

# Nitric Oxide Inhibits Angiotensin II-induced Migration of Rat Aortic Smooth Muscle Cell

## Role of Cyclic-Nucleotides and Angiotensin<sub>1</sub> 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 Boydens Chamber (transwell inserts with gelatin-coated polycarbonate membranes, 8  $\mu$ m 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 adenylyl cyclase) or by (R)-*p*-adenosine-3',5'-cyclic phosphorothioate (an inhibitor of the cAMP-dependent protein kinase). Low concentrations of the subtype AT<sub>1</sub>-receptor antagonist CGP 48933, but not the sub-

type AT<sub>2</sub>-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 AT<sub>1</sub> 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)<sup>1</sup> 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

This work was presented at the American Heart Association Council for High Blood Pressure Research, San Francisco, CA, September 1993 and is published in abstract form (1993. *Hypertension (Dallas)*. 22:412a).

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Received for publication 20 April 1994 and accepted in revised form 31 March 1995.

*J. Clin. Invest.*

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0021-9738/95/07/0141/09 \$2.00

Volume 96, July 1995, 141–149

1. *Abbreviations used in this paper:* AII, angiotensin II; AT<sub>1</sub>, angiotensin<sub>1</sub>-receptor subtype; AT<sub>2</sub>, angiotensin<sub>2</sub>-receptor subtype; DDA, 2',5'-dideoxyadenosine; HPF, high-power field; KT, KT5823; L-NAME, *N*-nitro *L*-arginine methyl ester; LY, LY 83583; NO, nitric oxide; Rp-cAMP, *R-p*-adenosine 3',5'-cyclic-phosphorothioate; SMC, smooth muscle cell; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase.

SMC migration. The rationale for this choice is that AII may be involved in the pathophysiology of several vascular abnormalities including intimal hyperplasia, hypertension-induced medial hypertrophy, and restenosis. For instance, AII stimulates media to intima migration of SMCs in deendothelized arteries (19) via activation of specific AII receptors (20–22). Several subtypes of AII receptors have been characterized (23), and both angiotensin<sub>1</sub>-receptor subtype (AT<sub>1</sub>) and angiotensin<sub>2</sub>-receptor subtype (AT<sub>2</sub>) appear to participate in AII-induced SMC proliferation. However, which AII receptors mediate AII-induced SMC migration has not been determined. Hence, a third objective of the current study was to elucidate the AII receptor subtype responsible for stimulating SMC migration.

## Methods

**Materials.** Male Sprague Dawley rats (6 wks old) were obtained from Versuchstierzucht, (Hannover, 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- $\mu$ m pore size, tissue culture treated) were obtained from Costar Europe Ltd. Angiotensin II (AII), sodium nitroprusside (SNP), 8-bromo-cGMP, L-arginine, *N*-nitro L-arginine methyl-ester (L-NAME), 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 83583 (LY) were purchased from Research Biochemicals Inc. (Natick, MA). 2',5'-Dideoxyadenosine (DDA) was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA), and KT5823 (KT) from Kamiya Biomedical Co. (Thousand Oaks, CA). Interleukin-1 $\beta$  (human recombinant, sp act 10 U/ng), was purchased from Boehringer Mannheim GmbH Biochemica (Mannheim, Germany). CGP 48933 and CGP 42112 were generous gifts from Ciba Geigy (Basel, Switzerland). [<sup>3</sup>H]thymidine (sp act 80 Ci/mmol) was 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 ascending thoracic aorta (25), and SMC purity was characterized by immunofluorescence staining with smooth muscle specific antismooth muscle  $\alpha$ -actin 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, AII-receptor antagonists, LY83583, and KT5823 on AII-induced SMC migration.** Migration assays were performed using a modified Boydens 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  $\mu$ l) of SMCs ( $0.5 \times 10^5$ ) 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), AT<sub>1</sub>-receptor antagonist CGP 48933 (29) ( $10^{-12}$  –  $10^{-4}$  M), and AT<sub>2</sub> receptor antagonist CGP 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  $10^{-9}$  M) or SNP ( $10^{-10}$  and  $10^{-8}$  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 AII to the lower chamber. The transwell chambers were then incubated for 4 h under standard culture conditions; nonmigrating SMCs

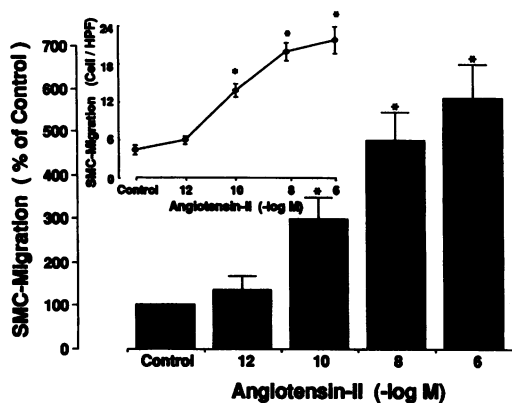
on the top of the membrane were carefully removed by gentle scraping. SMCs at the the lower surface of the membrane were fixed with a gradient of alcohol (70–100%) and stained with hematoxylin. The filters were removed, and the number of cells migrating to the lower surface of the filter were evaluated microscopically by counting the number of stained nuclei per high power field (HPF;  $\times 400$ ). 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 AII-induced migration, effects of SNAP ( $10^{-8}$  M [7, 8]) on AII ( $10^{-6}$  M)-induced migration were studied in the presence and absence of hemoglobin ( $5 \times 10^{-5}$  M [31]) or superoxide dismutase (100 U/ml [7, 8, 11, 16, 31, 32]). The effects of freshly prepared solutions of SNAP ( $10^{-6}$  and  $10^{-9}$  M) or solutions preincubated for 48 h (to allow prior release of NO from the molecule) on AII ( $10^{-6}$  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 AII ( $10^{-8}$  M)-induced migration, to confirm that the effects of SNP were due to NO. Additionally, the effects of SNAP ( $10^{-10}$  and  $10^{-7}$  M) on PDGF-BB ( $10^{-8}$  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  $0.5 \times 10^5$  SMCs added to the transwell inserts. Migration was initiated by adding angiotensin II ( $10^{-8}$  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 AII-induced SMC migration via generation of cGMP or cAMP, the effects of SNAP ( $10^{-7}$  M), 8-bromo-cGMP ( $10^{-5}$  M), and 8-bromo-cAMP ( $10^{-5}$  M) on AII ( $10^{-8}$  M)-induced SMC migration were studied in the presence and absence of LY-83583 (10  $\mu$ M; inhibitor of soluble guanylate cyclase [34]), KT5823 (10  $\mu$ M; preferential inhibitor of cGMP-dependent protein kinase [35]), DDA (100  $\mu$ M; inhibitor of adenylate cyclase [36]), Rp-cAMP (10  $\mu$ M; preferential inhibitor of cAMP-dependent protein kinase [37]). Additionally, to determine whether cGMP and cAMP mediate their antimigratory effects on AII-induced SMC migration via cGMP- or cAMP-dependent kinase, inhibition of AII-induced SMC-migration by 8-bromo-cGMP ( $10^{-5}$  M) and 8-bromo-cAMP ( $10^{-5}$  M) was studied in the presence and absence of KT5823 (10  $\mu$ M) and Rp-cAMP (10  $\mu$ M).

To test the validity of visual cell counting, additional experiments were carried out in parallel using SMCs prelabeled with [<sup>3</sup>H]thymidine (37). Briefly, 48 h before the the experiment, confluent cultures of SMC were labeled with [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci/ml) in presence of FCS (5%). On the day of the experiment, the cells were washed four times with DME to wash out free [<sup>3</sup>H]thymidine. Cells were then trypsinized, washed and counted, and migration in response to AII ( $10^{-6}$  M) was studied in the presence and absence of SNAP ( $10^{-6}$  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  $\beta$ -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  $\div$  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-1 $\beta$ -Induced nitric oxide on angiotensin II-induced*

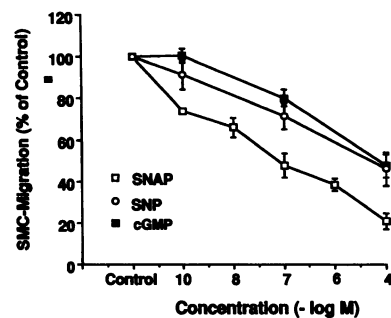


**Figure 1.** Concentration–response relationship of smooth muscle cell migration in response to angiotensin II. Results (mean±SEM) are expressed as percent increase in cell migration as compared to untreated control, defined as migration in absence of AII ( $n = 4$  experiments, each in triplicates). AII-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 ( $\times 400$ ). The basal migration in the different experiments was  $5 \pm 2$  (mean±SEM),  $3.7 \pm 1.0$ ,  $4.8 \pm 0.5$ , and  $4 \pm 0.8$ ,  $\times 400$  (HPF;  $5 \times 10^4$  cells/filter). AII 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- $1\beta$  were used to assess the effects SMC-derived NO (39–41) on AII-induced migration. Briefly, 24 h before the migration study, confluent SMCs were washed with arginine-free media (RPMI-1640), substituted with 10% arginine-deficient FCS and then treated with 4 ng/ml interleukin- $1\beta$  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 [ $10 \mu\text{M}$ ] to inhibit prostaglandin synthesis and 0.4% FCS). SMCs were then suspended in arginine-free medium containing either L-arginine ( $60 \mu\text{M}$ ), L-arginine ( $60 \mu\text{M}$ ) plus L-NAME ( $200 \mu\text{M}$ ), or D-arginine ( $200 \mu\text{M}$ ). Aliquots ( $0.5 \times 10^5$  cells) of interleukin- $1\beta$  treated or untreated cells were added to inserts and AII-induced migration studied by adding arginine-free medium containing AII ( $10^{-8}$  M) with or without L-arginine, D-arginine, or L-arginine plus L-NAME. 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- $1\beta$  on SMC, cell viability was evaluated by trypan blue exclusion in each experiment.

To evaluate whether IL- $1\beta$  stimulated NO modulates AII-induced SMC migration via guanylate cyclase and/or adenylate cyclase pathway, effects of AII ( $10^{-8}$  M) on the migration of IL- $1\beta$  treated SMC was investigated in the presence and absence of LY 83583 ( $10 \mu\text{M}$ ), KT5823 ( $10 \mu\text{M}$ ), DDA ( $100 \mu\text{M}$ ), or Rp-cAMP ( $10 \mu\text{M}$ ). Briefly, aliquots ( $0.5 \times 10^5$  cells) of IL- $1\beta$ -treated cells, with or without L-NAME, LY 83583, KT5823, DDA, or Rp-cAMP, were added to inserts, and AII-induced migration studied by adding arginine-free medium containing AII ( $10^{-8}$  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.



**Figure 2.** Concentration–response relationship for the inhibition of AII ( $10^{-8}$  M)-induced SMC migration by SNAP, SNP, and 8-bromo-cGMP. Results (mean±SEM) are expressed as percentage of control, where 100% is defined as migration in presence of AII alone ( $n = 6$  experiments, each in

replicates). SMC migration in response to  $10^{-8}$  M AII alone (control) in the different experiments was  $20.5 \pm 4$  (mean±SEM),  $27.6 \pm 4$ ,  $33 \pm 4.4$ ,  $35 \pm 5$ ,  $29 \pm 6$ , and  $23 \pm 3.2$ ,  $\times 400$  (HPF;  $5 \times 10^4$  cells/filter). SNAP, SNP, and 8-bromo-cGMP significantly inhibited AII-induced migration in a concentration-dependent manner ( $P < 0.05$ ).

## Results

**Effect of angiotensin II on smooth muscle cell migration.** Under quiescent (untreated) conditions, very few SMCs ( $5 \pm 2$  cells/HPF) migrated (Fig. 1). Addition of AII ( $10^{-12}$  –  $10^{-6}$  M) stimulated migration in a concentration-dependent manner ( $P < 0.05$  vs control). This increase in migration already occurred at  $10^{-10}$  M AII and reached maximal values at  $10^{-6}$  M of AII ( $576 \pm 40\%$  of untreated control;  $n = 4$  experiments;  $P < 0.01$ ).

To confirm that cell proliferation did not contribute to AII-induced migration, SMCs were added directly to the wells and treated in parallel with AII and counted after 4 h. No difference in cell number was observed between AII-treated and untreated groups ( $n = 6$ ; data not shown).

**Effect of chemically derived nitric oxide on angiotensin II-induced migration.** As compared to AII ( $10^{-8}$  M) alone, addition of AII together with SNP or SNAP to the lower chamber inhibited migration in a concentration-dependent manner (Fig. 2,  $n = 6$ ;  $P < 0.05$ ). As compared to SNP, SNAP was more potent in inhibiting AII-induced migration (Fig. 2;  $P < 0.05$ ). AII ( $10^{-8}$  M)-induced migration was also inhibited when SNAP or SNP 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 AII-induced migration ( $P < 0.05$ ) in a concentration-dependent manner (Fig. 2;  $P < 0.05$ ).

Hemoglobin reduced the inhibitory effects of SNAP on AII-induced migration (Fig. 3 A;  $P < 0.05$ ), whereas SOD further enhanced its effects (Fig. 3 A;  $P < 0.05$ ). AII-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  $\sim 5$  h; [7, 8, 24]), on AII-induced migration were evaluated. Freshly prepared SNAP ( $10^{-9}$  and  $10^{-6}$  M) inhibited AII-induced SMC migration by  $31.4 \pm 4.4\%$  and  $62 \pm 4\%$ , 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 SNP 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 AII-induced SMC migration were studied. As compared with SMCs treated with AII alone,

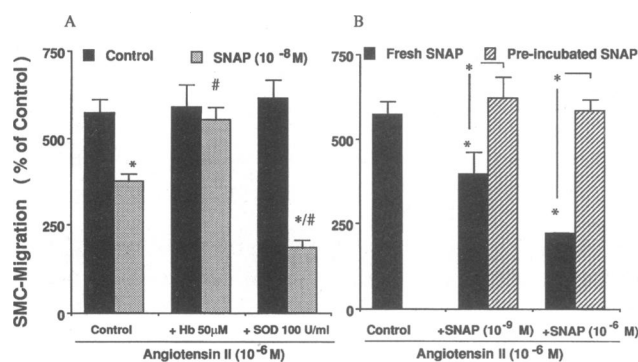
**Table 1. Inhibition of Angiotensin II-induced Migration of Smooth Muscle Cells by SNAP and SNP**

Treatments	SMC-migration (percentage of control)		
	Lower chamber	Upper chamber	Lower and upper chamber
<b>SNAP</b>			
10 <sup>-10</sup> M	18±3.8*	27±4.7*	26±6.8*
10 <sup>-8</sup> M	40±5.9*	59±6.5*	45±7.5*
<b>SNP</b>			
10 <sup>-10</sup> M	11±3.4	15±5.7*	17±6*
10 <sup>-8</sup> M	26±6.3*	31±4.8*	34±7.7*

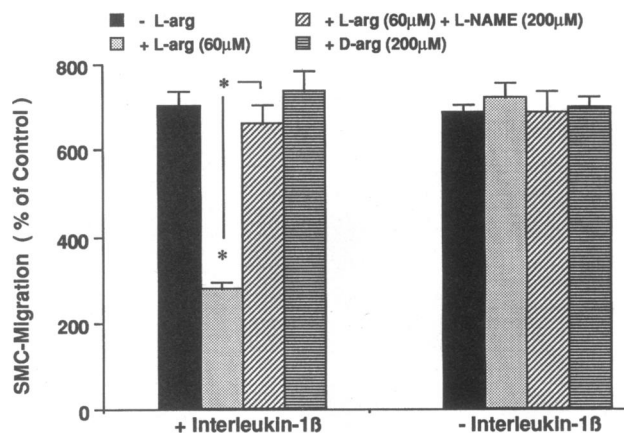
SNAP and SNP were added to the upper, lower, or both upper and lower parts of the modified Boydens Chamber. SNAP and SNP inhibited ( $P < 0.05$ ) AII-induced migration irrespective of chamber site to which they were added. Data are expressed as means±SEM. \* Significantly different from AII-treated group alone. SMC migration in response to 10<sup>-8</sup> M AII in different experiments was 23.3±4 (mean±SEM), 26±4.4, and 21.4±2.7, ×400 (HPF; 5 × 10<sup>4</sup> cells/filter).

10<sup>-10</sup> and 10<sup>-7</sup> M SNP inhibited AII-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 AII-induced SMC migration, and at a concentration of 10<sup>-7</sup> M, inhibited SMC migration by only 4±5% (NS).

The migration activity (percentage of control) in response to AII (10<sup>-6</sup> M), as well as inhibition of AII-induced migration by SNAP (10<sup>-10</sup> and 10<sup>-6</sup> 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 3. (A)** Effect of SNAP (10<sup>-8</sup> M) on 10<sup>-6</sup> M AII-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 AII-induced migration by SNAP; # Significant inhibition of migration ( $P < 0.05$ ) in absence of hemoglobin or superoxide dismutase. **(B)** Inhibitory effect of SNAP (10<sup>-6</sup> and 10<sup>-9</sup> M), freshly prepared and preincubated for 48 h, on AII (10<sup>-6</sup> 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 AII-induced SMC migration in absence of SNAP.



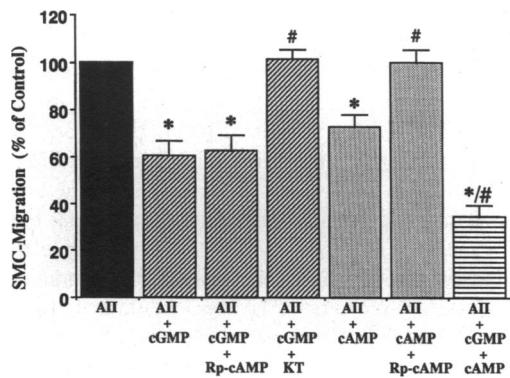
**Figure 4.** Bar graph showing the effects of AII (10<sup>-8</sup> M) on the migration of SMC-treated or not treated with interleukin-1β. SMCs were incubated for 24 h with or without interleukin-1β (4 ng/ml), and migration in response to AII was evaluated in the presence and absence of L-arginine (60 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 AII. AII-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 AII-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 AII-induced migration in both cases. \* Significant difference ( $P < 0.05$ ) from treatment with AII 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 AII-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<sup>-8</sup> M), PDGF-BB + SNAP (10<sup>-10</sup> M) and PDGF-BB + SNAP (10<sup>-7</sup> 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 AII-induced migration.

**Effect of smooth muscle cell-derived nitric oxide on angiotensin II-induced migration.** In contrast to untreated SMCs, AII-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 AII-induced migration was observed in the presence of D-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)-induced inhibition of AII-induced SMC migration in interleukin-1β-treated SMCs ( $P < 0.05$ ). AII-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, AII-induced migration was also inhibited by 59±15% in SMCs preincubated with forskolin to induce intracellular

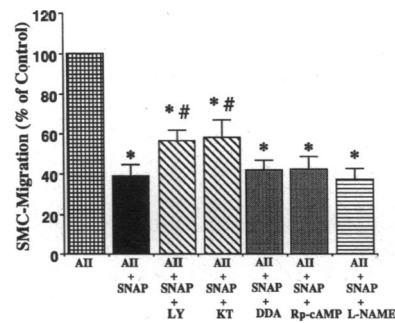


**Figure 5.** Effect of 8-bromo-cGMP (*cGMP*;  $10^{-5}$  M), 8-bromo-cAMP (*cAMP*;  $10^{-5}$  M) and 8-bromo-cGMP ( $10^{-5}$  M) + 8-bromo-cAMP ( $10^{-5}$  M) on AII ( $10^{-8}$  M)-induced SMC migration in presence and absence of Rp-cAMP ( $10 \mu\text{M}$ ) and KT ( $10 \mu\text{M}$ ). Results (mean $\pm$ SEM;  $n = 4$  experiments, each in replicates) are expressed as percentage of control (cells treated with AII [ $10^{-8}$  M] alone). The number of cells migrating in controls were  $21 \pm 3$ ,  $32 \pm 4$ ,  $40 \pm 5$ , and  $31 \pm 3.5$ ,  $\times 400$  (HPF;  $5 \times 10^4$  cells/filter). \* Significant inhibition of AII-induced migration (control;  $P < 0.05$ ); # Significantly different from 8-bromo-cAMP and 8-bromo-GMP induced-inhibition of AII-induced migration ( $P < 0.05$ ).

cAMP ( $P < 0.05$  vs control). Both 8-bromo-cGMP ( $10^{-5}$  M) and 8-bromo-cAMP ( $10^{-5}$  M) similarly inhibited AII ( $10^{-8}$  M)-induced SMC-migration ( $P < 0.05$ ; Fig. 5). Furthermore, combined treatment with 8-bromo-cGMP + 8-bromo-cAMP inhibited AII-induced migration in an additive fashion ( $P < 0.05$ ; Fig. 5). The inhibitory effects of 8-bromo-cAMP on AII-induced migration were blocked by Rp-cAMP ( $P < 0.01$ ; Fig. 5). Furthermore, the inhibitory effects of 8-bromo-cGMP on AII-induced migration were abrogated by KT5823 ( $P < 0.05$ ), but not by Rp-cAMP (Fig. 5), suggesting that 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 AII-induced migration.** Inhibition of  $10^{-8}$  M AII-induced SMC migration was partially, but significantly, reversed by the inhibitor of soluble guanylate cyclase LY 83583 ( $[34] 10 \mu\text{M}$ ) and the inhibitor of cGMP-dependent protein kinase KT5823 ( $[35] 10 \mu\text{M}$ ;  $P < 0.05$ ; Fig. 6). However, DDA ( $100 \mu\text{M}$ ; adenylate cyclase inhibitor [36]), Rp-cAMP ( $10 \mu\text{M}$ ; cAMP-dependent protein kinase inhibitor [37]) and L-NAME ( $200 \mu\text{M}$ ) did not modulate the inhibitory effects of SNAP on AII-induced migration (Fig. 6).

In contrast to untreated SMCs, AII-induced migration of SMCs pretreated with interleukin- $1\beta$  was inhibited in the presence of L-arginine ( $60 \mu\text{M}$ ; Fig. 7;  $P < 0.01$ ), an effect which was abrogated by L-NAME ( $200 \mu\text{M}$ ). L-arginine ( $60 \mu\text{M}$ ) induced inhibition of AII-induced SMC migration in interleukin- $1\beta$ -treated SMCs was partially, and significantly, reversed in the presence of Rp-cAMPs ( $10 \mu\text{M}$ ;  $P < 0.05$ ), LY 83583 ( $10 \mu\text{M}$ ;  $P < 0.05$ ) or KT5823 ( $10 \mu\text{M}$ ;  $P < 0.05$ ), but not in the presence of DDA ( $100 \mu\text{M}$ ; Fig. 7). The number of SMCs migrating in response to AII ( $10^{-8}$  M) in the presence of L-arginine $\pm$ DDA, Rp-cAMPs, LY 83583, or KT5823 was (percent of control $\pm$ SEM): 100% in the absence of L-arginine;  $43 \pm 5\%$  in presence of L-arginine ( $P < 0.05$  vs - L-arginine),



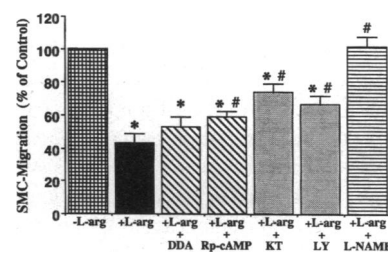
**Figure 6.** Effect of SNAP ( $10^{-7}$  M) on AII ( $10^{-8}$  M)-induced SMC-migration in the presence and absence of LY ( $10 \mu\text{M}$ ), KT ( $10 \mu\text{M}$ ), DDA ( $100 \mu\text{M}$ ), Rp-cAMP ( $10 \mu\text{M}$ ), or L-NAME ( $200 \mu\text{M}$ ). SNAP inhibited AII-induced SMC migration ( $P < 0.05$ ). Its inhibitory effect were partially reversed by LY ( $P < 0.05$ ), and KT ( $P < 0.05$ ), but not by DDA, Rp-cAMP, or L-NAME ( $P > 0.05$ ). Results are expressed as percentage of control, i.e., SMCs migrating in response to AII alone (mean $\pm$ SEM;  $n = 4$  experiments, each in replicates). The number of cells migrating in controls are same as in Fig. 6. \* Significantly different from SMC migration induced by AII (control;  $P < 0.05$ ). # Significantly different ( $P < 0.05$ ) from SMC-migration in presence of SNAP ( $10^{-7}$  M) + AII ( $10^{-8}$  M).

$53 \pm 6\%$  in presence of L-arginine + DDA (NS vs plus L-arginine);  $59 \pm 3\%$  in presence of L-arginine plus Rp-cAMP ( $P < 0.05$ );  $67 \pm 5\%$  in presence of L-arginine + LY 83583 ( $P < 0.05$ ) and  $74 \pm 5\%$  in presence of L-arginine + KT5823 ( $P < 0.05$ ; Fig. 7).

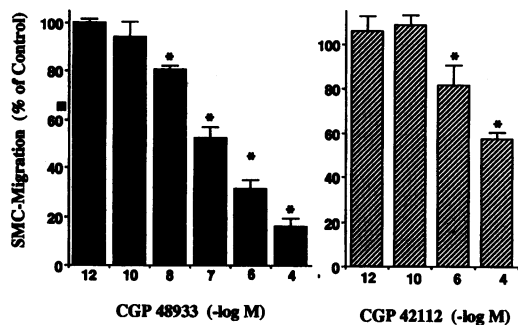
**Effects of  $AT_1$  and  $AT_2$  receptor antagonists on angiotensin II-induced migration.** The  $AT_1$ -receptor antagonist CGP 48933 inhibited AII ( $10^{-8}$  M)-induced SMC migration in a concentration-dependent manner (Fig. 8;  $P < 0.05$ ). Concentrations as low as  $10^{-8}$  M inhibited AII-induced migration by  $19 \pm 3\%$  ( $P < 0.05$ ). In contrast to CGP 48933, the  $AT_2$ -receptor antagonist CGP 42112 failed to inhibit AII-induced SMC migration at low concentrations ( $10^{-12}$  -  $10^{-7}$  M); however, at high concentration ( $10^{-6}$  and  $10^{-4}$  M) CGP42112 significantly inhibited AII-induced SMC migration by  $19 \pm 7$  and  $43 \pm 5\%$  respectively ( $P < 0.05$ ; Fig. 8).

## Discussion

The present study demonstrates that nitric oxide is a potent inhibitor of AII-induced migration. Both NO derived from nitro-



**Figure 7.** Bar graph showing the effects of L-arginine on AII ( $10^{-8}$  M)-induced migration of SMCs treated with IL- $1\beta$  for 24 h, in the presence and absence of DDA ( $100 \mu\text{M}$ ), Rp-cAMP ( $10 \mu\text{M}$ ), KT ( $10 \mu\text{M}$ ), LY ( $10 \mu\text{M}$ ), and L-NAME ( $200 \mu\text{M}$ ). Migration activity (mean $\pm$ SEM,  $n = 4$  experiments, each in duplicates) are expressed as percentage of control, i.e., IL- $1\beta$ -treated SMCs exposed to AII in absence of L-arginine. The number of cells migrating in controls were  $29 \pm 4$ ,  $37.5 \pm 5$ ,  $31 \pm 4$ , and  $27 \pm 4.4$ ,  $\times 400$  (HPF;  $5 \times 10^4$  cells/filter). \* Significantly different from the control (-L-arginine + AII;  $P < 0.05$ ); # Significant reversal of antimigratory effects of L-arginine ( $60 \mu\text{M}$ ) on AII-induced SMC-migration ( $P < 0.05$ ). Presence of L-arginine inhibited AII-induced SMC migration, and this inhibitory effect was partly, but significantly, reversed in presence of Rp-cAMP, KT, and LY ( $P < 0.05$ ). L-NAME completely reversed L-arginine-induced inhibition of AII-induced SMC migration ( $P < 0.05$ ).



**Figure 8.** Concentration–response relationship for the inhibition of AII ( $10^{-8}$  M)–induced SMC migration by AT<sub>1</sub> receptor antagonist CGP 48933 and AT<sub>2</sub> receptor antagonist CGP 42112. Results (mean±SEM) are expressed as percentage of control, where 100% is defined as migration in presence of AII alone ( $n = 3$  experiments, each in replicates). The migration in control experiments was  $31 \pm 3$  (mean±SEM),  $28 \pm 4$ , and  $32 \pm 3$ ,  $\times 400$  (HPF;  $5 \times 10^4$  cells/filter). Both, CGP 48933 and CGP 42112 significantly inhibited AII-induced migration in a concentration-dependent manner ( $P < 0.05$ ). As compared to CGP 48933, only high concentrations (nonspecific) of CGP 42112 inhibited AII-induced SMC migration.

vasodilators, as well as NO generated endogenously by SMCs stimulated with interleukin- $1\beta$ , inhibited AII-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, AII-induced SMC migration was inhibited by low concentrations of AT<sub>1</sub>-receptor antagonist CGP 48933 but not the AT<sub>2</sub>-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 AII 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 adventia (1). In the present study, migration was assessed by monitoring the movement of the cells towards AII (1, 42).

AII-induced migration in a concentration range ( $10^{-12}$  –  $10^{-10}$  M) much lower than that reported to induce SMC growth ( $10^{-8}$  –  $10^{-7}$  M [1, 2, 7, 8, 11, 26]). 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 AII levels. Although several AII receptor subtypes exist, AT<sub>1</sub> and AT<sub>2</sub> 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 AII on vascular smooth muscle cells are mediated through the AT<sub>1</sub> receptor subtype (20, 21), although the involvement of AT<sub>2</sub> receptor has also been suggested (22). However, the effects of these AT<sub>1</sub> and AT<sub>2</sub> antagonists on AII-induced SMC migration have not been reported. Our observation that low concentrations of the AT<sub>1</sub> receptor antagonist CGP 48933, but not of the AT<sub>2</sub> receptor antagonist CGP 42112 were able to inhibit AII induced SMC migration, suggests that AII induces SMC migration via AT<sub>1</sub> receptors. The weak inhibitory effect of CGP 42112 on migration at high concentrations is likely due to nonselective interactions with the AT<sub>1</sub>-receptor subtype (20, 29, 30).

Nitrovasodilators such as SNP generate NO spontaneously and by intracellularly catalyzed reactions, whereas SNAP releases NO spontaneously in aqueous media (24, 45). NO generated from SNAP inhibited AII-induced migration at very low concentrations ( $10^{-10}$  M). As compared to SNP, SNAP was more potent in inhibiting AII-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 ( $10^{-7}$  M) required to effectively inhibit migration was orders of magnitude lower than those ( $10^{-4}$  M) required to inhibit DNA synthesis in SMCs (7, 8). Similar to 8-bromo-cGMP, the concentration of 8-bromo-cAMP ( $10^{-5}$  M) which significantly inhibited AII-induced migration is one to two orders of magnitude lower than the concentration required to inhibit cell proliferation ( $10^{-4}$  –  $10^{-3}$  M [2, 46]). This is also in agreement with our unpublished observation that  $10^{-4}$  –  $10^{-3}$  M 8-bromo-cAMP, but not  $10^{-5}$  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 AII 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 AII were required to inhibit or induce migration, respectively, it is possible that even small changes in local AII 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 AII-induced migration by the nitrovasodilators was indeed due to NO generation. Direct effects of hemoglobin or superoxide dismutase can be excluded as AII-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 AII-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 AII-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 antimigratory effects of SNAP were partly reversed by the soluble guanylate 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 adenylate cyclase pathway) were unable to reverse the inhibitory effects of SNAP on AII-induced migration. 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 observations that increases in cGMP production in response to chemically derived NO are several magnitudes higher than the marginal 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 (mitochondrial) respiration,  $P_{O_2}$ , 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 studies were conducted in interleukin- $1\beta$  treated SMCs. Interleukin- $1\beta$  induces NO synthase in SMCs and causes a  $\geq 20$ -fold increase in NO release (41). In the presence of L-arginine, AII-induced migration was markedly inhibited in interleukin- $1\beta$  treated SMCs. Since in the absence of L-arginine AII-induced migration was similar in interleukin- $1\beta$ -treated and untreated cells, 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- $1\beta$  in the presence of L-arginine (60  $\mu$ M). 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 interleukin- $1\beta$  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-stimulated cells mediate its antimigratory effects via cAMP or cGMP pathways, functional studies were conducted using the inhibitors 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; however, 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- $1\beta$  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- $1\beta$  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- $1\beta$  (50). Hence, it is possible that in cytokine-treated SMCs, increases in cAMP, at least in part, contribute to the antimigratory effects.

In IL- $1\beta$ -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 KT5823 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 enzymes, which importantly regulate cell function, including the movement of the cells. Indeed, NO is known to inhibit mitochondrial 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 underlying 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-operated 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 endothelium 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

(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 AT<sub>1</sub> antagonist, cAMP, cGMP, as well as NO generated from cytokine treated SMCs or nitrovasodilators inhibit AII-induced SMC migration. AT<sub>1</sub> antagonist such as CGP 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.

## Acknowledgments

The authors would like to acknowledge the technical assistance of Delbert G. Gillespie.

This research work was supported from a grant from Swiss National research foundation (SNF# 32-325 41.91) and an educational grant of Ciba-Geigy, Basel/Switzerland. Dr. R. K. Dubey was a visiting scientist and was supported by a personal grant from the Roche Research Foundation, Basel/Switzerland; and by the National Institutes of Health grant (HL-35909 and HL-14192).

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