Overexpression of Human Endothelial Nitric Oxide Synthase in Rat Vascular Smooth Muscle Cells and in Balloon-Injured Carotid Artery

Lihua Chen, Günter Daum, Reza Forough, Monika Clowes, Ulrich Walter, Alexander W. Clowes

Abstract—Endothelial cells in normal blood vessels might prevent the unscheduled proliferation of smooth muscle cells (SMCs) by the expression of cell migration and growth inhibitors. NO, a potent vasodilator, generated by endothelium-specific constitutive NO synthase (ecNOS) might be such an inhibitor. To test this hypothesis, we overexpressed human ecNOS in syngeneic rat arterial SMCs using retrovirus-mediated gene transfer. Compared with SMCs transduced with vector alone (LXSN SMCs), DNA synthesis and cell proliferation were inhibited in the ecNOS-expressing SMCs (LCNSN SMCs). Basal and stimulated (by the calcium ionophore A23187) secretion of NO and intracellular cGMP were increased in LCNSN SMCs. N^{ω}-Nitro-L-arginine (L-NA), an inhibitor of NO synthesis, enhanced the proliferation of LCNSN SMCs but had no effect on LXSN SMCs. LCNSN SMCs seeded onto the luminal surface of balloon-injured rat carotid arteries inhibited neointimal formation by 37% and induced marked dilatation (3-fold increase in vessel diameter) at 2 weeks compared with LXSN SMC–seeded arteries. Orally administered L-NA blocked these changes. Phosphorylation of vasodilator-stimulated phosphoprotein, which is regulated in part by NO, was elevated in LCNSN SMCs and in LCNSN SMC–seeded arteries. This study demonstrates that NO generation by ecNOS inhibits SMC proliferation in vitro and modulates vascular tone locally in vivo. **(*Circ Res*. 1998;82:862-870.)**

Key Words: nitric oxide ■ endothelial nitric oxide synthase ■ proliferation ■ vasodilatation ■ vasodilator-stimulated phosphoprotein

Smooth muscle cell growth and migration are triggered by growth factors released by arterial injury, including basic fibroblast growth factor[1] and platelet-derived growth factor.[2] In an intact vessel, these growth factors do not induce SMC response,[3–5] perhaps because of the presence of the endothelium. Besides its function as a barrier, the endothelium also secretes inhibitory factors that might prevent the SMC response to mitogens.

A major function of the endothelium is to regulate vascular tone via the production of NO by ecNOS,[6–8] which is mediated by soluble guanylyl cyclase and cGMP. In addition to vasodilatation, NO might be a negative regulator of other SMC functions. Recent in vivo studies show that L-arginine feeding, oral delivery of NO donors, and local transfer of ecNOS DNA inhibits atherosclerotic lesions and intimal thickening after balloon injury.[9–13] Furthermore, increased blood flow inhibits neointimal formation, and treatment with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester abolishes this effect, suggesting the involvement of NO.[14]

In vitro, NO donors inhibit SMC proliferation.[15,16] NO can also inhibit platelet adhesion and aggregation[17,18] as well as stimulate metalloproteinase enzyme activity.[19,20] Recent studies by several groups showed that NO inhibits SMC and endothelial cell migration in vitro.[21–23] These results support the conclusion that NO might inhibit SMC proliferation, migration, or matrix deposition during neointimal formation. The expression of ecNOS by the endothelium might therefore prevent SMC activation as well as stimulate vasorelaxation.

To test this hypothesis, we transduced human ecNOS cDNA into SMCs using a retroviral vector.[24] The endothelium of rat carotid arteries was removed and replaced by ecNOS-expressing SMCs. The phosphorylation of VASP, a common substrate of both cGMP- and cAMP-dependent protein kinases (PKG and PKA, respectively),[25,26] was used as a biochemical marker of the intracellular effect of NO in our system in vitro and in vivo.

Materials and Methods

Materials

Male Fischer 344 rats (250 to 300 g) were obtained from Simonsen Laboratories (Gilroy, Calif). A full-length human ecNOS cDNA was kindly provided by Dr James K. Liao at Brigham and Women's Hospital, Boston, Mass. The mouse monoclonal anti-human ecNOS and anti-mouse iNOS antibodies were purchased from Transduction Laboratories. The antibody to mouse iNOS recognizes rat iNOS. The calcium ionophore A23187, L-NA, and other chemical reagents were purchased from Sigma Chemical Co. A cGMP radioimmunoassay kit was purchased from Amersham Co. BrdU and an anti-BrdU antibody were purchased from Boehringer-Mannheim Co.

Received September 23, 1997; accepted February 10, 1998.

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Selected Abbreviations and Acronyms

BrdU = bromodeoxyuridine
ecNOS = endothelium-specific constitutive NOS
IEL = internal elastic lamina
iNOS = inducible NOS
L-NA = N^{ω}-nitro-L-arginine
LCNSN = transduced with ecNOS
LXSN = transduced with vector alone
NOS = NO synthase
NOx = NO_2^- plus NO_3^-
PKA, PKG = protein kinase A and G
SMC = smooth muscle cell
VASP = vasodilator-stimulated phosphoprotein

Cell Culture

Rat SMC cultures were prepared by enzymatic digestion of aortas from Fischer rats as described[24] and propagated in DMEM containing 10% FBS (GIBCO Laboratories) in 5% CO_2 at 37°C. Cells were used between passages 7 and 15.

Retroviral Vectors

The retroviral construct containing human ecNOS (LCNSN) was made by insertion of the human ecNOS gene into the unique *Eco*RI site of the parental retroviral vector LXSN provided by A.D. Miller (Fred Hutchinson Cancer Research Center, Seattle, Wash).[27] LXSN has a *neo* (neomycin phosphotransferase) gene to allow selection of the transformants in the presence of G418 (GIBCO Laboratories) after transfection.

Packaging Cell Preparation and SMC Transduction

The packaging cell transfection and SMC transduction were performed as described.[24] Briefly, both constructs (LCNSN and LXSN) were transfected into PE501 ecotropic packaging cells, and the viral particles were used to infect the amphotropic PA317 packaging cells. Replication-defective retroviruses were obtained at a titer of 5×10^5 colony-forming units per milliliter. Early-passage (P7) SMCs were transduced with LCNSN or LXSN virus for a period of 24 hours in the presence of hexadimethrine bromide (4 μg/mL) and selected in G418 (1 mg/mL).

Northern Blotting

Total RNA was extracted from cultured LCNSN- and LXSN-transfected SMCs using guanidinium isothiocyanate and phenol extraction.[28] The RNA (20 μg) was size-fractionated by electrophoresis on a 1% agarose–3% formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled human ecNOS cDNA probe. Hybridization and washes were performed at 65°C as described.[29] Extracts of baboon endothelial cells were used as a positive control.

Western Blotting

For determination of ecNOS protein expression, the proteins from cultured SMCs or frozen arteries were extracted as described.[30] The same amount of protein was size-fractionated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad, Richmond, Calif), and probed as described with the mouse monoclonal antibodies to ecNOS or iNOS overnight, followed by an alkaline phosphatase–conjugated anti-mouse antibody (Promega). The color development was performed according to the manufacturer's protocol.

VASP Phosphorylation

Cultured SMCs and frozen seeded rat arteries were extracted in HEB buffer (25 mmol/L HEPES [pH 7.5], 5 mmol/L EDTA, 5 mmol/L EGTA, 150 mmol/L NaCl, 100 mmol/L $Na_4P_2O_7$, 50 mmol/L NaF, 1 mmol/L benzamidine, 1% Triton X-100, 10% glycerol, 0.1% β-mercaptoethanol, 1 μg/mL pepstatin A, 5 μg/mL leupeptin, and 5 μg/mL aprotinin).[31] Protein samples were heated in boiling water for 5 minutes, then size-fractionated in 8% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. After blocking in 1% BSA, the membrane was incubated with a rabbit polyclonal anti-VASP antibody overnight and subsequently with an alkaline phosphatase–conjugated anti-rabbit antibody. The color development was performed according to the manufacturer's protocol (Promega). Relative protein quantification was performed by using a Hewlett-Packard C2521A scanner, Adobe Photoshop 3.0 software, and MD ImageQuant 3.3 software. VASP phosphorylation was measured by quantifying (by SDS-PAGE) the shift from the 46-kD to the 50-kD form of this protein that was due to VASP phosphorylation at Ser157.[26]

Measurement of NO Production

The production of NO was evaluated by measuring nitrite (NO_2^-) and nitrate (NO_3^-) using a chemiluminescence NO analyzer as described.[32] Briefly, the cells were washed gently three times with modified Krebs-HEPES buffer (99 mmol/L NaCl, 4.69 mmol/L KCl, 1.87 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.2 mmol/L K_2HPO_4, 20 mmol/L sodium HEPES, and 1.1 mmol/L D-glucose, pH 7.35) and then incubated in Krebs-HEPES buffer with or without 10 μmol/L calcium ionophore A23187 (Sigma) at 37°C for 1 hour. Nitrate was used as a standard to calibrate the chemiluminescence NO analyzer (model 2107, Dasibi Environmental Inc). The amount of NOx was normalized to the protein content in the respective samples.

Measurement of Intracellular cGMP Level

The cells were seeded at 1×10^6 cells per plate in 60-mm plates. The cells were extracted, and the intracellular cGMP was determined by radioimmunoassay according to the manufacturer's protocol (Amersham Co).

[^{3}H]Thymidine Incorporation

SMCs were seeded at 10 000 cells per well in 12-well plates and starved in serum-free media for 48 to 72 hours. Then the cells were incubated in fresh DMEM containing 1 μCi [^{3}H]thymidine (NEN) with or without 10% FBS and an ecNOS inhibitor, L-NA. After 24 hours, cells were rinsed with PBS (pH 7.4), cold 10% trichloroacetic acid was added, and the DNA was extracted with 0.1 mol/L NaOH. The radioactivity of an aliquot of the extract was determined by scintillation counting.

SMC Seeding of Rat Carotid Artery

SMC seeding was performed as described.[24] Male Fischer 344 rats (250 to 300 g) were anesthetized, and the left carotid artery was surgically exposed. The distal half of the common carotid artery was isolated, and the endothelium was stripped by the passage of a 2F balloon catheter (V. Mueller) introduced through an arteriotomy in the external branch. Approximately 1×10^5 transduced SMCs in 0.04 mL culture medium were infused into the isolated carotid segment and left for 15 minutes. The external carotid was then ligated after removal of the catheter, the blood flow was restored, and the wound was closed. All surgical procedures were performed according to the *Principles of Laboratory Animal Care* and the *Guild for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 86–23, revised 1985).

Tissue Preparation, Morphology, Morphometry, and Measurement of SMC Proliferation and Endothelial Regeneration

After various time intervals, animals were killed, and the arteries were flushed clear of blood with Ringer's lactate solution, fixed by perfusion with 10% neutral buffered formalin (pH 7.4) at 100 mm Hg, excised, and processed in paraffin for histology and immunocytochemistry.[24,33] Measurements of luminal, intimal, and

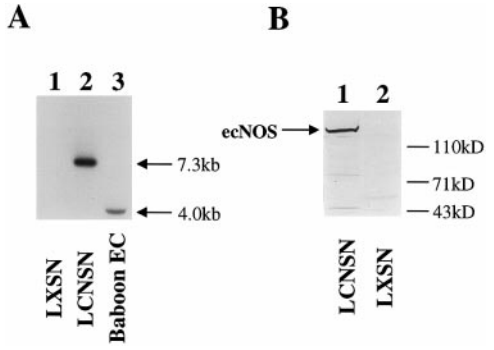

Figure 1. Expression of ecNOS in LCNSN SMCs detected by Northern and Western blotting**.** A, Northern blot probed with human ecNOS cDNA reveals a chimeric mRNA (7.3 kb) in LCNSN SMCs (lane 2) but not in LXSN SMCs (lane 1). The 4-kb ecNOS mRNA was detected in baboon endothelial cells (ECs) (lane 3). B, Western blot demonstration of ecNOS protein (138 kd) in LCNSN SMCs (lane 1) but not in LXSN control cells (lane 2).

medial areas as well as internal elastic length were made on two cross sections per rat taken from the middle of each carotid segment. BrdU (50 mg per rat) was given subcutaneously 24 hours before termination of the experiments. Immunocytochemistry was performed as described[33] using an anti-BrdU antibody, the mouse monoclonal antibodies to ecNOS or iNOS, and the Vectastain Elite ABC kit (Vector Laboratories). The BrdU labeling index [(number of nuclei with positive BrdU labeling/total nuclei)×100] was obtained by counting the number of BrdU-labeled nuclei, dividing by the total number of nuclei, and multiplying by 100. Arteries intended for measurement of ecNOS expression and VASP phosphorylation analysis were freshly frozen in liquid nitrogen and stored at −70°C. For measurement of endothelial regeneration, Evans blue in PBS (60 mg/kg, pH 7.4) was injected via tail vein 60 minutes before the rats were killed. The arteries were removed, and the endothelial regeneration was assessed as described.[34]

Statistics

All values are expressed as mean±SD. Comparisons between the two groups (LCNSN and LXSN) were made using the Mann-Whitney nonparametric test, and statistically significant difference was set at $P<.05$.

Results

Expression of ecNOS in Transduced SMCs

LCNSN SMCs expressed the appropriate ecNOS mRNA and protein, whereas the control cells (LXSN SMCs) did not (Figure 1). The larger size (7.3 kb) of the mRNA in the LCNSN SMCs was due to the fact that the transcript in the transduced cells included the *neo* gene (Figure 1A). Hybridization with GAPDH revealed that there was no significant difference in RNA loading (data not shown). On the Western blot, the anti-ecNOS antibody recognized the 140-kD ecNOS protein in extracts of LCNSN SMCs but not LXSN SMCs (Figure 1B). The expression of ecNOS protein was stable as the cells were propagated from passage 7 to passage 15 in the absence of G418.

SMCs have the ability to express iNOS[35] on stimulation with cytokines. However, iNOS mRNA and protein were not detected in LCNSN or LXSN SMCs in the presence of FBS (data not shown).

NO Production in LCNSN SMCs

ecNOS activity in transduced SMCs was estimated by measuring the secretion of NO (Figure 2A). The LCNSN SMCs

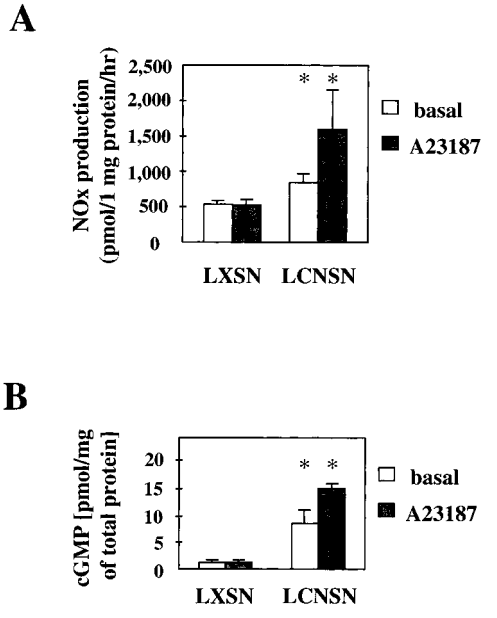

Figure 2. Basal and A23187-stimulated NO production and intracellular cGMP levels in LCNSN SMCs**.** A, NO production in cultured LCNSN SMCs and control LXSN SMCs. The cells were incubated in modified Krebs-HEPES buffer with or without 10 μmol/L A23187 at 37°C for 1 hour. NOx was measured by a chemiluminescence NO analyzer. B, Intracellular cGMP levels in cultured LCNSN SMCs and control LXSN SMCs, either basal or stimulated by 10 μmol/L A23187 at 37°C for 1 hour. cGMP levels were determined in the acid-soluble fractions. NOx production and cGMP levels were normalized to the protein content in the respective samples. *$P<.05$ (n=5).

secrete increased amounts of NO (843.0±150.2 pmol/mg protein) compared with LXSN SMCs (534.8±53.6 pmol/mg protein). The calcium ionophore A23187 increased NO production by 2- to 3-fold (1596.8±522.1 pmol/mg protein) over the basal level in LCNSN SMCs, whereas there was no effect on LXSN SMCs (525.8±27.6 pmol/mg protein).

Intracellular cGMP Levels in LCNSN SMCs

NO activates guanylyl cyclase to generate intracellular cGMP in many cells, including SMCs.[36] The intracellular cGMP concentrations in LCNSN SMCs were increased (Figure 2B) under basal (8.19±2.75 pmol/mg of protein) and A23187-stimulated conditions (14.71±1.03 pmol/mg of protein) compared with LXSN SMCs (1.17±0.12 and 1.22±0.03 pmol/mg of protein, respectively). The elevation of the intracellular cGMP concentration indicates that NO generated by retrovirally transduced ecNOS was capable of activating guanylyl cyclase.

Decreased Proliferation of LCNSN

LCNSN SMC growth was decreased compared with LXSN SMC growth (Figure 3A) and could be reversed with a selective NOS inhibitor, L-NA. However, L-NA had no effect on LXSN SMCs under the experimental conditions.

In mitogenesis experiments, thymidine incorporation in LCNSN SMCs in response to 10% FBS was decreased and was stimulated by the administration of L-NA (Figure 3B). LXSN SMCs were not affected by L-NA. These results

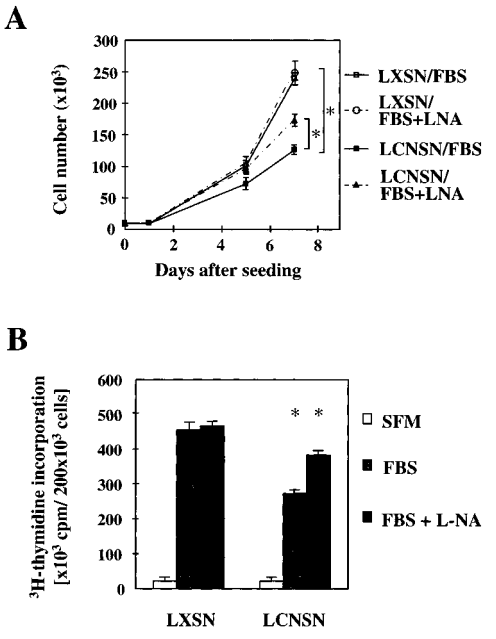

Figure 3. Growth and mitogenesis of LCNSN SMCs. A, Growth of cultured LCNSN SMCs and control LXSN SMCs. Cells (1×10^4) were seeded in 12-well plates in DMEM with 10% FBS with or without L-NA (0.4 mmol/L) and cultured at 37°C with 5% CO_2. The medium was changed on days 2, 4, and 6, and mean cell number (±SD) was recorded. The statistical significance was observed between LCNSN/FBS and LXSN/FBS and between LCNSN/FBS and LCNSN/FBS+L-NA at days 5 and 7. B, Mitogenesis of cultured LCNSN SMCs and control LXSN SMCs. Cells (2×10^5) were seeded and cultured overnight in DMEM with 10% FBS at 37°C with 5% CO_2. Then the cells were starved in DMEM. After 48 or 72 hours, medium was replaced by DMEM with 10% FBS containing [^{3}H]thymidine (1 μCi per well) with or without L-NA (0.4 mmol/L). The cells were harvested after 24 hours, and [^{3}H]thymidine incorporation was measured. SFM indicates serum-free medium. The graphs are representatives of three independent experiments, and each sample has at least three replicates in each experiment. *$P<.05$ (n=3).

suggest that NO might interfere with the mitogenic pathway stimulated by serum.

Effects of LCNSN SMCs on Rat Carotid Artery

To address the biological effects of NO in vivo on the vessel wall, we seeded the LCNSN SMCs onto the luminal surface of rat carotid arteries after balloon injury. In earlier studies, we have shown that seeded SMCs adhere to the denuded carotid artery within 10 minutes and that ≈10% of the cells remain at 1 and 2 weeks afterward (data not shown). These cells continue to express the transduced gene as late as 1 year later.[24] In the present study, ecNOS protein was overexpressed in LCNSN SMC–seeded arteries compared with LXSN SMC–seeded arteries (Figure 4). At 7 days, there were no significant differences in areas of lumen, intima, and media between LCNSN SMC–seeded arteries and LXSN SMC–seeded arteries (Table 1). At 14 days, histological examination and morphometry revealed marked vasodilatation in LCNSN SMC–seeded vessels (Figure 5). The luminal area of these vessels was increased nearly 3-fold (Table 1). The vessel perimeter measured at the level of the IEL (IEL length) was also increased in LCNSN SMC–seeded vessels (LCNSN, 2.03±0.07 mm; LXSN, 1.59±0.01 mm). The neointimal area of LCNSN SMC–seeded vessels was reduced, but not significantly, compared with the control vessels (Table 1). The medial areas in LCNSN SMC–and LXSN SMC–seeded vessels were not significantly different (Table 1). To demonstrate that the dilatation effect was due to NO produced by seeded LCNSN SMCs, we fed L-NA (10 mg/kg per day in drinking water) to rats seeded with LCNSN SMCs to suppress NO production. The results showed that L-NA can partially reverse the dilatation effect in arteries seeded with LCNSN SMCs (Table 2). However, there were no significant changes in intimal and medial areas.

Immunocytochemistry studies revealed that the seeded LCNSN SMCs were localized in the intima of the balloon-injured artery at 2 days after seeding (Figure 4A). At 2 weeks, LCNSN SMCs were integrated into the abluminal side of the intima intermixed with SMCs derived from the media (Figure 4B). LXSN SMC–seeded arteries showed no staining for ecNOS (Figure 4C). These results are consistent with early observations of balloon-injured arteries seeded with retrovirally transduced SMCs.[24] Another form of NOS, iNOS, has been found to be induced in vascular SMCs of the injured arteries in response to the various cytokines produced at the injured site.[37] To investigate whether iNOS is induced in the seeded vessels, Western blot and immunocytochemical analyses were performed. The results showed that iNOS expression is detectable in both LCNSN SMC–and LXSN SMC–seeded arteries at 7 days but not at 14 days after seeding (Figure 6).

TABLE 1. Morphometric Analysis of LCNSN SMC– and LXSN SMC–Seeded Rat Carotid Arteries

	Area, mm²	
	7 Days	14 Days
LCNSN		
Lumen	0.13±0.02	0.18±0.01*
Intima	0.13±0.01	0.12±0.02
Media	0.11±0.01	0.11±0.01
LXSN		
Lumen	0.14±0.03	0.05±0.01
Intima	0.16±0.02	0.19±0.06
Media	0.14±0.01	0.13±0.01

Carotid arteries seeded with LCNSN and LXSN SMCs were fixed by perfusion at 7 and 14 days (n=5 in each group), and luminal, intimal, and medial areas were measured by planimetry on histological cross sections. Values are mean±SD.

*$P<.05$ vs control (LXSN).

TABLE 2. Morphometric Analysis of Vessels From L-NA–Treated Rats

	Area, mm²		
	Lumen	Intima	Media
L-NA (10 mg/kg per day)	0.09±0.03*	0.16±0.02	0.14±0.02
Control (water)	0.18±0.02	0.13±0.01	0.12±0.01

Animals were treated with L-NA (10 mg/kg per day in drinking water) or vehicle (control, water) after LCNSN SMCs were seeded onto the carotid arteries (n=5 in each group). The vessels were fixed by perfusion at 14 days. Values are mean±SD.

*$P<.05$ vs control.

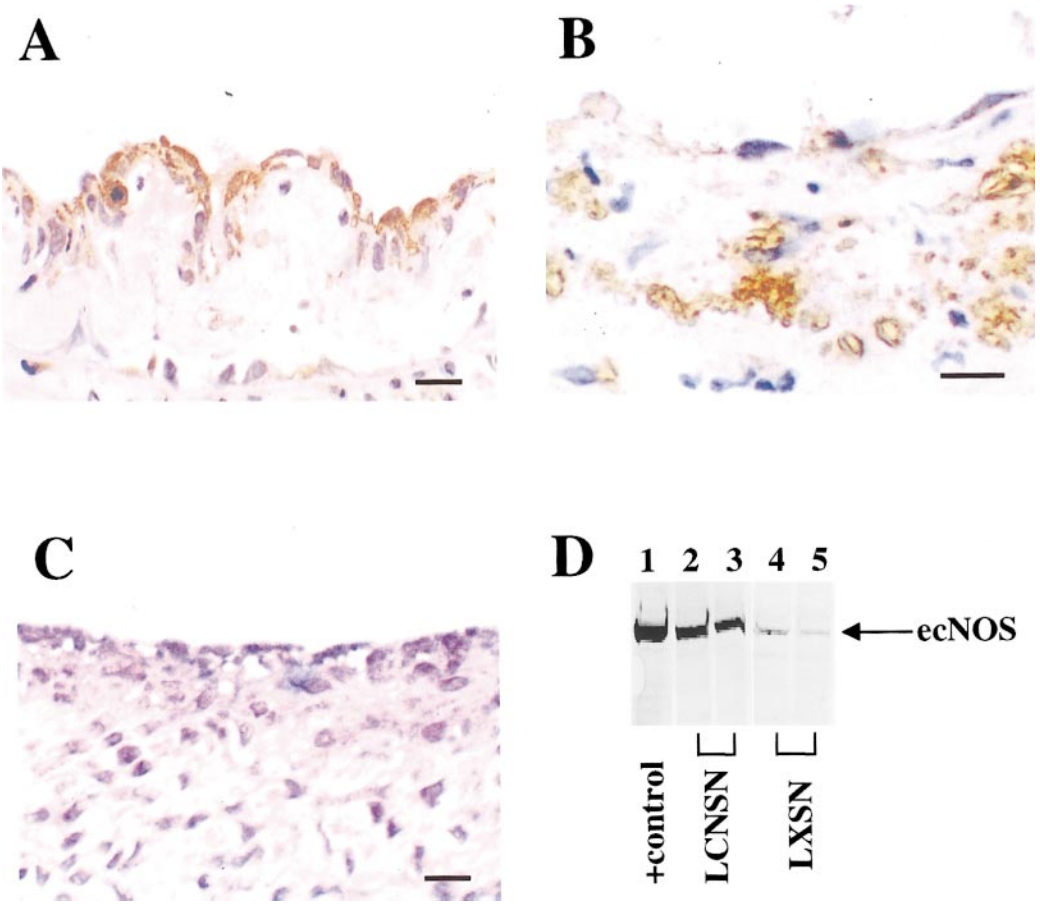

Figure 4. Localization of seeded LCNSN SMCs and expression of ecNOS proteins in LCNSN SMC–seeded arteries detected by immunocytochemistry and Western blotting**.** A through C, Brown staining shows ecNOS-expressing SMCs in LCNSN SMC–seeded arteries at 2 days (A) and 14 days (B). Fourteen-day artery seeded with LXSN SMC shows no staining for ecNOS (C). Nuclei are stained blue. The bars represent 20 μm. D, ecNOS protein was overexpressed in LCNSN SMC–seeded arteries at 14 days after seeding (lanes 2 and 3) but not in control LXSN SMC–seeded arteries (lanes 4 and 5). Lanes 2 through 5 represent arteries from four different animals. Lane 1 is a positive control for ecNOS protein provided by Transduction Laboratories.

After balloon injury, SMCs in the media proliferate and then migrate into the intima.[32] In order to examine the effect of local NO production on SMC proliferation in the injured vessels, BrdU was administered during the 24 hours before the animals were killed for study. At 2 and 14 days, the BrdU labeling indices in the media and intima were the same in LCNSN SMC–and LXSN SMC–seeded vessels (Table 3).

Endothelial regeneration was impaired in LCNSN SMC–seeded arteries at 2 weeks after seeding (LCNSN, 1.4±0.5 mm; LXSN, 3.6±1.1 mm; $P<.05$). This result is consistent with the observation in vitro that NO inhibits endothelial cell proliferation[38] and migration.[23] At 4 weeks, there were no significant differences in the areas of lumen, intima, and media, in IEL length, and in endothelial regeneration between LCNSN SMC–and LXSN SMC–seeded vessels (LCNSN, 10.2±3.3 mm; LXSN, 14.4±1.3 mm).

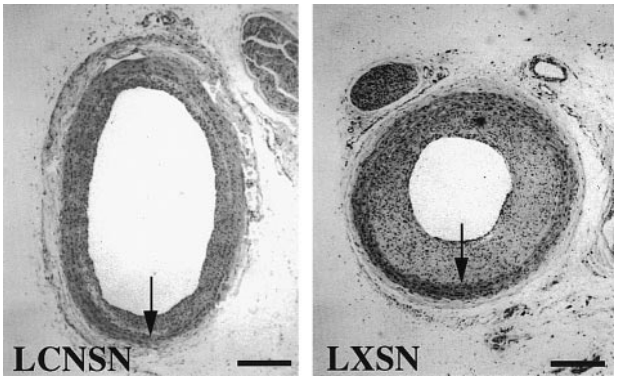

Figure 5. Effects of seeded LCNSN SMCs on balloon-injured arteries at 2 weeks. Histological cross sections of injured arteries seeded with LCNSN SMCs (left) and LXSN SMCs (right). The arrows indicate IEL. Bar=0.2 mm.

TABLE 3. SMC Proliferation in Seeded Carotid Arteries

	BrdU Labeling Index, % Labeled Cells			
	LCNSN		LXSN	
	Intima	Media	Intima	Media
2 Days	55.0±7.5	5.0±4.0	66.8±13.5	8.6±6.8
14 Days	7.0±0.9	1.1±0.2	7.9±0.8	0.9±0.1

Mean BrdU labeling index of SMCs in the intima and media of seeded arteries (n=4 for each group at 2 days and n=5 for each group at 14 days). Values are mean±SD. No statistical significance was found between LCNSN and LXSN groups at either 2 days or 14 days.

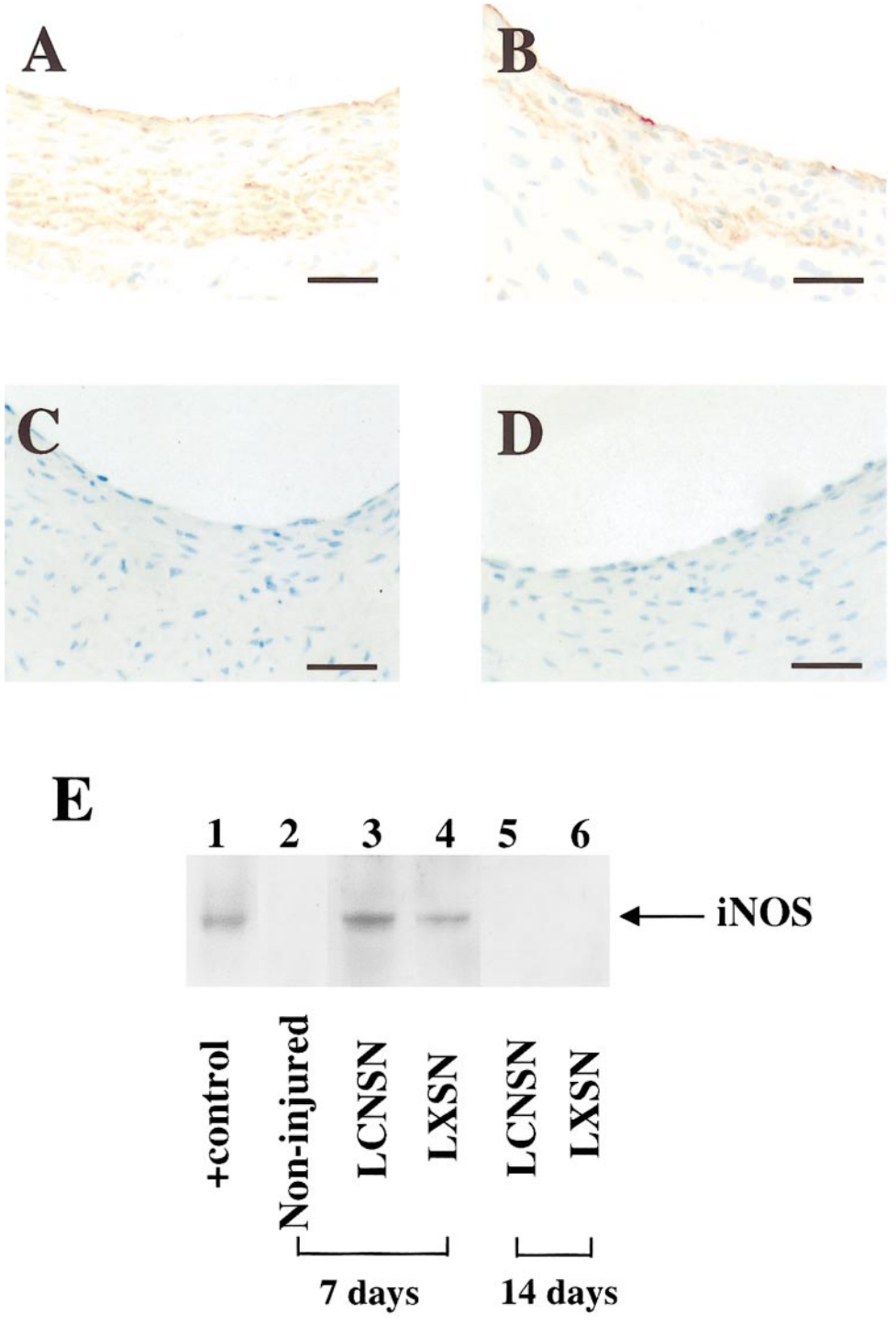

Figure 6. Localization and expression of iNOS protein on SMC-seeded arteries detected by immunocytochemistry and Western blotting. A through D, Brown staining shows iNOS-expressing SMCs in both LCNSN SMC– and LXSN SMC–seeded arteries at 7 days (A and B) but not 14 days (C and D). Nuclei are stained green. Bars=50 μm. E, iNOS expression in LCNSN SMC– and LXSN SMC–seeded arteries at 7 days (lanes 3 and 4) but not at 14 days (lanes 5 and 6) after seeding. Lane 1 is a positive control for iNOS protein provided by Transduction Laboratories. Lane 2 is the lysate from the contralateral uninjured artery.

Enhanced VASP Phosphorylation in Cultured LCNSN SMCs and LCNSN SMC–Seeded Arteries

VASP, a proline-rich protein substrate of both PKG and PKA, is expressed in most mammalian cell types and tissues including platelets, endothelial cells, and vascular SMCs. It is well established that agents that stimulate the NO/cGMP and prostaglandin/cAMP signal transduction pathways increase VASP phosphorylation in human platelets and other cells.[25,26] Functional evidence indicates that VASP is the crucial factor involved in the enhancement of actin filament formation.[39,40] In preliminary studies, we found that NO and prostaglandins increase VASP phosphorylation in human and rat SMCs in vitro (L. Chen, C. Nehls, G. Daum, U. Walter, and A. Clowes, unpublished data, 1997) and that injury of the rat carotid

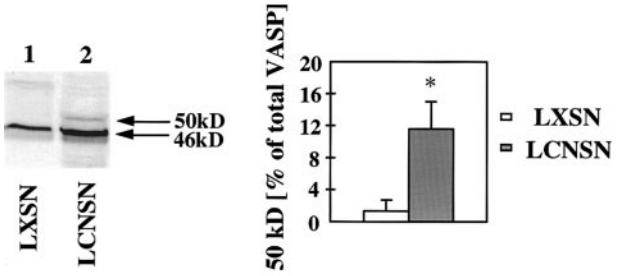

Figure 7. Left, VASP phosphorylation in LCNSN SMCs detected by Western blot**.** VASP phosphorylation was increased in cultured LCNSN SMCs (lane 2) compared with control LXSN SMCs (lane 1). The 50-kD band represents the VASP form phosphorylated at Ser157. Right, Relative density of the 50-kD band (percentage of total VASP) for three experiments. *$P<.05$ (n=3).

artery causes a decrease in VASP phosphorylation in vivo (data not shown). An increased level of VASP phosphorylation was found in LCNSN SMCs compared with LXSN SMCs (Figure 7). In LCNSN SMC–seeded vessels, we found that the level of VASP phosphorylation was increased compared with LXSN SMC–seeded vessels at 7 and 14 days after seeding (Figure 8).

Discussion

Our observations show that the human ecNOS gene introduced into rat SMCs via a retroviral vector behaves partially as it does in endothelial cells. It generates NO under resting conditions in amounts comparable to NO production in endothelial cells and is stimulated in response to the calcium ionophore A23187. Furthermore, the elevation of intracellular cGMP and phosphorylation of VASP in LCNSN SMCs demonstrates that NO generated by the human ecNOS is probably acting through endogenous guanylyl cyclase and cGMP-dependent protein kinase.

The inhibition of proliferation and mitogenesis in LCNSN SMCs in vitro is consistent with published observations that exogenous NO or NO-generating reagents inhibit SMC proliferation and mitogenesis.[15–16,41] Inhibition of DNA synthesis is detected when the LCNSN SMCs are stimulated with 10% FBS but not under serum-free conditions. This finding indicates that NO might interrupt the mitogenic pathway initiated by serum. Our observation that a NOS inhibitor, L-NA, can abolish this inhibitory effect strongly suggests that the inhibition of proliferation and mitogenesis of LCNSN SMCs is due to the increase in NO. NO has been found to have a very broad antiproliferative effect in vitro in many cell types, including neuronal cells,[42] endothelial cells,[21] mouse macrophage-like cells,[43] and hepatocytes[44] as well as SMCs. The mechanism of the inhibitory effect by NO is still unknown. Although most studies suggest that NO functions through the cGMP-dependent protein kinase pathway, it is also possible that NO and cGMP might have growth-inhibitory effects by activating cAMP-dependent kinase. In neuronal PC12 cells, NO triggers a switch to growth arrest during differentiation,[42] whereas in mouse macrophage-like cells, it blocks the cell cycle in the early G_2+M phase.[43] The present study shows that NO might inhibit the entry into the cell cycle induced by mitogens. Several recent studies have shown that NO can induce apoptosis in vitro in several cell systems, including SMCs,[45,46] mouse macrophage-like cells,[47] and chondrocytes,[48] and that the induction might be through a cGMP-dependent mechanism[45,46] or through p53 accumulation or both.[49] However, we could not detect an increase in cell death in the LCNSN SMCs using trypan blue staining over time (data not shown).

The NO produced by LCNSN SMCs in vessels caused vessel dilatation at 2 weeks after seeding. However, neointimal formation and SMC proliferation in the media and the neointima were not significantly affected. This result might be due to the induction of endogenous iNOS in injured rat carotid arteries.[37] iNOS was expressed at 7 days, but not at 14 days, after seeding in both LCNSN SMC– and LXSN SMC–seeded arteries (Figure 6). iNOS has an ability to generate much larger amounts of NO compared with ecNOS.[35,50] The large amounts of NO produced by iNOS locally might overshadow the inhibitory effects caused by seeded LCNSN SMCs. In fact, the luminal diameter in 7-day vessels was the same in experimental and control vessels (Table 1). In addition, the NO generated by iNOS might suppress the enzymatic activity of the ecNOS.[51] The vasodilatory effect of seeded LCNSN SMCs could be documented at 14 days (Figure 5 and Table 1), since at that time iNOS expression had ceased (Figure 6). It is of note that in association with the decrease in iNOS expression, the control LXSN SMC–seeded vessels contracted and exhibited a decrease in luminal diameter at 14 days compared with 7 days.

Recent studies have shown that the response of SMCs to NO might depend on their phenotype.[52,53] Basic fibroblast

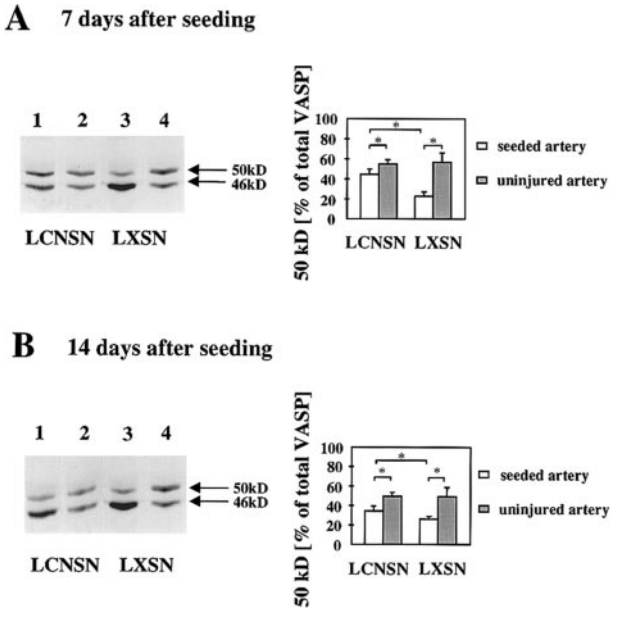

Figure 8. VASP phosphorylation in LCNSN SMC–seeded arteries detected by Western blot. A, VASP phosphorylation at 7 days in LCNSN SMC–seeded artery (lane 1) and contralateral uninjured artery (lane 2) and in LXSN SMC–seeded artery (lane 3) and contralateral uninjured artery (lane 4). B, VASP phosphorylation at 14 days in LCNSN SMC–seeded artery (lane 1) and contralateral uninjured artery (lane 2) and in LXSN SMC–seeded artery (lane 3) and contralateral uninjured artery (lane 4). VASP phosphorylation was expressed as the relative density of the 50-kD band (percentage of total VASP). *$P<.05$ (n=5).

growth factor–induced mitogenesis was found to be enhanced by NO in primary but not in subcultured rat SMCs.[52] This observation might explain why overexpression of ecNOS inhibited SMC proliferation in vitro but not in vivo. Another possibility is that the growth-inhibitory effect of NO on SMCs is dose dependent[16] and that the concentrations of NO-generating reagents and cGMP analogues required to inhibit SMC proliferation and migration are significantly greater than those required to relax vessels.[54] NO generated by seeded LCNSN SMCs might not be sufficient to inhibit the proliferation of the endogenous medial SMCs activated by balloon injury.

Our observation differs from the findings reported by other investigators.[12,55] In the study of von der Leyen et al,[12] ecNOS was expressed transiently by transfecting the injured vessel wall with a mixture of ecNOS cDNA, Sendai virus, and liposomes. This could account for the inhibition of the neointimal formation in these experiments, since NO production in the media SMCs locally might be high enough to inhibit medial SMC proliferation. In the study of Tzeng et al,[55] iNOS cDNA was expressed in injured vessels. The sustained production of NO generated by iNOS has been shown to inhibit several enzymes, such as ribonucleotide reductase, a key enzyme for DNA synthesis, and to damage DNA by highly toxic peroxynitrite.[50] Thus, sustained iNOS expression could kill SMCs as well as suppress SMC growth. NO can induce apoptosis in vitro in several cell types[45–48] and block apoptosis in others.[56,57] In the present study, BrdU labeling indices (Table 3) and the total nuclear number (data not shown) in LCNSN SMC– and LXSN SMC–seeded vessels were the same. This result makes it very unlikely that ecNOS expression was causing significant apoptosis.

Vasospasm is observed at 2 weeks in injured vessels and disappears at later times (4 weeks).[34] This phenomenon might be due to the development of SMC-rich intima in the absence of a luminal source of ecNOS. The observation in our experiments that LCNSN SMC–seeded vessels are dilated at 2 weeks and contracted by L-NA is clear evidence that ecNOS-generated NO blocked the vasospasm. The difference between LCNSN SMC– and LXSN SMC–seeded vessels was lost at 4 weeks. This finding might be due to the fact that NO produced by regenerated endothelium caused relaxation of the vessels. The evidence that endothelial regeneration proceeded at an increased rate in LXSN SMC–seeded vessels supports this conclusion. The fact that there were no differences in VASP phosphorylation at 4 weeks (data not shown) between LCNSN SMC– and LXSN SMC–seeded vessels also supports this conclusion.

The observation of the enhanced VASP phosphorylation in LCNSN SMC– and LCNSN SMC–seeded arteries (Figures 7 and 8) provides a link between the effect of NO on intimal hyperplasia and SMC cytoskeleton organization. Indeed VASP phosphorylation has been found to correlate closely with inhibition of fibrinogen binding to intact platelets.[58] Fibrinogen receptor activation plays an important role in platelet aggregation, and the integrin binding of fibrinogen might be affected by the state of VASP phosphorylation (inside out signaling). VASP, a proline-rich protein substrate of both PKG and PKA, is associated with focal adhesions, cell-cell contacts, and highly dynamic membrane regions that link signal transduction pathways and elements regulating cell motility and is an important regulator of the actin cytoskeleton.[38,39,59] VASP could therefore be a good candidate to mediate some effects of PKA and PKG on SMC motility, since it contains selective phosphorylation sites for both protein kinases. In addition, the activation of PKA by cGMP might be responsible in part for inhibition of SMC function by NO.[54]

The results of the present study demonstrate that rat SMCs expressing human ecNOS and seeded into denuded carotid arteries can perform some endothelial functions (vasodilatation) but not all (eg, inhibition of SMC growth). To inhibit SMC growth throughout the artery, increased expression of NO and additional inhibitors (eg, prostaglandins/prostacyclin) may be needed. From a clinical perspective, the strategy of luminal expression of ecNOS may have negative and positive effects. On the one hand, it might suppress endothelial regeneration; on the other hand, it might promote luminal dilatation.

Acknowledgments

This study was supported by a National Institutes of Health grant (HL-52459). We thank Holly Lea for excellent morphometric analysis and assistance with the in vivo experiments and Trevina Wang, Selina Vergel, Sarah MacKellar, and Tim Peterson for technical assistance.

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