Published in "Journal of Cardiovascular Pharmacology 52(4): 369-374, 2008"
which should be cited to refer to this work.

Endothelial Nitric Oxide Synthase Gene Transfer Inhibits Human Smooth Muscle Cell Migration via Inhibition of Rho A

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Abstract: Smooth muscle cell (SMC) migration contributes to vascular remodeling. Nitric oxide (NO) produced via endothelial NO synthase (eNOS) inhibits SMC migration. This study analyzes signal transduction mechanisms of SMC migration targeted by NO. SMCs were cultured from human saphenous veins, and cell migration was studied using Boyden chambers. PDGF-BB (0.1 to 10 ng/ml) stimulated SMC migration in a concentration-dependent manner, which was inhibited by adenoviral-mediated overexpression of eNOS and by the NO donor diethylentriamine NONOate (DETANO, 10^{-5} to 10^{-3} mol/L). NO release was enhanced in eNOS-transduced SMCs, and L-NAME blunted the effect of eNOS overexpression on migration. PDGF-BB (10 ng/ml) activated Rho A, which was inhibited by the overexpression of eNOS by DETANO and by 8 bromo-cGMP. The inhibitory effect of DETANO on Rho A activity was prevented by the cGMP-dependant kinase inhibitor. Furthermore, inhibition of Rho A by C3 exoenzyme and inhibition of ROCK by Y-27632 diminished cell migration stimulated by PDGF-BB. Finally, in the cells overexpressing constitutively active ROCK mutant (CAT), DETANO failed to prevent PDGF-BB–induced SMC migration. In conclusion, NO inhibits human SMC migration via blockade of the Rho A pathway.

Key Words: gene transfer, nitric oxide, eNOS, smooth muscle cell migration

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This study was supported by the Swiss National Foundation (4037-055166/1 and 32-67202.01 to TFL and 31-63811.00 to ZY), the Postgraduate Course of the Medical Faculty of the University of Zürich, the ADUMED Foundation (TFL), Max Cloetta Foundation and Hartmann-Müller Stiftung (Zürich) (ZY).

The authors state that they have no financial interest in the products mentioned within this article.

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INTRODUCTION

Migration of smooth muscle cells (SMCs) is an essential event in vascular remodeling and thus in the development of vascular diseases such as atherosclerosis, restenosis, and bypass vein graft disease.[1–3] Many growth factors such as PDGF, bFGF, and thrombin are potent mediators of cell migration.[4–6] Although an interaction of SMCs with extracellular matrix has been shown to play an important role in SMC migration,[7] intracellular signal transduction pathways activated by migratory stimuli are mostly undefined.

Stimulation of SMCs with growth factors or chemoattractants is associated with activation of an array of signal transduction pathways including p44/p42^{mapk}, p70^{S6K}, and the small GTP binding protein Rho GTPases activated either through tyrosine kinase receptors or G-protein coupled receptors.[8–10] These molecules transmit extracellular growth signals into the cell nucleus, thereby activating transcription factors and regulating downstream gene expression and cellular responses. The Rho GTPases form a novel subgroup of the Ras superfamily of 20- to 30-kDa GTP-binding proteins. Rho GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state and are involved in transcriptional regulation and cell proliferation/migration control. Rho A is one member of a distinct group of Rho-like proteins.[10]

NO produced from L-arginine via endothelial nitric oxide synthase (eNOS) is a potent vasodilator, platelet inhibitor, and reduces SMC proliferation and migration.[11,12] NO can affect proteins involved in cell cycle regulation and in turn induce cell cycle arrest.[13–15] However, it is still unclear through which signal transduction pathways NO regulates SMC migration. Therefore, the aim of this study focused on the intracellular signal transduction pathways of cell migration targeted by NO in human SMCs, with particular emphasis on Rho A pathway.

METHODS

Materials

All materials for cell culture were purchased from GibcoBRL (Basel, Switzerland). *Clostridium Botulinum* C3 exoenzyme,[16] DietylentriamineNONOate (DETANO),[17] and KT5823[18] were from Calbiochem (Luzern, Switzerland).

Rabbit polyclonal antibodies against phospho-p44/p42^{mapk} and phospho-p70^{S6K} were from New England BioLabs (Allschwil, Switzerland); Rho A (SC-119) was purchased from Santa Cruz Biotechnology Inc. (Basel, Switzerland). Mouse monoclonal antibody against human eNOS (N30020) was from Transduction Laboratories (Basel, Switzerland). The ROCK inhibitor Y-27632[16] was kindly provided by Welfide Corporation, Osaka, Japan.

Recombinant Adenovirus

An adenoviral vector for expression of human placental alkaline phosphatase (AdhpAP) and a control virus without transgene (AdΔE1) were derived from Ad5 sub360 and prepared as described.[19] A similar virus for expression of eNOS (adeNOS) was provided by Dr. Stephan Janssens, Leuven, Belgium. Expression of adeNOS was driven by a CMV promoter, while the AdΔE1 had no insert (for details see reference 15). Adenoviral vectors for expression of constitutively active ROCK mutant (CAT) and LacZ were generated using the CMV promoter in the pEF-BOS-myc-RB/PH (TT) vector as described elsewhere.[20] The titer of purified viruses was determined by plaque assay on 293 cells.

Culture of Human SMCs

SMCs were cultured from saphenous veins (SVs) obtained from patients undergoing coronary bypass surgery using an explant technique.[21] The patients gave informed consent in accordance with the Declaration of Helsinki. The cells were cultured in DMEM containing 20% FCS supplemented with 20 mmol/l L-glutamine and 10 mmol/l HEPES buffer solution, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (95% air/5% CO_2) at 37°C. Culture medium was replaced every 3 days. Cells were passaged by cell dissociation solution. Experiments were performed on passage 4–7. SMCs were characterized by their typical morphological pattern and by indirect immunofluorescence staining by using specific mouse monoclonal antibodies against human SMC α-actin.[21]

Gene Transfer

SMCs were incubated with a viral titer of 1000 pfu/cell for 1 hour in DMEM containing 2% FCS at 37°C in a humidified atmosphere and then kept in DMEM with 10% FCS for 24 hours. The transfer efficiency was analyzed using AdhpAP and determined 24 hours after infection by fixing the cells in 1.25% glutaraldehyde (Sigma, Buchs, Switzerland) and staining with NBT/BCIP (GIBCO) as described.[19] The eNOS expression was analyzed by Western blot. The cells for further experiments were then kept in serum-free medium for 24 to 48 hours. SMCs infected with AdΔE1 or LacZ and uninfected cells served as negative controls for all experiments.

Migration Assay

Vascular smooth muscle cells were grown to confluence in DMEM medium supplemented with 10% fetal bovine serum. Cells were detached from the plate by trypsinization, and migration efficiency was tested using a 48-well Boyden chamber (Neuroprobe Inc., Cabin John, MD) in the presence of 10 ng/ml PDGF as a chemotactic agent. A total of 25,000 cells per group were used for migration experiments. For adenoviral transduction, cells were seeded at 40% confluence and left in the incubator for 24 hours. Cells were transfected as described and treated with or without the eNOS inhibitor L-NAME, the Rho A inhibitor exoenzyme-C3 or the ROCK inhibitor Y-27632 at indicated concentrations for 30 minutes. Cells were allowed to migrate for 5 hours at 37°C/5%CO_2. Nonmigrated cells were removed from the upper side with a cell scraper, and migrated cells from the bottom side were fixed in ice-cold methanol, stained with Diffquick solution I and II (Medion Diagnostics, switzerland), and counted on 4 different fields as described.[22]

Activation of p44/42^{mapk} and p70^{s6k}

Quiescent human saphenous vein SMC rested in serum-free medium for 48 hours were stimulated with PDGF-BB (10 ng/ml). After stimulation, the cells were washed with ice-cold PBS and harvested with cold extraction buffer (120 mmol/l sodium chloride, 50 mmol/l Tris, 20 mmol/l sodium fluoride, 1 mmol/l benzamidine, 1 mmol/l DTT, 1 mmol/l EDTA, 6 mmol/l EGTA, 15 mmol/l sodium pyrophosphate, 0.8 μg/ml leupeptin, 30 mmol/l p-nitrophenylphosphate, 0.1 mmol/l PMSF, and 1% Nonidet P40). Twenty micrograms of cell extracts were boiled at 95°C for 10 minutes in Laemmli SDS-PAGE sample buffer (50 mmol/l Tris-Cl, 100 mmol/l DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and subjected to 10% SDS-PAGE gels for electrophoresis. Proteins were then transferred onto Immobilon-P filter papers (Millipore AG) with a semidry transfer unit. Equal loading was controlled by staining with Ponceau S. Membranes were then blocked by 5% skim milk in PBS-Tween buffer (0.1% Tween 20) for 1 hour and incubated with the primary antibodies (the anti-phospho-p44/42^{mapk} antibody, 1:1000; anti-phospho-p70^{S6K} antibody, 1:1000). The immunoreactive bands were detected by use of an enhanced chemiluminescence (ECL) system (Amersham).

Rho A Membrane Translocation

Confluent and quiescent SMCs were stimulated with PDGF-BB (10 ng/ml). The cells were then washed twice with cold PBS and then harvested in PBS buffer containing 2 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, and 1 μmol/l leupeptin. The cells were then disrupted by brief sonication on ice. The samples were then centrifuged at 500 × g for 10 minutes at 4ºC to remove the nucleus. The membrane and cytosolic portions were then separated by centrifugation at 100,000 × g for 1 hour at 4ºC (Airfuge; Beckman Instruments, Inc., Nyon, Switzerland). The cell membranes were washed once with the above-mentioned buffer and then resuspended in buffer containing 100 mmol/l Tris-HCl, 300 mmol/l NaCl, 1% Triton X-100, and 0.1% SDS containing 2 mmol/l EDTA, 2 mmol/l PMSF, and 1 μmol/l leupeptin. Equal amounts of protein (10 μg) were loaded into 12% SDS-PAGE gels and applied to electrophoresis. Western blot was then performed with the antibody against Rho A (1:1000).

Rho A Activity

Rho A activity was determined by pull-down assay. The cell lysates were incubated with agarose-conjugated Rhotekin Rho Binding Domain (Upstate Biotechnology, Lake Placid, NY)

for 45 minutes. The agarose beads were collected and run in 12% SDS-PAGE gels. Western blot was performed using the antibody against Rho A.

Expression of eNOS

eNOS expression was analyzed by Western blot. The SMCs were kept in DMEM containing 10% FCS for 24 hours after transduction. Cells were then harvested with cold extraction buffer, and 20 μg of protein was loaded on to 8% SDS-PAGE as described above. The eNOS antibody (1:600) was used as the primary antibody.

Nitric Oxide Release

SMCs were grown in DMEM medium supplemented with 10% fetal bovine serum. A total of 70,000 cells were seeded in a 12-well plate at 40% confluence and incubated for 24 hours before transfection. After transfection, 10% DMEM was replaced, and cells were incubated for additional 24 hours. Cells were prepared according to the manufacturer's instructions in the presence of DETC (Noxygen, Germany) and FeSO4. Nitric oxide levels were measured with e-scan (BRUKER). Data were calculated and represented in nanomoles/minute/number of cells.

Statistical Analyses

Data are presented as mean ± SEM. Rho A translocation was expressed as percent increase above control. In all experiments, n equals the number of patients from which cells were obtained; in the migration assay, every experiment was done in triplicates. Statistical analyses were performed with unpaired *t* test between 2 groups and analysis of variance among more than 3 groups. $P < 0.05$ was considered significant.

RESULTS

Overexpression of eNOS Inhibits SMC Migration

In cultured SMCs, PDGF-BB (0.1, 1, and 10 ng/ml) stimulated cell migration in a concentration-dependent manner (8 ± 2, 28 ± 3, and 76 ± 4 cells/field of vision compared to control 6 ± 2; $P < 0.05$ for 1 and 10 ng/ml). Cell migration stimulated by PDGF (10 ng/ml) was concentration dependently inhibited by the NO donor, DETANO (Figure 1A). Overexpression of eNOS in SMCs was achieved with an adenoviral vector. A viral titer of 1000 pfu/cell was used and resulted in 99% transfected SMCs (data not shown). Expression of eNOS in SMCs was further evidenced by Western blot (Figure 1B insert). eNOS overexpression significantly reduced PDGF-induced cell migration (Figure 1B). Although the control adenovirus (AdΔE1) tended to have some inhibitory effects, this did not reach statistical significance. Exposure of SMCs to AdΔE1 or AdeNOS did not exert any cytotoxic effects as determined by LDH release (n = 3; P = not significant; data not shown). Adenoviral overexpression of eNOS enhanced NO release from SMCs (n = 4; $P < 0.05$; data not shown). The inhibitory effect of adeNOS on SMC migration was blunted by L-NAME (Figure 1C).

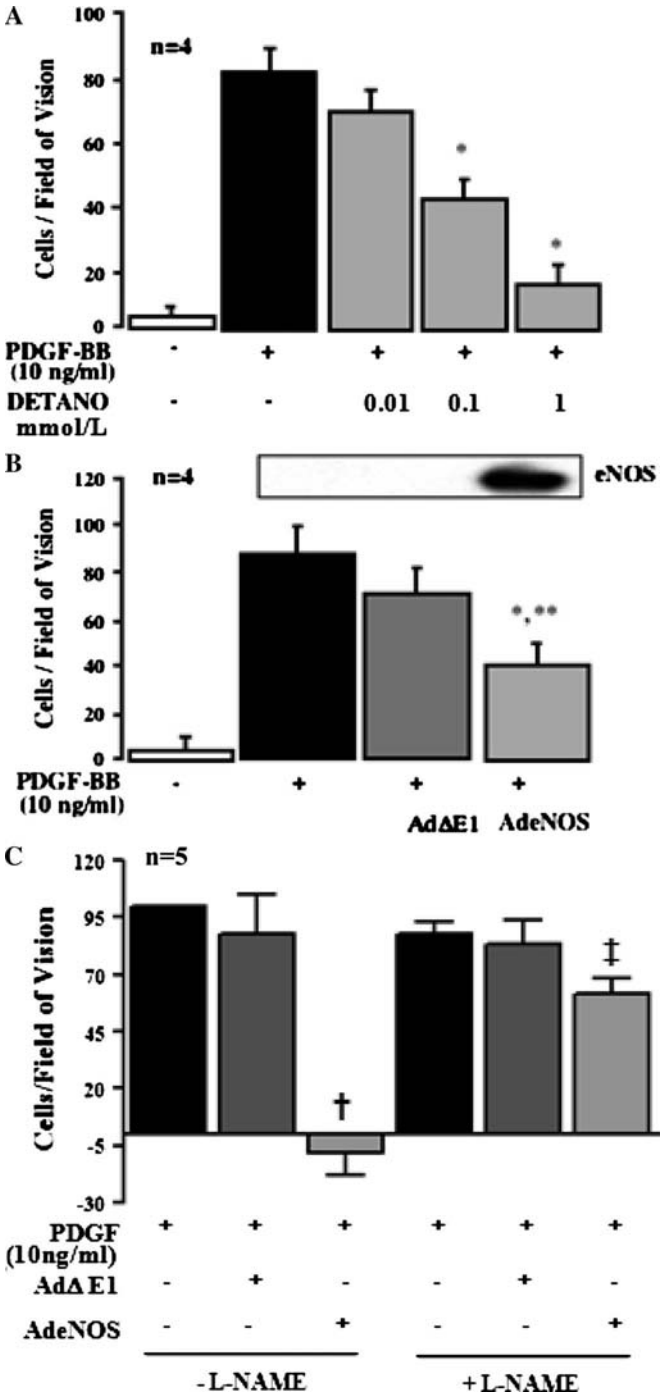

FIGURE 1. NO inhibits human SMC migration. (A) Treatment of the cells with the NO donor DETANO (10^{-5} to 10^{-3} mol/l) inhibited the migratory activity of SMCs in response to PDGF-BB (10 ng/ml, 4 hours) in a concentration-dependent manner. (B) Migration to PDGF-BB (10 ng/ml) was also inhibited by adenoviral eNOS (AdeNOS) transfer. eNOS gene expression in human SMCs was evidenced by Western blot (insert, n = 4). (C) L-NAME (3×10^{-4} mol/L) blunted the effect of AdeNOS on SMC migration. *$P < 0.05$ versus PDGF alone. **$P < 0.05$ versus empty vector. †$P < 0.001$ versus PDGF alone. ‡$P < 0.001$ versus PDGF without L-NAME.

NO Inhibits SMC Migration Via Inhibition of Rho A

The cell migration stimulated by PDGF-BB was associated with activation of various signal transduction pathways such as Rho A, p44/42^{mapk}, and p70^{s6k}. PDGF-BB (10 ng/ml) time-dependently stimulated Rho A membrane accumulation, which is the crucial step for activation of the GTPase (Figure 2A). Activation of Rho A reached the maximum at 30 to 60 minutes (Figure 2A). We could not detect significant changes of Rho A in cytosolic fraction, possibly due to a large amount of Rho A in cytoplasm. The Rho A membrane accumulation stimulated by PDGF-BB (10 ng/ml, 30 minutes) was prevented by DETANO (10^{-4} mol/l) (Figure 2B) and by eNOS overexpression (Figure 2C), although the vector alone also had some nonsignificant effect. Rho A activity was also evaluated with pull-down assay. Similar to the membrane translocation assay, PDGF-BB (10 ng/ml) also increased Rho A activity in SMCs, with the maximal effect at 30 minutes after the stimulation (Figure 3A). Total Rho A amount was not affected by PDGF until 60 minutes. The increase in Rho A activity stimulated by

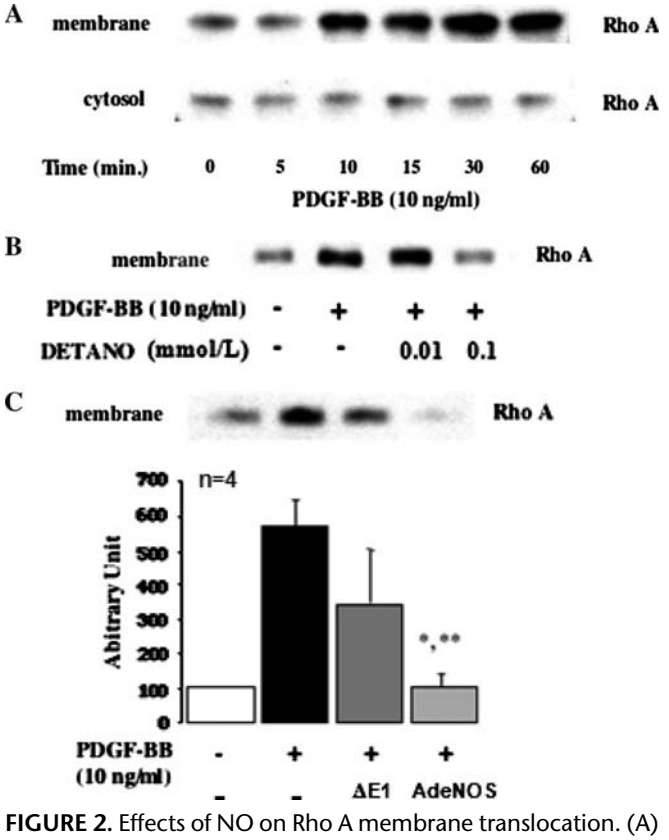

FIGURE 2. Effects of NO on Rho A membrane translocation. (A) Membrane translocation of Rho A was stimulated by PDGF-BB (10 ng/ml) in a time-dependent manner, which reached the maximum at 30 minutes. (B and C) Rho A translocation in response to PDGF-BB (10 ng/ml, 30 minutes) was reduced by the NO donor DETANO (10^{-4} mol/l) or by adenoviral eNOS gene transfer. *$P < 0.05$ versus PDGF alone. **$P < 0.05$ versus empty vector.

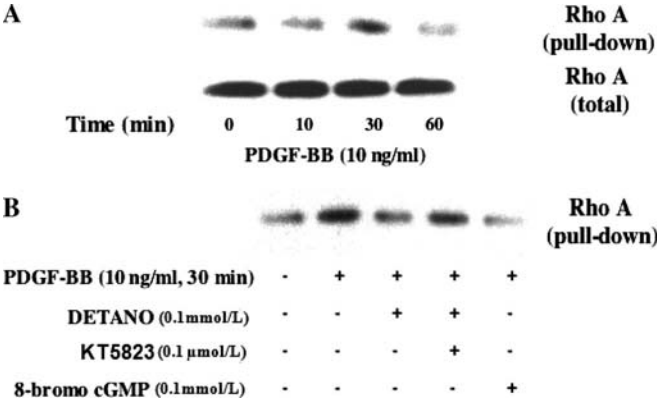

FIGURE 3. Effects of NO on Rho A activity. (A) Activity of Rho A determined by pull-down assay was stimulated by PDGF-BB (10 ng/ml) in a time-dependent manner, which reached the maximum at 30 minutes. (B) Rho A activity in response to PDGF-BB (10 ng/ml, 30 minutes) was reduced by the NO donor DETANO (10^{-4} mol/l) and 8-bromo cGMP (10^{-4} mol/l). cGMP-dependent kinase inhibitor KT5823 blocked the inhibitory effect of DETANO on Rho A activity. Similar results were obtained in 3 independent experiments.

PDGF-BB (10 ng/ml, 30 minutes) was prevented by DETANO (10^{-4} mol/l) and 8-bromo cGMP (10^{-4} mol/l) (Figure 3B). The inhibition of cGMP-dependent kinase by KT5823 (10^{-7} mol/l) blocked the suppressive effect of DETANO on Rho A activity (Figure 3B).

In addition to Rho A, PDGF-BB also activated p44/42^{mapk} and p70^{s6k} in cultured SMCs. PDGF-BB (10 ng/ml, 10 minutes) phosphorylated p44/42^{mapk} and p70^{s6k} as demonstrated by Western blots using specific antibodies against phospho-p44/42^{mapk} and phospho-p70^{s6k}. The NO donor DETANO (10^{-4} mol/l) alone stimulated, rather than inhibited, p44/42^{mapk}, but it did not influence the activation of p44/42^{mapk} or p70^{s6k} in response to PDGF-BB (10 ng/ml, data not shown).

Inhibition of Rho A and ROCK Inhibits SMC Migration

The migratory activity of human SMCs stimulated by PDGF-BB (10 ng/ml) was reduced by the Rho A inhibitor 50 μg/ml C3 exoenzyme (Figure 4A) as well as by the downstream kinase ROCK inhibitor Y-27632 (Figure 4B).

Overexpression of Constitutively Active ROCK Mutant Blocks the Effect of NO

To get direct evidence that the inhibitory effect of NO on SMC migration is mediated by inhibition of Rho/ROCK pathway, we investigated the effect of overexpression of constitutively active ROCK mutant (CAT). DETANO (10^{-4} mol/l) again prevented PDGF-BB–induced SMC migration in the cells infected with control adenoviruses for expression of LacZ. On the other hand, DETANO failed to prevent PDGF-BB-induced SMC migration in the cells overexpressing constitutively active ROCK mutant (CAT) (Figure 5).

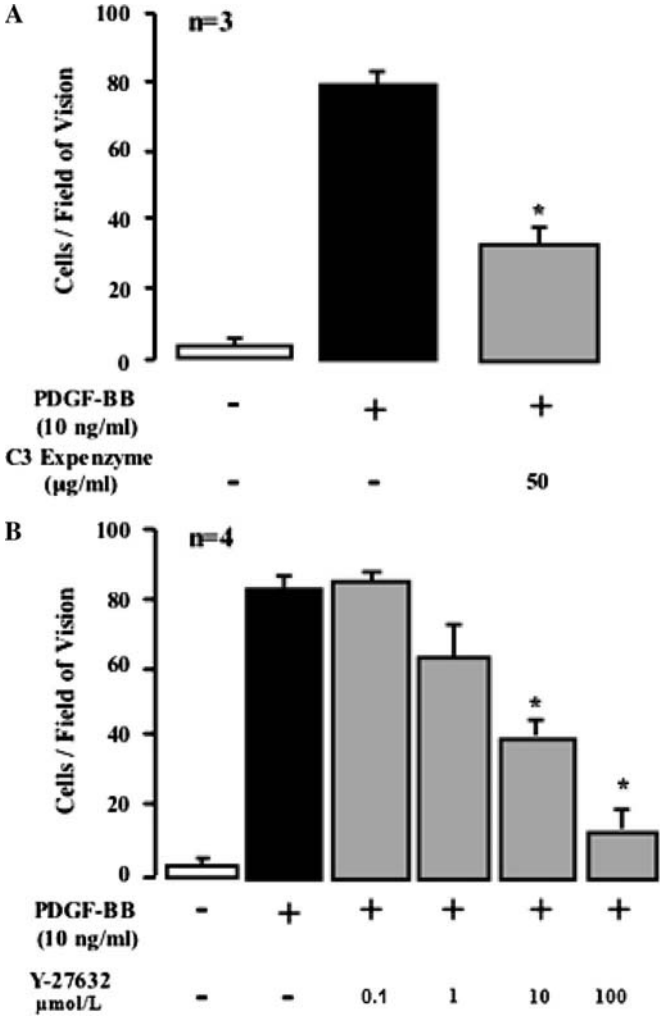

FIGURE 4. Inhibition of Rho A and ROCK suppresses SMC migration. PDGF-BB (10 ng/ml) increased the number of migrated cells within 4 hours, which was inhibited (A) by a specific Rho A inhibitor C3 exoenzyme (50 µg/ml) or (B) by a downstream kinase ROCK inhibitor Y-27632 in a concentration-dependent manner. *$P < 0.05$ versus PDGF alone.

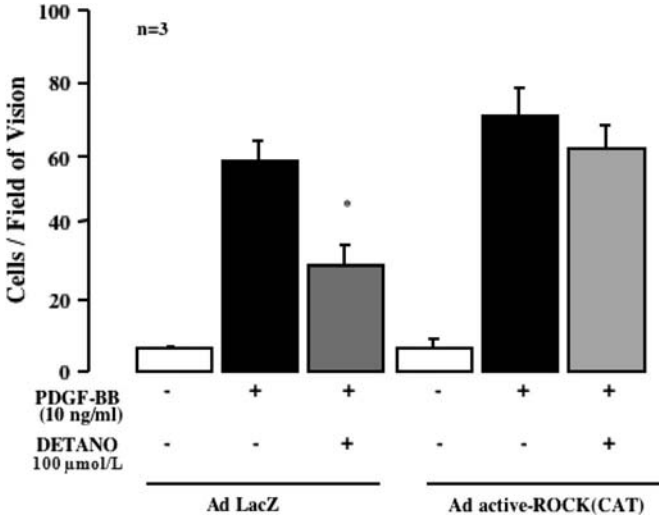

FIGURE 5. Overexpression of constitutively active ROCK mutant blocks the inhibitory effect of NO. In the cells overexpressing active ROCK mutant (CAT), the inhibitory effect of DETANO (10^{-4} mol/l) on SMC migration induced by PDGF BB (10 ng/ml, 4 hours) was not observed. *$P < 0.05$ versus PDGF alone.

DISCUSSION

In human SMCs, NO provided by the overexpression of eNOS gene with a recombinant adenovirus vector or added directly through an NO donor inhibits the cell migration in response to PDGF via inhibition of the Rho A pathway.

SMC migration essentially contributes to intimal thickening and vascular remodeling in atherosclerosis, restenosis, and venous bypass graft disease.[1–3] Almost all peptide growth factors, such as PDGF, are also important activators of SMC migration.[1–3] In this study, we selected PDGF as a migratory signal and obtained concentration-dependent stimulation of human SMC migration. We and the other group reported that NO inhibits migration of rat and human arterial SMCs.[11,12] We used DETANO as a pharmacological source of NO because this molecule exhibits very slow and prolonged release kinetics of the free radical, which is most suitable for cell culture experiments.[21] Overexpression of eNOS provides a novel concept for the inhibition of SMC proliferation and migration in vascular diseases such as restenosis and venous bypass graft disease.[23–25] Using an adenoviral vector, we successfully transferred eNOS into human vascular SMCs as demonstrated by Western blot. Previous studies demonstrated a very high transfer rate (99%) under these experimental conditions,[19] which was also achieved in the current experiments. As in previous studies, the control virus did exert some nonspecific effects; however, the pronounced effects of the eNOS adenovirus can be blocked by L-NAME,[15] demonstrating that NO indeed is involved.

Expression of eNOS in saphenous vein SMCs was associated with important biological effects. Indeed, eNOS gene transfer markedly reduced PDGF-induced migration of SMCs. Although the empty virus tended to have some effects as well, this did not reach statistical significance. PDGF-induced migration of SMCs was associated with activation of a series of intracellular signal transduction pathways such as $p44/42^{mapk}$, $p70^{s6k}$, and Rho A. In particular, PDGF increased the activity of Rho A as well as its membrane accumulation. High local levels of NO provided either pharmacologically with DETANO or via adenovirus-mediated eNOS gene transfer reduced the activity and the translocation of Rho A. In addition to Rho A, PDGF-BB also activated $p44/42^{mapk}$ or $p70^{s6k}$ in cultured SMCs. However, NO did not inhibit the activation of either $p44/42^{mapk}$ or $p70^{s6k}$ in response to PDGF-BB, suggesting that the effects of NO on the RhoA pathway are very specific. Evidence for the involvement of the Rho A pathway as a mediator of the antimigratory effects of NO was further strengthened by experiments with the specific inhibitors and the constitutively active ROCK mutant. Indeed, consistent with the report by the other group,[26] the migratory activity of PDGF in SMCs was reduced in the presence of the

Rho A inhibitor C3 exozyme[27] as well as the ROCK inhitor Y-27632[28]; conversely, the effects of NO were prevented by the overexpression of constitutively active ROCK mutant (CAT).

In addition, we investigated the mechanistic insights into how NO blocks Rho A activity. In general, the effects of NO on cellular functions are mediated by cGMP-dependent and independent mechanisms. We hypothesized that cGMP pathway might mediate the inhibitory action of NO on Rho A activity. Indeed, the cGMP analogue, 8-bromo cGMP prevented the increase in Rho A activity stimulated by PDGF and the cGMP-kinase inhibitor, KT5823 blocked the suppressive effect of NO on Rho A activity, suggesting that cGMP/cGMP-dependent kinase pathway plays an important role in this action. It was recently reported that cGMP-dependent kinase directly phosphorylates and inactivates Rho A.[29] The same mechanism may be involved in the inhibition of Rho A activity by NO in human SMCs.

This study demonstrated that the Rho A pathway is a target of NO to exert its antimigratory effects in human vascular SMCs. This therefore provides a new therapeutic approach for the treatment of human vascular disease associated with increased migration of SMCs, such as restenosis and venous bypass graft disease.

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