Nitric Oxide and C-Type Atrial Natriuretic Peptide Stimulate Primary Aortic Smooth Muscle Cell Migration via a cGMP-Dependent Mechanism

Relationship to Microfilament Dissociation and Altered Cell Morphology

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Abstract—Migration of aortic smooth muscle cells is thought to be of essential importance in vascular restenosis, remodeling, and angiogenesis. Recent studies have shown that NO donors inhibit the migration of subcultured aortic smooth muscle cells. However, there is evidence that NO elicits opposite effects on cell proliferation in primary versus subcultured cells, indicating fundamental differences among different models of aortic smooth muscle cell cultures. The purpose of the current study was to investigate the effect of NO donors on migration of primary cultures of rat aortic smooth muscle cells and to compare and contrast their response with those in subcultured cells. A second purpose was to investigate some of the underlying mechanisms associated with NO-induced effects on cell migration. We report that 2 NO donors, S-nitroso-N-acetylpenicillamine (SNAP) and 2,2-(hydroxynitrosohydrazino)bis-ethanamine, stimulated the migration of primary cells in a wounded-culture model as well as in a transwell migration model. The effect of NO donors was mimicked by 2 cGMP analogues and C-type natriuretic peptide and blocked by a specific inhibitor of guanylyl cyclase, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one, indicating the involvement of cGMP as second messenger. Moreover, neither NO donors nor cGMP analogues altered migration of primary cultures stimulated by either FBS or angiotensin II. In contrast to its effect in primary cultures, SNAP did not alter basal or stimulated migration of subcultured cells, except at a relatively high concentration of 1 mmol/L, at which migration was inhibited. The migration-stimulatory effect of NO donors and cGMP was associated with altered cell morphology and dissociation of actin filaments, consistent with recent studies indicating that cell morphology and cytoskeletal organization influence cell migration. The results suggest the possible involvement of NO-induced cell migration in vascular injury or remodeling, representing conditions in which vascular NO levels would be expected to be elevated. (Circ Res. 1999;84:655-667.)

Key Words: nitric oxide □ migration □ vascular smooth muscle □ cGMP

Migration and proliferation of vascular smooth muscle cells are considered to be essential events in the development of atherosclerosis and vascular stenosis.1,2 The primary event in the development of vascular pathology is thought to involve injury to the endothelium, leading to a response that may be similar to wound healing and requiring proliferation and migration of vascular smooth muscle cells.3-5 There is also evidence indicating a requirement for NO in flow-induced vascular remodeling involving medial hyperplasia.6

NO is an autacoid produced by endothelial cells, vascular smooth muscle cells, and inflammatory cells. Endothelial cells express a constitutively active isozyme of nitric oxide synthase (NOS; endothelial NOS), whereas endothelial cells as well as smooth muscle cells, macrophages, and lymphocytes have the capacity to express an isozyme of NOS that can be upregulated under the influence of various cytokines (inducible NOS; iNOS).7 Moreover, injury has been reported to elicit upregulation of iNOS in vascular smooth muscle cells.5-10

The effects of NO on cultured vascular smooth muscle cell proliferation are complex. Early studies from several laboratories, including our own, reported that relatively high concentrations (submillimolar to millimolar) of NO donors inhibited cell proliferation of subcultured vascular smooth muscle cells from rat or rabbit.11-13 More recent studies have revealed additional complexity as exemplified by a report from our laboratory indicating that the effect of NO on cell proliferation depends on the type of cell used for experiments. Specifically, in primary aortic smooth muscle cells isolated from adult rats, NO donors and atrial natriuretic peptides amplify fibroblast growth factor- or epidermal growth fac-
tor–elicited cell proliferation.\textsuperscript{14,15} In contrast, NO donors inhibit cell proliferation in subcultured cells from adult rats or in primary cells from newborn rats.\textsuperscript{16} That the phenotype of vascular smooth muscle cells is altered on repetitive subculture is well established.\textsuperscript{17,18} Thus, the type of proliferative response of vascular smooth muscle cells to NO correlates with specific cell phenotypes.

At least 2 laboratories have reported that NO decreases the migration of vascular smooth muscle cells. Accordingly, Dubey et al\textsuperscript{19} reported that explanted subcultured aortic cells from rats expressed lower levels of migration in a Boyden chamber assay when treated with nanomolar levels of NO donors or cGMP. On the other hand, Sarkar et al\textsuperscript{20} reported that submillimolar concentrations of NO donors inhibited cell migration of subcultured aortic cells in a cell culture–wounding assay. Both aforementioned studies were performed using subcultured and presumably relatively dedifferentiated vascular smooth muscle cells.

Because of the aforementioned culture-dependent differential effects of NO on cell proliferation, we felt that it would be useful to compare and contrast the effects of NO on cell migration in primary versus subcultured aortic smooth muscle cells. We report that in contrast to results obtained in subcultured cells, NO donors and natriuretic peptides stimulate migration in primary aortic smooth muscle cell cultures, as demonstrated via 2 independent assays. Moreover, we show that this effect is attributable to increased cGMP levels elicited by NO or atrial natriuretic peptide and is associated with microfilament dissociation and altered cell morphology.

## Materials and Methods

### Materials

DMEM/F-12 and FBS were from Gibco-BRL, GMP-8-(4-chlorophenylthio)triethylammonium salt (8-pCPT-cGMP) and 2,2-dichlorophenylthio)triethylammonium salt (8-pCPT-cGMP) were purchased from Alexis Corp. 8-Bromo (Br)–cGMP, 8-Br-cAMP, hydroxyurea, penicillin, streptomycin, 3-nitro-L-arginine methyl ester (NAM), protein kinase A inhibitor, and antibody against smooth muscle–type myosin heavy chain were purchased from Sigma. cGMP kinase heptapeptide inhibitor, and antibody against smooth muscle–type myosin heavy chain were purchased from Alexis Corp. Aortic smooth muscle cells were isolated as previously described.\textsuperscript{14} Isolated cells were seeded in DMEM/F-12 supplemented with penicillin (50 U/mL), streptomycin (50 \mu g/mL), insulin (5 \mu g/mL), transferrin (5 \mu g/mL), and selenous acid (5 \mu g/mL), at a density of \(2 \times 10^4\) cells/cm\(^2\), and were grown to confluence in the presence of 10% FBS in a humidified atmosphere of 5% CO\(_2\)/95% air. Cells were identified as smooth muscle by the presence of \(\alpha\)-smooth muscle actin (not shown) and smooth muscle–type myosin heavy chain (see Figure 9). For studies using subcultured cells, the cells were rinsed once with balanced salt solution, detached with trypsin, and subcultured until obtaining the required passage (passages 12 through 15).

### Vascular Smooth Muscle Cell Isolation and Culture via Enzymatic Dissociation

Male Sprague-Dawley rats (weighing 100 to 150 g) were obtained from Charles River Laboratories. Aortic smooth muscle cells were isolated as previously described.\textsuperscript{14} Isolated cells were seeded in DMEM/F-12 supplemented with penicillin (50 U/mL), streptomycin (50 \mu g/mL), insulin (5 \mu g/mL), transferrin (5 \mu g/mL), and selenous acid (5 \mu g/mL), at a density of \(2 \times 10^4\) cells/cm\(^2\). The cells were then grown to confluence in the presence of 10% FBS in a humidified atmosphere of 5% CO\(_2\)/95% air. Cells were identified as smooth muscle by the presence of \(\alpha\)-smooth muscle actin (not shown) and smooth muscle–type myosin heavy chain (see Figure 9).

For studies using subcultured cells, the cells were rinsed once with balanced salt solution, detached with trypsin, and subcultured until obtaining the required passage (passages 12 through 15).

### Vascular Smooth Muscle Cell Isolation and Culture via the Explant Method

To obtain cells by the explant method, rat thoracic aortas were excised, and the adventitias were removed by preliminary enzymatic dissociation and mechanical denudation as described previously.\textsuperscript{14} After rinsing, aortas were chopped into small pieces \(\approx 3\) mm in size, placed in a small volume of DMEM/F-12 (containing 10% serum) and allowed to attach to the culture surface. Smooth muscle cells grew out from the arterial tissues and after several weeks formed a confluent layer. Primary cultures were subcultured via the use of trypsin-EDTA.

### Migration Assays

Two types of migration assays were used. In the first, migration was measured using a monolayer-wounding protocol in which cells migrated from a confluent area into an area that was mechanically denuded of cells.\textsuperscript{21} In the second type of assay, collagen-coated transwell tissue culture inserts composed of a polycarbonate membrane containing 8-\mu m pores were used. This assay method determines cell chemotaxis.\textsuperscript{22}

For monolayer-wounding migration assays, confluent cells were treated with serum-free medium containing hydroxyurea (5 \mu mol/L) for 24 hours before the start of the experiments. Hydroxyurea was used to prevent proliferation of cells via a measure also used by Sarkar et al.\textsuperscript{20} The inclusion of hydroxyurea was considered to be necessary to eliminate potential effects of NO and cGMP on cell proliferation, as both NO and cGMP are able to alter smooth muscle proliferation.\textsuperscript{11–14} The capacity of hydroxyurea to prevent proliferation was verified by showing that it inhibited thymidine incorporation by >99% in these cells. A few experiments performed in the absence of hydroxyurea gave results qualitatively similar to those performed in the presence of hydroxyurea, suggesting that this reagent did not have a significant effect on cell migration. After incubation with hydroxyurea, cells were rinsed with DMEM/F-12, and a scratch of \(\approx 20\) mm thickness was made with a sterile single-edged razor blade. The cells were then incubated with the experimental medium (also containing hydroxyurea) for 24 hours. After this time period, the plates were removed, rinsed twice with PBS, fixed with formaldehyde (3.7%):Triton X-100 (2% solution), stained with crystal violet, and analyzed by image analysis (via NIH Image software). The number of cells migrating was then calculated as cell number per millimeter scratch.

For transwell cell migration assays, cells were subcultured once, before seeding into the apical (upper) chamber of the transwells (\(\approx 60,000\) cells per well). In most experiments the lower chamber contained the experimental reagents, although similar results were obtained in a few experiments in which reagents were added to both chambers. Cells were allowed to migrate for 24 hours, after which the inserts were removed. The nonmigrating cells in the upper layer were then removed with a cotton swab. To stain the cells embedded in the bottom membrane, the inserts were submerged in 3.7% formaldehyde solution for 10 minutes followed by 0.4% hematoxylin for 5 minutes. The number of migrated cells was measured by counting the number of stained nuclei per high-power field in a microscope (\(\times 200\)). Each sample was counted randomly in 3 separate locations in the center of the membrane and the smooth muscle cell migration activity reported as number of cells migrated per field of view.

### Measurement of cGMP-Dependent Protein Kinase Protein Levels

cGMP-dependent protein kinase levels in primary, passaged, or explanted cultures were measured by Western blotting. Dissociated cells were used at passage 0 (ie, primary cultures), 3 (for comparison with explanted cells), or 20. Explanted cells were used at passage 7. Although cGMP-dependent protein kinase protein levels were not measured for every cell isolation, routine measurement of cGMP-dependent protein kinase levels was performed at every few isolations to ensure that each cell isolation continued to express cGMP-dependent protein kinase.
For comparison of cGMP-dependent protein kinase levels in explanted versus dissociated cells, cells were plated in 60-mm culture dishes and grown to confluence. Cells were lysed using 300 μL of lysis buffer containing (in mmol/L) Tris-HCl 250, EDTA 2, sodium orthovanadate 2, pyrophosphate 10, and 4-(2-aminophenyl)-benzenesulfonyl fluoride 1 and 4% SDS and 10% glycerol, followed by agitation on a plate shaker for 10 minutes, after which the cells were scraped off and transferred to microcentrifuge tubes. The lysates were then boiled and centrifuged at 4°C to separate the supernatant and pellet. Samples (normalized for protein) from supernatants were prepared for SDS-PAGE by adding Laemmli buffer (4×). After separation by SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene fluoride membrane (Immobilon-P), and the membrane was incubated at room temperature for 1 hour in blocking buffer (PBS containing 0.1% Tween-20 and 3% BSA) to block nonspecific binding. For detection of cGMP-dependent protein kinase, membranes were incubated with rabbit anticiGMP-dependent protein kinase antibody (prepared by this laboratory and used at a dilution of 1:3000 in blocking buffer) and 3% BSA) to block nonspecific binding. For detection of cGMP-dependent protein kinase, membranes were incubated with rabbit anti–cGMP-dependent protein kinase antibody (prepared by this laboratory and used at a dilution of 1:5000) and then washed and incubated with peroxidase-linked goat anti-rabbit IgG (1:5000). The membranes were then washed 4 times with PBS and 3% BSA to block nonspecific binding. For detection of cGMP-dependent protein kinase activity, the membranes were then washed and incubated with peroxidase-linked goat anti-rabbit IgG (1:5000) and 1 hour at room temperature. After washing 4 times with PBS containing 0.1% Tween-20, proteins were visualized using an enhanced chemiluminescence system (NEN Life Sciences).

Measurement of cGMP-Dependent Protein Kinase Activity

Cell lysates were obtained as described above. cGMP-dependent protein kinase activity was measured by a modification of the method described by Colbran et al.23 Briefly, cell lysates were incubated with G kinase heptapeptide substrate (0.15 mg/mL) in the presence of 4 μCi [γ-32P]ATP and protein kinase A inhibitor (1.2 mg/mL). The lysates were incubated without or with 8-Br-cGMP (10 μmol/L) at 30°C to activate cGMP-dependent protein kinase. EDTA (6 mmol/L) was added to terminate the reaction after 10 minutes of incubation. Results were expressed as cpm 32P per micromgram protein.

Staining of F-Actin With Rhodamine Phalloidin

Cells were grown in Nunc chamber slides in the presence of DMEM/F-12 until ~60% confluent. After serum deprivation for 48 hours, the cells were treated for 1 hour with the experimental medium containing or lacking SNAP. Cells were then fixed and permeabilized with 3.7% formaldehyde:2% Triton X-100. After washing with PBS (containing 1% BSA), the cells were incubated with rhodamine phallolidin (Molecular Probes). After removal of rhodamine phallolidin, slides were inverted onto a microscope slide, a protective cover shield (Vectra) was added, and the cells were examined by fluorescence microscopy.

Statistical Analysis

Migration was expressed as the number of cells migrated per mm scratch (wound) or per high-power field (transwell) or as percentage of control migration. Differences between groups were analyzed by paired Student t test or 1-way ANOVA for data expressed as number of cells migrated, followed by Dunnett test for individual comparisons between groups, or 2-way ANOVA for data expressed as percentage of control migration, followed by Dunnett test for individual comparisons. Differences were considered significant at P<0.05. All experiments were repeated at least 3 times.

Results

NO Donors Stimulate Cell Migration in Primary Aortic Smooth Muscle Cells

We first examined the effect of NO donors and DETA-NO on migration, using the cell monolayer–wounding assay. Both SNAP and DETA-NO serve as donors of NO,25,26 and the relatively long half-life of each (SNAP, ~5 hours; DETA-NO, ~56 hours) is such that these NO donors can be used to investigate the effect of NO over long incubation periods.

In primary cultures (passage 0), 100 μmol/L SNAP increased cell migration in a wounded-culture model, as shown in Figure 1A. Figure 1B provides the results of a quantitative assessment indicating that 2 NO donors, SNAP (30 μmol/L) and DETA-NO (30 μmol/L), both increased basal cell migration in a wounded-culture model by 3- to 4-fold. In contrast, N-acetylpenicillamine (NAP), a substance that is structurally identical to SNAP except for the NO moiety, failed to have a significant effect (Figure 1B). This supports the view that the migration-stimulatory effect of SNAP was related to its capacity to provide NO. The increase in migration induced by SNAP (1 to 300 μmol/L) was concentration dependent, as shown in Figure 1C. Moreover, the maximal migration-stimulatory effect of SNAP was similar in magnitude to that elicited by 1% FBS or 100 nmol/L angiotensin II (compare Figures 1C and 2A).

NO Donors Fail to Influence Angiotensin- or Serum-Stimulated Migration in Primary Cultures

As expected, 1% FBS and 100 nmol/L angiotensin II each enhanced basal migration by 4- to 5-fold (Figure 2A). However, SNAP had no further statistically significant effect on migration in cells stimulated with either FBS (Figure 2B) or angiotensin II (Figure 2C), even at a relatively high concentration of 1 mmol/L.

cGMP Analogues Stimulate Migration in Primary Cultures

The next experiments were done to test the hypothesis that the migration-stimulatory effect of NO is mediated, at least in part, via cGMP. The capacity of NO to increase cGMP in vascular smooth muscle cells is well established, and this aspect was specifically verified in our cells (results not shown). As shown in Figure 3A, 2 cGMP analogues, 8-Br-cGMP (30 μmol/L) and 8-pCPT-cGMP (30 μmol/L), both increased migration in a wounded-culture model of primary cells. Moreover, the increase in migration elicited by 8-pCPT-cGMP (0.01 μmol/L to 300 μmol/L) was concentration dependent (Figure 3B). These results are consistent with the notion that the effect of NO on migration is at least in part mediated by cGMP as the second messenger.

cAMP Analogue 8-Br-cAMP Fails to Stimulate Migration in Primary Cultures

The next experiments were done to test whether the motogenic effect of cGMP in primary cells could be due to cross-activation of protein kinase A. We therefore determined the effect of the cAMP analogue 8-Br-cAMP (10 μmol/L to 1 mmol/L) and observed that this agent failed to stimulate migration in primary cultures (results not shown). Thus, it is unlikely that cross-activation of cGMP with protein kinase A could be responsible for the motogenic effect of cGMP.

Guanyl Cyclase Inhibitor ODQ Blocks the Migration-Stimulatory Effect of SNAP

To further test the involvement of the cGMP pathway in the migration-stimulatory effect of NO, we treated primary cul-
tures with or without SNAP and in the presence or absence of ODQ, a selective inhibitor of guanyl cyclase. ODQ alone elicited a slight increase in migration that was not statistically significant (Figure 4). However, ODQ completely blocked the migration-stimulatory effect of SNAP. Separately, we verified that ODQ completely blocked the increase of cGMP elicited by SNAP, as measured by immunoassay (results not shown). These results further support the notion that cGMP is an essential mediator of NO-elicited cell migration.

Atrial Natriuretic Peptide CNP Enhances Basal but Not Serum-Stimulated Cell Migration in Primary Cultures

It has been reported that cultured endothelial cells contain and release C-type natriuretic peptide (CNP), indicating the potential presence in vivo of natriuretic peptide in close proximity to smooth muscle cells. This natriuretic peptide is also thought to signal via the cGMP system and would therefore be expected to mimic the migration-stimulatory effect of NO. Indeed, as shown in Figure 5, CNP increased cell migration in a wounded-culture model of primary cells in a concentration-dependent fashion. On the other hand, CNP was ineffective in cells stimulated with 1% FBS, similar to the lack of effect of NO in the presence of serum (not shown). These results provide the third type of experimental evidence indicating that cGMP is likely to be the second messenger in NO-elicited cell migration.

**Figure 1.** Cell migration stimulated by NO donors in a wounded-culture model of primary aortic smooth muscle cells. A, Phase contrast view showing enhanced cell migration induced by SNAP. Results are representative of at least 5 independent experiments. B, Stimulation of cell migration induced by NO donors. Results are mean±SE from 5 independent experiments. *P<0.05 as compared with results in serum-free medium via ANOVA followed by Dunnett test. C, Concentration dependence of SNAP-induced cell migration in this model. Results are expressed as percentage of migration in the absence of SNAP and are mean±SE from 5 independent experiments. The migration index in the absence of SNAP was 8.93±0.74 cells/mm. *P<0.05 relative to results in the absence of SNAP, as determined by 2-way ANOVA, followed by Dunnett test.

**NO Donor and cGMP Analogue Enhance Cell Migration in a Chemotaxis Chamber**

The experiments described above were performed using a wounded-culture assay that represents cell migration in the absence of a chemotactic gradient. To determine whether these results were specific for migration in assays lacking a chemotactic gradient or whether they could also be demonstrated in a chemotactic chamber assay, we measured the effects of SNAP using a transwell migration assay. Accordingly, we found that both SNAP and 8-pCPT-cGMP increased transwell basal migration by ~2-fold (Figure 6A). These results indicate that the migration-stimulatory effect of cGMP agonists was not dependent on the specific type of cell migration assay used, and they also provide further support to the notion that the migratory effect of NO in this model system was mediated by cGMP as the second messenger. Furthermore, the SNAP analogue lacking NO, NAP, again failed to stimulate migration, which supports the view that the
The effect of SNAP was related to its capacity to supply NO (Figure 6A). In separate experiments, we measured the effect of SNAP in FBS-stimulated cell migration in a transwell migration assay and, as with the wounded-culture model, we again found no significant effect of SNAP in the presence of FBS (Figure 6B).

cGMP Agonists Fail to Stimulate Migration in Subcultured Cells

In contrast to their capacity to stimulate migration in primary cultures, neither SNAP nor DETA-NO significantly increased migration in a wounded-culture model of subcultured cells originally obtained by enzymatic dissociation of rat aorta (passages 12 through 15) (Figure 7A). Similarly, 8-Br-cGMP (30 μmol/L to 1 mmol/L) had no significant effect on migration in this model (Figure 7B). To determine whether the lack of effect of cGMP agonists was related to the specific migration assay used, we also tested the effect of these agonists in a chemotaxis chamber. As shown in Figure 7C,
neither SNAP nor 8-Br-cGMP nor the inactive analogue NAP had a significant effect on cell migration in a transwell chamber assay.

Two previous studies have reported that cGMP agonists decrease serum or peptide hormone–stimulated cell migration. Because NO did not have a significant effect on FBS- or angiotensin II–stimulated cell migration of primary cultures, we considered it important to compare our results with those obtained from models used in earlier studies. Previous studies had used 2 different subcultured cell models for these experiments. Sarkar et al20 used subcultured cells that had been originally isolated via enzymatic dissociation of rat aortas. Using this model, we investigated whether NO donors had an effect on migration stimulated by 1% FBS or angiotensin II (100 nmol/L). As shown in Figure 7D, in a wounded-culture model, 1% FBS and 100 nmol/L angiotensin II both enhanced migration by ≈2-fold. However, SNAP had no significant effect on FBS-stimulated migration, except for a tendency toward inhibition at >100 μmol/L (Figure 7F).

Dubey et al19 used a model of explanted subcultured aortic smooth muscle cells in which they observed that NO donors at nanomolar levels inhibited angiotensin II–induced cell migration in a transwell system. Results given in Figure 8 indicate that in a similar experimental model and over the concentration range used by Dubey et al,19 SNAP had no

**Figure 4.** Blockade of NO-induced stimulation of cell migration by guanyl cyclase inhibitor ODQ in a wounded-culture model of primary aortic smooth muscle cells. Results are expressed as percentage of migration in serum-free medium and are mean±SE of 7 independent experiments. The migration index in serum-free medium was 11.53±1.25 cells/mm.

**Figure 5.** Concentration-dependent increase of cell migration induced by CNP in a wounded-culture model of primary aortic smooth muscle cells. Results are expressed as percentage of control of migration in serum-free medium and are mean±SE from 5 independent experiments. The migration index in serum-free medium was 10.7±1.74 cells/mm. *P<0.05 relative to results in the absence of CNP, as determined by 2-way ANOVA, followed by Dunnett test.

**Figure 6.** A, Stimulation of primary aortic smooth muscle cell migration induced by NO donor, cGMP analogue, or NO donor analogue in a transwell migration chamber. Results are expressed as percentage of control of migration in serum-free medium and are mean±SE from 4 independent experiments. The migration index in serum-free medium was 42±12.2 cells per high-power field. *P<0.05 as compared with results in serum-free medium via 2-way ANOVA followed by Dunnett test. B, Lack of effect of SNAP on transwell migration of primary aortic smooth muscle cells stimulated with 1% FBS. Results are mean±SE from 4 independent experiments. The difference between migration in the presence of serum versus serum plus SNAP was not statistically significant.
Figure 7. Cell migration in a wounded-culture model of subcultured aortic smooth muscle cells. A, Lack of effect of NO donors on cell migration. Cells were originally isolated via enzymatic dissociation of aortic tissue and were used in passages 12 through 15. Results are mean±SE from 6 independent experiments. None of the differences was statistically significant. B, Lack of effect of cGMP analogue on cell migration. Cells were originally isolated via enzymatic dissociation of aortic tissue and were used in passages 12 through 15. Results are mean±SE from 6 independent experiments. None of the differences was statistically significant. C, Lack of effect of SNAP, SNAP analogue, or cGMP analogue on transwell migration of subcultured aortic smooth muscle cells. Cells were originally isolated via enzymatic dissociation of aortic tissue and were used in passages 12 through 15. Results are mean±SE from 4 independent experiments. None of the differences was statistically significant. D, Stimulation of cell migration by FBS and angiotensin II. Cells were originally isolated via enzymatic dissociation of aortic tissue and were used in passages 12 through 15. Results are mean±SE from 6 independent experiments. E, Inhibition of cell migration by high concentration of SNAP in cells stimulated by FBS. Cells were originally isolated by enzymatic dissociation of aortic tissue and were used in passages 12 through 15. Results are mean±SE from 4 independent experiments. *P<0.05 as compared with migration in the absence of SNAP, via ANOVA followed by Dunnett test. F, Lack of effect of SNAP in cells stimulated by angiotensin II. Cells were used in passages 12 through 15. Results are mean±SE from 4 independent experiments. None of the differences was statistically significant as determined by ANOVA.
statistically significant effect, although there was a tendency toward decreased migration at the lowest concentrations of SNAP.

**Migrating Cells Express Smooth Muscle–Type Myosin Heavy Chain**

Seidel et al\(^{31,32}\) have recently reported that only cells expressing a nonmuscle myosin heavy chain phenotype undergo migration and proliferation in a model of cultured cells from canine carotid arteries. The specific marker used by Seidel et al\(^{31,32}\) for identification of vascular smooth muscle cells was the smooth muscle myosin heavy chain isoform. We were therefore interested in identifying the myosin heavy chain phenotype of migratory cells in our experimental model. As shown in Figure 9, virtually all cells migrating in response to SNAP (100 mmol/L) in a wounded-culture model expressed smooth muscle–type myosin heavy chain, as determined by specific immunocytochemical staining, thus identifying these cells as vascular smooth muscle in origin.

**Comparison of cGMP-Dependent Protein Kinase Levels in Primary Versus Subcultured Cells**

The levels of cGMP-dependent protein kinase have been reported to be downregulated in subcultured vascular smooth muscle cells.\(^{33,34}\) We therefore wondered whether the migratory differences observed between primary cultures and various models of subcultured cells were related to down-regulation of cGMP-dependent protein kinase. Thus, we measured cGMP-dependent protein kinase levels via Western blotting and cGMP-dependent protein activity via phosphorylation of a specific peptide substrate.

As shown in Figure 10A, we found that primary and early-passage cultures had the highest levels of cGMP-dependent protein kinase as measured by Western blotting. Cells at passage 3 originally derived by enzymatic dissociation of aortic medial tissue had slightly reduced levels of cGMP-dependent protein kinase, although the levels continued to be remarkably high even up to passage 20 in cells originally derived via enzymatic dissociation.

![Figure 8](image-url)  
**Figure 8.** Lack of effect of SNAP on transwell migration of subcultured aortic smooth muscle cells stimulated by angiotensin II. Cells were originally isolated via explant culture from aortic tissue and were used in passages 3 to 6. Results are expressed as percentage of migration (mean ± SE, n = 6) in the absence of SNAP. The migration index in the absence of SNAP was 7.55 ± 3.22 cells per high-power field. None of the differences was statistically significant as determined by 2-way ANOVA.

![Figure 9](image-url)  
**Figure 9.** Immunochemical staining of migrating cells stimulated with SNAP (100 μmol/L) for smooth muscle–type myosin heavy chain. A, Staining with nonspecific antibody. B, Staining with antibody specific for smooth muscle myosin heavy chain. The second antibody used was conjugated to horseradish peroxidase. Color development was done via the use of 3,3’-diaminobenzidine/hydrogen peroxide treatment. Magnification ×62.5. Similar results were obtained in 2 additional experiments.

![Figure 10](image-url)  
**Figure 10.** Levels of cGMP-dependent protein kinase in primary and subcultured cells as measured by Western blot. Densitometric analysis of bands (via NIH Image software) was as follows (in arbitrary units): primary cells derived from enzymatic dissociation (p0/dissoc.), 174.5; passage 3 cells derived from enzymatic dissociation (p3/dissoc.), 145.4; passage 20 cells derived from enzymatic dissociation (p20/dissoc.), 101.3; and passage 7 cells derived from explant culture (p7/expl.), 18.9. Similar results were obtained in 2 additional experiments.
contrast, explanted cells at passage 7 had kinase levels only \( \approx 10\% \) of those in primary cultures.

Similarly, measurement of cGMP-dependent protein kinase activity indicated that both primary and subcultured cells (passages 12 through 14) expressed significant amounts of activity as shown by increased phosphorylation of a specific substrate in the presence of \( 10 \mu\text{mol/L} \) 8-Br-cGMP. Thus, in primary cells, protein kinase activity was increased from \( 499 \pm 103 \text{ cpm/\mu g protein} \) to \( 1201 \pm 217 \text{ cpm/\mu g protein} \) by 8-Br-cGMP \((n=5, P<0.05 \text{ via paired } t\text{ test})\), whereas enzyme activity in dissociated subcultured cells (passages 12 through 14) was increased from \( 391 \pm 93 \text{ cpm/\mu g protein} \) to \( 999 \pm 230 \text{ cpm/\mu g protein} \) \((n=5, P<0.05 \text{ via paired } t\text{ test})\). In contrast, in explanted cells tested at passages 4 through 7, 8-Br-cGMP (10 \( \mu\text{mol/L} \)) failed to stimulate protein kinase activity as indicated by basal activity of \( 424 \pm 26 \text{ cpm/\mu g protein} \) and cGMP-stimulated activity of \( 471 \pm 77 \text{ cpm/\mu g protein} \) \((n=5, P>0.05 \text{ by paired } t\text{ test})\). Thus, the lack of a stimulatory effect of cGMP agonists on cell migration in subcultured cells derived from enzymatically dissociated primary cultures cannot be attributed to the lack of cGMP-dependent protein kinase. However, our observation that explants contain insignificant levels of cGMP-dependent protein kinase is consistent with the possibility that the lack of response of cells from explant cultures to NO may be related to the relatively low levels of cGMP-dependent protein kinase in this model.

**SNAP Induces Alteration of Cell Morphology and Cytoskeletal Reorganization**

Cell shape and cytoskeletal organization play important roles in mediating cell migration. Reduced cell spreading and actin filament dissociation is associated with increased cell migration in both vascular smooth muscle cells and fibroblasts.35–37 We therefore determined the effect of SNAP on cell morphology and actin filament organization. As shown in Figure 11, SNAP elicited cell rounding, an effect that was antagonized by the guanyl cyclase inhibitor ODQ, consistent with a cGMP-mediated mechanism associated with cell migration.
To investigate possible cross-activation of protein kinase A by cGMP, we also examined the effect of 8-Br-cAMP on cell morphology. We found that 8-Br-cAMP (100 μmol/L) did not elicit cell rounding of primary smooth muscle cells (results not shown). Cross-activation of PKA by cGMP is therefore unlikely to account for the effect of cGMP agonists on morphology. Because increased migration is also associated with actin filament disassembly, we also determined the levels of actin filament organization. As depicted in Figure 12, SNAP caused significant actin filament disassembly, an effect that was also blocked by ODQ and mimicked by 8-pCPT-cGMP.

Discussion

Smooth muscle cell migration is thought to play an important role in the response to vascular injury. Moreover, there is evidence implicating NO in the control of vascular morphology and remodeling. Although 2 studies, performed in subcultured cells, have reported that NO decreases basal or stimulated vascular smooth muscle cell migration, there have been no studies targeting the response of primary cultures. We felt that the distinction between primary and subcultured cells could be of importance, by analogy to the finding that the mitogenic response of cultured vascular smooth muscle cells to NO is variable. Accordingly, our laboratory has reported that cGMP agonists, including NO, enhance the mitogenic effect of fibroblast growth factor or epidermal growth factor in primary cultures of aortic smooth muscle cells from adult rat, in contrast to the inhibitory effect of NO in subcultured cells or in primary cultures from newborn rat, as also demonstrated by our laboratory and others. Thus, the principal objective of this work was to determine the effect of NO on migration of primary cultured vascular smooth muscle cells isolated from adult rat aortas. Because cGMP is an important second messenger for transduction of many of the effects induced by NO, we also wanted to evaluate the contribution of cGMP in NO-induced responses.

The principal new finding communicated in this report is that 2 chemically dissimilar NO donors, SNAP and DETA-NO, increase basal migration but have no effect on stimulated migration in primary cultures of aortic smooth muscle cells isolated from adult rats. In contrast, neither NO donor has a significant effect on cell migration of subcultured cells, except at supraphysiological concentrations at which they inhibit migration. Moreover, we demonstrate that the effect of SNAP in primary cultures is specifically related to its capacity to function as NO donor because the related analogue NAP lacking NO is completely inactive. Furthermore, the motogenic effect of NO is independent of the type of cell migration assay used. That is, NO stimulates cell migration in...
a wounded-culture model representing chemokinetic cell movement as well as in a transwell filter model representing chemotaxis. It should be noted that the migration-stimulatory effect of NO is entirely unrelated to cell proliferation, as the experiments were for the most part done in cells treated with hydroxyurea to prevent DNA synthesis and hence cell proliferation.

The migration-stimulatory effect of NO is not unique to vascular smooth muscle cells, because a similar effect of NO on macrophages, endothelial cells, and epithelial cells has been reported. Moreover, the effect of NO on endothelial migration is thought to be an important contributor to angiogenesis, and our results support the possibility that the capacity of NO to stimulate vascular smooth muscle cell migration may play a role in angiogenesis, independent of its role on endothelial cells.

That NO-induced cell migration was mediated by cGMP as the second messenger was shown by 3 independent criteria. First, 2 different cGMP analogues, 8-Br-cGMP and 8-pCPT-cGMP, mimicked the migration-stimulatory effect of NO. Second, the natriuretic peptide CNP, which is well established to increase cGMP in vascular smooth muscle cells, stimulated cell migration. Third, a selective inhibitor of soluble guanylyl cyclase, ODQ, blocked the migration-stimulatory effect of NO. Although the degree of selectivity of ODQ may be questioned, the use of 3 independent approaches to evaluate the role of cGMP provides strong support for this cyclic nucleotide as the second messenger mediating the stimulatory effect of NO. That an analogue of cAMP, 8-Br-cAMP, failed to stimulate migration also reduces the likelihood that the effect of cGMP can be attributed to cross-activation of protein kinase A.

Although NO increased basal migration, it failed to increase serum or angiotensin II–elicited migration. A possible explanation for the lack of effect of NO in the presence of other motogenic factors may be the convergence of mechanisms related to cell migration elicited by NO versus other motogenic factors such as serum or angiotensin II.

The migration-stimulatory effect of NO was associated with reduced cell spreading and decreased levels of actin stress fibers. These effects were also mimicked by cGMP and blocked by ODQ, further supporting the involvement of cGMP as second messenger. These results are consistent with the notion that shape change, actin stress fiber disassembly, and migration may be causally related. It is interesting to note that reduced cell spreading has been associated with increased cell migration in fibroblasts. Similarly, actin stress fiber disassembly has been associated with increased migration in vascular smooth muscle cells. NO was also shown to decrease cell adhesion in cultured mesangial cells, and a preliminary experiment indicates the existence of a similar effect in primary aortic smooth muscle cell cultures. We therefore speculate that NO-induced cytoskeletal rearrangements may be causally related to increased cell migration in a model of primary aortic smooth muscle cells.

It should be noted that the current findings are in contrast to previous studies that have reported that NO inhibits basal or stimulated migration of subcultured aortic smooth muscle cells. In the current study, we have also observed that treatment of subcultured cells with high concentrations of NO donor decreases cell migration in at least 1 model of cultured cells. The factors that could explain the difference between the NO-induced migratory responses in primary versus subcultured cells are not apparent. However, it is well established that vascular smooth muscle cells express altered cytoskeletal and contractile phenotypes on subculture.

Because cell migration involves active participation of actomyosin contractile filaments, a plausible explanation for the differential responses of primary versus subcultured vascular smooth muscle cells is based on altered actin and myosin expression. Indeed, consistent with findings by other investigators, we have found that the levels of α-smooth muscle actin are significantly decreased on subculture (not shown). An alternative explanation, not exclusive to the one involving cytoskeletal changes, is related to the decrease of cGMP-dependent protein kinase. Thus, it has been reported that in rat vascular smooth muscle cells there is a loss of type I protein kinase G (cGMP-dependent protein kinase) and a decrease in cGMP responsiveness on subculturing. By passage 6, cGMP-dependent protein kinase levels were reduced from 400 ng/mg to ~50 ng/mg, an 8-fold decrease, and in passages >8, levels of cGMP-dependent protein kinase were undetectable. However, in our hands, cGMP-dependent protein kinase levels remained relatively high, even up to 20 passages, at least in enzymatically dissociated subcultured cells, ruling out the possibility that the demonstrated differences could be due to the lack of cGMP-dependent protein kinase. Furthermore, cGMP-dependent protein kinase activity was found to be similar for both primary and subcultured cells. However, it seems possible that the lack of response of subcultured cells derived from explants may be related to the relatively low levels of cGMP-dependent protein kinase in these cells. Moreover, the role of cGMP in mediating NO-induced motogenic versus antimotogenic activity appears to be variable, as Sarkar et al found that only 20% to 30% of the antimotogenic response to NO in subcultured cells was attributable to cGMP, whereas we found a greater apparent involvement of cGMP, as evidenced by the complete blockade of NO-induced migration by the guanylyl cyclase inhibitor ODQ.

Seidel et al have recently reported that vascular smooth muscle cells from canine carotid arteries lack the capacity to migrate and proliferate. In contrast, our findings indicate that cells expressing smooth muscle–type myosin heavy chain, by definition considered to be vascular smooth muscle cells, do have the capacity to migrate. Moreover, smooth muscle–type myosin heavy chain levels are not notably decreased on subculture (not shown), which suggests that vascular smooth muscle cells isolated from rat aorta have the capacity to proliferate. A plausible explanation for these divergent results is based on differential characteristics of vascular smooth muscle cells isolated from dog carotid versus rat aorta.

The current results suggest that the effect of NO on vascular smooth muscle migration in vivo may depend on the cytoskeletal phenotype of migrating cells. Thus, medial cells that express a highly differentiated phenotype may be induced to migrate under the influence of either endothelium-derived NO or in response to NO generated by iNOS. The increase of iNOS in...
vascular injury \cite{18-20} is consistent with this possibility. Similarly, several groups have reported that increased arterial flow elicits an increase of endothelium-derived vascular NO and cGMP levels.\cite{20,35} Thus, NO-stimulated cell migration could serve as a wound-repair mechanism or it could also be involved in vascular remodeling that has been shown to occur in the presence of altered arterial flow.\cite{6} A similar role can also be attributed to CNP on the basis of its existence in endothelial cells.\cite{28,29} On the other hand, in cells expressing a less differentiated cytoskeletal phenotype, perhaps arising in the neointima after vascular injury and after migration of cells from the media,\cite{49} high levels of NO may inhibit further migration in response to other motogens, as demonstrated by previous studies.\cite{19,20} Whereas the motogenic response may serve to repair vascular injury or contribute to vascular remodeling, the anti-motogenic response may limit the extent of vascular smooth muscle migration and may contribute to the well-established capacity of NO to limit neointimal formation after vascular injury.\cite{52,53}

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