Central Role of the MAPK Pathway in Ang II–Mediated DNA Synthesis and Migration in Rat Vascular Smooth Muscle Cells

Xiao-Ping Xi, Kristof Graf, Stephan Goetze, Eckart Fleck, Willa A. Hsueh, Ronald E. Law

Abstract—Angiotensin II (Ang II) promotes vascular smooth muscle cell (VSMC) growth and migration, but the signaling pathways mediating these VSMC behaviors critical to restenosis and atherosclerosis are not completely known. The purpose of the present investigation was to define the role of mitogen-activated protein kinase (MAPK) in Ang II–induced DNA synthesis, migration, and c-fos induction in VSMCs. PD 98059, a synthetic inhibitor of MAPK kinase, or antisense oligodeoxynucleotides (ODNs) to deplete extracellular signal–regulated kinase (ERK)1 and ERK2 MAPKs, were used to inhibit MAPK signaling. PD 98059 at 30 μmol/L reduced Ang II–induced MAPK activity by 69% (P<0.01). Under these conditions, Ang II–induced DNA synthesis was completely inhibited (P<0.01), and Ang II–directed migration was attenuated by 76% (P<0.05). In contrast, induction of c-fos by Ang II was only partially suppressed (58% inhibition, P<0.01). Antisense ODNs against the initiation site of rat ERK1 and ERK2 MAPK mRNAs reduced corresponding protein levels by 63% (P<0.01) and completely inhibited MAPK activation by either Ang II (1 μmol/L) or 10% serum. Antisense ODNs (0.4 μmol/L) completely inhibited Ang II–induced DNA synthesis (P<0.01), decreased migration by 47% (P<0.01), and reduced c-fos induction by 40% (P<0.01 versus control ODN–transfected VSMCs). The Ang II type 1 (AT1)–receptor blocker irbesartan completely blocked DNA synthesis, migration, MAPK activation, and c-fos induction by Ang II in VSMCs. These results demonstrate that activation of MAPK plays a crucial role in Ang II–directed migration and DNA synthesis through the AT1 receptor. In contrast, Ang II–mediated c-fos induction and migration were only partially inhibited by either antisense ODNs or PD 98059, suggesting that other pathways in addition to the MAPK pathway may be involved in these actions of Ang II. We conclude that MAPK is a critical regulatory factor for Ang II–mediated migration and growth in VSMCs. Ang II–induced DNA synthesis showed a stronger MAPK dependence than did Ang II–directed migration or c-fos induction.

Key Words: vascular smooth muscle cells • migration • angiotensin II • DNA synthesis • mitogen-activated protein kinase

Angiotensin II (Ang II) is an important growth factor and chemoattractant for vascular smooth muscle cells (VSMCs). These effects implicate a role for Ang II in the development of restenosis, atherosclerosis, and hypertension. Although there are at least 4 Ang II receptor subtypes, the stimulation of growth and migration by Ang II in VSMCs is mediated by signal transduction through the Ang II type 1 (AT1) receptor. This receptor is a member of the superfamily of G protein–coupled receptors that contain 7 transmembrane helices. Signal transduction after activation of the AT1 receptor is complex and includes the following: (1) stimulation of phosphatidylinositol-specific phospholipase that generates inositol trisphosphate and diacylglycerol, (2) mobilization of intracellular calcium, (3) activation of protein kinase C (PKC), and (4) activation of the extracellular signal–regulated kinase (ERK)1 and ERK2 mitogen-activated protein kinase (MAPK) pathway. Identification of the functions associated with these pathways is important toward developing therapeutic strategies designed to mitigate Ang II’s damaging effects on the vasculature.

Activation of the MAPK pathway leading to the induction of the proto-oncogene c-fos and other early growth-response genes has emerged as a common theme for peptide growth factor signal transduction in a variety of cell lineages. MAPKs are serine-threonine protein kinases that transduce extracellular signals into the nucleus by phosphorylation and activation of transcription factors that regulate the expression of genes required for growth. More recently, we demon-
strated that the MAPK pathway is essential not only for basic fibroblast growth factor (bFGF)-induced growth of VSMCs\textsuperscript{10} but also for platelet-derived growth factor-BB (PDGF)-directed migration of VSMCs.\textsuperscript{11} Taken together, these studies suggest that MAPK represents a critical step in the regulation of both growth and migration of VSMCs. Therefore, a highly relevant issue was the role of MAPK in Ang II regulation of these processes. We used 2 independent approaches to inhibit the MAPK pathway: (1) a selective inhibitor of MAPK kinase (MEK), PD 98059, to prevent the phosphorylation and activation of MAPK\textsuperscript{12,13} and (2) antisense oligodeoxynucleotides (ODNs) to deplete cellular levels of the ERK1 and ERK2 MAPKs. Using either method, we were able to substantially diminish the growth and migration responses of VSMCs to Ang II. Irbesartan and losartan, 2 AT\textsubscript{1}-receptor blockers, completely suppressed Ang II–induced DNA synthesis and migration in VSMCs. These findings suggest that MAPK integrates the signals for both growth and migration through the AT\textsubscript{1} receptor and therefore constitutes an attractive target for future therapeutic intervention.

**Methods**

Materials were obtained from the following suppliers: Dulbecco’s modified Eagle’s medium (DMEM), glutamine, antibiotics, HEPES, dimethyl sulfoxide (DMSO), FBS, myelin basic protein, and monoclonal antibody against SM \( \alpha \)-actin from Sigma Chemical Co; Ang II from Bachem; culture plasticware from Becton-Dickinson; the Transwell chambers from Costar; and \([\text{P}^{32}]\text{ATP}\) from ICN. Sprague-Dawley rats were obtained from Charles River (Wilmington, Mass). Antibody against rat-ERK1 (K-23) that cross-reacts with ERK2 was obtained from Santa Cruz Biotech. Antibody against rat ERK2 that does not cross-react with ERK1 was obtained from Upstate Biotechnology. The MEK inhibitor PD 98059 was kindly provided by Dr Alan R. Saltiel (Parke-Davis, Ann Arbor, Mich). The AT\textsubscript{1}-receptor blocker irbesartan was a generous gift from Bristol Myers Squibb (Princeton, NJ). The AT\textsubscript{1}-receptor blocker losartan was a generous gift from Merck (West Point, Pa). The AT\textsubscript{1}-receptor blocker PD 123319 was provided by Parke-Davis. All compounds were dissolved in DMSO at 100 mmol/L. The institutional animal care and use committee approved the procedures involving animals.

**Cell Culture**

Rat aortic SMCs were prepared from thoracic aortas of 2- to 3-month-old Sprague-Dawley rats by the explant technique.\textsuperscript{14} The cells were cultured in DMEM containing 10% FBS, 150 mmol/L HEPES, 100 U/mL penicillin, 100 \( \mu \)g/mL streptomycin, and 200 mmol/L glucose. The purity and identity of the SMC cultures were verified by using a monoclonal antibody against SM \( \alpha \)-actin. Flow cytometry revealed purity of 96% to 98.5%. For all experiments, early-passaged (4 or less) rat VSMCs were grown to 60% to 70% confluence and made quiescent by serum starvation for at least 16 hours, when MAPK activity or DNA synthesis was assayed. When used, PD 98059, PD 123319, irbesartan, or losartan was added 30 minutes before the addition of Ang II. For all data shown, each experiment was performed in duplicate or triplicate and were repeated at least 3 times.

**Measurement of DNA Synthesis**

Incorporation of the thymidine analogue BrdU was measured to determine the effect of Ang II on DNA synthesis. VSMCs (2 to 4 passages) were placed in 96-well culture plates at a concentration of 10,000 cells/well. Cells were starved in serum-free medium (100 \( \mu \)L/well DMEM) for 16 to 32 hours. Cells were then preincubated with or without inhibitors for 30 minutes in serum-free medium before addition of Ang II. After 20 hours, 15 \( \mu \)mol/L BrdU was added to each well, and the cells were incubated for an additional 6 hours and then fixed. Quantification of BrdU incorporation was performed using a commercially available ELISA (Boehringer Mannheim).

**Liposomal Transfection With Antisense ODNs**

The antisense phosphorothioate-modified ODN was a 17-mer (\( 5'\text{-GCGGCGCggCggCgCcat-3'} \)) directed against the initiation of the translation start site of rat ERK1 and ERK2 mRNAs, which have the identical sequence at this site.\textsuperscript{15} These ODNs have been used successfully to downregulate MAPK expression in rat cardiac myocytes\textsuperscript{16} and in 3T3 cells.\textsuperscript{16} Sense (\( 5'\text{-AttgCggCggCgCggCggCgC-3'} \)) and scrambled (\( 5'\text{-CcggCggCgcCggCgcC-3'} \)) controls were used. All ODNs were synthesized at the Microsequencing Core Facility of the University of Southern California with the use of an automated DNA synthesizer (Applied Biosystems). ODNs were purified on OP cartridges (Applied Biosystems), dried down, and resuspended in sterile water.

Transfection with ODNs was performed in DMEM/serum-free medium with lipofectin at a final concentration of 10 \( \mu \)g/mL for 6 hours at 37°C. Medium was then replaced with lipofectin-free DMEM plus 1% FBS containing the original ODN concentration. Cells were then incubated for an additional 42 hours at 37°C before the addition of Ang II or 10% FBS to induce MAPK activity or DNA synthesis. For measurement of DNA synthesis, transfections were performed in 96-well plates at concentrations described above. For migration experiments, 100,000 cells were plated into the upper compartment of gelatin-coated Transwell chambers in DMEM +10% FBS 20 hours before transfection. VSMCs were transfected with ODNs for 48 hours, and migration was induced by addition of Ang II to the lower compartment.

**MAP In-Gel Kinase Assay**

MAPK activity was measured by the in-gel kinase assay as described elsewhere.\textsuperscript{17} The cell extracts (5 to 10 \( \mu \)g) were separated by SDS-polyacrylamide gel electrophoresis through a gel containing 0.4 mg/mL myelin basic protein as a substrate of MAPK. The SDS was then washed away, and the gel proteins were denatured, renatured, and phosphorylated in the gel with [\( ^{32} \text{P} \)]ATP. Autoradiograms were analyzed for quantification of MAPK activity by densitometric analysis using National Institutes of Health (NIH) Image 1.60 software for Macintosh personal computers.

**Migration**

Migration experiments were performed as described previously.\textsuperscript{10} VSMC migration was examined in Transwell cell culture chambers by using a gelatin-coated polycarbonate membrane with 8-\( \mu \)m pores. Preconfluent SMCs were suspended in DMEM–0.4% FBS to a concentration of 5.0 \( \times \)10\textsuperscript{5} cells/mL. Cells were pretreated with PD 98059, irbesartan, losartan, or vehicle for 30 minutes at 20°C. The MEK inhibitor PD 98059 and the AT\textsubscript{1}-receptor blockers irbesartan and losartan were added at the indicated concentrations to both the upper and lower compartments. DMEM–0.4% FBS (0.6 mL) was added to the upper compartment, and cells were then incubated at 37°C (95% air–5% CO\textsubscript{2}). Migration was induced by addition of Ang II at a final concentration of 1 \( \mu \)mol/L to the lower compartment. After 4 hours, the filters were fixed with methanol (10 minutes at 4°C), followed by counterstaining with hematoxylin. The number of VSMCs per 320\textsuperscript{2} high-power field that had migrated to the lower surface of the filters was determined microscopically. Four randomly chosen high-power fields were counted per filter. Experiments were performed in duplicate or triplicate and were repeated at least 3 times.

**Immunoblotting**

Cells were washed twice with ice-cold PBS and lysed in the same buffer as used in the in-gel kinase assay. Equal amounts of proteins (15 \( \mu \)g) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by using a Bio-Rad transblotter. Nonspecific binding was blocked by using 5% fat-free milk powder. Membranes were incubated with a rabbit polyclonal anti-ERK1 (1 \( \mu \)g/mL), which also recognizes ERK2, or a rabbit polyclonal anti-ERK2 antibody that does not cross-react with ERK1 for 1 hour in blocking solution. Blots were washed and incubated for
another hour with a goat anti-rabbit horseradish peroxidase–conjugated antibody (1:500, Amersham Life Science Inc) before final development with the ECL detection system (Amersham).

**Isolation and Analysis of RNA**
Total RNA was isolated from VSMCs by guanidinium isothiocyanate followed by phenol-chloroform extraction.17 RNA was size-fractionated by electrophoresis through a denaturing 1% agarose gel, transferred to nitrocellulose membranes, and hybridized with cDNA fractionated by electrophoresis through a denaturing 1% agarose gel, and hybridized with cDNA probes labeled with [32P]dCTP (3000 μCi/mmol) by random priming. The ERK cDNA probe was kindly provided by Dr Ellis Levine (University of California at Irvine School of Medicine). The cDNA for c-fos was from Dr Jill Norman (UCLA School of Medicine). The hybridization signals of the specific mRNAs of interest were normalized to those of Chinese hamster ovary clone B (CHOB) to correct for differences in loading or transfer. CHOB cDNA was originally isolated from CHO cells and corresponds to an mRNA ubiquitously expressed in mammalian tissues that does not exhibit regulation as a function of growth or development.18 Quantification of Northern blots was performed by densitometric analysis using NIH Image 1.60 software for Macintosh personal computers. Several autoradiographic film exposures (from 12 hours to 7 days) were used to ensure that the density of the signals was linear on each film.

**Statistics**
ANOVA and paired or unpaired t test were performed for statistical analysis, as appropriate. P values <0.05 were considered statistically significant. Data are expressed as mean±SEM.

**Results**

**Effect of the MEK Inhibitor PD 98059 on Ang II–Induced MAPK Activation, DNA Synthesis, and Migration**
PD 98059 inhibited Ang II–induced MAPK activity in rat VSMCs in a concentration-dependent manner (Figure 1A and 1B). Incubation with 1 μmol/L PD 98059 reduced Ang II–induced MAPK activation by 42% (P<0.01 versus Ang II alone), whereas 30 μmol/L PD 98059 reduced it by 69% (P<0.01 versus Ang II alone). Preincubation with irbesartan (100 μmol/L) completely prevented Ang II–induced MAPK activation (Figure 1C and 1D).

The effect of inhibiting MAPK activation on Ang II–induced DNA synthesis is shown in Figure 2A. Ang II at 1 μmol/L modestly increased DNA synthesis by ~30% (27.6±6.5%, P<0.05), relative to the basal DNA synthesis observed for quiescent VSMCs as measured by incorporation of the nucleotide analogue BrdU. Ang II–induced DNA synthesis was completely inhibited by 30 μmol/L PD 98059 (86.7±7.2% of quiescent VSMCs). Cells treated with 30 μmol/L PD 98059 alone also showed a statistically significant (P<0.05) reduction in DNA synthesis (20% to 25% inhibition) when compared with untreated, quiescent controls (data not shown). This effect of PD 98059 may reflect the partial MAPK dependence of the unknown pathways mediating basal DNA synthesis in serum-deprived VSMCs. Basal MAPK activity in quiescent VSMCs, however, was almost undetectable (Figure 1A and 1C), which argues against the importance of this pathway in basal DNA synthesis. These data, therefore, may indicate that PD 98059 targets another protein, in addition to the ERK MAPKs, involved in the pathways mediating basal DNA synthesis.

Ang II–stimulated DNA synthesis was mediated by signal transduction through the AT1 receptor, since it was completely suppressed when VSMCs were treated with the AT1-receptor antagonist irbesartan (93.5±5.6%, P<0.02 versus Ang II alone; Figure 2A). In contrast the AT2-receptor–specific antagonist PD 123319 had no effect on Ang II–induced DNA synthesis (Figure 2A). Losartan or irbesartan when present at 100 μmol/L had no statistically significant effect on basal DNA synthesis in quiescent, control VSMCs (data not shown).

PD 98059 also inhibited Ang II–stimulated migration of VSMCs (Figure 2B). Ang II increased VSMC migration...
2.3 ± 0.2-fold compared with unstimulated cells. Inhibition of the MAPK pathway by PD 98059 resulted in partial inhibition of migration at 1 μmol/L (35% inhibition) and 10 μmol/L (56% inhibition). At 30 μmol/L PD 98059, we observed a 76% inhibition of Ang II–directed migration (P < 0.05 versus Ang II alone; Figure 2B). Ang II–directed migration was inhibited in a dose-dependent manner by both irbesartan and losartan, 2 different AT1-receptor blockers (Figure 2C). When each blocker was added in 100-fold molar excess over Ang II, a near-complete inhibition of Ang II–directed migration was observed (irbesartan, 100 ± 6% inhibition, P < 0.05 versus Ang II alone; losartan, 91 ± 4% inhibition, P < 0.05 versus Ang II alone). Similar to our findings for Ang II–stimulated DNA synthesis (Figure 2A), the presence of the AT2-receptor blocker PD 123319 had no effect on Ang II–directed migration.

Depletion of MAPK by Antisense ODNs Against ERK1 and ERK2 mRNAs

Although several studies have detailed the high selectivity of PD 98059 for inhibiting activation of the MAPKs ERK1 and
ERK2 without a significant effect on a variety of other protein kinases important for signal transduction,12,13 we pursued an alternative strategy of inhibiting the MAPK pathway by transfecting VSMCs with ERK1 and ERK2 antisense ODNs. A representative Northern blot of RNA isolated from VSMCs that were transfected with an antisense ODN and a scrambled-sequence control ODN having a base composition identical to the antisense ODN (0.4 μmol/L) is shown in Figure 4. For both ERK1 and ERK2, mRNA levels were reduced after a 48-hour treatment with antisense ODNs when compared with cells treated with either sense or scrambled control ODNs. Decreased ERK1 and ERK2 mRNA levels in the presence of antisense ODNs are consistent with their degradation by an RNase H–based mechanism.19 A decrease in MAPK mRNA levels was observed at 0.4 μmol/L antisense ODN, whereas lower concentrations of this ODN did not have a consistent effect. When VSMCs were treated with any of the ODNs at concentrations >0.4 μmol/L, we frequently observed toxicity for each of the 3 tested sequences. All subsequent experiments, therefore, were performed at ODN concentrations of 0.4 μmol/L and lower.

The effect of MAPK antisense ODNs on MAPK protein levels is shown in Figure 5. VSMCs were transfected with antisense, sense, or scrambled ODNs in serum-free medium for 5 hours, after which transfected cells were refed with complete medium containing 1% FBS for another 42 hours. The presence of 1% FBS was required to protect against ODN-mediated cytotoxicity. Transfection with antisense ODN resulted in a concentration-dependent reduction of ERK1 and ERK2 protein levels, measured using either an antibody to ERK1 that recognizes both ERKs (Figure 5A, upper panel) or an antibody specific for ERK2 (Figure 5A, lower panel). VSMCs transfected with sense or scrambled control ODNs showed no changes in the amounts of ERK1 and ERK2 proteins (Figure 5A). Treatment with 0.4 μmol/L antisense ODNs reduced ERK1 and ERK2 levels by 61% when compared with cells treated with lipofectin alone (P<0.01) and by 64% when compared with scrambled ODN transfection (P<0.01). In all experiments, ERK1 protein levels exhibited a 10% to 20% greater decline than observed for ERK2 at 0.4 μmol/L antisense ODN.

The representative in-gel kinase assay presented in Figure 6A shows that both Ang II and serum induced MAPK activity; densitometric analysis demonstrated an ≈2-fold increase in activity (Figure 6B, P<0.01 versus control). Stimulating antisense-treated VSMCs with either Ang II or serum failed to appreciably increase MAPK activity above its basal, unstimulated level. Cells transfected with either sense or scrambled ODNs showed that a 2-fold increase in MAPK activity was induced by Ang II or serum. This 2-fold induction in MAPK activity is similar to that observed in lipofectin-treated cells that were not exposed to ODN (data not shown). The relatively high background of MAPK activity in controls (no ODN, no lipofectin) in Figure 6 compared with the low basal activity in Figure 1A is likely due to the fact that 1% FBS, instead of 0.4% FBS, was present during the serum-starvation step to limit cytotoxic effects in experimental groups treated with antisense, sense, or scrambled ODNs. Using either 0% or 0.4% FBS during starvation of ODN-treated VSMCs resulted in extensive cytotoxicity (data not shown). Immunoblotting of the extracts used for the in-gel kinase assay depicted in Figure 6A showed...
MAPK Regulates VSMC DNA Synthesis and Migration

MAPK Antisense ODNs Specifically Inhibit Ang II–Induced DNA Synthesis, Migration, and c-fos Induction in VSMCs

Ang II–induced DNA synthesis was completely inhibited in VSMCs treated with 0.1 or 0.4 μmol/L antisense ODNs for 48 hours before stimulation (Figure 7A). Basal DNA synthesis was also significantly inhibited by antisense ODNs to MAPK at both 0.1 and 0.4 μmol/L, suggesting that the pathways responsible for this activity are also MAPK dependent. Some of the basal DNA synthesis is likely due to pathways responsible for this activity are also MAPK dependent. Some of the basal DNA synthesis is likely due to stimulation by the presence of 1% serum in the assay. MAPK activation by serum stimulation was also severely impaired in antisense ODN–treated VSMCs (Figure 6). No significant effect on Ang II–induced DNA synthesis was observed in VSMCs transfected with 0.4 μmol/L sense ODN, scrambled ODN, or lipofectin alone (Figure 7A).

MAPK antisense ODN treatment also inhibited Ang II–mediated migration. The data in Figure 7B demonstrate a significant inhibition of Ang II–directed migration in cells treated for 48 hours with 0.1 or 0.4 μmol/L (inhibited by 29% compared with lipofectin-treated controls; P<0.05) and 0.4 μmol/L of MAPK antisense ODN (inhibited by 47% compared with lipofectin alone; P<0.01). VSMCs treated with 0.4 μmol/L of either scrambled or sense control ODNs migrated toward Ang II to the same extent as lipofectin-treated controls.

Induction of the early growth-response cellular proto-oncogene c-fos by Ang II in VSMCs is at least partially dependent on MAPK activation, as shown by experiments with the MEK inhibitor PD 98059 (Figure 3). In Figure 8A, a representative Northern blot shows that the strong induction of c-fos mRNA by Ang II at 30 minutes was inhibited in VSMCs treated with 0.4 μmol/L ERK1 antisense ODN for 48 hours. The presence of 1% serum during the starvation step did not result in a significant induction of c-fos expression in quiescent VSMCs. This result is in marked contrast to the high basal MAPK activity observed for quiescent cells in Figure 6A. Activation of MAPK, therefore, may be necessary but not sufficient for c-fos induction in these cells. Quantification of autoradiograms from 4 separate experiments revealed that depletion of MAPK levels by antisense ODN resulted in a 40% inhibition (P<0.05) of Ang II–induced c-fos expression (Figure 8B).

Discussion

The present study demonstrates for the first time that both Ang II–mediated DNA synthesis and migration are regulated by a common signaling pathway through the AT1 receptor, which involves the activation of MAPK. We have used the pharmacological inhibitor PD 98059 to selectively inhibit MAPK activation by Ang II. PD 98059 selectively inhibits MEK, a dual-specificity kinase, which activates MAPK by phosphorylation. Previous studies have demonstrated that PD 98059 has no activity against a variety of other signaling molecules, including p70 S6 kinase, phospholipase C, Raf kinase, cAMP-dependent kinase, PKC, v-Src, epidermal growth factor–receptor kinase, the PDGF-receptor kinase, and the phosphatidylinositol 3-kinase. At the highest concentration tested, PD 98059 inhibited Ang II–induced MAPK activation by 70% and completely suppressed Ang II–induced DNA synthesis. This finding is in accord with a recent study by Wilkie et al,21 who also observed that PD 98059 inhibited Ang II–induced DNA synthesis to a greater extent than MAPK in rat VSMCs.

Despite the apparent high selectivity of PD 98059 to inhibit the MAPK pathway, it is not possible to completely exclude its activity against other cellular targets. The lack of concordance in the inhibitor’s effects on Ang II–induced DNA synthesis and MAPK activation may reflect the presence of additional PD 98059–sensitive processes required for VSMC growth. Our
finding that PD 98059 inhibited basal DNA synthesis in quiescent VSMCs, which have no detectable MAPK activity, may be additional evidence that the MEK inhibitor is not completely specific for the MAPK pathway. We therefore used antisense ODNs to MAPK as an alternative approach to block that pathway. Antisense ODNs against the ERK1 and ERK2 MAPK mRNAs diminished corresponding protein levels by \( \approx 65\% \), which prevented significant MAPK activation in quiescent VSMCs stimulated with Ang II.

The antisense ODNs used in this study were 17-mers directed against the translation initiation site, which is conserved between both ERK1 and ERK2 mRNAs in the rat. More important, these ODNs have been used successfully by Glennon et al. in rat cardiac myocytes under conditions where sense or scrambled control ODNs did not affect MAPK levels. Migration of rat aortic VSMCs toward Ang II was inhibited after 48-hour transfection with antisense ODNs against ERK1 and ERK2 MAPK, whereas control ODNs or lipofectin treatment did not affect VSMC migration (n=6, mean±SEM expressed as % of Ang II–directed migration; \( \ast P<0.01 \) versus lipofectin-treated, Ang II–stimulated [lipofectin] cells).

Figure 7. A, MAPK antisense ODNs specifically inhibit Ang II (AII)–induced DNA synthesis as measured by BrdU incorporation. Ang II (1 \( \mu \text{mol/L} \)) significantly increased DNA synthesis (no lipofectin [Lipof]), measured by BrdU incorporation, which was not affected by lipofectin treatment alone. Antisense ODNs inhibited DNA synthesis in a concentration-dependent manner, whereas sense or scrambled ODNs did not affect BrdU incorporation (n=6, mean±SEM expressed as % of unstimulated cells: no Ang II, no lipofectin; \( \ast P<0.01 \) versus lipofectin). B, MAPK antisense ODNs specifically inhibit Ang II–induced migration.

Figure 8. MAPK antisense ODNs partially suppress Ang II (AII)–induced c-fos expression: Northern blot analysis showing the effect of antisense ODNs on Ang II–mediated c-fos mRNA induction. VSMCs were treated with 1 \( \mu\text{mol/L} \) Ang II for 30 minutes. A, Representative autoradiogram of a single experiment. The lower lane represents the signal for CHOB, an internal standard for the RNA loading (see Methods). Lipof indicates transfection with lipofectin alone. B, Densitometric analysis of Northern blots from 3 different experiments (mean±SEM; \( \ast P<0.05 \) versus Ang II alone). arb. U. indicates arbitrary units.

for 1 hour incorporated the ODN into both the nuclear and the cytoplasmic compartments. A similar high efficiency of transfection can be inferred from our results, which showed that MAPK protein levels were depleted by 65% in antisense ODN–treated VSMCs compared with sense and scrambled ODNs (Figure 5). It should be noted that lipofectin treatment of VSMCs consistently resulted in increased basal MAPK activity relative to quiescent cells that were not exposed to this agent (cf Figure 1A and Figure 6A). This result is likely due to the presence of 1% serum during the 48 hours of transfection that was necessary to minimize VSMC cytotoxicity resulting from ODN treatment. This high background MAPK activity may account for the surprising finding that neither Ang II nor serum further induced MAPK in antisense ODN–treated VSMCs; despite the presence of significant MAPK protein (\( \approx 35\% \) relative to sense and scrambled control). Alternatively, the inability of the remaining pool of MAPK proteins to be activated by Ang II or serum may reflect a nonspecific effect of antisense ODNs on an upstream component of the MAPK cascade.

Ang II induces VSMC growth by initiating signaling through the AT_1 receptor, which leads to increased intracellular \( \text{Ca}^{2+} \), activation of PKC and MAPK, and the transcriptional activation of c-fos. Activation of the transcription factor ETS-like-1 (ELK-1), after phosphorylation by MAPK at serine and threonine residues in its C-terminal transactivation domain, is important for transcription regulated through the serum-response element (SRE) in the c-fos promoter. This series of events is followed by an increase in DNA synthesis. From these studies a cause-and-effect relationship has been inferred between Ang II–induced MAPK
activation, c-fos induction, and DNA synthesis in VSMCs. To date, no study has directly tested this relationship by specifically inhibiting MAPK activation or by examining the effect on both Ang II–induced DNA synthesis and c-fos induction.

The present experiments suggest that blocking the MAPK pathway is sufficient to completely inhibit Ang II–induced DNA synthesis in VSMCs, despite significant residual induction of c-fos. Incomplete inhibition of c-fos induction after blocking the MAPK pathway is not a surprising result. After Ang II binds to the AT1 receptor, signal transduction diverges into multiple intracellular branch pathways. Activation of PKC1,2 and/or the Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) pathway25 by Ang II represents 2 additional signaling branches that can trigger c-fos transcription. Although recent studies have shown that PKC activation may precede and converge on the MAPK pathway through the phosphorylation of RAF or MEK,1,2,26 the JAK/STAT pathway appears to regulate c-fos transcription through a different regulatory element, the SIE (c-sis-inducible element).27 Because signaling through the JAK/STAT pathway utilizes both MAPK-dependent and -independent mechanisms,28 we did not expect that Ang II–induced c-fos expression would be completely suppressed by PD 98059 or MAPK antisense ODNs. Consistent with prior studies, we observed that blocking Ang II signaling with an AT1-receptor antagonist, such as irbesartan, completely suppressed c-fos induction by Ang II.

Partial inhibition of MAPK-dependent c-fos induction could block DNA synthesis if required threshold levels of c-fos could be provided only through combined signaling of MAPK to the SRE and of JAK/STAT to the SIE. The fact that c-fos knockout transgenic mice survive and have not been reported to exhibit vascular pathology indicates that c-fos induction is not obligatory for VSMC growth.29 Our data suggest that signaling through the MAPK pathway is necessary for the induction of DNA synthesis in rat VSMCs, but 2 recent reports suggest that MAPK activation alone is not sufficient to initiate DNA synthesis31; coactivation of both MAPK and the JAK/STAT pathways appears to be required.30 Other pathways, including phosphatidylinositol 3-kinase p70 S6 kinase,31 may also be involved in Ang II–mediated nuclear signaling to c-fos.

The second important finding in this study is the requirement of the MAPK pathway for Ang II–mediated migration of VSMCs. In contrast to Ang IIB–induced growth, very little is understood concerning the signaling events mediating Ang II–directed migration. Similar to the previous work of Dubey and colleagues,32 we found that Ang II stimulates VSMC migration through the AT1 receptor. The same authors also reported that cAMP analogues and agents that increase intracellular cAMP inhibited Ang II–induced VSMC migration.32 This observation is consistent with our finding that Ang II–induced migration is at least partially MAPK dependent, because several reports describe antagonistic behavior between the cAMP and MAPK pathways.

Our data do not conclusively establish whether chemotactic signaling through the AT1 receptor in VSMCs is exclusively through MAPK and does not in addition involve MAPK-independent pathways. As the data summarized in the Table show, PD 98059 at 30 μmol/L inhibited migration by 76%, which was correlated with a 69% inhibition of MAPK activity. Even very high concentrations of PD 98059, up to 50 μmol/L, did not consistently result in total inhibition of Ang II–induced MAPK activity (X.-P.X. et al, unpublished observations, 1998). Interestingly, the incomplete inhibition of MAPK activity achieved with 30 μmol/L PD 98059 was sufficient to completely block Ang II–stimulated DNA synthesis. In contrast to PD 98059, antisense ODNs to ERK1 and ERK2 prevented any significant increase in MAPK activity in Ang II–stimulated cells. Under these conditions, Ang II–directed migration was only partially inhibited, consistent with the presence of an AT1 receptor–mediated, MAPK-independent chemotactic signaling pathway in VSMCs. A caveat to this interpretation is the high basal MAPK activity present in ODN-treated VSMCs maintained in 1% FBS (Figure 6A). In these cells, the additional, modest, 2-fold induction of MAPK activity by Ang II may not have been rate limiting for transduction of a chemotactic signal, thereby resulting in only partial inhibition of Ang II–directed migration. In support of this interpretation, MAPK antisense ODNs were also less effective than PD 98059 in inhibiting Ang II–induced c-fos expression (40% versus 58% inhibition, the Table). Additional studies are required to determine whether other signaling proteins in the phosphatidylinositol 3-kinase or PKC family are important for Ang II–regulated VSMC migration.

We have also recently shown that PDGF-directed migration of human and rat VSMCs is MAPK dependent.31 Our finding that MAPK is critical for VSMC migration is in contrast to similar studies in fibroblasts, in which phosphatidylinositol 3-kinase α7 kinase may also be involved in Ang II–mediated nuclear signaling to c-fos.

Ang II is a relatively weak mitogen and chemoattractant for VSMCs in comparison with other growth factors, such as PDGF and bFGF. Moreover, the concentrations of Ang II required to elicit VSMC responses in vitro are often not physiological, frequently, 100 nmol/L or 1 μmol/L.21,36–39 Ang II, however, may play a more prominent role in vivo to promote vascular lesion formation. Prolonged infusion of Ang II stimulates VSMC DNA synthesis and increases neointimal cross-sectional area, which is mediated through the AT1 receptor.40,41 Prominent vascular effects of Ang II may result from synergism between the AT1 receptor and other growth factor receptor–signaling pathways. For example, mitogenic signaling pathways in VSMCs triggered by oleic acid or Ang II have been recently shown to interact synergistically through an MAPK-dependent mechanism.22

### Table: Effect of Inhibiting the MAPK Pathway on Ang II Signaling in VSMCs

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<th>Maximum Observed Inhibition, %</th>
<th>PD 98059</th>
<th>ERK 1/2 Antisense ODN</th>
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<td>MAPK activity</td>
<td>69</td>
<td>100</td>
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<tr>
<td>DNA synthesis</td>
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<tr>
<td>Migration</td>
<td>76</td>
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<tr>
<td>c-fos induction</td>
<td>58</td>
<td>40</td>
</tr>
</tbody>
</table>
Strategies targeting the MAPK pathway, therefore, may permit the development of new therapeutic agents for the treatment of vascular diseases. Restenosis and atherosclerosis are vascular diseases that may result in part from dysregulated VSMC growth and migration induced by various stimuli. Enhanced VSMC migration and the induction of hypertrophy and proliferation are important mechanisms that contribute to the development of vascular lesions in peripheral and coronary artery disease. It has been shown that angiotensins-converting enzyme inhibitors and AT1-receptor blockers prevent neointima formation in the rat after balloon injury, which underscores the role of the renin-angiotensin system in the regulation of vascular remodeling events. Studies in other species, however, ended with diverse results. Although trials using angiotensin-converting enzyme inhibitors failed to prevent restenosis after coronary angioplasty treatment of vascular diseases. Restenosis and atherosclerosis are vascular diseases that may result in part from dysregulation of cardiovascular disease in humans. These contrary results are likely due in part to the action of other growth factors besides Ang II, such as PDGF or bFGF, in vascular remodeling. Thus, targeting the critical signaling steps common to the action of multiple growth factors could be more useful than inhibition of a single growth factor pathway. We recently demonstrated that the antidiabetic drug troglitazone, which also targets the MAPK pathway, prevents neointima formation in rat aortas after balloon injury by inhibiting growth factor–induced proliferation and migration. The present findings further underscore the important and central role of the MAPK pathway in regulating cellular mechanisms that lead to vascular remodeling.

Acknowledgments

This study was supported by a research grant from the National Heart, Lung, and Blood Institute, NIH, Bethesda, Md (RO1 HL58328), to Willa A. Hsueh. The authors wish to thank Dolores Mendoza and Janie Teran for their secretarial contributions.

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Central Role of the MAPK Pathway in Ang II–Mediated DNA Synthesis and Migration in Rat Vascular Smooth Muscle Cells
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*Arterioscler Thromb Vasc Biol.* 1999;19:73-82
doi: 10.1161/01.ATV.19.1.73

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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