Mechanism of eNOS gene transfer inhibition of vascular smooth muscle cell proliferation

FIONA M. D’SOUZA,1 RODNEY L. SPARKS,1 HUIYING CHEN,1 PHILIP J. KADOWITZ,2 AND JAMES R. JETER, JR.1
1Departments of Structural and Cellular Biology and 2Pharmacology, Tulane University Health Sciences Center, New Orleans, Louisiana 70112

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D’Souza, Fiona M., Rodney L. Sparks, Huiying Chen, Philip J. Kadowitz, and James R. Jeter, Jr. Mechanism of eNOS gene transfer inhibition of vascular smooth muscle cell proliferation. Am J Physiol Cell Physiol 284: C191–C199, 2003. First published September 18; 10.1152/ajpcell.00179.2002.—Endothelial nitric oxide synthase (eNOS) is responsible for the production of nitric oxide (NO) in blood vessels. NO has been shown to be involved in the inhibition of vascular smooth muscle cell (VSMC) proliferation. In the present study, the eNOS gene was transferred into rat aortic smooth muscle cells by using an adenoviral vector, and the effect of endogenously produced NO on VSMC proliferation was investigated. The presence of eNOS in eNOS-transfected cells was confirmed by immunocytochemistry and Western blot analysis. eNOS transfection resulted in inhibition of VSMC proliferation. This effect was accompanied by increased levels of p53 and p21. The increased levels of p53 and p21 observed in eNOS-transfected cells were reduced in the presence of the PKA inhibitor. These data suggest that p21 and p53 play a role in the inhibition of proliferation in eNOS-transfected cells and that levels of these two proteins are regulated by PKA.

The endothelium serves as the principal physiological source of nitric oxide (NO) in blood vessels (4, 5, 31). NO has been shown to have an inhibitory effect on vascular smooth muscle cell (VSMC) proliferation (12, 23). Endothelial damage and the disruption in NO production decrease the inhibitory effect of NO on VSMC proliferation. After endothelial injury, VSMCs undergo a “phenotypic modulation” and migrate from the media to the intima where they begin to proliferate and produce collagen (1). This results in vessel fibrosis, decreased cross-sectional area, and restenosis (4).

Endothelial nitric oxide synthase (eNOS) catalyzes the conversion of L-arginine to L-citrulline and NO (16). NO then diffuses from the endothelium into adjacent VSMCs and binds to the heme moiety of soluble guanylate cyclase (16). This binding results in activation of the enzyme and an increase in smooth muscle cGMP concentration (14, 16, 24). Pig coronary arteries treated with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) showed increased cGMP levels, whereas cAMP levels were not altered (21). Studies by Cornwell et al. (6) revealed that in primary explants of aortic smooth muscle treated with either IL-1β or NO donors, cGMP levels, but not cAMP levels, were increased. This study also demonstrated that, in the presence of an NO donor, when the activity of protein kinase A (PKA), but not that of protein kinase G (PKG) was inhibited, VSMC proliferation in both treated and controls was similar (6). However, when PKG activity alone was inhibited in NO donor-treated cells, proliferation was also inhibited. It was, therefore, proposed that the increased cGMP levels in these cells were sufficient to activate PKA and that cGMP-activation of PKA resulted in the inhibition of VSMC proliferation (6). However, the mechanism by which PKA acts to inhibit VSMC proliferation remains to be elucidated.

A potential downstream target for PKA is p21, also known as WAF1, CIP1, or SDI1. p21 itself is a downstream target for the p53 tumor suppressor gene (10). p53 has also been implicated in controlling cell passage (8, 9). In some cell types the induction of p21 has been shown to be p53 dependent (10, 28), whereas in other cell types this has been found not to depend on p53 (18, 28). The p21 gene has been reported to have a p53 transcriptional motif that enhances transcription of a number of genes, including p21 (27). p21 binds to cyclin/CDK complexes, blocking their activity, and results in the cell being unable to progress from the G1 to the S phase (15). Exogenous NO has been reported to affect the activity of p21 late in the G1 phase (18). In a study in VSMCs in vitro, p21 expression was increased by treatment with the NO donor SNAP (14). In another study, the p21 gene was transfected in vivo into porcine arteries injured by a balloon catheterization procedure and produced a 35% reduction in VSMC proliferation and a reduction in intimal hyperplasia (37). However, in platelet-derived growth factor (PDGF)-treated...
guinea pig coronary artery VSMCs transfected with the eNOS gene, a significant increase in p21 was not observed with the inhibition of VSMC proliferation (33). Thus the role of p21 in NO-inhibited induction of VSMC proliferation is uncertain. In the present study, we attempted to determine whether these cell cycle inhibitory proteins are involved in NO-induced inhibition of proliferation in eNOS-transfected aortic VSMCs. Recent studies of VSMC proliferation have focused on the transfer of specific genes, such as eNOS, p21, or retinoblastoma (Rb), in vivo or in vitro (3, 20, 30, 33). In the present study, the eNOS gene was transfected into rat aortic VSMCs in vitro, and the effect of eNOS transfection on cell proliferation was studied. It has been reported that PKA plays an important role in the inhibition of vascular VSMC proliferation following NO donor treatment (6). However, the PKA targets involved in the inhibition of VSMC proliferation have not yet been determined. The present study investigated the role of PKA in the inhibition of proliferation in eNOS-transfected cells and the involvement of the downstream effector molecules p53 and p21 in this process.

METHODS

Cell culture and cell transfection. Rat aortic VSMCs were obtained from male Sprague-Dawley rats weighing ~350–500 g. The rats were anesthetized with pentobarbital sodium (35 mg/kg ip), and the aorta was removed and placed in tissue culture medium 199 (Sigma Chemical, St. Louis, MO) (17). The vessel was incubated in a collagenase solution (200 U/ml collagenase type I, 0.4 mg/ml trypsin inhibitor) for 30 min at 37°C. The adventitia was removed, and the aorta was cut longitudinally. A sterile cotton swab was used to disrupt the endothelial lining. The vessel was then minced into small pieces and placed in a collagenase-elastase solution (200 U/ml collagenase type I, 15 U/ml elastase, type III) for 2 h. The tissue was washed with culture medium 199 supplemented with 10% FBS containing penicillin (100 U/ml) and streptomycin (200 μg/ml). The tissue pieces were plated on a 25-cm² cell culture flask. The flask was placed in a humidified incubator (95% air-5% CO₂) at 37°C. The tissue segments were allowed to attach for 5–7 days, after which the medium in the flask was aspirated. Fresh medium supplemented with FBS containing penicillin and streptomycin was added. Upon reaching 70% confluency, the cells were passed by trypsinization (0.25% trypsin, 0.053 mM EDTA; GIBCO, Grand Island, NY). The identity of the cells was confirmed by the typical “hill-and-valley” appearance exhibited by VSMCs. In addition, immunohistochemical studies showed that >95% of the cells exposed to an NO donor stained positive for smooth muscle-specific α-actin.

The cells were transfected with replication-deficient recombinant adenoviruses encoding either nuclear targeted β-galactosidase (AdCMVβgal) or eNOS (AdCMVeNOS). Both adenoviruses were driven by the cytomegalovirus (CMV) promoter. The adenoviral vectors were prepared by the University of Iowa Gene Vector Core Laboratory (Iowa City, IA).

Cell proliferation. In all proliferation studies, 6 × 10⁴ cells were plated in each well of a six-well cell culture dish (Costar, Corning, NY). The cells were transfected with 150, 300, or 450 multiplicity of infection units (MOI) of AdCMVeNOS. Cell numbers were measured over the course of 4 days. Once the optimal dose of the eNOS vector transfection was determined in pilot studies, subsequent experiments were conducted with 300 MOI AdCMVeNOS. Cell numbers in β-galactosidase-transfected cells and nontransfected cells were compared to ensure that the adenoviral vector itself had no effect on proliferation.

Detection of eNOS. After transfection, the presence of intracellular eNOS was determined by using immunocytochemistry and Western blot analysis. For immunocytochemical detection, passage II cells were plated on single-chambered slides (Nunc, Naperville, IL). Nontransfected and cells transfected with AdCMVβgal or AdCMVeNOS were used for the immunohistochemical detection of eNOS. eNOS expression was determined on days 2 and 4 following transfection. Immunohistochemistry was performed by using the Immunocruz staining system (Santa Cruz Biotechnology, Santa Cruz, CA). Medium was aspirated from the chamber slides, and the cells were fixed in methanol (−10°C) for 5 min. To quench endogenous peroxidase activity, we incubated the fixed cells in a 0.5% solution of hydrogen peroxide in PBS for 7 min. Cells were washed in PBS and then incubated in serum-free medium for 20 min to prevent nonspecific binding. The cells were then fixed immediately with the primary antibody eNOS (1:50) (Transduction Laboratories, San Diego, CA) for 2 h. The cells were washed in PBS and treated with biotinylated secondary antibody for 30 min, followed by treatment with horseradish peroxidase (HRP)-streptavidin complex. The cells were then exposed to HRP solution for 10 min. The cells were dehydrated by using 95% and 100% ethanol and xylene. The slides were coveredslipped and viewed under a light microscope. The number of cells transfected was determined by counting immunopositive cells.

Western blot analysis for eNOS was carried out on passage II cells, as described in Cell culture and cell transfection. The cells were plated, and nontransfected cells and cells transfected with either β-galactosidase or eNOS were used in these experiments. The cells were allowed to grow for 4 days after transfection, and cell counts were obtained daily. The cells were harvested by trypsination and lysed with a hypotonic buffer (10 mM Tris, 1.5 mM MgCl₂, 1 mM PMSF). Protein levels were quantified by using a bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL). Equivalent quantities of protein (20 μg) from each sample were electrophoresed on a 4–20% gradient gel. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Amersham, Amersham, UK) for 1 h by using a Hoefer model TE 50 setup (Hoefer Scientific, San Francisco, CA). The membrane was blocked with 5% milk in Tris-buffered saline containing 0.1% Tween (TBST) for 1 h. The membrane was washed in TBST three times for 10 min each and was incubated with anti-eNOS monoclonal antibody (Transduction Laboratories) at a dilution of 1:500 for 1 h. After the membrane was washed in TBST, the bound antibody was detected with labeled anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology). eNOS was visualized by using an enhanced chemiluminescence kit (ECL; Amersham) and BioMax MR-1 film (Eastman Kodak, Rochester, NY).

Inhibition of eNOS activity with t-NAME. The effect of the NOS inhibitor Nω-nitro-l-arginine methyl ester (l-NAME) on eNOS-transfected cells was investigated. The effect of l-NAME on nontransfected cells was first determined by treating passage II rat aortic VSMCs with concentrations of 10⁻³, 10⁻⁴, and 10⁻⁵ M, and a dose-response study was then carried out in eNOS-transfected cells. After it was determined that 10⁻⁴ M was an optimal dose in pilot studies, the cells were left untreated or were transfected with AdCMVeNOS or AdCMVβgal. In the transfection experi-
ments, the cells were preincubated with L-NAME for 30 min before transfection, and cell counts were obtained over the course of 4 days.

To determine the role of PKA on NO-induced inhibition of proliferation, we treated eNOS-transfected cells with the PKA inhibitor Rp-8-bromo-adenosine 3’,5’-cyclic monophosphate (Rp-8-BrCAMP). A dose-response study (25, 50, and 100 μM) for Rp-8-BrCAMP was conducted within a range previously reported to have good specificity for PKA (6, 13, 22). After the optimal dose (100 μM) was determined, the cells were either left untreated or transfected with eNOS in the presence or absence of Rp-8-BrCAMP. Cell counts were obtained daily for 4 days, after which time the cells were harvested. NO is known to increase intracellular levels of cGMP, resulting in the activation of PKG (7). To determine the role of PKG in the NO-induced inhibition of proliferation in eNOS-transfected cells, we treated the cells with the PKG inhibitor Rp-8-bromo-guanosine 3’,5’-cyclic monophosphothioate (Rp-8-BrGMPS). A dose-response study with concentrations of 25, 50, and 100 μM for Rp-8-BrGMPS was conducted.

Effect of eNOS transfection and inhibition of PKA on expression of p21 and p53. Passage II rat aortic VSMCs were either left untreated or transfected with β-galactosidase or eNOS. One group of eNOS-transfected cells was treated with Rp-8-BrCAMP (100 μM). The cells were treated for 4 days and then harvested and lysed. Protein levels were quantified, and equivalent amounts of protein (20 μg) for each group were run on a 4–20% gradient gel. Western blot analysis was used to detect p21 and p53 levels. Monoclonal mouse anti-p21 IgG (Santa Cruz Biotechnology) was used as the primary antibody for the detection of p21, whereas monoclonal rabbit anti-p53 IgG (Santa Cruz Biotechnology) was used to detect p53. Bound antibody was detected with anti-mouse IgG-HRP and anti-rabbit IgG-HRP secondary antibodies. Bound antibody was visualized using the ECL chemiluminescence kit (Amersham).

Detection of apoptosis in eNOS-transfected cells. Studies were conducted to determine whether apoptosis was associated with increases in p21 and p53 levels in eNOS-transfected cells. The trypan blue exclusion test and Western blot analysis were used to access viability and the presence of apoptosis in cells infected with eNOS. Cells were exposed to AdCMVeNOS for 4 days, after which cell viability was determined. Cell viability was found to be >95% in rat aortic VSMCs transfected with AdCMVeNOS at 300 MOI. Cell proliferation was significantly inhibited at this dose of the adenoviral vector. For these studies, passage II rat aortic VSMCs were plated in six-well culture dishes. After attachment, the medium was aspirated and cells were either untreated or infected with AdCMVβgal at 300 MOI. Noninfected cells were grown to either 95% confluence over a 4-day period. At 30% confluence, the cells were in log-phase growth. eNOS-transfected cells were incubated at 37°C for 4 days, after which the cells were trypsinized and lysed in hypotonic buffer. Protein samples were quantified by using the BCA protein assay (Pierce), and 20 μg of protein were loaded in each well in a 4–20% gradient gel. Western blot analysis was conducted by using anti-Bax IgG (Transduction Labs).

Statistical analysis. Cells were counted by using an Olympus microscope. Protein assays were carried out by using a Labsystems Multiskan MS plate reader (Labsystems, Franklin, MA). Western blot autoradiographs were scanned with an AlphaEase program (Alpha Innotech, San Leandro, CA). The data were analyzed statistically by using a one-way ANOVA followed by post hoc analysis with Tukey’s test.

RESULTS

Proliferation studies in transfected cells. As shown in Fig. 1A, after treatment with 150 MOI AdCMVeNOS, cell proliferation was significantly inhibited by day 4. Treatment with 300 MOI AdCMVeNOS also significantly inhibited cell proliferation compared with nontransfected cells. The inhibition of proliferation was maintained over the 4-day period of the experiment. At 450 MOI AdCMVeNOS, cell proliferation was significantly inhibited but cell numbers decreased by day 4 (Fig. 1A). In subsequent studies, the cells were treated with 300 MOI AdCMVeNOS. Figure 1B shows cell counts carried out in control and β-galactosidase-transfected rat aortic VSMCs over a 4-day period. There was no significant difference between the two groups, and the doubling time for the AdCMVβgal-transfected cells was the same as that observed in control cells.

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Fig. 1: A: effect of AdCMVeNOS on cell number over a 4-day period. Passage II rat aortic vascular smooth muscle cells (VSMCs) were plated in 6-well cell culture dishes. Cells were untreated (control) or transfected with 150, 300, or 450 multiplicity of infection (MOI) AdCMVeNOS. B: effect of AdCMVβgal on cell proliferation. Passage II rat aortic VSMCs were plated in 6-well cell culture dishes. Cells were untreated (control) or transfected with 300 MOI AdCMVβgal. Cell counts were obtained daily. Data were analyzed by 1-way ANOVA followed by post hoc analysis with Tukey’s test. Each point represents the mean cell count ± SE (n = 4), *P < 0.01; **P < 0.001 vs. control.
Detection of eNOS. Immunohistochemical detection of eNOS was conducted on VSMCs transfected with 300 MOI AdCMVeNOS. eNOS-transfected cells were compared with nontransfected and β-galactosidase-transfected cells on day 4 following transfer of the eNOS gene, and these results are shown in Fig. 2.

Immunocytochemistry was performed, and the cells transfected with AdCMVeNOS revealed the presence of eNOS staining on day 4 (Fig. 2). Similar results were obtained on days 1, 2, and 3 (data not shown). Transfection efficiency was between 80 and 90%, and corresponding nontransfected and β-galactosidase-transfected cells did not show the presence of eNOS staining.

The Western blot analysis was used to detect the presence of eNOS in nontransfected cells, cells transfected with β-galactosidase, and cells transfected with AdCMVeNOS for 4 days. Figure 3A is representative of a typical Western blot for eNOS. Figure 3B summarizes mean data from three experiments, each carried out in triplicate. Equivalent quantities of protein (20 mg) were loaded in all wells for electrophoresis. The cells transfected with AdCMVeNOS showed the presence of eNOS protein, whereas eNOS protein was not detected in nontransfected and β-galactosidase-transfected cells (Fig. 3).

Inhibition of eNOS activity with L-NAME. In eNOS-transfected cells, addition of L-NAME caused a significant reduction of NO-induced inhibition of proliferation at concentrations of 10⁻³, 10⁻⁴, and 10⁻⁵ M (Fig. 4A). Figure 4B shows the effect of L-NAME (10⁻⁴ M) on eNOS- and β-galactosidase-transfected cells. L-NAME did not appear to have an effect on β-galactosidase-transfected cells, whereas it prevented the inhibition of proliferation in eNOS-transfected cells (Fig. 4B).

Effect of Rp-8-BrcAMPS and Rp-8-BrcGMPS on rat aortic VSMC proliferation. Figure 5A shows the effect of three concentrations (25, 50, and 100 μM) of Rp-8-BrcAMPS on proliferation in eNOS-transfected cells. A dose of 100 μM Rp-8-BrcAMPS was found to block the inhibition of proliferation (Fig. 5A). As shown in Fig. 5B, by day 4 cell numbers in eNOS-transfected cells were significantly lower than cell numbers in nontransfected and β-galactosidase-transfected cells. This finding suggests that Rp-8-BrcAMPS blocked the PKA-mediated inhibitory effect of eNOS-generated NO on VSMC proliferation (Fig. 5B).

Rp-8-BrcGMPS at 25 and 50 μM did not appear to have any effect on VSMC proliferation in nontransfected VSMC (Fig. 6A). At 100 μM, Rp-8-BrcGMPS cell proliferation was inhibited but cell viability decreased (data not shown). The effect of 25 and 50 μM Rp-8-
BrcGMPS in eNOS-transfected was investigated (Fig. 6B). No loss of cell viability was observed in cells treated with these concentrations of inhibitor. In eNOS-transfected cells, Rp-8-BrcGMPS at 25 or 50 μM inhibited proliferation in VSMC similar to that seen in VSMC transfected with eNOS alone (Fig. 6B). Cell proliferation in eNOS-transfected cells was not significantly different from that observed in eNOS-transfected cells treated with Rp-8-BrcGMPS. However, cell numbers were significantly different from untreated controls.

The difference in results from eNOS-transfected cells exposed to Rp-8-BrcAMPS or Rp-8-BrcGMPS suggests that in these cells, Rp-8-BrcAMPS is selective for PKA and Rp-8-BrcGMPS is selective for PKG in the concentrations selected.

Expression of p21 and p53 in eNOS-transfected cells. Figure 7A is representative of a typical Western blot for p21 from three experiments, each run in triplicate. Transfer of the eNOS gene to rat aortic VSMCs resulted in an upregulation of p21. The levels of p21 were significantly higher in eNOS-transfected cells compared with levels in nontransfected and β-galactosidase-transfected cells. In cells transfected with eNOS and treated with Rp-8-BrcAMPS, p21 levels were found to be significantly lower than in eNOS-transfected cells and not significantly different from levels in

Fig. 5. Effect of Rp-8-bromoadenosine 3', 5'-cyclic monophosphothioate (Rp-8-BrcAMPS), a PKA inhibitor (PKAi), on cell growth in eNOS-transfected cells. A: dose-response relationship for Rp-8-BrcAMPS. Cells were untreated or treated with Rp-8-BrcAMPS for 4 days. B: passage II cells were untreated (control), transfected with eNOS, or transfected with eNOS in the presence of Rp-8-BrcAMPS (100 μM; eNOS + PKAi). Cell counts were obtained daily over a 4-day period. Each point represents the mean cell count ± SE (n = 3). Data were analyzed by 1-way ANOVA followed by post hoc analysis with Tukey’s test. *P < 0.01 vs. control or eNOS + PKAi. **P < 0.001 vs. control.

Fig. 4. Effect of Nω-nitro-ω-arginine methyl ester (l-NNAME) on eNOS-transfected cells. A: dose-response relationship for l-NNAME in eNOS-transfected cells. Passage II rat aortic VSMCs were transfected with 300 MOI AdCMV eNOS. Bars represent data from day 4 following treatment. Each point represents the mean cell count ± SE (n = 3). Data were analyzed by 1-way ANOVA followed by post hoc analysis with Tukey’s test. *P < 0.05; **P < 0.001 vs. control. #P < 0.05; ##P < 0.001 vs. eNOS-transfected cells. B: effect of l-NNAME on proliferation in eNOS-transfected cells. β-Galactosidase and eNOS-transfected cells were treated with l-NNAME (10^-4 M). Bars represent cell numbers from day 4 following treatment. Each point represents the mean cell count ± SE (n = 3). Data were analyzed by 1-way ANOVA followed by post hoc analysis with Tukey’s test. *P < 0.05 vs. AdCMV/βgal. **P < 0.001 vs. control, β-galactosidase (βgal) + l-NNAME, or eNOS + l-NNAME.
nontransfected and in β-galactosidase-transfected cells (Fig. 7).

Figure 8A shows a typical Western blot for p53, and Fig. 8B summarizes the mean data for Western blot analysis from three experiments, each carried out in triplicate. Western blot analysis showed that p53 levels were significantly increased in eNOS-transfected cells compared with levels in nontransfected cells (Fig. 8, A and B). Whereas levels of p53 in eNOS-transfected cells tended to be higher than those in β-galactosidase-transfected cells and eNOS-transfected cells treated with Rp-8-BrcAMPS, these differences were not statistically significant (Fig. 8B).

**Determination of apoptosis in eNOS-transfected cells.**

The trypan blue exclusion test on nontransfected cells (control) and eNOS-transfected cells showed that cell viability in both control and eNOS-transfected cells was >95%. There was no significant difference in cell viability between the two groups (data not shown). Additionally, the occurrence of apoptosis in eNOS-transfected cells was determined by using flow cytometry. Results of the flow cytometry experiments showed no increase in hypodiploidy in transfected cells compared with control cells (data not shown).

Figure 9A shows a representative Western blot for Bax, and Fig. 9B summarizes the mean data from Western blot analysis from three experiments, each carried out in triplicate. Levels of Bax in eNOS-transfected and nontransfected cells at 30% confluency were significantly lower than levels in nontransfected cells at 95% confluency, which are in a stationary phase
EFFECT OF eNOS GENE ON SMOOTH MUSCLE PROLIFERATION

A

Bax (21 kDa)

Lane
AdCMV eNOS

1 2 3

Control(95%)
Control(30%)
AdCMV eNOS

% of control

Treatment

Fig. 9. Analysis of Bax expression in eNOS-transfected cells. Blot in A is representative of a typical Western blot analysis for Bax. Lane 1, nontransfected passage II cells at 95% confluency; lane 2, nontransfected cells at 30% confluency; and lane 3, cells transfected with 300 MOI AdCMVeNOS. eNOS-transfected passage II cells were harvested 4 days after transfection. Typical confluency of eNOS-transfected cells by day 4 is 50%. B: autoradiographs for Western blot analyses were quantified by scanning densitometry. Bars represent summary of data from 3 experiments, each carried out in triplicate. Results are means ± SE expressed as a percentage of control. Data were analyzed by 1-way ANOVA followed by post hoc analysis with Tukey’s test. *P < 0.001 vs. control (95%).

(Fig. 9B). Nontransfected cells at 30% confluence showed levels of Bax similar to levels in eNOS-transfected cells. eNOS-transfected cells are typically inhibited at the 50% confluency level. Thus Bax levels in the eNOS-transfected cells (at 50% confluence) were equivalent to levels in nontransfected cells that were in log-phase growth (30% confluence). These results indicate that there was no significant increase in apoptosis in eNOS-transfected cells compared with levels in nontransfected control cells in log-phase growth.

DISCUSSION

Results of the present study show that transfection of rat aortic VSMCs with 300 MOI AdCMVeNOS significantly inhibited smooth muscle cell proliferation. This effect was sustained through the course of the experiment without affecting cell viability. At the 450 MOI dose of AdCMVeNOS, cell numbers decreased below control level by day 4. These results suggest that doses of AdCMVeNOS higher than 300 MOI could be cytotoxic, and, therefore, the 300 MOI dose was used in all subsequent experiments. The extent of eNOS transfection was determined by immunocytochemical studies, and the presence of dark brown staining confirmed the presence of the eNOS gene. Transfection efficiency for this adenoviral vector was between 80 and 90%, and the presence of eNOS was detected in cells as early as 1 day after transfection. Western blot analysis and immunocytochemistry demonstrated the presence of eNOS protein, and eNOS protein and staining were not detected in nontransfected cells or in β-galactosidase transfected cells. To ensure that inhibition of proliferation occurred as a result of NO production in eNOS-transfected cells, we investigated the effect of the NOS inhibitor L-NAME. Inhibition of proliferation in eNOS-transfected cells was reduced by L-NAME, suggesting that eNOS overexpression increased the production of NO and providing support for the hypothesis that the inhibition of VSMC proliferation by eNOS gene transfer results from NO formation.

In addition to being associated with the inhibition of VSMC proliferation, NO has also been found to play a role in apoptosis. It has been reported that NO-induced upregulation of Fas was associated with increased apoptosis in rat and human VSMCs (11). However, in another study, NO was shown to inhibit Fas-induced apoptosis in human leukocytes (29). To ensure that transfer of the eNOS gene to rat aortic VSMCs was not cytotoxic, we evaluated cell viability using the trypan blue exclusion test and showed that at the dose (300 MOI) and transfection level observed, cell viability in nontransfected and eNOS-transfected cells was >95% on day 4. This finding indicated that at the concentration used in these studies, eNOS transfection was not cytotoxic to rat aortic VSMCs. In addition, Western blot analysis was performed to detect the apoptosis promoter Bax. Bax expression in eNOS-transfected cells was found to be equivalent to expression levels in proliferating, nontransfected cells (30% confluence) in log-phase growth. Bax levels in eNOS-transfected cells were lower than levels in nontransfected cells in the stationary phase (95% confluence).

In the present study, eNOS transfection resulted in growth arrest in rat aortic VSMCs. Whereas adenoviral transfer of the eNOS gene was found to have an inhibitory effect on proliferation, β-galactosidase transfection had no effect on proliferation, and cell numbers in AdCMVβgal-transfected cells were equivalent to numbers in nontransfected cells up to 4 days after gene transfer. This finding indicated that the inhibition of proliferation observed in eNOS transfected cells was not dependent on the adenoviral vector but, rather, on the introduction and biological activity of the eNOS gene itself.

A major advantage of adenoviral gene transfer is that the localization of the gene can be achieved in organs, such as the lung, without having systemic effects (2). Studies involving the transfer of genes like p21 (37) and eNOS (36) suggest that gene transfection could serve as a possible approach to the treatment of intimal hyperplasia following angioplasty. eNOS transfection has also been shown to have other potential therapeutic uses. eNOS transfection to the lung of the mouse in vivo has been shown to reduce pulmonary vascular resistance. In that study, Champion et al. (2) demonstrated that eNOS transfection was able to se-
ectively reduce pulmonary pressor responses and to reduce the development of pulmonary hypertension. In another study, the eNOS gene was transferred to the common carotid artery in rabbits (30), and in a manner similar to that observed in our study, Ooboshi et al. (30) were also able to detect eNOS gene expression in transfected arteries by using immunohistochemical techniques. In their study, in which arteries precontracted with phenylephrine were used, eNOS-transfected vessels showed a greater relaxation response to acetylcholine compared with responses in nontransfected and β-galactosidase-transfected arteries (30). This finding suggests that eNOS transfection enhanced the production of NO, which mediated the increased vasorelaxant response (30). Thus eNOS transfection and NO overproduction may be a useful technique for the study of NO-mediated vascular responses.

In addition to studies involving p21 and eNOS gene transfection, the effects of Rb gene transfer have also been investigated (3). Cell proliferation was inhibited in rat aortic VSMCs transfected with the Rb gene in vitro (3). In that same study, in vivo arterial transfection with the Rb gene at the time of balloon angioplasty was found to significantly reduce neointimal thickening in rat carotid and porcine femoral arteries (3). While gene transfer of downstream effector molecules, such as p21 and Rb, could be useful in inhibiting VSMC proliferation associated with intimal hyperplasia, it is interesting to note that NO formed by eNOS has been shown to be effective in inhibiting VSMC proliferation and also for maintaining cells in a contractile phenotype (35, 36).

In determination of the role of p21 in PDGF-stimulated guinea pig coronary arteries, p21 protein levels did not increase in response to eNOS transfection (34). However, in the present study, an increase in p21 levels in eNOS-transfected rat aortic VSMCs was observed. The differential effects of eNOS on p21 expression may be attributed to the fact that the VSMCs used in the two studies were obtained from different sources. Another possible reason for this difference could be that PDGF was used to stimulate smooth muscle growth in the study of Sharma et al. (33), whereas this cytokine was not added in our studies. In contrast to the results of Sharma et al. (33), an increase in p21 levels, but not p53 expression, has been reported in studies using human umbilical VSMCs treated with the NO donor SNAP (18). In our studies, an induction of both p53 and p21 was observed in eNOS-transfected rat aortic VSMCs. An increase in p21 levels was also reported in a study by Sato et al. (32). In that study, eNOS gene transfer to VSMCs resulted in an upregulation of p21 and p27 levels. A similar upregulation in p21 was also reported in VSMCs exposed to SNAP or after inducible nitric oxide synthase (iNOS) gene transfer (26). In a more recent study, Ishida et al. (19) demonstrated that, contrary to results of their previous report, p53 was involved in the mediation of NO-induced expression of p21 in VSMCs. In the more recent publication, Ishida et al. (19) attributed this discrepancy to the use of a different lysis buffer. The latter study was conducted with a stronger detergent, which resulted in more efficient extraction of p53 (19).

The study of Ishida et al. (19) reported that whereas p21 induction was dependent on p53 upregulation, it was not dependent on increases in cGMP concentration. The role of cGMP in NO-mediated inhibition of proliferation is uncertain. Cornwell et al. (6) proposed a cross-activation of PKA by cGMP. A study by Tanner et al. (34) indicated that the inhibitory effect of eNOS overexpression on human aortic VSMC proliferation was cGMP independent, whereas levels of cGMP were found to have increased in eNOS-transduced porcine coronary artery VSMCs (32). Kibbe et al. (25) determined that overexpression of iNOS inhibited VSMC proliferation in a cGMP-independent manner. It is possible that the involvement of cGMP may be dependent on the source of NO (NO donor as opposed to transfection with the NOS gene) or on the NOS isoform transfected into the cell. Thus, while the upstream regulators of PKA are still uncertain, our study indicates that PKA plays an important role in eNOS-induced inhibition of VSMC proliferation. In their study, Ishida et al. (19) also studied the effect of the PKA inhibitor Rp-8-Br-cAMPS in SNAP-treated cells. They reported that at a concentration of 50 μM, the PKA inhibitor did not attenuate the effect of SNAP on [3H]TdR incorporation (19). In our study, the use of Rp-8-Br-cAMPS at a concentration of 50 μM did not prevent the inhibition of proliferation in eNOS-transfected cells. However, our data show that when eNOS-transfected cells were exposed to a 100 μM concentration of Rp-8-Br-cAMPS, the cells proliferated at the same rate as nontransfected cells. However, when eNOS-transfected cells were exposed to Rp-8-Br-cGMPS, there was no effect on the inhibition of proliferation. These data suggest that PKA, but not PKG, was involved in NO-induced inhibition of proliferation. Whereas eNOS-transfected cells had increased p21 and p53 levels, eNOS-transfected cells in the presence of Rp-8-Br-cAMPS showed a decrease in levels of these proteins. These data suggest that p21 and p53 were involved in NO-induced inhibition of proliferation and that the induction of these proteins was mediated by PKA. The induction of p21 and p53 was not found to be associated with apoptosis but, rather, with the inhibitory effect of NO on VSMC proliferation.

The present results suggest that PKA may be involved in the inhibition of proliferation in eNOS-transfected VSMCs by targeting the downstream effector molecules p53 and p21. At the transfection level attained in our studies, apoptosis did not appear to be a factor in the increased expression of p53 in eNOS-transfected cells. Whether PKA directly activates p53 and p21 or acts through another pathway remains to be determined, as do the upstream effector pathways for PKA. In conclusion, our results along with those from other studies suggest that eNOS gene transfer may have great potential for use in the treatment of cardiovascular diseases.
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REFERENCES


