NOS gene transfer inhibits expression of cell cycle regulatory molecules in vascular smooth muscle cells

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The major goal of the present study was to test the hypothesis that eNOS transfer into VSM cells inhibits platelet-derived growth factor (BB homodimer) (PDGF-BB)-stimulated cell proliferation by inhibiting growth factor-induced expression of cell cycle progression mol-

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MATERIALS AND METHODS

Materials. Chemicals and materials were obtained from the following sources: TRIzol (total RNA isolation reagent), and fibronectin (human plasma) from GIBCO BRL; L-[2,3,4,5-3H]-arginine (63 Ci/mmol), [3H]thymidine (2 Ci/mmol), [3H]labeled adenoviral vectors demonstrated expression in 70–90% of cells, indicating that adenoviral vectors are highly efficient in transducing the eNOS into cultured coronary artery VSM cells. We have shown that eNOS expression in VSM cells inhibited the PDGF-BB-stimulated DNA synthesis that is associated with the inhibition of cyclin A and PCNA expression. A preliminary report of these findings has been published (31).

Enzyme assays. NOS enzyme activity was determined as described previously (18) using the spectrophotometric method of Sorensen et al. (29). NOS activity was measured at 37°C, and data was presented as the mean ± SEM for between 3 and 10 transfections per condition. NOS activity was expressed as pmol of L-arginine converted to L-citrulline/min.

Adenoviral vector (Ad5/RSVeNOS). We have used the replication-deficient recombinant adenoviral vector Ad5/RSVeNOS containing the bovine aortic eNOS (22) to transfer the eNOS into coronary artery VSM cells. Adenoviral vectors containing transgene were prepared by the University of Iowa Vector Core as described elsewhere (23, 30) and obtained from Dr. Donald Heistad, Department of Internal Medicine, University of Iowa. The DNA constructs of replication-deficient adenovirus comprise a full-length copy of the adenovirus genome in which the eNOS, lacZ, and green fluorescent protein (gfp) expression cassette is incorporated at the site of E1 region deletions. In this cassette, a Rous sarcoma virus (RSV) promoter to drive transcription of the eNOS precedes eNOS. A polyadenylation sequence of SV40 is cloned downstream of eNOS. For each vector, high titer adenoviral stocks were prepared by double cesium gradient purification, and virus titer (PFU) was determined by standard methods. Virus preparations were suspended in phosphate-buffered saline containing 3% sucrose and stored at −70°C until used.

Cell culture. Coronary artery VSM cells were cultured from guinea pigs (4–6 mo old, 600–800 g body wt) obtained from a local supplier. Hearts were removed from guinea pigs anesthetized with xylazine (1 mg/kg im) and ketamine (80 mg/kg ip) and kept in Ham's F-12 culture medium. VSM cells were cultured according to procedures established in our laboratory (2) and were used up to the 8th passage from two batches in this study. The purity of VSM cells was confirmed by immunocytochemical localization of smooth muscle-specific α-actin using monoclonal antibodies against the NH2-terminal decapetide of a smooth muscle α-actin (2).

Adenovirus-mediated eNOS transfer into coronary artery VSM cells. Cultured coronary artery VSM cells were transfected with eNOS, gfp, or lacZ as described earlier (30). Briefly, VSM cells in passages 3–8 were plated in cell culture dishes. Three to six days after plating was completed, VSM cells were transfected by incubation with Ad5/RSVeNOS [from 1 to 200 multiplicity of infection (MOI)] in DMEM supplemented with 0.1% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone (serum-free defined medium). After the appropriate incubation time (30 min–2 h), the virus-containing culture medium was removed and replaced with fresh virus-free and serum-free culture medium supplemented with 0.1% BSA for 48 h. The extent of eNOS expression was quantitated after 48 h of gene transfer by Northern blotting. Western blotting, citrulline assay, and immunocytochemical localization.

Northern blotting. Total RNA was extracted from control (gfp-transfected) and eNOS-transfected VSM cells as described previously using a commercial guanidinium isothiocyanate reagent (TRIzol, GIBCO BRL) (2, 29). RNA was quantified spectrophotometrically, and equal amounts of denatured RNA samples were separated by electrophoresis on 1.0% agarose/formaldehyde gels and transferred by capillary action to a Nitran membrane (Schleicher and Schuell).

Western blotting. VSM cells were harvested into 1× PBS (pH 7.4) containing calcium-calmodulin and 200 µM L-NNA. Western blotting detection kits were purchased from Amersham Pharmacia Biotechnology.

Cultured VSM cells were transfected with Ad5/RSVeNOS, and eNOS expression was measured by Western blot analysis. The eNOS protein was detected by incubation with a mouse anti-eNOS monoclonal antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG. Band intensity was determined using the ImageQuant software (Molecular Dynamics) and normalized to tubulin expression.

Western blot analysis. The gfp- and eNOS-transfected VSM cells were serum starved for 48 h and then stimulated with 10 ng/ml PDGF-BB for the indicated time periods. After PDGF stimulation, cells were washed with ice-cold phosphate-buffered saline and then lysed in 1 ml of lysis buffer containing 10 mM...
Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 50 mM sodium fluoride, 5 mM pyrophosphate, 0.2 mM sodium vanadate, 1 mM phenylmethyl/sulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 10% glycerol, 1% Triton X-100, and 0.5% NP-40 as described earlier (10, 11). The cell lysate was centrifuged at maximum speed for 10 min at 4°C using a microcentrifuge. The supernatant was collected, and the protein concentration was measured using Bio-Rad reagent. Equal volumes of 2× sample buffer were added to the supernatant and boiled for 5 min. Equal amounts of proteins (10–20 µg) from all samples were separated on 10% polyacrylamide gels, blotted onto polyvinylidene difluoride membranes. To detect specific protein expression, the membranes were blocked in 5% nonfat milk, incubated with antibody against NOS-3 (1:500), PDGF-β receptor (1:500), PY20 (1:3,000), cdk2 (1:1,000), cyclin A (1:200), cyclin E (1:500), p27 (1:500), p21 (1:200), or PCNA (1:200) for 1–1.5 h, and then incubated with goat anti-rabbit IgG-HRP (1:10,000) or goat anti-mouse IgG-HRP (1:500) for 1 h. Labeled peroxidase activity was detected using ECL Western blotting detection kit. For multiple blotting for different proteins on the same membrane, the membrane was stripped with the following stripping buffer (1% Triton X-100 containing 0.1% BSA). Nonspecific binding was determined in the presence of 20 ng/ml unlabeled human recombinant PDGF-BB and was subtracted to calculate specific binding. Scatchard analysis was carried out to calculate binding constants.

[3H]Thymidine incorporation. [3H]Thymidine incorporation was carried out using semiconfluent VSM cells essentially as described earlier (2, 29). Cells were cultured in 24-multiwell dishes, and 2–3 days after subculture the cells were transfected with Ad5/RSVeNOS (as described in Adenovirus-mediated eNOS transfer into coronary artery VSM cells) in serum-free medium and maintained in these serum-free conditions for 48 h in DMEM containing 0.1% BSA. Control or lacZ-transfected cells were also kept in DMEM-BSA for 48 h. Quiescent cells were stimulated with PDGF-BB for 20 h to initiate DNA synthesis. After the growth factor-containing medium was removed, cells were incubated in DMEM-BSA for 4 h with 1 µCi/ml [3H]thymidine to quantitate DNA synthesis. TCA-insoluble cellular material was solubilized in 1 N NaOH at 37°C for 60 min, and an equal volume of 1 N acetic acid was added. [3H]Thymidine incorporation was determined by liquid scintillation spectrometry.

125I-labeled PDGF binding. PDGF-β receptor number and affinity was measured using semiconfluent quiescent VSM cells by radioreceptor assay using 125I-labeled PDGF-BB essentially as described by Bowen-Pope and Ross (3). Cells were cultured in 24-multiwell dishes, and 2–3 days after subculture the cells were transfected with eNOS or control vector carrying reporter gene gfp (as described in Adenovirus-mediated eNOS transfer into coronary artery VSM cells) in serum-free medium and maintained in serum-free conditions for 48 h in DMEM containing 0.1% BSA. Serum-starved cells were washed once with ice-cold binding medium (DMEM containing 0.2% BSA) and incubated with increasing concentration of 125I-labeled PDGF-BB (0.1–6 ng/ml) in 1 ml of ice-cold binding medium. Binding assays were carried out in triplicate by incubating the tris buffer on ice with gentle shaking for 5 h. Binding assays were terminated by four washes (2 ml each well) with ice-cold binding medium and cell-associated 125I-labeled PDGF-BB extracted with 1 ml of solubilization buffer (1% Triton X-100 containing 0.1% BSA). Nonspecific binding was determined in the presence of 20 ng/ml unlabeled human recombinant PDGF-BB and was subtracted to calculate specific binding. Scatchard analysis was carried out to calculate binding constants.

Statistical analysis. Results are means ± SE. Statistical analysis was carried out using StatView software on a Macintosh computer. Student's t-test was used to evaluate differences between the treatment groups that were considered significant at P < 0.05.

RESULTS

Efficiency of adenovirus-mediated eNOS transfer into VSM cells. Incubation of cultured guinea pig coronary artery VSM cells with Ad5/RSVeNOS viral construct for 60 min at 37°C produced a dose (MOI or PFU per cell)-dependent increase in both mRNA levels (Fig. 1A) and eNOS enzyme activity (Fig. 1B). A significant increase in NOS enzyme activity was observed as low as 1 PFU/cell of virus construct, and enzyme activity increased with increasing virus concentration up to the maximum 200 PFU/cell tested in this study (Fig. 1).

![Fig. 1. Effect of increasing Ad5/RSVeNOS vector concentration on efficiency of endothelial nitric oxide synthase (eNOS) gene transfer into vascular smooth muscle (VSM) cells. Cultured coronary artery VSM cells were incubated with different concentrations of Ad5/RSVeNOS for 60 min, the medium was changed, and cells were reincubated for 48 h at 37°C. A: increase in eNOS mRNA levels in transfected cells from a representative experiment. Total RNA was extracted, and mRNA levels for eNOS was measured by Northern blotting using 32P-labeled eNOS probe as described in MATERIALS AND METHODS. Ethidium bromide-stained 18S RNA is given to compare RNA loading in different treatment groups. Similar results were obtained in two other experiments. MOI, multiplicity of infection. B: effect of virus concentration on eNOS activity as measured by citrulline assay. Results represent specific calcium-dependent NOS activity from a representative experiment. Similar concentration response was observed in one more experiment](http://ajpheart.physiology.org/DownloadedFrom)
eNOS mRNA levels remained elevated for up to 2 wk after transfection of VSM cells with eNOS viral construct (data not shown). Calcium-dependent NOS activity in VSM cells infected with 20 PFU/cell of Ad5/RSVeNOS for 30 min was significantly greater (P < 0.05) than the calcium-dependent NOS activity observed in bovine aortic endothelial cells (48 ± 8 pmol·mg⁻¹·min⁻¹ in bovine aortic endothelial cells vs. 147 ± 13 pmol·mg⁻¹·min⁻¹ in eNOS-transfected VSM cells; n = 3). We could not detect calcium-dependent NOS enzyme activity or eNOS mRNA in nontransfected or control vector (carrying lacZ or gfp) transfected cells.

NOS enzyme activity in eNOS-transfected VSM cells was completely dependent on the presence of calcium in the assay medium, and no activity was observed in the presence of EGTA (Fig. 2A). In nontransfected or lacZ-transfected coronary artery VSM cells, we could not detect measurable calcium-dependent or calcium-independent NOS activity (Fig. 2A). L-NNA, a specific inhibitor of NOS, completely inhibited NOS activity in eNOS-transfected VSM cells (data not shown) and was used as a control in all assays for eNOS activity measurements. Expression of eNOS enzyme activity increased with increasing time of incubation of VSM cells with the Ad5/RSVeNOS viral construct (100 PFU/cell) and reached near maximum in 60–120 min (Fig. 2B). A brief 10-min incubation period of VSM cells with 100 PFU/cell of eNOS viral construct resulted in expression of significant enzyme activity measured 48 h after the infection of cells with eNOS viral construct (Fig. 2B).

Immunocytochemical localization of eNOS protein in transfected cells revealed robust eNOS protein expression in 70–90% of VSM cells after a 30-min incubation period with 100 PFU/cell of Ad5/RSVeNOS viral construct (data not shown). In eNOS-transfected VSM cells, there was robust expression of eNOS protein that was completely absent in control vector carrying gfp-transfected cells (Fig. 3). These results demonstrate that the Ad5/RSVeNOS viral construct rapidly and efficiently transferred a functional eNOS into cultured coronary artery smooth muscle cells.

eNOS transfer inhibits PDGF-BB-stimulated VSM cell proliferation. PDGF released from platelet aggregation at restenotic or atherosclerotic lesions is an important chemoattractant and a proliferative agent for VSM cells (2, 29). We have shown earlier that cultured VSM cells express predominantly PDGF-β receptors (8). To test the effect of eNOS transfer on VSM cell proliferation, we measured [³H]thymidine incorporation as an index of DNA synthesis and cell proliferation. [³H]thymidine incorporation in VSM cells was measured 3 days after the infection with the viral constructs. PDGF-BB stimulation of both Ad5/RSVlacZ- and Ad5/RSVeNOS-infected cells was associated with dose-dependent increase in [³H]thymidine incorporation during the 20 h of stimulation (Fig. 4). This time point was chosen because the doubling time for guinea pig coronary artery smooth muscle cells is 16–20 h in the presence of 10% fetal bovine serum (2). eNOS transfer significantly (P < 0.5) inhibited PDGF-stimulated [³H]thymidine incorporation compared with lacZ control vector-transfected cells (Fig. 4). Removal of L-arginine 24 h before PDGF stimulation reversed eNOS transfer-mediated inhibition of thymidine incorporation (data not shown). The extent of inhibition was 40–50% at both concentrations of PDGF-BB.

Effect of eNOS transfer on PDGF-β receptor number and autophosphorylation. To understand the molecular mechanisms of NO-mediated inhibition of VSM cell proliferation, we have investigated the effects of eNOS transfer into VSM cells on PDGF receptor number and activation. We have previously shown that cultured VSM cells express predominantly PDGF-β receptors (8). Therefore, we first checked the effect of eNOS transfer on PDGF-β-receptor levels. eNOS transfer did not significantly alter PDGF-β-receptor number or affinity compared with gfp-transfected cells [maximal binding (in fmol/10⁶ cells) = 120 ± 23 gfp vs. 134 ± 15 eNOS; dissociation constant (in pM) = 77 ± 6 gfp vs. 84 ± 4 eNOS; means ± SE, n = 4]. Similarly, Western
analysis demonstrated that PDGF-β-receptor density was slightly increased in eNOS-transfected VSM cells compared with gfp-transfected cells, but the differences did not reach statistical significance (Fig. 3). The amount of PDGF-β receptor rapidly decreased on PDGF-BB stimulation (70% decrease in 2 h), implying rapid internalization and degradation of the ligand-receptor complex (Fig. 3); however, no significant differences were observed between eNOS- and gfp-transfected VSM cells in the time course of receptor internalization and degradation. Activation of PDGF-β receptors was estimated by quantifying receptor autophosphorylation after PDGF-BB stimulation (Fig. 3). PDGF-BB stimulation of VSM cells rapidly increased PDGF-β receptor tyrosine phosphorylation that reached a maximum in 5 min (first time point analyzed) and then decreased rapidly. No significant differences were observed in the receptor autophosphorylation in eNOS- and gfp-transfected VSM cells (Fig. 3). These results suggest that eNOS transfer does not alter PDGF-β-receptor activation in VSM cells and that signaling events responsible for NO-mediated inhibition of VSM cell proliferation are downstream of receptor activation.

**Fig. 3.** Effect of eNOS transfer on eNOS, platelet-derived growth factor (PDGF)-β-receptor levels and PDGF-β receptor tyrosine phosphorylation. VSM cells were transfected with 20 MOI of either eNOS- or gfp-carrying vectors. Cells were serum starved for 48–72 h and then stimulated with 10 ng/ml PDGF-BB for indicated times. Cells were then lysed and 15 µg cell lysate from each treatment group was analyzed by Western blotting (left). Antibody dilutions used were NOS-3 (1:500), PDGF-β receptor (1:500), and PY 20: phosphotyrosine (1:3,000). Densitometric analysis of density of bands for PDGF-β receptor (n = 4) and PDGF-β receptor tyrosine phosphorylation PY 20 (n = 3) are given at right, where 1 min represents 0 time unstimulated cells.

**Fig. 4.** Effect of eNOS transfer on PDGF-BB-stimulated [3H]thymidine incorporation in VSM cells. Confluent VSM cells grown in 100-mm culture dishes were transfected with 100 MOI of Ad5/RSveNOS virus or control vector Ad5/RSVlacZ for 30 min. [3H]thymidine incorporation was measured as described in MATERIALS AND METHODS. Results are means ± SE (n = 3) of [3H]thymidine incorporated (cpm per 10^6 cells). * Significant differences (P < 0.05) between lacZ- and eNOS-transfected VSM cells.
subsequent activation and inactivation of cyclin-dependent kinases (9, 12, 19). Recently, it has been shown that differentiation and proliferation of postmitotic VSM cells in response to injury in rat carotid arteries is accompanied by temporally and spatially coordinated expression of cyclins A and E, cdk2, and PCNA (35). Therefore, we used Western blot analysis to determine the effects of eNOS transfer on PDGF-BB-stimulated expression of cyclins A and E and cdk2. In the initial set of experiments, we tested the effect of PDGF-BB stimulation on the expression of these molecules at 0, 2, 4, 6, 12, 16, and 24 h after addition of 10 ng/ml PDGF to the 72-h serum-deprived cells. In this set of experiments, we observed that cyclin E and A were maximally stimulated in 4–8 h and then remained elevated above basal levels. Therefore, subsequent experiments were undertaken at shorter time intervals. Levels of cdk2 were high in quiescent VSM cells and did not increase significantly on PDGF-BB stimulation (Fig. 5). eNOS transfer did not alter cdk2 levels compared with gfp-transfected cells. Cyclin A was expressed at low levels in nonstimulated cells, and its expression increased rapidly on PDGF-BB stimulation. Increase in cyclin A expression in response to PDGF-BB was biphasic; it first peaked at 30 min and then slightly declined and was followed by a second peak starting at 6–8 h (Fig. 5). eNOS transfer significantly decreased (P < 0.05) PDGF-BB-stimulated expression of cyclin A at 30 min, 2 h, 4 h, and 8 h after stimulation. Cyclin E was also expressed in quiescent VSM cells at low levels and it rapidly increased on PDGF-BB stimulation, reaching a maximum in gfp-transfected cells at 30 min and then remained elevated above basal levels up to 8 h (Fig. 5). PDGF-BB-stimulated expression of cyclin E was markedly delayed in eNOS-transfected VSM cells compared with gfp-transfected cells (Fig. 5). However, maximum expression was comparable between both eNOS- and gfp-transfected cells at 2, 4, and 8 h after PDGF-BB stimulation.

Effect of eNOS transfer on p27, p21, and PCNA expression in VSM cells. In addition to the positive regulation of cdks by specific cyclins, cdk activities are also negatively regulated by CKI proteins (5, 12, 33).
These inhibitory proteins bind to and inactivate the cyclin-cdk complexes. p21 (also known as CIP1/WAF1/CAP20 and SD11) and p27 (also known as KIP1) are related proteins that preferentially bind to and inhibit cdk2-cyclin E, cdk2-cyclin A, and cdk4-cyclin D activities (12, 19). Overexpression of p27 (12, 19, 33) and p21 (5, 12) blocks entry of various cell types into the S phase in response to growth factors and oncogenic transformation, suggesting that CKI-dependent regulation of cdk2 plays a crucial role in cell cycle progression from G1 to S phase. Because CKIs are regulated positively and negatively at the transcription level by positive and negative stimuli of cell proliferation (12, 19), we tested whether NO may increase the expression of p27 or p21 in VSM cells to produce cell cycle arrest of VSM cells.

Initially, we observed that p27 expression was high in the G0/G1 phase cell cycle-arrested cells, and levels started to decrease 2 h after PDGF-BB stimulation. p27 expression reached steady-state levels (50% decrease) in 6–12 h and then remained low up to 24 h (data not shown). Therefore, subsequent experiments were planned at shorter time intervals to determine the real time course of decrease in p27 expression after PDGF-BB stimulation (Fig. 6). Levels of p27 were lower in eNOS-transfected cells compared with gfp-transfected cells; however, differences between the two groups were not statistically significant except at the 10-min interval after PDGF-BB stimulation (Fig. 6). In both treatment groups, p27 levels decreased by 50% 8 h after PDGF-BB stimulation. These data would suggest that altered expression of p27 in eNOS-transfected cells is not involved in NO-mediated inhibition of VSM cell proliferation.

In contrast to p27, levels of p21 are very low in quiescent VSM cells. In these experiments, 60–80 µg protein/lane were resolved to test the effect of eNOS transfer on p21 expression. p21 levels increased initially after the addition of PDGF-BB and...
G1/S phase of the cell cycle after growth factor stimulation plays an important role in PCNA expression during the phosphorylation of retinoblastoma gene product (pRB). Moreover, it has been suggested that cdk-mediated silencing during the S phase and cell proliferation (21). In addition to cyclins and cdk2, growth factor-stimulated expression of PCNA, a cofactor for DNA synthesis, plays an important role in DNA synthesis and cell proliferation (21). PCNA was expressed at low levels in quiescent VSM cells and increased gradually, reaching maximum levels at 4-8 h after PDGF-BB stimulation, and remained elevated above the levels observed in nonstimulated cells for up to 24 h (data not shown). PDGF-BB-stimulated PCNA expression was significantly inhibited (P < 0.05) in eNOS-transfected cells compared with gfp-transfected cells at 10 min, 30 min, 2 h, 4 h, and 8 h (Fig. 6). PCNA expression in eNOS-transfected VSM cells was at least 50% lower compared with that in gfp-transfected control cells. These results would suggest that decreased expression of PCNA in response to PDGF-BB stimulation in eNOS-transfected cells may, in part, explain the inhibitory effects of eNOS expression on VSM cell DNA synthesis and proliferation.

DISCUSSION

The major findings of the present study are 1) eNOS transfer into VSM cells inhibits PDGF-stimulated DNA synthesis; and 2) eNOS transfection into VSM cells inhibits PDGF-stimulated expression of cell cycle progression molecules like cyclin A and PCNA and delays the expression of cyclin E. These changes in PDGF-BB-mediated signaling events in eNOS-transfected VSM cells appear to be downstream of PDGF-β-receptor activation because neither the PDGF-β receptor number and affinity (measured by radioreceptor assay) nor the receptor density and autophosphorylation (measured by Western blotting) are altered in VSM cells expressing the eNOS compared with cells expressing the gfp reporter gene. These results demonstrate that cell cycle regulatory molecules are targets of the NO action in VSM cells and may explain, at least in part, the molecular mechanism of NO-mediated inhibition of VSM cell proliferation in response to PDGF.

Several studies have demonstrated that pharmacological intervention with NO donors and high concentrations of L-arginine inhibit neointima formation in injured arteries (14, 17). In cultured VSM cells, NO donors inhibit VSM cell proliferation (18, 28). Von der Leyen et al. (34) demonstrated that in vivo eNOS transfer into balloon-injured rat carotid arteries using Sendai virus/liposomes system can restore normal NO production and inhibit neointima formation by 70%. Similarly, we have recently demonstrated that adenovirus-mediated eNOS transfer into medial VSM cells of balloon-injured rat common carotid artery significantly inhibits intima hyperplasia up to 4 wk after injury by augmenting the recovery of injury-induced downregulation of paxillin in VSM cells (11). Results presented here confirm and extend these observations in an in vitro system using VSM cells cultured from the coronary artery. eNOS transfer into coronary artery VSM cells significantly inhibited PDGF-stimulated DNA synthesis, an indicator of VSM cell proliferation (Fig. 4).

The effects of eNOS transfer on VSM cell proliferation are due to NO-mediated inhibition of cell cycle regulatory molecules expression rather than selection of cells expressing specific differential phenotypes after infection with adenovirus containing eNOS or reporter genes for the following reasons. First, cells are infected with viral vectors for a short time, and the experiments are completed in <48-72 h after gene transfer in the absence of serum, conditions, and time not sufficient for clonal expansion of differentiated VSM cell phenotype. Second, we (30) have shown earlier that transfection of VSM cells with adenovirus vector containing lacZ reporter gene has no effect on VSM cells survival or morphology up to 3 wk after gene transfer. Third, similarly we have demonstrated that eNOS transfection-mediated inhibition of VSM cell migration and paxillin tyrosine phosphorylation in response to PDGF are reversed by L-NNa, a specific inhibitor of NOS (10). Finally, we (11) have also demonstrated that eNOS transfer at the time of vascular injury significantly (P < 0.05) inhibited neointima formation at 2 and 4 wk after vascular injury. All this information taken together would indicate that eNOS transfer-mediated inhibition of VSM cell proliferation and migration is due to NO-mediated inhibition of specific signaling events.

As discussed above, it is well documented that NO inhibits VSM cell proliferation; however, the molecular mechanisms of NO-mediated inhibition of PDGF-stimulated VSM cell proliferation are not well understood. Treatment of VSM cells with antisense oligonucleotides to protooncogenes (13) and positive cell cycle control genes (1, 20, 21) (in vitro as well as in vivo) inhibit growth factor-stimulated as well as injury-induced VSM cell proliferation and neointima formation. Similarly, gene transfer of negative cell cycle control molecules has been shown to inhibit VSM cell proliferation and neointima formation (5, 6). Moreover, many agents that inhibit mammalian cell proliferation have been shown to inhibit G1/S transition by inhibiting cyclin-dependent kinase activity (9, 12, 19, 33). A recent study has demonstrated that vascular injury leads to time-dependent expression of cyclin A, cyclin E, and PCNA, suggesting an important role for these molecules in injury-induced VSM cell proliferation (35). Thus regulation of cell cycle regulatory molecules expression by NO may explain eNOS transfer-mediated inhibition of VSM cell proliferation.

Our results show that eNOS transfection in VSM cells inhibited PDGF BB-stimulated expression of cyclin A and delayed the time course of cyclin E expression (Fig. 5); both of these cyclins are essential for the activation of cdk2 and progression of cell cycle from the
G1/S phase (9, 12, 19). Recently, Ishida et al. (16) observed that while NO donors decrease the message levels for cyclin A and cyclin E, they do not affect their protein levels in response to fetal bovine serum or fibroblast growth factor stimulation of VSM cells. They have further shown that NO donors increase p21 expression that may be responsible for the decrease in cdk2 activity. It is possible that sustained increase in the high levels of NO produced by NO donors and subsequent metabolism of NO to peroxynitrite may produce p53 induction in response to DNA damage that can lead to p21 expression as demonstrated in other cell types (15). Data presented in this paper show that p21 is expressed at very low levels in cultured VSM cells, and its expression is slightly but not significantly increased by eNOS transfer. These differences in our study and that of Ishida et al. (16) could be due to differences in the time and dose of NO exposure. Alternatively, it is possible that culture conditions and growth medium may lead to differential expression of p21. Moreover, recent efforts to demonstrate p21 protein expression in quiescent cultured VSM cells or in medial smooth muscle cells in vivo have produced negative results (32, 35). In addition, it has been shown that p27 levels are not altered during VSM cell proliferation in response to growth factors or vascular injury, suggesting that under normal circumstances p21 may not play an important role in regulating VSM cell cycle (32, 35).

In addition to p21, another cdk inhibitor, p27, has been shown to play an important role in the restriction point control of the fibroblast cell cycle (7, 9, 12, 19, 33). p27 levels were high in G0/G1-arrested VSM cells and decreased on PDGF-BB stimulation with a 50% decrease observed after 8 h of treatment that persisted up to 24 h. These findings are consistent with those reported in nontransformed fibroblasts where growth arrest has been shown to upregulate p27 expression, whereas growth stimulation decreased p27 expression (7). However, we did not find a significant change in p27 levels in eNOS-transfected cells, except at early time points, compared with gfp vector-transfected control cells. These results suggest that although p27 may play a role in regulating VSM cell cycle progression (since its levels decrease as cells progress in the G0/G1 phase of the cell cycle on PDGF-BB stimulation), regulation of p27 is not a target of NO-mediated inhibition of cell cycle engine.

Data presented in this paper also show that PDGF-BB-stimulated expression of PCNA was decreased by 50% or more in eNOS-transfected VSM cells compared with gfp vector-transfected control cells (Fig. 6). Because PCNA is a cofactor for DNA polymerase-δ, the eNOS transfer-mediated decrease in PCNA may inhibit DNA replication in the S phase of the cell cycle and thus inhibit or prolong the cell cycle (12). This decrease in PCNA expression in eNOS-transfected VSM cells could be, in part, responsible for the cytostatic effects of eNOS expression in VSM cells.

In summary, we have shown that functional eNOS transfer into VSM cells did not alter PDGF-β-receptor protein levels or receptor autophosphorylation. eNOS transfer significantly decreased cyclin A and PCNA expression and markedly delayed cyclin E expression in response to PDGF-BB stimulation. These data suggest that signaling events affected by NO are downstream of PDGF receptor activation and that NO-mediated delay in the expression of cyclin E along with the inhibition of cyclins A and PCNA expression may play an important role in the inhibition of PDGF-BB-stimulated cell cycle progression.

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