Src Is an Important Mediator of Extracellular Signal–Regulated Kinase 1/2–Dependent Growth Signaling by Angiotensin II in Smooth Muscle Cells From Resistance Arteries of Hypertensive Patients

Rhian M. Touyz, Gang He, Xiao-Hua Wu, Jeong Bae Park, Mohammed El Mabrouk, Ernesto L. Schiffrin

Abstract—The role of c-Src in growth signaling by angiotensin (Ang) II was investigated in vascular smooth muscle cells (VSMCs) from arteries of hypertensive patients. c-Src and extracellular signal–regulated kinase 1/2 (ERK1/2) activity, proto-oncogene expression, activating protein-1 (AP-1) DNA-binding activity, and DNA and protein synthesis were studied in Ang II–stimulated VSMCs derived from small peripheral resistance arteries of normotensive subjects (NTs, n=5) and age-matched untreated hypertensive patients (HTs, n=10). Ang II type 1 (AT₁) and type 2 (AT₂) receptor status was also assessed. Ang II dose-dependently increased the synthesis of DNA and protein, with enhanced effects in VSMCs from HTs. PD 098,059, a selective inhibitor of the ERK1/2 pathway, attenuated Ang II–stimulated growth in HTs. The effects of PD 098,059 were greater in HTs than in NTs. In NTs, Ang II transiently increased ERK1/2 phosphorylation, whereas in HTs, Ang II–stimulated actions were augmented and sustained. PP2, a selective Src inhibitor, reduced ERK1/2 activity and normalized ERK1/2 responses in HTs. Ang II–induced c-Src phosphorylation was 2- to 3-fold greater in HTs than in NTs. In HTs but not NTs, kinase activation was followed by overexpression of c-fos and enhanced AP-1 DNA-binding activity. PD 098,059 and PP2 attenuated these responses. AT₁ receptor expression was similar in NTs and HTs. In HT cells transfected with c-fos antisense oligodeoxynucleotide, Ang II–stimulated growth was reduced compared with sense oligodeoxynucleotide. Our findings suggest that augmented Ang II–stimulated VSMC growth is mediated via hyperactivation of c-Src–regulated ERK1/2-dependent pathways, leading to overexpression of c-fos mRNA and enhanced AP-1 DNA-binding activity. Because AT₁ receptor expression was unaltered in HTs, increased Ang II signaling may be a postreceptor phenomenon. These data define a signal transduction pathway whereby Ang II mediates exaggerated growth in VSMCs from HTs. (Hypertension. 2001;38:56-64.)

Key Words: receptors, angiotensin • signal transduction • arteries, resistance • hypertension, essential

Essential hypertension is characterized by abnormally increased peripheral vascular resistance, which is due in large part to functional and structural alterations of resistance arteries.1,2 Vascular structural changes in hypertension (vascular remodeling) include a smaller lumen and an increased media to lumen ratio and are due to rearrangement of existing tissue around a smaller lumen (eutrophic remodeling) and/or to growth-related processes with an increased cell number (hyperplasia or proliferation) and increased cell size (hypertrophy [hypertrophic remodeling]).1,2 Vascular remodeling in hypertension has an important angiotensin (Ang) II–dependent component, as evidenced by studies demonstrating that treatment of hypertensive patients with ACE inhibitors or Ang II type 1 (AT₁) receptor antagonists corrects small artery structure and endothelial dysfunction.3–5 The trophic actions of Ang II appear to be direct and independent of hemodynamic changes.5,6 Ang II, originally described as a pressor agent, is now considered an important growth factor for vascular smooth muscle cells (VSMCs). In cultured rat aortic cells, Ang II acts primarily as a hypertrophic agent,7,8 whereas in human VSMCs, it induces both hyperplasia and hypertrophy.9–11 Growth effects of Ang II may be direct or indirect through transactivation of growth factor receptors, such as the epidermal growth factor receptor and the platelet-derived growth factor receptor, or via increased production of other vasoactive agents and growth factors.12 Many signal transduction pathways mediate Ang II growth actions, including the activation of Janus kinase (which phosphorylates and activates signal transducers and activators of transcription factors) and the stimulation of the mitogen-activated protein (MAP) kinase pathway.13–15 Multiple mammalian MAP kinases have been identified, of which the extracellular signal–regulated kinase (ERK) cascade is the best characterized.16

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Ang II–activated ERK1/2 is responsible for the induction of early growth response genes, whose family includes the proto-oncogenes c-fos, c-jun, and c-myc.17,18 Protein products of these genes are involved in signaling cascades resulting in the growth response. Dysregulation of Ang II–mediated ERK1/2 signal transduction contributes to pathological cellular growth processes underlying vascular remodeling in experimental hypertension. Glomerular MAP kinase activity and c-fos gene expression are enhanced in Ang II–induced hypertension,19,20 and in spontaneously hypertensive rats (SHR), VSMC growth and ERK activity are increased in vivo and in vitro.21–23

Proximal mediators of vascular ERK activation by Ang II have not been fully elucidated, and mechanisms responsible for ERK1/2 dysregulation in hypertension are unclear. We propose that Src family nonreceptor tyrosine kinases are important upstream regulators of ERK1/2 and that alterations in c-Src activation could underlie abnormal growth signaling by Ang II in hypertension. This is based on findings that c-Src is expressed in VSMCs and that the Src family of proteins tyrosine kinases that characteristically interact with transmembrane tyrosine kinase receptors also interact functionally with AT1 receptors.24,25 Downstream targets of c-Src include p21ras, which activates MAP kinase kinase 1/2 (MEK1/2), which in turn phosphorylates ERK.25 Src also modulates cytosolic Ca2+, which is important in ERK1/2 hyperactivation in SHR.22,26,27 Many Src-related kinases have been identified, of which the 60-kDa c-Src is the prototype.25

To our knowledge, there are no data regarding the role of Src in Ang II–mediated ERK1/2 signaling in human essential hypertension. In the present study, we report the novel findings that augmented growth of VSMCs from small arteries of hypertensive patients is associated with increased c-Src–dependent ERK1/2-activated signaling pathways that enhance c-fos but not c-myc or c-jun mRNA expression. Furthermore, by use of cells transfected with c-fos antisense oligodeoxyribonucleotides (ODNs), it has been demonstrated that ERK1/2-induced overexpression of the c-fos gene is essential for exaggerated growth responses. AT1 receptor status was unaltered in hypertensive patients, suggesting that augmented Ang II signaling in hypertension may be a postreceptor phenomenon. Our data define a signal transduction pathway whereby Ang II–stimulated VSMCs could induce the DNA and protein synthesis required for cell growth and vascular remodeling in hypertension.

**Methods**

**Subjects and Patients**

Normotensive subjects and patients with untreated essential hypertension (age 30 to 65 years) were recruited at the Hypertension Clinic of the Clinical Research Institute of Montreal (IRCM). The study was approved by the IRCM Ethics Committee. All subjects gave written consent. Control subjects had blood pressure (BP) <140/85 mm Hg. BP was measured on at least 6 occasions, and patients were diagnosed as hypertensive if recumbent diastolic BP was >90 mm Hg on at least 3 occasions. Clinic sitting BP was measured after 15 minutes of rest, and daytime ambulatory BP was recorded for 12 hours with a model 90207 Spacelabs recorder. Exclusion criteria included smoking >5 cigarettes/day, abnormal fasting blood glucose, serum creatinine >150 μmol/L, and systemic diseases. Essential hypertension was diagnosed by the absence of secondary hypertension and normal serum electrolytes, urinalysis, and abdominal echogram.

Figure 1. Ang II effects on [3H]thymidine and [3H]leucine incorporation. In some experiments, cells were cotreated with PD 098,059 (10−5 mol/L). Bar graphs demonstrate the difference between Ang II–induced and Ang II + PD 098,059–induced [3H]thymidine and [3H]leucine incorporation. Results are mean±SEM. Experiments were performed 5 to 10 times in triplicate. ∗P<0.05 and ∗∗P<0.01 vs normotensive group; †P<0.05 and ††P<0.01 vs Ang II counterpart; and ‡P<0.05 and ‡‡P<0.01.
Cell Culture

Gluteal biopsies of subcutaneous fat of 1.0-3.0 cm³ were obtained under local anesthetic. Arteries were microdissected immediately after the biopsy was performed. Vessels were mounted on a myograph, and morphometric parameters were measured as previously described. Arteries with diameters <300 μm (corresponding to resistance arteries) were used for culture. VSMCs were isolated and cultured as described. Purity of cell culture was confirmed immunocytochemically. Experiments were performed by using cells between passages 2 and 6.

Determination of DNA and Protein Synthesis

DNA synthesis, evaluated by measuring [3H]thymidine incorporation, was considered a marker of hyperplasia, and protein synthesis, determined by measuring [3H]leucine incorporation, was considered a marker of hypertrophy, as we previously described. Quiescent cells were stimulated for 30 hours with Ang II (10⁻¹² to 10⁻⁵ mol/L) in the absence and presence of the selective MEK1/2 inhibitor, PD 098,059 (10⁻⁵ mol/L). Western Blotting

Cells were stimulated with Ang II in the absence and presence of PD 098,059, PP2 (a selective Src inhibitor), losartan (10⁻⁵ mol/L), or PD 123,319 (10⁻⁵ mol/L). Cells were prepared for Western blot analysis as described. ERK1/2 phosphorylation was detected with a phospho-specific ERK antibody (1:1000, New England Biolabs Inc), c-Src phosphorylation was determined with an anti-c-Src antibody (1:750) that recognizes the autophosphorylation site of pp60c-Src (Upstate Biotechnology), c-fos was assessed with a polyclonal anti-c-fos antibody (1:1000, Santa Cruz Biotechnology Inc), and AT₁ receptor expression was detected with a polyclonal AT₁ antibody (1:7000, Chemicon Inc). Immune complexes were visualized by use of enhanced chemiluminescence.

Immunoprecipitation and c-Src Immune Complex Kinase Assay

Immunoprecipitation of c-Src and the kinase assay were based on methods described by Ishida et al. For immunoprecipitation, 300 to 500 μg cellular protein was incubated overnight at 4°C with 2 μg monoclonal anti-mouse Src antibody (clone 327, Calbiochem). Antibody complexes were collected by incubation with Protein G Plus-Agarose beads (Santa Cruz Biotechnology Inc), and immunoprecipitates were collected after centrifugation. Precipitates were washed and collected by centrifugation and then suspended in kinase reaction buffer containing 50 μmol/L ATP in the absence or presence of acid-denatured rabbit muscle enolase (25 to 50 μg). The kinase reaction was initiated by adding 10 μCi of [γ-³²P]ATP (specific activity 3000 mCi/mmol) and terminated after 10 minutes by addition of SDS-PAGE buffer. Samples were boiled for 5 minutes and subjected to SDS-PAGE (8%). Gels were dried and exposed, and radioactive bands were quantified by use of the Image Quant program.

Reverse Transcription–Polymerase Chain Reaction

Expression of AT₁, and Ang II type 2 (AT₂) receptors and of c-fos, c-myc, and c-jun mRNA was measured by reverse transcription (RT)–polymerase chain reaction (PCR) as we previously described. For amplification of AT₁ receptor cDNA, the sense primer 5'-GTAGC CAAAG TCACC TGCAT-3' and the antisense primer 3'-TATCG AATAA AATTG TTAAC GGACT-5' were used. For amplification of AT₂ receptor cDNA, the sense primer was 5'-ACCTG CATGA GTGTT GATAGG-3', and the antisense primer was 3'-ACTTCA ATATC GTCAGT AACTGGAC-5'. For c-fos cDNA amplification, the sense primer 5'-GGGAA CCAAG TCACC TGCAT-3' and the antisense primer 3'-TACTC GTCAGT AACTGGAC-5' were used. For c-myc cDNA amplification, the sense primer 5'-CTGAA GGAGA AGGAA AAACT AGA-3' and the antisense primer was 3'-TTAAC AATTG TTAAC GGACT-5'. c-jun cDNA

Figure 2. Effects of PP2 on Ang II–induced ERK1/2 phosphorylation. Top panel, Representative Western blots. p-Erk1 and p-Erk2 indicate phosphorylated ERK1 and ERK2, respectively. Bottom panel, Time course of ERK1/2 phosphorylation. Results are mean±SEM of 4 to 6 experiments. *P<0.05 vs normotensive, Ang II alone; **P<0.01 vs other groups.
amplification, the sense primer was 5'-CAAGT GCCGA AAAAG GAAG-3', and the antisense primer was 3'-GTCGT CAACG TTTGT AAAAC-5'. For GAPDH, the sense primer was 5'-GCCAA AAGGG TCTATC ATCT-3', and the antisense primer was 3'-TGTCA GGTAC GGTAG TGACG-5'. The amplification protocol involved denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds for 30 cycles. Under these conditions, the reaction occurred in the linear phase. After amplification, PCR products were electrophoresed (1.5% agarose gel). Bands corresponding to RT-PCR products were visualized by UV light, and intensities were measured densitometrically.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from VSMCs according to previously described methods. The probe (containing an activating protein-1 [AP-1] binding site) was made by annealing 2 single-stranded DNA probes: 5'-CGCTTGATGACTCAGCCGGAAC-3' and 5'-CGAGTTCCGGCTGAGTCATCAAGCG-3'. The labeling was carried out in 10 μL of 1× T4 PNK buffer (GIBCO-BRL) containing 1 pmol cold probe, 13 μCi of [γ-32p]ATP, and 20 U T4 polynucleotide kinase at 37°C for 10 minutes. Under these conditions, the reaction occurred in the linear phase. After amplification, PCR products were electrophoresed (1.5% agarose gel). Bands corresponding to RT-PCR products were visualized by UV light, and intensities were measured densitometrically.

Liposomal Transfection With c-fos Antisense Oligonucleotide

Cells from hypertensive patients were used for transfection studies. The antisense phosphorothioated ODN (5'-CCGAGAACATCATCGTGGCG-3') was directed against the translation initiation site of c-fos mRNA. Corresponding sense ODN (5'-CGCAGCACTGATCTTCTCGG-3') was used as a control. Transfection was performed with 50 nmol/L ODN performed in serum-free antibiotic-free DMEM with 3 μg/mL Lipofectamine reagent (GIBCO-BRL) for 5 hours at 37°C in 5% CO2/95% air. Culture medium was replaced with Lipofectamine-free DMEM, and VSMCs were cultured for 30 to 36 hours. DNA synthesis and c-fos expression were measured.
Data Analysis

Ang II–stimulated effects were determined as the percent increase over control, with the control normalized to 100%. Each experiment was performed at least 4 times. Results are presented as mean ± SEM and compared by ANOVA or by the Student’s t test where appropriate. The Tukey-Kramer correction was used to compensate for multiple testing. A value of *P*, 0.05 was considered to be significant.

Results

Patient Characteristics and Vascular Morphology

BP was significantly higher (*P*, 0.01) in the hypertensive group (clinic BP 136 ± 3/96 ± 1.5 mm Hg, ambulatory BP 141 ± 2/94 ± 1.3 mm Hg) than in the normotensive group (clinic BP 121 ± 3/79 ± 0.9 mm Hg). The media/lumen ratio was increased (*P*, 0.01) in arteries from hypertensive patients (8.1 ± 0.36%) compared with arteries from normotensive subjects (6.0 ± 0.5%).

Effects of Ang II on DNA and Protein Synthesis

Ang II dose-dependently increased [3H]thymidine and [3H]leucine incorporation, with significantly enhanced responses (*P*, <0.01) in cells from hypertensive patients (Figure 1). Treatment of VSMCs with the selective MEK1/2 inhibitor, PD 098,059, did not alter basal [3H]thymidine or [3H]leucine incorporation. However, PD 098,059 attenuated Ang II–stimulated DNA and protein synthesis in the control group and normalized responses in the hypertensive group (Figure 1), indicating that Ang II–stimulated growth is mediated in large part via ERK-dependent signaling pathways and that the effect of ERK1/2 inhibition is greater in cells from hypertensive patients than from normotensive subjects.

Effect of Ang II on Phosphorylation of ERK and c-Src

ERK1/2 protein expression was not different in cells from normotensive and hypertensive individuals (data not shown). However, ERK1/2 phosphorylation, which was dose-dependently increased by Ang II, was significantly augmented in cells from hypertensive compared with normotensive individuals (Figures 2 and 3). Both the magnitude of effect and the duration of activation were greater in the hypertensive group. At 10 minutes, ERK1/2 was almost completely dephosphorylated in the normotensive subjects, whereas in the hypertensive patients it remained significantly phosphorylated and was still elevated 30 minutes after stimulation. PD 098,059 did not alter basal ERK phosphorylation but significantly inhibited Ang II–stimulated effects (Figure 3). Losartan, but not PD 123,319, reduced Ang II–induced ERK activation. ERK1/2 phosphorylation was attenuated by the selective Src inhibitor, PP2 (Figure 2). The effect was greater in VSMCs from hypertensive patients (Figure 2), indicating that Src modulates ERK1/2 activity and that Src dysregulation may underlie ERK1/2 hyperactivation in hypertension. To further examine vascular Src status, Ang II–induced phosphorylation was assessed with an antibody that recognizes the autophosphorylation site of c-Src, and kinase activity was assessed by measuring enolase phosphorylation. Ang II rapidly increased c-Src phosphorylation and activity in both groups (Figure 4, Table 1). Responses in the
control group peaked at 30 seconds and reached prestimulated levels by 5 minutes. In the hypertensive group, maximal effects were observed at 1 minute and were sustained for up to 10 minutes (Figure 4).

**Effects of Ang II on Proto-oncogene Expression and AP-1 DNA-Binding Activity**

Ang II increased c-fos mRNA with significantly greater effects \( (P<0.01) \) in hypertensive patients than in normal subjects (Figure 5). Expression of c-fos was higher in hypertensive patients \( (249 \pm 15\%) \) than in normotensive subjects \( (152 \pm 17\%) \). Ang II–induced c-myc gene expression was not significantly different in normotensive and hypertensive individuals, and c-jun mRNA expression was only modestly increased by Ang II in both groups (Figure 5). PD 098,059 reduced Ang II–induced proto-oncogene expression, particularly in the hypertensive group. Having demonstrated the important role of c-fos in Ang II signaling in hypertension, we investigated in greater detail the downstream effects of c-fos by assessing AP-1 binding activity with the use of nuclear extracts from hypertensive patients. AP-1 DNA binding activity was markedly increased after 60 minutes of stimulation (Figure 6). The reaction was specific, inasmuch as the addition of an excess amount of cold AP-1 but not the cold nonspecific ODN abolished the signal. AP-1 is a sequence-specific transcriptional activator composed of jun and fos subunits. To determine whether the binding complex contained c-fos and c-jun, a supershift assay was performed. Incubation with antibodies to c-fos and c-jun elicited a supershift complex, suggesting the presence of these proteins in the binding complex (Figure 6, bottom panel).

**Effects of c-fos Antisense ODN on DNA Synthesis and c-fos Expression**

To investigate the role of increased c-fos expression in Ang II–mediated growth responses in hypertension, we examined the effects of c-fos antisense ODN on DNA synthesis. Treatment with antisense c-fos ODN significantly reduced \( (P<0.05) \) agonist-stimulated c-fos protein expression and \( [3\text{H}] \)thymidine incorporation (Figure 7). Sense ODN did not alter Ang II–mediated actions (Figure 7).

**AT\(_1\) and AT\(_2\) Receptor Expression**

To verify whether altered Ang II signaling is due to changes in Ang II receptor status, we determined the expression of AT\(_1\) and AT\(_2\) receptors at the mRNA and protein levels, in unstimulated cells, and in cells exposed to Ang II \( (10^{-7} \text{ mol/L}) \). Expression of AT\(_2\) receptors was undetectable in unstimulated and stimulated cells from both groups of subjects. AT\(_1\) receptor expression was similar in cells from normotensive subjects and hypertensive patients, and Ang II treatment did not influence mRNA or protein expression in either group (Table 2).

**Discussion**

VSMC growth is associated with numerous vascular diseases, including medial hypertrophy in hypertension. Among the

### Table 1. Phosphorylation of Enolase by Ang II (10\(^{-7}\) mol/L) in VSMCs From Normotensive and Hypertensive Subjects

<table>
<thead>
<tr>
<th>Time of Stimulation</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
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<tbody>
<tr>
<td>30 s</td>
<td>135(\pm)10%</td>
<td>239(\pm)17%*</td>
</tr>
<tr>
<td>1 min</td>
<td>159(\pm)15%</td>
<td>288(\pm)25%†</td>
</tr>
<tr>
<td>5 min</td>
<td>152(\pm)29%</td>
<td>277(\pm)23%*</td>
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Values are expressed as percentage of control and are mean\(\pm\)SEM.

*\(P<0.05\) and †\(P<0.01\) vs normotensive counterpart.

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![Figure 5](image-url) RT-PCR analysis of c-fos, c-myc, and c-jun mRNA expression. Cells were treated with Ang II \( (10^{-7} \text{ mol/L}) \) with or without Los or PD123. Results are mean\(\pm\)SEM of 6 to 9 experiments. C(−) indicates negative control. *\(P<0.05\) and **\(P<0.01\) vs control (C); + +\(P<0.01\) vs Ang II counterpart (30 minutes).
many humoral factors contributing to the hypertrophic response, Ang II seems to be one of the most important. Molecular mechanisms whereby Ang II modulates cell growth in hypertension have not been fully elucidated, but the present study demonstrates that dysregulation of Src-dependent growth-signaling events is important. Although one cannot directly extrapolate findings obtained in in vitro conditions to in vivo situations, examination of cultured cell preparations provides information on cell behavior and regulation, which would otherwise be impossible to investigate. Furthermore, isolated cells allow for dissection and investigation of subcellular signaling pathways controlling biological cellular responses that may be functional in intact tissue. We demonstrate that cells derived from peripheral resistance arteries from hypertensive patients exhibit potentiated growth responses to Ang II, and we report the novel findings that mechanisms underlying these effects are dependent on enhanced activation of Src-dependent ERK-mediated signaling pathways. Upregulation of these kinases is associated with increased proto-oncogene expression, particularly c-fos, and enhanced AP-1 DNA-binding activity. Our findings demonstrate a signal transduction pathway through which activated VSMCs could contribute to structural changes in hypertension.

Enhanced growth-promoting effects of Ang II have been reported in cardiomyocytes and VSMCs from SHR.\textsuperscript{33,34} To our knowledge, the present study demonstrates for the first time that Ang II–induced [\textsuperscript{3}H]thymidine and [\textsuperscript{3}H]leucine incorporation are increased in VSMCs from resistance arteries of hypertensive patients. Inhibition of the ERK1/2 pathway by PD 098,059 decreased cell growth and normalized responses in the hypertensive group, indicating a pivotal role for ERK1/2 in VSMC growth in hypertension. Data from experimental models support our findings. In SHR, aortic and renal ERK1/2 phosphorylation is increased, and in acute hypertension, both ERK1/2 and c-Jun N-terminal kinase are transiently activated.\textsuperscript{19,20} Lucchesi et al.\textsuperscript{22} described a hypertensive signal transduction phenotype in VSMCs from SHR,

\[ \text{Figure 6. Analysis of AP-1 binding activity in nuclear extracts from cells from hypertensive patients. Top and middle panels, Representative gel mobility shift assay (top) and corresponding bar graphs (middle). C(–) indicates negative control (lane 1); C indicates control conditions, in which cells were stimulated with vehicle only (lane 2). Cells were stimulated with Ang II (10^{-8} to 10^{-6} mol/L) (lanes 3 to 5) for 1 hour with or without PD98 (10^{-5} mol/L) (lane 6). In lane 7, cells were stimulated with Ang II (10^{-6} mol/L) incubated with a radioligand containing an AP-1 binding site in the presence of specific competitor (S.C) or nonspecific competitor (NS.C) (lane 8). Bottom panel, Supershift analysis performed with specific anti–c-fos and anti–c-jun antibodies (1 \mu g). Normal IgG at the same concentration did not cause supershift of AP-1 binding activity. AP-1 indicates specific AP-1 binding complexes; NS, nonspecific binding. Figures are representative of 3 experiments.} \]

\[ \text{Figure 7. Graphs demonstrate effects of Ang II (10^{-7} mol/L) on [\textsuperscript{3}H]thymidine incorporation and c-fos expression in VSMCs from hypertensive patients transfected with antisense phosphorothioated ODN and sense ODN. In control experiments, cells were exposed to Lipofectamine reagent in the absence of ODN. Results are mean±SEM of 3 or 4 experiments. *P<0.05 and **P<0.01 vs basal counterpart; ++P<0.05 and +P<0.01 vs Ang II counterpart in other groups.} \]
whereas ERK1/2 activity in SHR showed a greater dependency on intracellular Ca\(^{2+}\) mobilization, and ERK1/2 inactivation after Ang II stimulation was more rapid than that found in WKY cells, possibly because of changes in MAP kinase phosphatase. In the present study, ERK1/2 phosphorylation in the hypertensive group was almost double that of the control group, and the kinetics of ERK1/2 activation were significantly different. Whereas Ang II–induced ERK1/2 phosphorylation was transient in normotensive subjects, it was sustained in the hypertensive patients. ERK1/2 expression in VSMCs from hypertensive and normotensive individuals was similar, indicating that increased ERK1/2 phosphorylation in hypertension is probably due to changes in regulation of kinase activity. A possible cause for this may be dysregulation of mediators that are upstream from MAP kinases, such as c-Src, which we previously demonstrated to be potently activated by AT\(_1\) receptors in human VSMCs. c-Src is required for vascular ERK activation by Ang II and is essential in agonist-stimulated cytoskeletal reorganization and signal transduction at focal adhesions. The functional role of this kinase in vascular growth processes in hypertension is unknown. Results in the present study demonstrate that Src inhibition, by PP2, reduces ERK1/2 phosphorylation and normalizes responses in the hypertensive group, suggesting that altered Src regulation may be an important cause of ERK1/2 hyperactivation in hypertension. PP2 inhibits multiple members of the Src family, so we cannot exclude the possibility that in addition to c-Src, other Src kinases may also be important in these processes. To further support the role of c-Src, we report the novel findings that Ang II–induced c-Src phosphorylation and kinase activity are markedly augmented in VSMCs from the arteries of hypertensive patients. Because peak phosphorylation is very rapid, occurring within seconds, increased c-Src activation may be one of the earliest signaling events modulating growth in hypertension. Mechanisms underlying vascular c-Src hyperactivity in hypertension are unclear, but this seems to be a postreceptor phenomenon, inasmuch as AT\(_1\) receptor expression was not increased in cells from hypertensive patients. We reported similar findings in rat VSMCs, in which AT\(_1\) receptor status was not different in cells from WKY and SHR, as assessed by binding studies and determination of mRNA and protein expression. The link between the AT\(_1\) receptor and Src remains to be clarified, but interaction between the G\(_{\beta}\)G\(_{\gamma}\) subunits, their associated kinases, and kinase substrates (Janus kinase 2 and possibly β-arrestin) may provide the signaling complex that activates and binds c-Src.

Various transcription factors and regulatory proteins are phosphorylated by MAP kinases. In the present study, ERK1/2 activation was followed by increased proto-oncogene expression. Protein products of these genes are involved in transcriptional control and lead to a cascade of gene activation important in the growth response. In VSMCs from hypertensive patients, Ang II–induced expression of c-fos mRNA, but not c-myc or c-jun, was augmented. Furthermore, c-fos expression was increased in the hypertensive group. Similar findings have been reported in cells from experimental models of hypertension. Expression of c-fos in the hypertensive group was normalized by PD 098,059, suggesting the upstream role of ERK1/2. Having demonstrated that Ang II–mediated c-fos is overexpressed in cells from hypertensive but not normotensive individuals, we performed further studies in the hypertensive group to verify the significance and importance of c-fos in growth signaling in hypertension. Our data demonstrate that Ang II markedly increased AP-1 DNA-binding activity in the hypertensive group and that the AP-1 binding complex contained c-fos protein, as shown by supershift analysis. Therefore, increased c-fos expression, after Src phosphorylation and ERK1/2 activation, seems to be important in the activation of transcription factor AP-1. The AP-1 complex binds to specific DNA elements (termed AP-1 binding sites) and stimulates DNA synthesis as well as transcription. The pivotal role of c-fos gene expression in VSMC growth regulation in hypertension was further demonstrated in cells transfected with c-fos antisense ODNs. Inhibition of c-fos mRNA translation by antisense c-fos transcription inhibited Ang II–stimulated c-fos protein expression and DNA synthesis. Taken together, these data suggest that Src-regulated ERK1/2-mediated c-fos overexpression is essential for enhanced VSMC growth in hypertension.

In summary, findings from the present study have demonstrated that VSMCs from the small arteries of patients with essential hypertension exhibit augmented growth responses to Ang II. These effects are mediated via upregulation of c-Src–dependent pathways, leading to increased ERK1/2 activation and overexpression of the c-fos gene, which appear to be critical in VSMC growth. To our knowledge, this is the first evidence indicating that vascular c-Src activity is enhanced in human hypertension, and we suggest that this abnormality in kinase regulation is a postreceptor phenome-

### TABLE 2. AT Receptor mRNA and Protein Expression in VSMCs From Normotensive and Hypertensive Subjects

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<tr>
<th>Receptor Subtype</th>
<th>Normotensive</th>
<th>Hypertensive</th>
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<tr>
<td><strong>mRNA expression</strong></td>
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<tr>
<td>Basal</td>
<td>0.54±0.13</td>
<td>0.44±0.17</td>
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<tr>
<td>30 min</td>
<td>0.41±0.03</td>
<td>0.51±0.10</td>
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<tr>
<td>1 h</td>
<td>0.47±0.02</td>
<td>0.58±0.22</td>
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<tr>
<td><strong>Protein expression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>2 h</td>
<td>113±22%</td>
<td>91±15%</td>
</tr>
<tr>
<td><strong>AT(_1) receptor mRNA expression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>1 h</td>
<td>Not detected</td>
<td>Not detected</td>
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<tr>
<td><strong>Protein expression</strong></td>
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<tr>
<td>Basal</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>2 h</td>
<td>Not detected</td>
<td>Not detected</td>
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Values are mean±SEM of 4 to 6 experiments. mRNA expression is reported as the ratio of expression of AT receptor to GAPDH, and protein expression is reported as the percent relative to basal, with basal normalized to 100%. Expression of AT\(_1\) receptors was not detected at the mRNA level or at the protein level. Cells were treated with 10\(^{-7}\) mol/L Ang II for varying periods.
non that may be a critical early proximal mediator of downstream aberrant signaling events in hypertension. Our data define a signal transduction cascade through which activated arterial smooth muscle cells could contribute to vascular remodeling in human hypertension.

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References

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