Enhanced Recovery of Injury-Caused Downregulation of Paxillin Protein by eNOS Gene Expression in Rat Carotid Artery

Mechanism of NO Inhibition of Intimal Hyperplasia?

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Abstract—Injury-caused dedifferentiation accompanied by proliferation and migration of smooth muscle cells (SMCs) is an important process in the development of the neointima. Nitric oxide (NO) stimulates differentiation and inhibits proliferation and migration of SMCs. Paxillin has been found to play an important role in cell differentiation, and its phosphorylation is regulated by NO in cultured SMCs. However, the regulation of paxillin by NO in the injured artery has not been investigated. Therefore, the aim of this study was to study the effects of in vivo endothelial NO synthase (eNOS) gene transfection on paxillin expression and intimal hyperplasia. A catheter balloon–denuded rat carotid artery was transfected in vivo with the replication-deficient adenovirus Ad5/RStVeNOS or with Ad5/RSVLacZ as the control. Transfected eNOS gene expression was determined by immunostaining, Western blot analysis, and citrulline assay. The expression of paxillin and its associated proteins was determined in injured arteries by Western blot analysis. The area of the intima and the ratio of intima to media were examined on cross sections by morphometry. The data showed that the expression of paxillin was significantly downregulated after injury. eNOS gene transfer showed no effect on paxillin downregulation 2 days after injury but significantly enhanced the recovery of paxillin protein 5 days and 2 weeks after injury. Vinculin, a paxillin-binding protein, was not altered by vascular injury or by eNOS gene transfer. eNOS gene transfer significantly inhibited intimal hyperplasia for up to 4 weeks. These results suggest that NO inhibition of intimal hyperplasia may be mediated by enhancing the recovery of injury-caused downregulation of paxillin. (Arterioscler Thromb Vasc Biol. 1999;19:147-152.)

Key Words: balloon-injured artery ▪ rats ▪ restenosis ▪ gene transfer ▪ paxillin ▪ vinculin

Paxillin is highly expressed in smooth muscle cells (SMCs) and is localized in dense plaques in vivo and at focal adhesion sites, the homologue of dense plaque, in cultured cells.1–5 At the focal adhesion sites, paxillin is associated with the actin filament–binding protein vinculin. Therefore, paxillin may play a role in “anchoring” actin filaments to dense plaques in SMCs through vinculin.6 In other words, paxillin may be involved in maintaining the integrity of the contractile apparatus in vascular SMCs. Paxillin is also a “docking” protein involved in organizing the signal transduction complex.4–6 Paxillin can directly associate with cytosolic protein tyrosine kinases, csk, crk, c-src, and focal adhesion kinase (FAK).6–9 Paxillin has multiple tyrosine phosphorylation sites that can be phosphorylated by csk, c-src, and FAK to form the src homology 2 domain (SH2).6,10 SH2, together with SH3 and the LIM domains (a conserved sequence of Lin-11, Isl-1, and Mec-3) of paxillin, mediates protein-protein interactions.6 Tyrosine phosphorylation of paxillin is associated with cytokines, mitogenic peptides, growth factors, and extracellular matrix protein–stimulated signal transduction, including the mitogen-activated protein kinase pathway that is involved in cell migration and proliferation.5,7,11

As a member of the LIM domain–containing proteins, paxillin may play an important role in the differentiation of vascular SMCs. For example, CRP1 (cysteine-rich-protein) is involved in the differentiation of chicken gizzard SMCs.12,13 SmCRP, an LIM protein cloned from rat aorta, has been found to play a role in the differentiation of vascular SMCs.14 Rhombotin-2 is required for erythroid differentiation during mouse development,15 and paxillin has also been found to be associated with the differentiation of HL-60 and neuronal cells.16,17

Taken together, paxillin may function as a structural protein to maintain the integrity of the SMC contractile apparatus and as a docking protein to potentially mediate signal transduction for SMC contraction, migration, proliferation, and possibly differentiation. The dedifferentiation accompanied by proliferation and migration of medial SMCs contributes to the formation of the neointima in advanced...
atherosclerosis and in postinjury restenosis. Therefore, regulation of paxillin expression may be involved in the development of vascular diseases.

Substantial evidence indicates that NO inhibits neointimal formation in injured arteries. NO inhibition of SMC migration and proliferation is at least partially responsible for this effect. A recent study found that NO regulates vascular SMC differentiation. Therefore, NO-mediated antiproliferative and antiinflammatory functions may occur through its stimulatory effect on SMC differentiation, since it has long been known that differentiated SMCs have less potential to proliferate and migrate. Although evidence suggests that NO may stimulate SMC differentiation through the cyclic GMP–dependent protein kinase pathway, the other possibilities have not been explored. As discussed earlier, paxillin may also be involved in vascular SMC differentiation. We have recently demonstrated that NO may inhibit vascular SMC migration and proliferation through its inhibition of paxillin tyrosine phosphorylation. Therefore, we postulate that paxillin may be a novel target for NO regulation of SMC migration, proliferation, and differentiation.

Therefore, in this study, we investigated the effect of replication-deficient adenovirus-mediated eNOS gene transfer on paxillin expression and neointimal formation in the injured rat carotid artery. Our results showed that paxillin was significantly downregulated after vascular injury. In vivo eNOS gene transfer to medial SMCs enhanced the recovery of injury-caused downregulation of paxillin protein. Furthermore, eNOS gene transfection significantly inhibited intimal hyperplasia. These results suggest that upregulation of paxillin may be at least partially responsible for NO-mediated inhibition of intimal hyperplasia.

Methods

Adenoviral Vectors

Replication-deficient, recombinant adenoviruses Ad5/RSVeNOS and Ad5/RSVLacZ were obtained from the Vector Core, University of Iowa College of Medicine, Iowa City. E1A, E1B, and E3 regions of the adenovirus were deleted to impair the ability of the virus to infect cells. For in vitro gene transfection, the adenoviral vectors were deleted to impair the ability of the virus to infect cells. For in vivo gene transfection, the adenoviral vectors were delivered to the animals. The adenoviral vectors were delivered to the animals.

In Vivo Gene Transfection

Adult, male Sprague-Dawley rats were housed and cared for according to National Institutes of Health (NIH; Bethesda, Md) guidelines for the animal care facility of the University of Iowa. Seventy-nine rats weighing 500 to 600 g were used for this study. In vivo gene transfer to catheter balloon-injured carotid arteries was performed essentially as described in previous reports. In brief, the rats were anesthetized by intraperitoneal injection of ketamine (10 mg/kg). The left carotid artery was exposed through a midline incision. A segment of the common carotid artery 1 cm long was isolated by temporary ligation of the proximal common carotid artery and proximal internal carotid artery. A 2F embolectomy balloon catheter was inserted into the isolated common carotid artery through an arteriotomy site in the external carotid artery. The balloon was inflated with air and drawn toward the arteriotomy 3 times to denude the endothelium. The injured artery was flushed with normal saline solution, and then 60 μL of 10 plaque-forming units per mL of Ad5/RSVeNOS or Ad5/RSVLacZ (control) was instilled into the injured arterial segment, which became distended and remained so for 30 minutes. Blood flow was restored after the viral vector was withdrawn. At various time periods after transfection, the animals were killed. The uninjured and injured arteries were processed for immunostaining. Western blotting analysis, β-galactosidase histochemistry, or histological examination.

β-Galactosidase Histochemistry and Immunostaining for eNOS

To examine transgene expression, 4 rats transfected with the LacZ gene were killed 2 days after transfection. The injured arteries were removed and fixed in freshly made 4% paraformaldehyde in PBS, 0.01 mol/L (pH 7.3), for 1 hour at 4°C. After being washed in PBS, the arteries were stained for β-galactosidase activity as previously described. The arteries were further fixed in the same fixative for an additional 4 hours. The arteries were then cut into transverse sections on a cryostat. The sections were examined and photographed under a light microscope. The right uninjured common carotid arteries were processed in the same way as controls. For examining the transgene expression in vivo, 7 days after gene transfection the arteries (n = 3) were removed and fixed in 4% paraformaldehyde. Then the samples were cut into transverse cryostat sections (7-μm thickness). eNOS was examined by immunofluorescence staining by using the procedure described under in vitro gene transfection.

Western Blotting Analysis

To examine the effects of vascular injury and eNOS gene transfection on the expression of paxillin, vinculin, and extracellular signal–regulated kinases (Erk’s), 3 groups of injured arteries (the injured but nontransfected, eNOS-transfected, and LacZ-transfected arteries) were collected 2 days (n = 4), 5 days (n = 6), and 2 weeks (n = 5) after transfection. The injured arteries were removed and quickly frozen in liquid N2 until use. The contralateral uninjured arteries were deendothelialized and frozen immediately after they were removed from the animals. These uninjured arteries were used as the controls. For Western blotting, the arteries were thawed, stripped of adventitia, minced, and then homogenized in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L sodium vanadate, 0.2 mmol/L PMSF, 1% Triton X-100, and 0.5% NP-40. The homogenate was centrifuged for 15 minutes at 4°C. The supernatant was collected and processed for SDS–polyacrylamide gel electrophoresis and Western blotting. The membrane was first blocked in 5% nonfat milk and then incubated with a specific first antibody for 1 to 1.5 hours and a

FMN, 4 μmol/L FAD, 1 μg calmodulin, 1 mmol/L calcium, and 40 to 80 μg total proteins in a final volume of 200 μL. For examining calcium-independent activity, 1 mmol/L EGTA was used instead of 1 mmol/L calcium. The reaction was carried out at 37°C for 10 minutes and was terminated by adding 5.5 mL of Dowex slurry (Dowex AG50W-X8, 100 to 200 mesh, Na+ form) to remove unconverted L-[14C]arginine. L-[14C]citrulline was measured with a liquid scintillation spectrometer.

In Vivo Gene Transfection

Rat aortic SMCs were isolated as previously reported. Cells were cultured to confluence and then transfected with a 100 multiplicity of infection (MOI) Ad5/RSVeNOS or Ad5/RSVLacZ for 2 hours. The transfected cells were further cultured for 48 hours in Dulbecco’s modified Eagle’s medium containing 0.2% BSA. Then the cells were processed for immunostaining for eNOS or citrulline assay for eNOS activity.

For immunostaining, cells were fixed in freshly made 4% paraformaldehyde for 1 hour. After being washed in PBS, the cells were stained for eNOS by indirect immunofluorescence labeling. In brief, cells were blocked with 10% normal goat serum in PBS for 1 hour, then incubated with a specific first antibody for 1 to 1.5 hours and a goat anti-rabbit IgG and examined by fluorescence microscopy. Following by incubation with polyclonal anti-eNOS antibodies or cells were blocked with 10% normal goat serum in PBS for 1 hour. After being washed in PBS, the cells were processed for immunostaining for eNOS or citrulline assay for eNOS decreased by measuring the conversion of L-[14C]arginine to L-[14C]citrulline. Cells were homogenized in 50 mmol/L Tris-HCl (pH 7.4) containing 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 12 mmol/L β-mercaptoethanol, 1 mmol/L PMSF, 3 μmol/L leupeptin, 1 μmol/L aprotinin, 1 μmol/L pepstatin, and 1 μmol/L soybean trypsin inhibitor. The reaction mixture contained 50 mmol/L Tris-HCl (pH 7.4), 5 μmol/L L-arginine, 0.25 μCi L-[14C]arginine, 0.5 mmol/L NADPH, 10 μmol/L tetrahydrobiopterin, 4 μmol/L

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peroxidase-conjugated secondary antibody for 1 hour. The peroxidase activity was examined using a chemiluminescence detection kit (Western view, Transduction Laboratory).

The dilution factors for the antibodies used in this study were as follows: rabbit anti-eNOS polyclonal antibodies (1:500, Santa Cruz Biotech Inc), monoclonal anti-paxillin (1:5000, Transduction Laboratory), monoclonal anti-vinculin (1:1000, Sigma), monoclonal anti-Erk (1:800, Santa Cruz Biotech Inc), goat anti-rabbit IgG–peroxidase (1:10 000, Sigma), and anti-mouse IgG–peroxidase (1:10 000, Sigma). For multiple blotting for different proteins on the same membrane, it was stripped with the stripping buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 100 mmol/L β-mercaptoethanol for 30 minutes at 65°C.

Histological Assessment of Intimal Hyperplasia

eNOS and LacZ gene–transfected arteries were harvested at 2 (n=5) and 4 (n=7) weeks after transfection. Twenty minutes before, the rats were injected intravenously with Evans blue (15 mg/0.5 mL saline per rat) to label the injured segments of left common carotid arteries. The morphometric analysis was done essentially as described by van der Leyen et al. The segment from the middle part of the labeled artery was removed. The specimens were fixed in formaldehyde, embedded in paraffin, cut into 7-μm transverse sections, and then stained with hematoxylin and eosin. Three sections were selected from each specimen. The areas of intima and media were measured with a computerized digital image analysis system (NIH image). The cross-sectional area of intima was obtained by subtracting lumen area from the area enclosed by the internal elastic membrane. The area of the media was obtained by subtracting the area enclosed by the external elastic membrane from that enclosed by the internal elastic membrane.

Statistical Analysis

All values are expressed as mean±SEM. Student’s t test was used to determine significant differences. A value of P<0.05 was considered significant.

Results

Transgene Expression in Injured Common Carotid Arteries

The efficiency of adenoviral vector–mediated gene transfer in injured rat carotid arteries was estimated on transverse histological sections of the arteries transfected with Ad5/RSVLacZ. The transfected and total cell numbers per section were counted by using 40 sections from 4 arteries 2 days after transfection. The estimated transfection efficiencies ranged from 25% to 40% among the 4 arteries (Figure 1A). No staining for LacZ was found in the contralateral uninjured arteries. The expression of transfected eNOS was examined by Western blotting analysis and immunostaining. The results showed that eNOS gene transfer resulted in a marked increase in eNOS gene expression compared with undetectable expression in LacZ-transfected arteries (Figure 1B). Immunofluorescence staining showed that the transfected eNOS was localized in the medial SMCs (Figure 1C). There was no eNOS staining found in LacZ-transfected arteries (Figure 1D). The functional activity of the transfected eNOS was characterized in cultured vascular SMCs. Immunofluorescence staining revealed that ≈90% of the cells express eNOS after 2-hour exposure to a 100 MOI Ad5/RSVeNOS (Figure 2A). Citrulline assay showed that eNOS gene transfection resulted in a marked increase in eNOS activity and that the activity was calcium dependent (Figure 2B). The activity was abolished in the presence of EGTA (Figure 2B). There was no detectable NOS activity in nontransfected and LacZ-
transfected cells. These data indicate that the transfected eNOS is functional.

Effects of Vascular Injury and eNOS Gene Transfection on Paxillin Expression

As previously reported, paxillin appears as 75- and 55-kDa bands.4,28 The 55-kDa band is believed to be the proteolytic segment of 75-kDa paxillin (Figure 3A). Paxillin protein was drastically downregulated 2 days after injury in LacZ and eNOS gene–transfected vessels compared with uninjured controls. Five days after injury, the paxillin level had slightly recovered in LacZ-transfected vessels but had greatly recovered in eNOS-transfected vessels compared with day 2 after injury (Figure 3A). There was no difference in paxillin expression between LacZ-transfected and injured but non-transfected arteries at days 2 and 5 after injury (data not shown). Two weeks after transfection, paxillin had further recovered in LacZ-transfected and injured but not in transfected arteries (Figure 3B). Still, there was more paxillin in eNOS-transfected arteries 2 weeks after transfection (Figure 3B). These observations indicate that eNOS gene transfection enhanced the recovery of the injury-caused downregulation of paxillin. Interestingly, the paxillin-associated cytoskeletal protein vinculin (116 kDa) was not altered either by vascular injury or by eNOS gene transfer (Figure 3A and 3B). We also examined the expression of Erk, which has been found to be an important component in signal transduction for cell proliferation and migration.34–36 Moreover, Erk is known to be activated by 2 paxillin-binding tyrosine kinases, FAK and src.37,38 We observed that both Erk 1 and Erk 2 were not changed at 2 days but were upregulated 5 days and 2 weeks after injury. eNOS gene transfection did not modulate Erk expression (Figure 3A and 3B).

Inhibition of Intimal Hyperplasia by eNOS Gene Transfection

Histological examination showed that eNOS gene transfer inhibited neointimal formation 2 weeks after injury and that this inhibition persisted for up to 4 weeks (Figure 4). We noticed that in some of the eNOS gene–transfected vessels, the neointima was unevenly formed (Figure 4E and 4F), which may be due to the uneven transfection of eNOS gene in the injured arteries. The inhibition of neointimal formation by eNOS gene transfer was further confirmed by morphometric analysis. The results showed that eNOS gene transfer reduced the neointimal area by 55.6% (0.224±0.015 versus 0.099±0.017 mm², n=7) at 2 weeks and by 43.3% (0.208±0.025 versus 0.118±0.009 mm², n=5) at 4 weeks and reduced the ratio of neointima to media by 56.9% (1.713±0.127 versus 0.739±0.111 mm², n=7) at 2 weeks and by 46.6% (1.91±0.126 versus 1.02±0.107 mm², n=5) at 4 weeks compared with LacZ gene–transfected control arteries (Figure 5).
Discussion

In this study, we demonstrated for the first time that in vivo eNOS gene transfection enhances the recovery of injury-caused downregulation of paxillin protein and results in sustained inhibition of intimal hyperplasia for up to 4 weeks. The downregulation of paxillin after injury and the enhanced recovery of injury-caused paxillin downregulation by eNOS gene transfer support the concept that paxillin may play an important role in vascular SMC differentiation. These results imply a novel mechanism for NO inhibition of intimal hyperplasia.

In response to injury, vascular SMCs undergo phenotypic modulation. They lose their properties of the contractile phenotype, such as SMC-specific a-actin and myosin heavy chain, dense bodies, and plaques and acquire a synthetic phenotype.18,19,39 Cells of synthetic phenotype contain abundant rough endoplasmic reticula and Golgi apparatus.19,39 Findings that SMC dedifferentiation is accompanied by miRNA upregulated. These changes are consistent with the previous report.24 It is interesting to note that in some of the eNOS gene–transfected vessels, the neointima was unevenly formed, which may be due to uneven gene transfection in the medial SMCs found in this study and others.31,41 The enhanced recovery of paxillin protein by eNOS gene transfer implies the recovery of paxillin-mediated inhibitory signaling for SMC migration and proliferation.

Adenovirus-mediated eNOS gene transfer to medial SMCs resulted in significant attenuation of neointimal formation, which is consistent with a previous report.28 It is interesting to note that in some of the eNOS gene–transfected vessels, the neointima was unevenly formed, which may be due to uneven gene transfection in the medial SMCs found in this study and others.31,41 The enhanced recovery of paxillin protein may be at least partially responsible for the inhibition of neointimal formation by eNOS gene transfer.

Acknowledgments

This study was supported by NIH grant HL-14388 and a grant-in-aid from the Iowa Heart Association. We greatly appreciate the help of the University of Iowa Vector Core for the preparation of virus vector used in this study. We thank Dr Mary J.C. Hendrix for helpful suggestions.

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doi: 10.1161/01.ATV.19.1.147

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