrrrrr-COOH), (e) biotinylated R7 (bR7: biotin-Aca-RRRRRRR-$CONH_2$), and (f) biotinylated hepta-L-lysine (bK7: biotin-Aca-KKKKKKK-$CONH_2$, K = L-lysine).

Recombinant rat recombinant interferon (IFN) -gamma, E. coli lipopolysaccharide (LPS) (0111:B4), and L-NMMA (N^G-monomethyl L-arginine) were purchased from Sigma Chemical (St Louis, MO, USA). A monoclonal anti-iNOS antibody was purchased from Transduction Laboratories (Lexington, KY, USA), and goat anti-mouse IgG antibody

conjugated with horseradish peroxidase was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA).

Cell Culture

Rat VSMC were prepared from the media layer of the thoracic aorta of Sprague-Dawley rats by the explant method. They were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified atmosphere containing 5% CO_2. After subconfluent growth, cells were obtained and cultured with MEM Select Amine Kit (Gibco BRL) to expose them to specific concentrations of extracellular free L-arginine. Experiments were performed using cultured cells at passage levels of 5–10.

Translocation of Biotin-Labeled Peptides Into VSMC

Unpublished observations by members of our group (JBR and CGF) indicate that in non-vascular cells in suspension, the uptake of arginine polymers is energy-dependent and requires that the peptides have a chain length of at least 6 residues. Accordingly, we performed experiments to confirm these observations in vascular cells. Rat VSMC were grown on glass microscope slide chambers (Nunc Inc, Naperville, IL, USA). Subconfluent cells were washed and placed in serum-free medium, and after 2 h, cells were treated with bR7, or bK7 (0.1 μmol/L, 1.0 μmol/L, 10 μmol/L), at 37°C for 30 min. To assess the role of endocytosis in cellular uptake of the peptides, experiments were performed at 4°C, and also in the presence of sodium azide (1.0%) for 30 min prior to exposure to the peptides. To assess the involvement of the basic amino acid transport system in the translocation of peptides into the cell, experiments were performed in the presence of excess extracellular L-arginine (10 mmol/L).

After 30 min of incubation with the peptides, cells were washed 3 times with phosphate-buffered saline (PBS), fixed for 5 min at –20°C in ethanol/acetone; washed 3 times in PBS; incubated for 30 min with a peroxidase suppressor (ImmunoPure, Pierce, Rockford, IL, USA) to block endogenous peroxidase activity and nonspecific binding; washed, and then incubated with 5 μg/ml of horseradish peroxidase (HRP) conjugated strepavidin (Pierce) for 30 min. Cells were washed 3 times with PBS, and a substrate of HRP, DAB (Sigma), was added to the cells. The reaction was terminated by washing in distilled water after a 60-s incubation with DAB. Cell preparations were observed by conventional light microscopy. This experimental protocol was repeated 3 times.

Stimulation of Cells With Interferon-Gamma and LPS

Cells were plated at a density of 5×10^3 per well into 96-well plates. For the experiments that assessed the effects of extracellular L-arginine concentration on NO synthesis from cytokine-stimulated VSMC, subconfluent cells were washed twice with arginine-free medium and then incubated for 24 h with the medium containing the desired concentration of L-arginine (0, 10 μmol/L, 100 μmol/L, 1 mmol/L, 10 mmol/L). After 24 h of incubation, the cells were then treated with a mixture of IFN-gamma (100 U/ml) and LPS (100 μg/ml) in the medium containing the same dose of L-arginine for another 24 h, and nitrite (NO_2) accumulation in the culture medium was quantified.

To study the effects of arginine polymers on NO production, subconfluent cells were pre-incubated with medium containing 100 μmol/L arginine for 24 h and then transiently pretreated with each arginine polymer for 30 min. After translocation, the cells were incubated with a mixture of IFN-gamma (100 U/ml) and LPS (100 μg/ml) in medium containing 100 μmol/L L-arginine for another 24 h. This experiment assessed the effects of the chain length of arginine polymer and polymer dose.

In some experiments, L-NMMA (1 mmol/L), a nitric oxide synthase inhibitor, was added to the medium.

NO_2 Measurement

Extracellular NO production was measured as its stable oxidative metabolite, nitrite (NO_2). At the end of each incubation, samples of the medium (80 μl) were collected, and NO_2 measurement was performed using the Griess reaction facilitated by a commercial colorimetric assay (Cayman Chemical, Ann Arbor, MI, USA). Values of NO_2 production were corrected with relative cell count assessed by a cell proliferation kit II (XTT) (Boehringer Mannheim, Germany).

Assay for iNOS Protein Expression

In order to clarify the effects of arginine polymer translocation on iNOS expression, the iNOS protein of rat VSMC was analyzed by western blotting. Samples were analyzed from nonstimulated cells or cells stimulated with IFN-gamma (100 U/ml) and LPS (100 μg/ml), or R7 (10 μmol/L) pretreated cells that were stimulated with IFN-gamma and LPS. Treated cells were washed twice with PBS, and total cell lysates were harvested in 150 μl of lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris/Cl, pH 8.0, 1% NP40, and 0.1% Sodium dodecyl Sulfate (SDS). Protein concentrations were measured using the Lowry method. Cell lysates containing 5 μg of protein were boiled for 5 min and separated on an 8.0% SDS-polyacrylamide minigel. Eluted proteins were electroblotted onto nitrocellulose membrane (HyBond, Amersham, UK) and the blots were incubated for 3 h with primary monoclonal antibodies against iNOS protein diluted 1:2,500 in TBS/Tween. The blots were then incubated with peroxidase labeled goat anti-mouse IgG in the same buffer for 1 h. Peroxidase labeled protein was visualized with an enhanced chemiluminescence detection system (Amersham) on X-ray film.

Statistical Analysis

Data were expressed as mean ± SEM. Comparison of the multiple groups was performed by one-way ANOVA followed by Scheffe's F test. A value of $p<0.05$ was considered statistically significant.

Results

Translocation of Short Polymers of Arginine Into Cultured VSMC

When cultured VSMC were treated with bK7 (10 μmol/L), no internalized biotin signal was observed (Fig 1A). On the other hand, even at the lowest concentration of bR7 (0.1 μ mol/L), internalized biotin was observed in all VSMC. Furthermore, incubation with 10 μ mol/L R7 showed very intense staining, not only in the cytoplasm, but also in the nucleus of all VSMC (Fig 1B,C). These findings indicated that R7 was very efficient at translocating across both the cytoplasmic and nuclear membranes of VSMC cells in culture, and acted as a carrier for a second

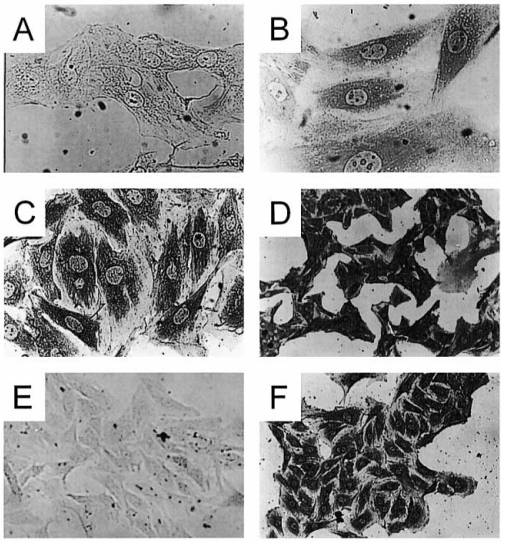

Fig 1. Translocation of biotin labeled heptaarginine (bR7) into cultured rat vascular smooth muscle cells (VSMC) that were treated with biotin-labeled peptide for 30 min. VSMC treated with (A) bK7 (10 μmol/L), (B) bR7 (0.1 μmol/L), (C) bR7 (10 μmol/L): Note intense staining in the nucleus as well as in the cytoplasm of all VSMC. (D) bR7 translocation into vascular tissue at 4°C shows no apparent reduction in efficacy. (E) Sodium azide inhibits R7 translocation, indicating that this is an energy-dependent process. (F) Translocation of bR7 was not inhibited by the addition of free L-arginine (10 mmol/L) to the media, indicating that the y+ transporter is not involved in polymer uptake. The photographs are representative of 3 separate experiments.

molecule, biotin.

To gain some insight into the mechanism(s) of intracellular transport, the effects of temperature and sodium azide on R7 translocation into vascular tissue were examined. When the experiments were performed at 4°C, no apparent reduction was observed in the efficacy of bR7 translocation (Fig 1D). On the other hand, when cells were exposed to 1% sodium azide for 30 min prior to incubation with bR7, neither cytoplasmic nor nuclear staining was observed (Fig 1E). Translocation of the bR7 was not inhibited by the addition of free L-arginine up to 10 mmol/L into the media (Fig 1F). These studies indicated that the cellular uptake of arginine polymers is an energy dependent process, but is not mediated by the basic amino acid transport system y+, or classical endocytotic pathways.

L-Arginine is Rate-Limiting for iNOS Activity

The effects of extracellular concentrations of L-arginine on NO production were assessed in cytokine-stimulated VSMC. No detectable NO_2 was measured in the medium of nonstimulated VSMC, but when cells were stimulated with a mixture of IFN-gamma (100 U/ml) and LPS (100 μmol/L), a significant amount of NO_2 in the medium was detected (14.7±0.3 μmol/L 10^5cells/24 h). There was a dose-dependent effect of IFN-gamma on NO biosynthesis as assessed by NO_2 accumulation in the media (Fig 2A).

Cells were stimulated with IFN-gamma (100 U/ml) and LPS (100 μg/ml) for 24 h to assess the dose-dependency on substrate availability. Increases in extracellular L-arginine led to a progressive increase in NO_2 synthesis by cytokine-stimulated VSMC over the range of 0 to 10 mmol/L (Fig 2B). This finding confirmed previous observations that

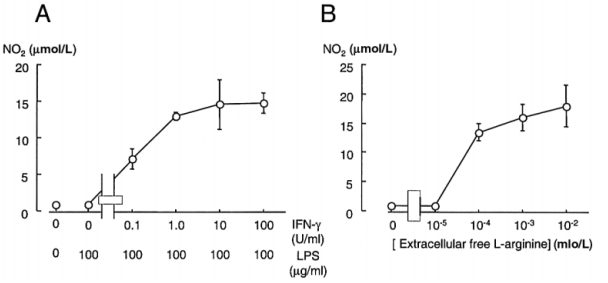

Fig 2. Dependency of NO biosynthesis on cytokine concentration and L-arginine availability. (A) Effects of IFN-gamma dose on NO production by rat vascular smooth muscle cells (VSMC). Cells were incubated in medium containing 400 μmol/L free L-arginine. (B) Effects of extracellular free L-arginine on NO production. VSMC were stimulated with a mixture of IFN-gamma (100 U/ml) and LPS (100 μg/ml). NO_2 accumulation in the culture medium was quantified after 24 h. NO_2 production was corrected as 10^5 cells.

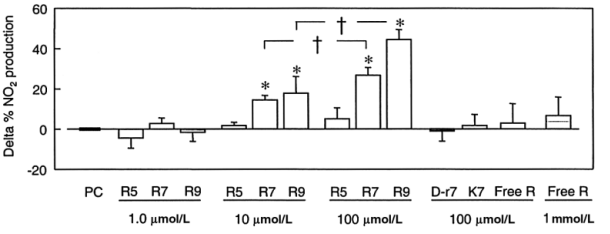

Fig 3. Effects of arginine polymer on NO synthesis by cytokine-stimulated vascular smooth muscle cells (VSMC). To evaluate the effects of dose and chain length on NO_2 production, rat VSMC were pretreated with arginine short polymers for 30 min, and then stimulated with a mixture of IFN-gamma (100 U/ml) and LPS (100 μg/ml). NO production is expressed as a percentage of that observed in vehicle treated cytokine-stimulated cells. R5, penta-L-arginine; R7, hepta-L-arginine; R9, nona-L-arginine; D-r7, hepta-D-arginine; K7, hepta-L-lysine. $^{*\dagger}p<0.05$ vs vehicle treated cells.

in cytokine-stimulated VSMC, L-arginine is a limiting factor for NO production.

Effects of Short Polymers of Arginine on NO Synthesis by Cytokine Stimulated VSMC

Based on the results of the preceding studies, medium containing 100 μmol/L L-arginine was used for the assessment of the effects of the arginine polymer translocation on NO synthesis of cytokine-stimulated VSMC.[10] When cells were incubated with R5 for 30 min, at concentrations up to 100 μmol/L, no significant enhancement of NO_2 production was observed. By contrast, R7 and R9 pretreatment for 30 min resulted in dose-dependent increases in NO_2 production in doses as low as 10 μmol/L. The degree of enhancement was significantly greater in cells treated with R9 than those treated with R7 (24±3.8 vs 44±5.2%, $p<0.05$). When VSMC were exposed to R7 for 5 min, a sustained increase in NO_2 biosynthesis was observed for 24 h. By contrast, exposure of VSMC to free L-arginine (100 μ mol/L, 1 mmol/L) for 5 min did not significantly increase NO biosynthesis. Treatment with D-r7 or K7 (100 μmol/L, 30 min) did not increase NO_2 production (Fig 3).

When R7 translocated cells were subsequently treated with the NO synthase inhibitor, L-NMMA, the enhancement of NO_2 production was abolished (Fig 4).

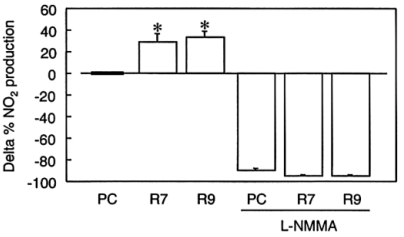

Fig 4. Effects of L-NMMA on NO production from arginine polymer treated vascular smooth muscle cells (VSMC). Rat VSMC were pretreated with each polymer for 30 min, and then stimulated with a mixture of IFN-gamma (100 U/ml) and LPS (100 μg/ml) with or without L-NMMA (1 mmol/L). NO production is expressed as a percentage of that observed in vehicle treated cytokine-stimulated cells. *$p<0.05$ vs vehicle treated cells.

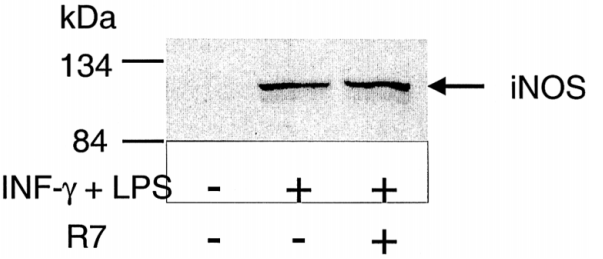

Fig 5. Western blots showing the effects of R7 translocation on iNOS protein expression. The blot is representative of 3 separate experiments.

Effects of Short Polymers of Arginine on iNOS Protein Expression

In order to clarify whether R7 translocation affected the protein expression of cytokine-stimulated VSMC, iNOS protein expression was examined by Western blotting. Inert rat VSMC did not express detectable iNOS protein (130 kDa). When the cells were stimulated by IFN-gamma and LPS, there was demonstrable expression of iNOS protein. R7 (10 μmol/L, 30 min exposure) had no effect on the expression levels of iNOS (Fig 5). Therefore, the enhancing of NO production by R7 or R9 was not because of an increase in iNOS expression.

Discussion

The salient findings of this study are: (1) short polymers of arginine with chain lengths of 7 or more amino acids were extraordinarily efficient at translocating into vascular cells, (2) cellular translocation was energy dependent, but did not involve classical endocytosis pathway, nor the basic amino acid transporter, and (3) cellular translocation of short polymers of arginine enhanced NO biosynthesis in activated VSMC.

We found that heptamers of arginine, but not heptamers of lysine, rapidly crossed the cytoplasmic and nuclear membranes of cultured VSMC and this was not because of the polycationic character of the peptide, but rather the guanidinium groups of arginine, because K7 did not enter cells effectively. The mechanism by which arginine oligopeptides translocate through the cell membrane is not clear, but we did find that the uptake was inhibited by sodium azide, and therefore required intracellular stores of active phosphates, which is consistent with energy requiring endocytosis. However, no impairment of translocation was observed at 4°C, which is incompatible with known endocytotic pathways. Furthermore, translocation of these peptides was not inhibited by the addition to the media of free L-arginine (up to 10 mmol/L). High concentrations of extracellular L-arginine would be expected to compete for binding to the y+ transporter, which is known to transport monomers of basic amino acids.[21] Because exogenous L-arginine did not block the effect of the arginine polymers, the latter are not utilizing the transport system y+ to gain intracellular access. This conclusion is further supported by the observation that a 5-min pretreatment of cells with R7 (10 μmol/L) caused a significant elevation of NO production for 24 h and this increase was not observed when cells were treated with high concentrations of free L-arginine (up to 1 mmol/L) for 5 min. Therefore, cellular uptake of the short polymers of arginine is uniquely efficient, with different kinetics than the y+ transport system.

In cells producing NO, consumption of intracellular L-arginine is maintained by 3 possible mechanisms: extracellular uptake, intracellular protein degradation, and de novo arginine synthesis, which has been termed the citrulline–NO cycle.[10] Among these mechanisms, extracellular transport is the most important for maintaining intracellular L-arginine levels in NO producing cells. In some pathological conditions, such as hypercholesterolemia and congestive heart failure, arginine import through the high-affinity membrane transport system y+ is impaired in vascular cells[22,23] and it seems possible this derangement results in a local shortage of L-arginine as well as decreased vascular NO production. Even in conditions where endothelium-dependent vasodilation is apparently normal (such as in the peripheral resistance vessels of patients with vasospastic angina), local NO synthesis may be reduced.[24] Accordingly, L-arginine supplementation by means of these short polymers of arginine may be useful in the setting of vascular diseases.

In preliminary studies, we have been exploring the utility of arginine polymers in myointimal hyperplasia, the vascular proliferative disorder observed after angioplasty or with vein graft bypass. Myointimal hyperplasia involves the migration and proliferation of VSMC in the intima accompanied by elaboration of the extracellular matrix.[25] NO limits the vascular lesion by inhibiting VSMC proliferation and by inducing VSMC apoptosis.[5,26] Vascular NO, derived largely from iNOS in the setting of vascular injury, may play an important role in suppressing VSMC hyperplasia.[1,2] In animal models of atherosclerosis, restenosis after angioplasty and vein graft disease, local iNOS protein expression in VSMC occurs within hours of the injury and persists for several weeks, and NO synthesis by means of iNOS also seems to be beneficial in these settings.[27–29] Accordingly, in the current study, we mimicked the activation of iNOS in VSMC using cytokine stimulation and under those conditions, extracellular free L-arginine, in a dose dependent manner, increased NO synthesis.

When arginine homopolymers were incubated with cytokine-stimulated vascular cells incubated in physiological levels of extracellular L-arginine, R7 and R9, but not R5, enhanced the accumulation of NO in cell culture medium. We also observed a significant difference in NO enhancement between the cultures treated with R7 or R9. Our group have previously demonstrated that a polymer chain length of 6 arginines is required for rapid cellular uptake,[18] and cellular uptake increases as a function of length from 6 to 9 amino acids. We speculate that this

significant difference is derived from the more efficient transport of a the longer polymer.

The translocation of R7 did not influence iNOS protein expression and therefore, the enhancement of NO production was not the result of a change of iNOS protein expression. It is more likely that, after entering the cells, the polymer is degraded into its constituent arginine monomers, which are then utilized by iNOS as substrate. This conclusion is supported by the observations that arginine polymers induce a dose-dependent increase of NO synthesis and that this effect is blocked by the NO synthase inhibitor, L-NMMA, as well as iNOS protein expression being unchanged by treatment with the arginine polymers.

We have observed that the short polymers of arginine even effectively enter vascular tissue segments. Furthermore, jugular vein graft segments pretreated with R7 produced more NO, and exhibited significantly less myointimal hyperplasia following vein graft bypass surgery in a rabbit model.[19] These beneficial effects seem to be derived from the mechanisms we have presented. Numerous reports indicate the utility of L-arginine supplementation to enhance NO biosynthesis in several vascular diseases;[14–17,30,31] however, there have been conflicting negative studies.[32,33] Although there are many factors influencing the outcome of L-arginine supplementation in vascular disorders, the degree of intracellular L-arginine elevation may limit its biological effects. We now report an alternative and exceedingly efficient mode of delivering L-arginine into vascular tissue and this approach could also be useful for enhancing delivery of L-arginine to the endothelium under conditions where L-arginine may be rate-limiting.[34]

The activation of iNOS may have complex effects on the growth of vascular lesions; for example, by inducing cell death, iNOS activation may contribute to the development of the 'necrotic core' of complex plaque. The NO or the peroxynitrite anion produced by iNOS in the vessel wall could induce apoptosis of vascular smooth muscle;[35,36] and furthermore, they may reduce collagen formation by vascular cells and activate metalloproteinases, which degrade extracellular matrix.[37,38] Conversely, antagonism of iNOS activity could promote platelet aggregation, leukocyte adherence, vasoconstriction, and proliferation of VSMC and macrophages. It may be that iNOS is a countervailing force in the accretion of atherosclerosis or restenosis. Furthermore, by reducing proliferation and promoting apoptosis of VSMC and macrophages in the lesion, iNOS activation may lead to plaque stabilization and even regression.[39]

To conclude, we found that a short polymer of L-arginine (R-7) is capable of rapidly crossing the cytoplasmic and nuclear membranes to stimulate NO production, using an active transport pathway that differs from the y+ uptake system and is not a classical endocytotic mechanism. These unique characteristics of R7 may be employed to enhance intracellular delivery of small molecules, proteins or genes fused or associated with these polymers, and to enhance local NO production.

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