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Nitric oxide (NO) and angiotensin II (AII) can effect vascular smooth muscle cell (SMC) proliferation. However, the effects of such agents on SMC migration, an equally important phenomenon with regard to vascular pathophysiology, have received little attention. The objectives of the present study were: (a) to determine whether NO inhibits AII-induced migration of vascular SMCs; (b) to investigate the mechanism of the interaction of NO and AII on SMC migration; and (c) to evaluate the AII receptor subtype that mediates AII-induced SMC migration. Migration of rat SMCs was evaluated using a modified Boydens Chamber (transwell inserts with gelatin-coated polycarbonate membranes, 8 microns pore size). AII stimulated SMC migration in a concentration-dependent manner, and this effect was inhibited by sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP). In the presence of L-arginine, but not D-arginine, IL-1 beta, an inducer of inducible NO synthase, also inhibited AII-induced SMC migration, and this effect was prevented by the NO-synthase inhibitor, N-nitro-L-arginine methyl ester. The effects of NO donors on AII-induced SMC migration were mimicked by 8-bromo-cGMP. Also, the antimigratory effects of SNAP were partially inhibited by LY83583 (an inhibitor of soluble guanylyl cyclase) and by KT5823 (an inhibitor of cGMP-dependent protein kinase). Although 8-bromo-cAMP (cAMP) also mimicked the antimigratory effects of NO donors, the antimigratory effects of SNAP were not altered by 2',5'-dideoxyadenosine (an inhibitor of […]

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Nitric Oxide Inhibits Angiotensin II–induced Migration of Rat Aortic Smooth Muscle Cell
Role of Cyclic-Nucleotides and Angiotensin, Receptors

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Abstract

Nitric oxide (NO) and angiotensin II (AII) can effect vascular smooth muscle cell (SMC) proliferation. However, the effects of such agents on SMC migration, an equally important phenomenon with regard to vascular pathophysiology, have received little attention. The objectives of the present study were: (a) to determine whether NO inhibits AII-induced migration of vascular SMCs; (b) to investigate the mechanism of the interaction of NO and AII on SMC migration; and (c) to evaluate the AII receptor subtype that mediates AII-induced SMC migration. Migration of rat SMCs was evaluated using a modified Boyden’s Chamber (transwell inserts with gelatin-coated polycarbonate membranes, 8 μm pore size). AII stimulated SMC migration in a concentration-dependent manner, and this effect was inhibited by sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP). In the presence of L-arginine, but not D-arginine, IL-1α, an inducer of inducible NO synthase, also inhibited AII-induced SMC migration, and this effect was prevented by the NO-synthase inhibitor, N-nitro-L-arginine methyl ester. The effects of NO donors on AII-induced SMC migration were mimicked by 8-bromo-cGMP. Also, the antimigratory effects of SNAP were partially inhibited by LY83583 (an inhibitor of soluble guanylyl cyclase) and by KTS823 (an inhibitor of cGMP-dependent protein kinase). Although 8-bromo-cAMP (cAMP) also mimicked the antimigratory effects of NO donors, the antimigratory effects of SNAP were not altered by 2',5'-dideoxyadenosine (an inhibitor of adenylyl cyclase) or by (R)-p-adenosine-3',5'-cyclic phosphorothioate (an inhibitor of the cAMP-dependent protein kinase). Low concentrations of the subtype AT1-receptor antagonist CGP 48933, but not the subtype AT2-receptor antagonist CGP 42112, blocked AII-induced SMC migration. These findings indicate that (a) NO inhibits AII-induced migration of vascular SMCs; (b) the antimigratory effect of NO is mediated in part via a cGMP-dependent mechanism; and (c) AII stimulates SMC migration via an AT1 receptor. (J. Clin. Invest. 1995. 96:141–149.)

Key words: nitric oxide • angiotensin II • cyclic GMP • cyclic AMP • CGP 48933

Introduction

Smooth muscle cell (SMC)1 migration and proliferation contribute importantly to pathological vascular structural changes such as vascular remodeling, medial hyperplasia, and neointimal formation associated with hypertension, atherosclerosis, and restenosis (1–6). Consequently, detailed knowledge of the processes that regulate SMC migration and proliferation is of great clinical importance.

The endothelium of healthy arteries releases nitric oxide (NO), an efficacious inhibitor of SMC proliferation (1, 2, 7, 8). Inasmuch as removal and/or dysfunction of endothelial cells results in migration, as well as proliferation of SMCs (9, 10), endogenous NO probably exerts a net inhibitory influence on migratory, as well as proliferative behavior of SMCs (9, 10). However, although the effects of NO on SMC proliferation have been studied previously (7, 8, 11), the effects of NO on SMC migration have not been evaluated. Accordingly, the first goal of this study was to determine whether NO inhibits vascular SMC migration.

The vasodilator (12–15) and antiproliferative (7, 8, 11) effects of NO are mediated mainly via activation of soluble guanylyl cyclase and generation of cGMP (16, 17), although participation of other mechanisms, including increases in cAMP, also have been suggested (18). Since the mechanism by which NO affects SMC migration was completely unexplored, a second goal of this study was to investigate how NO affects SMC migration. In this regard, the relative importance of the second messengers, cGMP and cAMP, was evaluated.

To investigate the antimigratory properties of NO, it was necessary to stimulate migration with an agonist, and in the present study angiotensin II (AII) was selected to stimulate

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1 Abbreviations used in this paper: AII, angiotensin II; AT1, angiotensin–receptor subtype; AT2, angiotensin–receptor subtype; DDA, 2',5'-dideoxyadenosine; HPF, high-power field; KT, KTS823; l-NAMe, N-nitro-L-arginine methyl ester; LY, LY 83583; NO, nitric oxide; Rp-cAMP, R-p-adenosine 3',5'-cyclic-phosphorothionate; SMC, smooth muscle cell; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase.

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SMC migration. The rationale for this choice is that AI may be involved in the pathophysiology of several vascular abnormalities including intimal hyperplasia, hypertension-induced medial hypertrophy, and restenosis. For instance, AI stimulates media to intima migration of SMCs in denuded arteries (19) via activation of specific AI receptors (20–22). Several subtypes of AI receptors have been characterized (23), and both angiotensin-receptor subtype (AT1) and angiotensin-II-receptor subtype (AT2) appear to participate in AI-induced SMC proliferation. However, which AI receptors mediate AI-induced SMC migration has not been determined. Hence, a third objective of the current study was to elucidate the AI receptor subtype responsible for stimulating SMC migration.

Methods

Materials. Male Sprague Dawley rats (6 wks old) were obtained from Versuchstierzucht, (Hanover, Germany). DME, HBSS, arginine-deficient RPMI-1640 medium, FCS, dialyzed FCS, penicillin, streptomycin, 0.25% trypsin-EDTA solution, and all tissue culture ware was purchased from Gibco Laboratories, Life Technologies A. G. (Basel, Switzerland). Coculture transwell chambers (8-μm pore size, tissue culture treated) were obtained from Costar Europe Ltd. Angiotensin II (AI), sodium nitroprusside (SNP), 8-bromo-cGMP, L-arginine, N-nitro-L-arginine methyl-ester (l-NAM), forskolin, hemoglobin, superoxide dismutase (SOD), indomethacin, gelatin, and PDGF-BB were purchased from Sigma Chemical Co. (St. Louis, MO). S-nitroso-N-acetylpenicillamine (SNAP) was synthesized and the purity determined as previously described (11, 24). 8-bromo-cAMP, (R)-p-adenosine 3',5'-cyclic phosphorothioate (Rp-cAMP), and LY 83853 (LY) were purchased from Amersham International (Zürich, Switzerland). All other chemicals used were of tissue culture or best grade available.

Cell culture. Aortic smooth muscle cells were cultured with the explant technique from the aortic thoracic aorta (25), and SMC purity was characterized by immunofluorescence staining with smooth muscle specific antisense RNA monoclonal antibodies (11, 26). SMCs were passaged by trypsinization, and cells at third passage were used for migration studies.

Protocols

Effect of nitric oxide, cGMP, cAMP, forskolin, AI-receptor antagonists, LY83583, and KTS823 on AI-induced SMC migration. Migration assays were performed using a modified Boyden chamber (27, 28). Briefly, SMCs in third passage were trypsinized, washed with DME containing 10% FCS, and suspended for 1 h in DME containing 0.4% FCS, to regain normal shape of SMCs. Aliquots (100 μl) of SMCs (0.5 × 10^5) suspended in DME containing 0.4% FCS were added to the transwell inserts precoated with 0.25% gelatin. Migration was induced by angiotensin II (10^{-12} – 10^{-6} M) with or without SNAP (10^{-10} – 10^{-4} M), SNP (10^{-10} – 10^{-4} M), 8-bromo-cGMP (10^{-10} – 10^{-4} M), and AT1-receptor antagonist CPG 48933 (29) (10^{-12} – 10^{-7} M), and AT2-receptor antagonist CPG 42112 (30) (10^{-12} – 10^{-4} M) added into the lower chamber (cluster plate) beneath the insert membrane. In some experiments, to assess whether the effects of chemically derived NO is vectorial or gradient dependent, SNAP (10^{-10} and 10^{-8} M) or SNP (10^{-10} and 10^{-4} M) were added along with the SMC either to the upper chamber or both upper and lower chambers of the insert and migration induced by addition of AI to the lower chamber. The transwell chambers were then incubated for 4 h under standard culture conditions; nonmigrating SMCs on the top of the membrane were carefully removed by gentle scraping. SMCs at the the lower surface of the membrane were fixed with a formaldehyde solution (70–100%) and stained with hematoxylin. After the filters were removed, the number of cells migrating to the lower surface of the filter were evaluated microscopically by counting the number of stained nuclei per high power field (HPF; ×400). All assays were performed in triplicate, and each sample counted randomly in three different areas in the center of the membrane. The SMC migration activity is reported as cells/HPF. Each experiment was repeated three to six times with separate cultures.

To assure specificity of the effects of SNAP on AI-induced migration, effects of SNAP (10^{-4} M [7, 8]) on AI (10^{-8} M)-induced migration were studied in the presence and absence of hemoglobin (5 × 10^{-5} M [31]) or superoxide dismutase (100 U/ml [7, 8, 11, 16, 31, 32]). The effects of freshly prepared solutions of SNAP (10^{-4} and 10^{-2} M) or solutions preincubated for 48 h (to allow prior release of NO from the molecule) on AI (10^{-8} M) induced migration were investigated. Furthermore, since SNP metabolizes into the cyanide metabolite, sodium thiocyanate (33), we also compared the effects of 10^{-10} and 10^{-7} M SNP and sodium thiocyanate on AI (10^{-8} M)-induced migration, to confirm that the effects of SNAP were due to NO. Additionally, the effects of SNAP (10^{-10} and 10^{-9} M) on PDGF-BB (10^{-7} M)-induced SMC migration were also investigated to assess whether NO also influences the migratory effects of other mitogens.

To evaluate effects of intracellular cAMP, SMCs were treated for 1 h with forskolin (10 nM) under standard conditions in DME substituted with 0.4% FCS. Pretreated SMCs were counted, and 0.5 × 10^5 SMCs added to the transwell inserts. Migration was initiated by adding angiotensin II (10^{-9} M) to the lower chamber and migration activity assessed. The controls were treated identically with DME containing 0.4% FCS. To determine whether NO induces its antimigratory effects on AI-induced SMC migration via generation of cGMP or cAMP, the effects of SNAP (10^{-4} M), 8-bromo-cGMP (10^{-5} M), and 8-bromo-cAMP (10^{-3} M) on AI (10^{-8} M)-induced SMC migration were studied in the presence and absence of LY-83583 (10 μM; inhibitor of soluble guanylate cyclase [34]), KTS823 (10 μM; preferential inhibitor of cGMP-dependent protein kinase [35]), DDA (100 μM; inhibitor of adenylyl cyclase [36]), Rp-cAMP (10 μM; preferential inhibitor of cAMP-dependent protein kinase [37]). Additionally, to determine whether cGMP and cAMP mediate their antimigratory effects on AI-induced SMC migration via cGMP- or cAMP-dependent kinase, inhibition of AI-induced SMC-migration by 8-bromo-cGMP (10^{-3} M) and 8-bromo-cAMP (10^{-3} M) was studied in the presence and absence of KTS823 (10 μM) and Rp-cAMP (10 μM).

To test the validity of visual cell counting, additional experiments were carried out in parallel using SMCs prelabeled with [3H]thymidine (37). Briefly, 48 h before the experiment, confluent cultures of SMC were labeled with [3H]thymidine (0.5 μCi/ml) in presence of FCS (5%). On the day of the experiment, the cells were washed four times with DME to wash out free [3H]thymidine. Cells were then trypsinized, washed and counted, and migration in response to AI (10^{-7} M) was studied in the presence and absence of SNAP (10^{-8} and 10^{-10} M) as described above. Aliquots of radiolabeled cells were directly placed in a scintillation vial to obtain total counts. Linear regression analysis showed excellent agreement between incorporated cpm and cell number. After 4 h incubation, cells from the upper surface of the filters were removed, and inserts were washed four times with PBS, placed in scintillation vials, digested with NaOH, neutralized with HCl, and counted after addition of scintillation fluid (Ready Solve HP; Beckman Instruments, Inc., Fullerton, CA) using a β-scintillation counter (38). Control filters to estimate background consisted of filters without cells, filters cleared of cells from the upper and lower surface of the membrane. Percentage of migrated cells was = migrated cells (cpm lower surface) – background + total cells added (total cpm) – background. In parallel experiments using unlabeled cells the migration was evaluated by cell counting (as described above) and the percent change in migration activity of the SMCs by the two procedures were compared.

Effect of IL-1β–Induced nitric oxide on angiotensin II–induced...
Figure 1. Concentration–response relationship of smooth muscle cell migration in response to angiotensin II. Results (mean±SEM) are expressed as percent increase in cell migration as compared to untreated control, defined as migration in absence of All (n = 4 experiments, each in triplicates). All-induced migration in a concentration-dependent manner (P < 0.05). The inserted figure shows the same effects (mean±SEM) from a representative experiment and expressed as cell number/high-power field (x400). The basal migration in the different experiments was 5 ± 2 (mean±SEM), 3.7 ± 1.0, 4.8 ± 0.5, and 4 ± 0.8, x400 (HPF; 5 x 10^6 cells/filter). All increased migration activity in a concentration-dependent manner (*P < 0.05). Similar results were obtained in four independent experiments.

SMC-migration. SMCs treated with interleukin-1β were used to assess the effects SMC-derived NO (39–41) on All-induced migration. Briefly, 24 h before the migration study, confluent SMCs were washed with arginine-free medium (RPMI-1640), substituted with 10% arginine-deficient FCS and then treated with 4 ng/ml interleukin-1β in RPMI-1640 containing 10% arginine-deficient FCS. After 24 h treatment, when maximal release of NO occurs from cultured SMC (39, 41), cells were trypsinized and washed with arginine-free medium (RPMI-1640 substituted with indomethacin [10 μM] to inhibit prostaglandin synthesis and 0.4% FCS). SMCs were then suspended in arginine-free medium containing either L-arginine (60 μM), L-arginine (60 μM) plus L-NNAME (200 μM), or D-arginine (200 μM). Aliquots (0.5 x 10^5 cells) of interleukin-1β treated or untreated cells were added to inserts and All-induced migration studied by adding arginine-free medium containing All (10^-4 M) with or without L-arginine, D-arginine, or L-arginine plus L-NNAME. Migration activity was assessed as described above. Each experiment was conducted in triplicate and repeated four times with separate cultures. To ensure specific effects of interleukin-1β on SMC, cell viability was evaluated by trypan blue exclusion in each experiment.

To evaluate whether IL-1β stimulated NO modulates All-induced SMC migration via guanylate cyclase and/or adenylate cyclase pathway, effects of All (10^-5 M) on the migration of IL-1β treated SMC was investigated in the presence and absence of LY 83583 (10 μM), KT5823 (10 μM), DDA (100 μM), or Rp-cAMP (10 μM). Briefly, aliquots (0.5 x 10^5 cells) of IL-1β–treated cells, with or without L-NNAME, LY 83583, KT5823, DDA, or Rp-cAMP, were added to inserts, and All-induced migration studied by adding arginine-free medium containing All (10^-5 M) with or without L-arginine. Migration activity was assessed as described above. Each experiment was conducted in replicates and repeated four times with separate cultures. To ensure specific effects of the different combinations on SMC, cell viability was evaluated by trypan blue exclusion in each experiment.

Statistics. All experiments were performed in replicates, triplicate, or quadruplicates. Data are presented as mean±SEM of three to six separate experiments. Statistical analysis was performed using ANOVA and paired t test as appropriate. A value of P < 0.05 was considered to be statistically significant.

Results

Effect of angiotensin II on smooth muscle cell migration. Under quiescent (untreated) conditions, very few SMCs (5±2 cells/HPF) migrated (Fig. 1). Addition of All (10^-12 – 10^-6 M) stimulated migration in a concentration-dependent manner (P < 0.05 vs control). This increase in migration already occurred at 10^-10 M All and reached maximal values at 10^-8 M of All (576±40% of untreated control; n = 4 experiments; P < 0.01). To confirm that cell proliferation did not contribute to All-induced migration, SMCs were added directly to the wells and treated in parallel with All and counted after 4 h. No difference in cell number was observed between All–treated and untreated groups (n = 6; data not shown).

Effect of chemically derived nitric oxide on angiotensin II-induced migration. As compared to All (10^-8 M) alone, addition of All together with SNAP or SNAP to the lower chamber inhibited migration in a concentration-dependent manner (Fig. 2, n = 6; P < 0.05). As compared to SNAP, SNAP was more potent in inhibiting All-induced migration (Fig. 2; P < 0.05). All (10^-3 M)–induced migration was also inhibited when SNAP or SOD were added along with the SMCs to either the upper chamber alone or both the upper and lower chambers of the insert (Table I). Similar to SNAP and SNP, 8-bromo-cGMP inhibited All-induced migration (P < 0.05) in a concentration-dependent manner (Fig. 2; P < 0.05).

Hemoglobin reduced the inhibitory effects of SNAP on All-induced migration (Fig. 3 A; P < 0.05), whereas SOD further enhanced its effects (Fig. 3 A; P < 0.05). All-induced migration was unaffected by hemoglobin or SOD alone (Fig. 3 A). To further confirm that the inhibition was due to NO generation and not due to the compound itself, effects of freshly prepared SNAP and SNAP preincubated for 48 h (SNAP spontaneously generates NO in aqueous media and has a half-life of ~ 5 h; [7, 8, 24]), on All-induced migration were evaluated. Freshly prepared SNAP (10^-9 and 10^-6 M) inhibited All-induced SMC migration by 31.4±4.4% and 62±4%, respectively (Fig. 3 B; P < 0.05), whereas preincubated SNAP had no inhibitory effect (Fig. 3 B; P < 0.05 vs control). Furthermore, to confirm that the effects of SNAP were due to NO and not due to its cyanide metabolites, the effects of equimolar concentrations of sodium thiocyanate (10^-10 and 10^-7 M) on All-induced SMC migration were studied. As compared with SMCs treated with All alone, replicates). SMC migration in response to 10^-8 M All alone (control) in the different experiments was 20±5±SEM). 27.6±4, 33±4.4, 35±5, 29±6, and 23±3.2, x400 (HPF; 5 x 10^6 cells/filter). SNAP, SNP, and 8-bromo-cGMP significantly inhibited All-induced migration in a concentration-dependent manner (P < 0.05).

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Table 1. Inhibition of Angiotensin II-induced Migration of Smooth Muscle Cells by SNAP and SNP

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Lower chamber</th>
<th>Upper chamber</th>
<th>Lower and upper chamber</th>
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<tr>
<td>SNAP</td>
<td></td>
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<tr>
<td>10^{-10} M</td>
<td>18±3.8*</td>
<td>27±4.7*</td>
<td>26±6.8*</td>
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<tr>
<td>10^{-8} M</td>
<td>40±5.9*</td>
<td>59±6.5*</td>
<td>45±7.5*</td>
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<td>SNP</td>
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<tr>
<td>10^{-10} M</td>
<td>11±3.4</td>
<td>15±5.7*</td>
<td>17±6*</td>
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<tr>
<td>10^{-8} M</td>
<td>26±6.3*</td>
<td>31±4.8*</td>
<td>34±7.7*</td>
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SNAP and SNP were added to the upper, lower, or both upper and lower parts of the modified Boyden's Chamber. SNAP and SNP inhibited (P < 0.05) All-induced migration irrespective of chamber site to which they were added. Data are expressed as means±SEM. * Significantly different from All-treated group alone. SMC migration in response to 10^{-10} M All in different experiments was 23±3.4% (mean±SEM), 26±4.4, and 21±2.7, ×400 (HPF; 5 × 10^6 cells/filter).

10^{-10} and 10^{-7} M SNP inhibited All-induced migration by (mean±SEM, n = 3) 12±2% and 45±9%, respectively, (P < 0.05 as compared to control), whereas equimolar concentrations of sodium thiocyanate did not significantly alter All-induced SMC migration, and at a concentration of 10^{-7} M, inhibited SMC migration by only 4±2% (NS).

The migration activity (percentage of control) in response to All (10^{-8} M), as well as inhibition of All-induced migration by SNAP (10^{-10} and 10^{-6} M), did not vary between the two assay systems (radiolabeled cells vs cell counting) and were comparable (P < 0.05). This finding validated the procedure for migration assay by cell counting and suggested that either procedure could be used to assess cell migration.

Figure 4. Bar graph showing the effects of All (10^{-8} M) on the migration of SMC-treated or not treated with interleukin-1β. SMCs were incubated for 24 h with or without interleukin-1β (4 ng/ml), and migration in response to All was evaluated in the presence and absence of L-arginine (60 μM), L-arginine (60 μM) + L-NAME (200 μM), and D-arginine (200 μM). Migration activity (mean±SEM; n = 4 experiments) is expressed as percentage of control, defined as cell migration in response to arginine-free media containing 0.4% arginine deficient FCS and no All. All-induced migration of interleukin-1β treated and untreated cells in absence of L-arginine (P < 0.05). In contrast to untreated cells, L-arginine inhibited All-induced migration in interleukin-1β-treated cells (P < 0.05), and this inhibitory effect of L-arginine was reversed by L-NAME (P < 0.05). D-arginine did not alter All-induced migration in both cases. * Significant difference (P < 0.05) from treatment with All in absence of L-arginine. Similar results were obtained in three independent experiments using separate cultures.

Trypan blue exclusion viability tests were carried out in cells treated in parallel to the migration studies. There was no loss in viability of cells (radiolabeled or unlabeled) treated with SNAP, SNP, or cGMP and 0.5% of the cells took up the dye.

In addition to inhibiting All-induced SMC migration, SNAP also inhibited SMC migration induced by PDGF-BB (P < 0.05). The migration activity of SMCs in response to PDGF-BB (10^{-8} M), PDGF-BB + SNAP (10^{-10} M) and PDGF-BB + SNAP (10^{-7} M) were (percentage of control; mean±SEM; n = 3) 627±188, 496±101, and 205±54%, respectively, suggesting that the antimigratory effects of NO were not limited to All-induced migration.

Figure 3. (A) Effect of SNAP (10^{-8} M) on 10^{-8} M All-induced SMC migration in presence and absence of hemoglobin (50 μM) or superoxide dismutase (100 U/ml). Results (mean±SEM; n = 4 experiments, each in triplicates) are expressed as percentage of untreated control and the number of cells migrating in controls were 5±0.4 cells/HPF; (n = 4 experiments). * Significant inhibition of All-induced migration by SNAP; ** Significantly different from SNAP-inhibited migration (P < 0.05) in absence of hemoglobin or superoxide dismutase. (B) Inhibitory effect of SNAP (10^{-8} and 10^{-10} M), freshly prepared and preincubated for 48 h, on All (10^{-8} M)-induced SMC migration. Results are expressed as percentage of untreated control, i.e., migration in presence of 0.4% FCS. * Significantly (P < 0.05) different from All-induced SMC migration in absence of SNAP.

Trypan blue exclusion viability tests were carried out in cells treated in parallel to the migration studies. There was no loss in viability of cells (radiolabeled or unlabeled) treated with SNAP, SNP, or cGMP and 0.5% of the cells took up the dye.

In addition to inhibiting All-induced SMC migration, SNAP also inhibited SMC migration induced by PDGF-BB (P < 0.05). The migration activity of SMCs in response to PDGF-BB (10^{-8} M), PDGF-BB + SNAP (10^{-10} M) and PDGF-BB + SNAP (10^{-7} M) were (percentage of control; mean±SEM; n = 3) 627±188, 496±101, and 205±54%, respectively, suggesting that the antimigratory effects of NO were not limited to All-induced migration.

Effect of smooth muscle cell-derived nitric oxide on angiotensin II-induced migration. In contrast to untreated SMCs, All-induced migration of SMCs pretreated with interleukin-1β was inhibited in the presence of L-arginine (60 μM; Fig. 4; P < 0.01). However, no inhibition of All-induced migration was observed in the presence of β-arginine (200 μM) or in the absence of L-arginine in both interleukin-1β--treated and untreated SMCs. Furthermore, L-NAME (200 μM) reversed L-arginine (60 μM) inhibition of All-induced SMC migration in interleukin-1β--treated SMCs (P < 0.05). All-induced migration of SMCs not treated with interleukin-1β was not modulated by the various treatments described above (Fig. 4).

Effects of forskolin, 8-bromo-cAMP and 8-bromo-cGMP on angiotensin II-induced migration. As compared to untreated controls, All-induced migration was also inhibited by 59±15% in SMCs preincubated with forskolin to induce intracellular...
cAMP (P < 0.05 vs control). Both 8-bromo-cGMP (10^{-3} M) and 8-bromo-cAMP (10^{-3} M) similarly inhibited All (10^{-4} M)-induced SMC-migration (P < 0.05; Fig. 5). Furthermore, combined treatment with 8-bromo-cGMP + 8-bromo-cAMP inhibited All-induced migration in an additive fashion (P < 0.05; Fig. 5). The inhibitory effects of 8-bromo-cAMP on All-induced migration were blocked by Rp-cAMP (P < 0.01; Fig. 5). Furthermore, the inhibitory effects of 8-bromo-cGMP and 8-bromo-cAMP induce their antimigratory effects specifically via cGMP-dependent and cAMP-dependent protein kinase pathways, respectively.

Effects of inhibitors of cGMP- or cAMP-dependent pathways on All-induced migration. Inhibition of 10^{−4} M All-induced SMC migration was partially, but significantly, reversed by the inhibitor of soluble guanylate cyclase LY 83583 (134) 10 μM and the inhibitor of cGMP-dependent protein kinase KT5823 (135) 10 μM; P < 0.05; Fig. 6). However, DDA (100 μM; adenylate cyclase inhibitor [36]), Rp-cAMP (10 μM; cAMP-dependent protein kinase inhibitor [37]) and L-NAME (200 μM) did not modulate the inhibitory effects of SNAP on All-induced migration (Fig. 6).

In contrast to untreated SMCs, All-induced migration of SMCs pretreated with interleukin-1β was inhibited in the presence of L-arginine (60 μM; Fig. 7; P < 0.01), an effect which was abrogated by L-NAME (200 μM). L-arginine (60 μM) induced All-induced SMC migration in interleukin-1β-treated SMCs was partially, and significantly, reversed in the presence of Rp-cAMPS (10 μM; P < 0.05), LY 83583 (10 μM; P < 0.05) or KT5823 (10 μM; P < 0.05), but not in the presence of DDA (100 μM; Fig. 7). The number of SMCs migrating in response to All (10^{-4} M) in the presence of L-arginine:DDA, Rp-cAMPS, LY 83583, or KT5823 was (percent of control±SEM): 100% in the absence of L-arginine; 43±5% in presence of L-arginine (P < 0.05 vs – L-arginine).

Discussion

The present study demonstrates that nitric oxide is a potent inhibitor of All-induced migration. Both NO derived from nitro-
vasodilators, as well as NO generated endogenously by SMCs stimulated with interleukin-1β, inhibited All-induced SMC migration. These antimigratory effects of NO were also mimicked by stable analogs of cGMP and cAMP, as well as by forskolin, a stimulator of intracellular cAMP levels. Furthermore, as compared to inhibitors of the adenylate cyclase pathway, inhibitors of the guanylate cyclase pathway were more effective in reducing the antimigratory effects of NO. Additionally, All-induced SMC migration was inhibited by low concentrations of AT1 receptor antagonist CGP 48933 but not the AT2 receptor antagonist CGP 42112.

Cell migration occurs during physiological processes such as embryogenesis, angiogenesis, and wound healing and during pathophysiological processes such as tumor metastasis and vascular disease. Cell migration involves regulated cell attachment, detachment, contraction, and cytoskeletal plasticity (1, 2, 27, 28, 42). Extracellular signals for migration include physical forces, autocrine/paracrine substances, and circulating hormones such as All and other polypeptide growth factors (1, 27, 28, 42). Cell migration is distinct from proliferation (1); indeed, nearly half of the migrating neointimal SMCs after balloon injury do not synthesize DNA (1, 43). Cell migration can be analyzed as chemokinesis (random migration) or chemotaxis (directed migration) (1). In atherosclerosis and hypertension, migration probably represents chemotaxis rather than chemokinesis, as SMCs migrate in a directed manner from the media to the intima, but not to the adventitia (1). In the present study, migration was assessed by monitoring the movement of the cells towards All (1, 42).

All-induced migration in a concentration range (10^{-12} \text{ - } 10^{-10} \text{ M}) much lower than that reported to induce SMC growth (10^{-8} \text{ - } 10^{-7} \text{ M}) (1, 2, 7, 8, 11, 261). Similar observations have been reported with platelet-derived growth factor (44). This is in line with the fact that migration is an earlier response than proliferation (1, 44) and supports the importance of this response at sites of increased local vascular All levels. Although several All receptor subtypes exist, AT1 and AT2 on smooth muscle appear most important (20-23). Recently, specific antagonists have become available (29, 30). Using specific antagonists it has been shown that the proliferative effects of All on vascular smooth muscle cells are mediated through the AT1 receptor subtype (20, 21), although the involvement of AT2 receptor has also been suggested (22). However, the effects of these AT1 and AT2 antagonists on All-induced SMC migration have not been reported. Our observation that low concentrations of the AT1 receptor antagonist CGP 48933, but not of the AT2 receptor antagonist CGP 42112 were able to inhibit All induced SMC migration, suggests that All induces SMC migration via AT1 receptors. The weak inhibitory effect of CGP 42112 on migration at high concentrations is likely due to nonselective interactions with the AT1 receptor subtype (20, 29, 30).

Nitrovasodilators such as SNA generate NO spontaneously and by intracellularly catalyzed reactions, whereas SNAP releases NO spontaneously in aqueous media (24, 45). NO generated from SNAP inhibited All-induced migration at very low concentrations (10^{-10} \text{ M}). As compared to SNAP, SNA was more potent in inhibiting All-induced migration, and these differences are most likely related to the different capacity of the compounds to generate NO (7, 8, 45). These inhibitory effects of the nitrovasodilators were also mimicked by stable analogs of cGMP (8-bromo-cGMP) and cAMP (8-bromo-cAMP), respectively. Again the concentrations of 8-bromo-cGMP (10^{-7} \text{ M}) required to effectively inhibit migration was orders of magnitude lower than those (10^{-4} \text{ M}) required to inhibit DNA synthesis in SMCs (7, 8). Similar to 8-bromo-cGMP, the concentration of 8-bromo-cAMP (10^{-5} \text{ M}) which significantly inhibited All-induced migration is one to two orders of magnitude lower than the concentration required to inhibit cell proliferation (10^{-6} \text{ - } 10^{-5} \text{ M}) [2, 46)]. This is also in agreement with our unpublished observation that 10^{-4} \text{ - } 10^{-3} \text{ M} 8-bromo-cAMP, but not 10^{-3} \text{ M}, inhibits 2.5% FCS-induced DNA synthesis. Some of these differences could be due to the experimental conditions. Specifically, in previous studies (7, 8) the effects of 8-bromo-cGMP on DNA synthesis induced by 5% FCS were studied, whereas All was used in our study. Most likely, however, this indicates that NO and its second messengers cGMP, as well as cAMP, inhibit SMC migration at much lower concentrations near or at the physiological range. Since low concentrations of NO, 8-bromo-cGMP, 8-bromo-cAMP and/or All were required to inhibit or induce migration, respectively, it is possible that even small changes in local All or NO levels trigger SMC migration from the media towards the intima.

The fact that the antimigratory effects of SNAP were reduced in the presence of the NO scavenger hemoglobin (31) and potentiated by SOD (which prevents the inactivation of NO by free radicals [7, 8, 16, 31, 32]) indicates that the inhibition of All-induced migration by the nitrovasodilators was indeed due to NO generation. Direct effects of hemoglobin or superoxide dismutase can be excluded as All-induced migration remained unaltered in their presence. The enhanced antimigratory effects of SNAP and SNP when added to the upper chamber, the inability of preincubated SNAP to inhibit All-induced migration (which already released all NO from its molecule at the time of the experiment, i.e., after 5 h [8, 24]), and the inability of sodium thiocyanate to inhibit All-induced migration further supports the interpretation that the antimigratory effects were indeed due to NO generation. Trypan blue exclusion experiments demonstrated that the antimigratory effects of NO generated by SNAP and SNP were not due to cell toxicity.
The observation that the inhibitory effects of the nitrovasodilators were mimicked by a stable analog of cGMP (8-bromo-
cGMP), suggest that cGMP in part mediates the inhibitory
effects of NO. In this regard, our observation that the antimigra-
tory effects of SNAP were partly reversed by the soluble guany-
late cyclase inhibitor LY 83583 (34), as well as by KT5823, a
preferential inhibitor of cGMP-dependent protein kinase (35),
also suggests that the antimigratory effects of NO, at least in
part, must be mediated by a cGMP-dependent mechanism, as
are the vasodilatory and antiproliferative effects of NO (7, 8,
16, 17).

Similar to NO and 8-bromo-cGMP, 8-bromo-cAMP also
attenuated AII-induced migration. The inhibitory effects of 8-
bromo-cAMP on AII-induced SMC migration were blocked by
Rp-cAMP an inhibitor of cAMP-dependent protein kinase.
However, in contrast to LY 83583 and KT5823, Rp-cAMP, and
DDA (inhibitors of the adenylyl cyclase pathway) were unable
to reverse the inhibitory effects of SNAP on AII-induced migra-
tion. Taken together these findings suggest that NO induces its
antimigratory effects at least in part via cGMP, but not cAMP,
generation. This conclusion is consistent with previous observa-
tions that increases in cGMP production in response to chemi-
cally derived NO are several magnitudes higher than the mar-
ginal increases reported for cAMP (47).

Although cGMP participates in the NO-induced inhibition of
migration, the fact that LY 83583 and KT8823 only partially
reversed the effects of chemically derived NO suggests that NO
may be inducing its antimigratory effects to some degree by
some other mechanism(s) independent of cGMP. Indeed cell
(matochondrial) respiration, Po2, extracellular matrix proteins
and integrin expression importantly contribute to the process
of cell movement (1) and these parameters are known to be
influenced by NO (48).

To investigate whether NO released from SMCs themselves
is capable of inhibiting migration, AII-induced migration stud-
ies were conducted in interleukin-1β treated SMCs. Interleu-
kin-1β induces NO synthase in SMCs and causes a ≈ 20-fold in-
crease in NO release (41). In the presence of L-arginine, AII-
induced migration was markedly inhibited in interleukin-1β
ofys MHC. Since in the absence of L-arginine AII-induced
migration was similar in interleukin-1β-treated and untreated
SMCs require extracellular L-arginine to synthesize NO
when stimulated with a cytokine. L-NAME, an inhibitor of NO
formation, abolished the inhibitory effect of interleukin-1β
in the presence of L-arginine (60 μM). As L-arginine and L-NAME
per se were unable to modulate AII-induced migration, these
effects must be related to a specific interaction with the L-
arginine pathway in cytokine-treated cells. In line with this
interpretation, AII-induced migration was not inhibited by in-
terleukin-1β in the presence of D-arginine. As indomethacin
was present in all experiments, a contribution of prostaglandins
can also be excluded (6, 10, 39).

To investigate whether NO generated from cytokine-stimu-
lated cells mediate its antimigratory effects via cAMP or cGMP
pathways, functional studies were conducted using the inhibi-
tors LY 83583, KT5823, DDA, and Rp-cAMPS. In contrast to
L-NAME, LY 83583 and KT5823, only partly reversed the
inhibitory effects of L-arginine on AII-induced migration.
Furthermore, Rp-cAMP, but not DDA, also partially reversed the
antimigratory effects observed in presence of L-arginine; how-
ever, the effects LY 83583 and KT5823 in reversing L-arginine-
induced inhibition of SMC migration were significantly greater
than those observed with Rp-cAMPS and DDA. IL-1β has been
shown to induce the generation of NO, cGMP as well as cAMP
(49). However, as compared to cGMP, the increase in cAMP
induced by IL-1β is much lower (49). Indeed, increases in
cAMP can also be mediated indirectly by factors other than NO
and cGMP which increase in response to IL-1β (50). Hence,
it is possible that in cytokine-treated SMCs, increases in cAMP,
at least in part, contribute to the antimigratory effects.

In IL-1β–treated SMCs, the inhibitors of cAMP-mediated
responses DDA and Rp-cAMP, marginally reversed L-arginine-
induced inhibition of AII-induced migration. However, they
were unable to inhibit the antimigratory effects of SNAP. It is
conceivable, therefore, that in cytokine-treated cells the cAMP-
dependent inhibition of migration was not due to NO or cGMP,
but rather some other secondary products. Additionally, the
observation that in cytokine-treated cells LY 83583 and KT8823
significantly reversed the effects of SNAP, as well as L-arginine-
induced inhibition of AII-induced migration, suggests that NO-
induced cGMP but not cAMP, at least in part, accounts for the
antimigratory effects of NO. Furthermore, it is possible that NO
directly interacts with the extracellular matrix proteins, adhesion
molecules (integrin expression), and respiratory chain en-
zymes, which importantly regulate cell function, including the
movement of the cells. Indeed, NO is known to inhibit mito-
ochondrial respiration (48), and extracellular matrix synthesis
(48). Hence it is conceivable that NO inhibits SMC migration
by directly interacting with these mechanisms. Alternatively,
NO is known to cause decreases in intracellular Ca++ (16, 48),
and Ca++ is known to influence the reassembly of the actin
filaments which in turn can regulate SMC migration.

Structural vascular changes are associated with hypertension
and atherosclerosis (1–3). In normal arteries, the endothelium
maintains SMC quiescence by synthesizing growth inhibitors
like NO and growth promoters like AII (1, 2, 9, 10, 51) in a
balanced fashion. Removal or damage of the endothelium, on
the other hand, results in migration and proliferation of SMC
(9, 10, 51) suggesting that an intact monolayer of confluent
endothelium normally has a net inhibitory influence on the un-
derlying SMCs (9, 10, 51). NO together with other substances
synthesized by endothelial cells most likely contributes to these
effects. Endothelial function is altered in atherosclerosis and
hypertension (3, 52). In particular, the basal and receptor-oper-
ated release of biologically active NO appears to be decreased
(52). Hence, decreased synthesis and/or increased breakdown
of NO could impair the inhibitory effects of the endothelium
and result in migration of SMCs and eventually in remodeling
and intimal thickening. While the latter appears to be important
in large conduit arteries exposed to hypertension, ballooning,
or atherosclerosis, remodeling (i.e., rearrangement of medial
SMC with no increase in cell number) occurs in hypertensive
resistance arteries (1, 2, 6, 53).

Smooth muscle cells synthesize NO only when stimulated
with cytokines or lipopolysaccharide (54). Increased cytokine
levels occur at sites of endothelial injury after ballooning,
in atherosclerosis and hypertension, and in vasculitis due to
infiltration of leukocytes (capable of generating cytokines [1,
2, 4, 40, 54]). Hence, NO synthesis by SMCs in response to
cytokines could represent a potential defense mechanism to
limit SMC migration from the media into the intima, at sites of
vessel injury. Furthermore, NO is also capable of inhibiting
SMC migration induced by PDGF-BB, a mitogen that similar to
AII increases at sites of damaged/abnormal vascular tissue

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(1, 2). It is conceivable that an impaired capacity of SMC to generate NO in response to cytokines, as it occurs in hypertension and atherosclerosis (52, 55), may facilitate SMC migration and proliferation and augment the response to injury of the vessel wall.

In conclusion, this is the first report providing direct evidence that AT1, antagonist, cAMP, cGMP, as well as NO generated from cytokine treated SMCs or nitrovasodilators inhibit ADP-induced SMC migration. AT1 antagonist such as CGB 48933 may be useful in reducing abnormal growth and proliferation of smooth muscle cells observed in hypertension and atherosclerosis. Similarly, nitric oxide donor molecules may exert beneficial effects on vascular structure.

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