Activation of Extracellular Signal–Regulated Kinases (ERK1/2) by Angiotensin II Is Dependent on c-Src in Vascular Smooth Muscle Cells

Mari Ishida, Takafumi Ishida, Sheila M. Thomas, Bradford C. Berk

Abstract—Among the angiotensin II (Ang II)–mediated signal events likely to be important in vascular smooth muscle cells (VSMCs) is activation of extracellular signal–regulated kinases 1 and 2 (ERK1/2). The upstream mediators by which Ang II activates ERK1/2 remain poorly defined. Recently, we showed that Ang II activated c-Src, a nonreceptor kinase, which is a candidate to mediate Ang II signal events. To determine whether c-Src is required for ERK1/2 activation by Ang II, we studied the effects of Src family–selective tyrosine kinase inhibitors on ERK1/2 activation and also studied Ang II–mediated signal events in Src-deficient and Src-overexpressing VSMCs. The tyrosine kinase inhibitors, genistein and CP-188,556, blocked Ang II–mediated ERK1/2 activation in rat VSMCs (rVSMCs). We derived Src-deficient VSMCs from the aortas of c-Src knockout mice (Src−/− mVSMCs). Basal ERK1/2 activity was lower, and activation of ERK1/2 by Ang II was significantly decreased in Src−/− mVSMCs compared with wild-type mVSMCs, whereas ERK1/2 protein expression and ERK1/2 activation by phorbol 12-myristate 13-acetate were similar. To examine the role of c-Src further, we overexpressed wild-type or dominant-negative c-Src in rVSMCs using retroviral vectors. ERK1/2 activation by Ang II was significantly increased in rVSMCs that overexpressed c-Src, whereas ERK1/2 activation by Ang II was significantly inhibited in rVSMCs that overexpressed dominant-negative c-Src compared with control rVSMCs. These findings demonstrate that c-Src activation is required for Ang II stimulation of ERK1/2 in VSMCs and suggest an important role for c-Src in Ang II–mediated signal transduction. (Circ Res. 1998;82:7-12.)

Key Words: tyrosine kinase ■ signal transduction ■ mitogen-activated protein kinase

Angiotensin II plays an important role in cardiovascular diseases associated with VSMC growth and vessel wall inflammation, such as atherosclerosis, restenosis following interventional procedures, and hypertension. Ang II has been shown to be important in the regulation of vascular tone and in hypertrophic growth of VSMCs, as well as hyperplastic growth of VSMCs from injured vessels. Among the signal events likely to be important in Ang II–mediated effects is activation of ERK1/2. ERK1 and ERK2 are serine/threonine protein kinases activated as an early response to a variety of stimuli involved in cellular growth, transformation, and differentiation. ERK1/2 activation is likely to be required to produce the effects of Ang II, such as vasoconstriction, stimulation of cytosolic phospholipase A2 and NAD(P)H oxidase, stimulation of Na⁺−H⁺ exchange, and VSMC growth.

The upstream mediators by which Ang II activates ERK1/2 remain poorly defined. Because many similarities exist between Ang II–mediated signal transduction and classic growth factor/cytokine–stimulated signal events, including protein tyrosine phosphorylation, it is likely that a tyrosine kinase is upstream from ERK1/2, as shown for platelet-derived growth factor and epidermal growth factor. A requirement for tyrosine kinases is also suggested by the inhibition of Ang II–mediated ERK1/2 activation by genistein. Recently, we have shown that Ang II activates c-Src, a nonreceptor kinase that is a candidate in the mediation of Ang II signal events.

To determine whether c-Src is required for ERK1/2 activation by Ang II, we studied the effects of Src family–selective tyrosine kinase inhibitors on ERK1/2 activation and Ang II–mediated signal events in c-Src–deficient and c-Src–overexpressing VSMCs.

Materials and Methods

Cell culture media, Immunoprecipitin, protein A agarose, and protein G agarose were purchased from Gibco-BRL. Monoclonal antibody against Src kinase (mAb 327) was purchased from Oncogene Science, Inc. Polyclonal Fyn antibody, polyclonal Lyn antibody, monoclonal Lck antibody, monoclonal anti-phosphotyrosine antibody (4G10), monoclonal anti-Src antibody, and anti-chicken Src monoclonal antibody (avian specific) were purchased from Upstate Biotechnology Inc. Polyclonal anti-phosphotyrosine antibody was provided by New England Biolabs. CP-118,556 (PP1) was kindly provided by Pfizer Inc.

The retroviral vector LXSN and the cell lines PE501 and NIH3T3 TK− were purchased from New England Biolabs.
were all generously provided by Dr A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, Wash. cDNA for WT and dominant-negative (kinase-inactive) c-Src was kindly provided by Dr S.A. Courtneidge, Sugen, Inc, Redwood City, Calif.17

Cell Culture
rVSMCs were isolated from the aorta of 200- to 250-g male Sprague-Dawley rats and maintained in DMEM supplemented with 0.1% calf serum for 48 hours, and the medium was changed 1 hour before the experiments.

Immunoprecipitation and Western Blot Analysis
Growth-arrested VSMCs were stimulated with 0.1% calf serum as described.1 Passage-6 to -16 rVSMCs at 70% to 80% confluence were growth-arrested by incubation in DMEM supplemented with 0.1% calf serum for 48 hours before use. mVSMCs were isolated from the aorta of 4- to 6-week-old C57BL (WT) and Src−/− mice, which are homozygous for a disruption in the c-Src gene,18 by enzymatic dissociation as previously described.1 More than 90% of cells stained positive for α-actin (sm-1). Cells were maintained in 75% MEM plus 25% HAM F-12 medium supplemented with 10% calf serum. Passage-6 to -13 mVSMCs at 70% to 80% confluence were growth-arrested by incubation in MEM/HAM F-12 supplemented with 0.1% calf serum for 48 hours, and the medium was changed 1 hour before the experiments.

Immunoprecipitation and Western Blot Analysis
Growth-arrested VSMCs were stimulated with Ang II as indicated in each experiment. Cells were lysed in NP-40 buffer (1% NP-40, 25 mmol/L Tris, pH 7.5, 50 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 137 mmol/L NaCl, 10% glycerol, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, and 10 μg/mL leupeptin), scraped off the dish, and centrifuged at 14 000 rpm at 4°C for 10 minutes, and protein concentrations of the supernatants were determined by DC protein assay (Bio-Rad). Lysates containing equal amounts of soluble proteins were precleared against immunoprecipitin and incubated with antibody overnight at 4°C. Antibody complexes were collected by incubation with protein G agarose for monoclonal antibody. Precipitates were washed 3 times in buffer containing 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% Triton X-100, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin or in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed. After incubation in blocking solution (GIBCO-BRL), membranes were incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C. Excess primary antibody was removed by washing the membranes in PBS containing 0.03% Tween 20. The blots were incubated with appropriate secondary antibodies for 1 hour. The membranes were washed, and proteins were detected by the ECL system (Amersham Life Science).

Immune Complex Kinase and In-Gel Kinase Assays
Growth-arrested VSMCs were lysed in RIPA buffer (10 mmol/L sodium phosphate, pH 7.0, 150 mmol/L NaCl, 50 mmol/L NaF, 2 mmol/L EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mmol/L sodium orthovanadate, 0.5 mmol/L dithiothreitol, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Immunoprecipitation was performed with a monoclonal Src antibody, mAb 327, and immunoprecipitates were washed 3 times as described above and twice in kinase reaction buffer (20 mmol/L Tris-HCl, pH 7.2, 25 mmol/L MgCl2, 5 mmol/L MnCl2, 0.4 mmol/L EGTA, 0.05 mmol/L sodium orthovanadate, and 0.4 mmol/L dithiothreitol). Src kinase assay was performed with the Src family kinase assay kit (UBI), which uses a specific peptide as substrate. ERK1/2 activity was analyzed using an in-gel kinase assay exactly as described previously.17 Data from autoradiograms were quantified by NIH Image 1.60 in the linear range of film exposure and are reported as mean±SEM. Statistical significance was determined by Student’s t test with values of P<0.05 considered significant.

Construction of Recombinant c-Src Retrovirus and rVSMC Transduction
c-Src cDNAs containing the complete regions of the WT and dominant-negative (kinase-inactive) chicken c-Src were cloned into the unique BamHI site of the retroviral vector LXSN to construct the recombinant WT and dominant-negative c-Src retroviral vector (LSSN or LDNSSN, respectively). The kinase-inactivation mutation in c-Src was Lys-295 to Met-295.19 Viral packaging was performed according to Miller and Rosenman15 using the PES01 cell line. Virus titers were 1.8×105 cfu/mL for PE/LSSN, 1.2×105 cfu/mL for PE/LDNSSN, and 1.7×105 cfu/mL for PE/LXSN. Sixteen-hour virus harvests from the PE/LSSN, PE/LDNSSN, and PE/LXSN cells were used to infect the rVSMCs. rVSMCs were plated at subconfluence and incubated with virus for 24 hours to infect the cells. rVSMCs infected with retrovirus were selected with DMEM/10% calf serum containing 0.6 mg/mL G418. Before the experiments, rVSMCs were serum-starved for 48 hours with DMEM/0.4% calf serum.

Results
ERK1/2 Activation by Ang II Is Blocked by Tyrosine Kinase Inhibitors in rVSMCs
ERK1/2 is rapidly and strongly activated by Ang II in rVSMCs.19 To determine the involvement of tyrosine kinases in Ang II–mediated ERK1/2 activation, we used the tyrosine kinase inhibitor genistein. ERK1/2 activation by Ang II was blocked by genistein pretreatment in a concentration–dependent manner (Fig 1A and 1B), suggesting the involvement of tyrosine kinases. We recently demonstrated that c-Src is activated rapidly in rVSMCs after Ang II stimulation.13 To determine the involvement of tyrosine kinases in Ang II–mediated ERK1/2 activation, we used the tyrosine kinase inhibitor genistein. ERK1/2 activation by Ang II was blocked by genistein pretreatment in a concentration–dependent manner (Fig 1A and 1B), suggesting the involvement of tyrosine kinases. We recently demonstrated that c-Src is activated rapidly in rVSMCs after Ang II stimulation.13 To determine whether c-Src is involved in Ang II–mediated ERK1/2 activation, we used CP-118,556. CP-118,556 is a pyrazolopyrimidine, interacts specifically with Src family kinases, inhibits Src family kinases preferentially compared with ZAP-70, JAK2, and the epidermal growth factor receptor.14 In rVSMCs, Ang II–mediated ERK1/2 activation was blocked by CP-118,556 in a concentration–dependent manner, with an IC50 of ≈10 μmol/L.13 CP-118,556 inhibits Src family kinases preferentially compared with ZAP-70, JAK2, and the epidermal growth factor receptor.14 In rVSMCs, Ang II–mediated ERK1/2 activation was blocked by CP-118,556 in a concentration–dependent manner, with an IC50 of ≈10 μmol/L (Fig 1C and 1D), suggesting that c-Src activation is necessary for ERK1/2 activation by Ang II. On the other hand, tyrosine phosphorylation of JAK–2 by Ang II was not inhibited by CP-118,556 at concentrations as high as 100 μmol/L (data not shown), confirming the specificity of CP-118,556 as an Src family–selective tyrosine kinase inhibitor.

Characterization of WT mVSMCs and Src−/− mVSMCs
To examine further the involvement of c-Src in ERK1/2 activation by Ang II, we derived mVSMCs from WT and Src−/− mice. To compare signal transduction in mVSMCs with...
rVSMCs, we examined Ang II stimulation of both c-Src and cellular protein tyrosine phosphorylation. c-Src was activated rapidly by Ang II in WT mVSMCs, as measured by an immune complex kinase assay, and had a time course and magnitude similar to those found with rVSMCs (Fig 2). As expected, c-Src was not expressed in Src−/− mVSMCs (Fig 2). To study Ang II–stimulated tyrosine phosphorylation, cells were stimulated with 100 nmol/L Ang II, and tyrosine-phosphorylated proteins were immunoprecipitated with monoclonal anti-phosphotyrosine antibody, 4G10 and immunoblotted with horseradish peroxidase–conjugated PY20 (anti-phosphotyrosine antibody). Arrowheads indicate Ang II–stimulated tyrosine-phosphorylated proteins in VSMCs. Asterisk indicates 50-kD tyrosine-phosphorylated protein equally present in all VSMCs. Results are representative of three experiments.

**ERK1/2 Activation by Ang II Is Decreased in Src−/− mVSMCs**

To measure ERK1/2 activity, mVSMCs were stimulated with Ang II, and cell lysates were analyzed by in-gel kinase assay with myelin basic protein as substrate. The time course for Ang II–mediated ERK1/2 activation in mVSMCs was similar to that in rVSMCs, with a peak at 5 minutes and a return to the control level within 30 minutes (data not shown). ERK1/2 protein expression was the same in WT and Src−/− mVSMCs, as shown by ERK1/2 Western blotting (Fig 4). However, basal ERK1/2 activity was lower, and activation of ERK1/2 by Ang II was significantly decreased in Src−/− mVSMCs. In contrast, ERK1/2 activation by PMA was the same in WT and Src−/− mVSMCs (Fig 4), implying that other components of the signal cascade, not dependent on c-Src, were intact. Activation of ERK1/2 in both WT and Src−/− mVSMCs was blocked by the AT1 receptor antagonist losartan (Fig 4). Ang II stimulated a concentration-dependent increase in ERK1/2 activation in WT mVSMCs (maximal 146.3±0.6% increase, Fig 5), with a peak at 300 nmol/L. In Src−/− mVSMCs, the magnitude of ERK1/2 activation was less, only a 74.6±4.9% increase at 300 nmol/L Ang II. It should be noted that the absolute ERK1/2 activity in Src−/− mVSMCs was 51.3±0.97% that in WT mVSMCs at 300 nmol/L because basal ERK1/2 activity was lower in Src−/− mVSMCs. These data indicate that c-Src is involved in ERK1/2 activation by Ang II and suggest that basal ERK1/2 activity is also regulated by c-Src. Although ERK1/2 activation by Ang II was dramatically decreased in Src−/− mVSMCs, the residual ERK1/2 activity raised the concern that other Src family kinases might be expressed in Src−/− mVSMCs to compensate for the absence of c-Src because Src family kinases have both specific
and overlapping functions.\textsuperscript{29} In fact, other Src family kinases, Fyn and Lyn, were expressed to a greater extent in Src\textsuperscript{2/2} mVSMCs compared with WT mVSMCs (Fig 6). To evaluate the roles of Fyn and Lyn, we blocked Src family kinase activity in Src\textsuperscript{2/2} mVSMCs with CP-188,556, an Src family–specific inhibitor. ERK1/2 activation by Ang II was completely inhibited by 1 \textmu mol/L CP-188,556 (Fig 7). At higher concentrations of CP-188,556 (10 and 100 \textmu mol/L), ERK1/2 activity was reduced below the control level, demonstrating that CP-188,556–sensitive kinases are responsible for basal ERK1/2 activity. These results suggest that other Src family kinases can partially substitute for c-Src as a mediator of Ang II signal transduction.

**ERK1/2 Activation by Ang II Is Dependent on the Presence of c-Src in rVSMCs Retrovirally Transduced With Exogenous WT c-Src or Dominant-Negative c-Src**

To confirm the significance of c-Src in ERK1/2 activation by Ang II, we overexpressed dominant-negative chicken c-Src or exogenous WT chicken c-Src in rVSMCs using retrovirus vectors (LDNSSN or LSSN, respectively). We chose the chicken c-Src gene to evaluate specifically how much exogenous gene was introduced by use of an avian-specific c-Src antibody. Both exogenous WT and dominant-negative chicken c-Srcs were overexpressed in transduced cells as

![Figure 4](link)

**Figure 4.** ERK1/2 activation by Ang II is decreased in Src\textsuperscript{2/2} mVSMCs. Growth-arrested mVSMCs were pretreated with 10 \textmu mol/L losartan (Los) for 30 minutes where indicated. Cells were then stimulated with 100 nmol/L Ang II for 5 minutes or with 200 nmol/L PMA for 5 minutes and lysed, and proteins were subjected to either in-gel kinase assay or Western blot with polyclonal ERK1/2 antibody as described. ERK1/2 activity after pretreatment with losartan alone was the same as control. Results are representative of three experiments.

![Figure 5](link)

**Figure 5.** Concentration response for Ang II–mediated ERK1/2 activation. Growth-arrested mVSMCs were stimulated with the indicated concentrations of Ang II for 5 minutes and lysed, and proteins were subjected to in-gel kinase assay. A, Representative autoradiogram of three separate experiments. B, Densitometric analysis of myelin basic protein phosphorylation by ERK1/2 from three separate experiments. Results were normalized to the untreated mVSMCs (control). Data are expressed as mean ± SEM.

![Figure 6](link)

**Figure 6.** Expression of Src family kinases in mVSMCs. Growth-arrested mVSMCs were lysed, equal amounts of protein were subjected to SDS-PAGE and transferred to membranes, and Western blotting was performed with the indicated antibodies.

![Figure 7](link)

**Figure 7.** ERK1/2 activation by Ang II in Src\textsuperscript{2/2} mVSMCs is blocked by CP-118,556 (CP). Growth-arrested Src\textsuperscript{2/2} mVSMCs were pretreated with the indicated concentrations of CP for 15 minutes and stimulated with 100 nmol/L Ang II for 5 minutes. Cells were lysed, and proteins were subjected to in-gel kinase assay as described. A, Representative autoradiogram of three separate experiments. The autoradiogram shown is a long exposure to highlight the effects of CP. B, Densitometric analysis of myelin basic protein phosphorylation by ERK1/2 from three separate experiments. Results were normalized to the untreated mVSMCs (control). Data are expressed as mean ± SEM.
Src family kinases have both specific and overlapping functions with respect to phosphorylation of cytoskeletal proteins: cortactin and tensin phosphorylation are completely c-Src dependent, whereas focal adhesion kinase and paxillin phosphorylation are dependent on both c-Src and Fyn. Finally, it should be noted that other tyrosine kinases, such as Pyk2 (also termed CADTK or RAFTK), may be involved in Ang II–mediated ERK1/2 activation.21,22

The present study suggests that the Gq-coupled AT1 receptor activates ERK1/2 via a pathway that differs significantly from the Gqβ,γ pathway proposed for Gq-coupled receptors, such as the m2-muscarinic receptors. For Gq-coupled receptors, ERK1/2 activation has been shown to require Gqβ,γ25-27 and c-Src is involved in Gqβ,γ-mediated ERK1/2 activation.21,28 In addition, Luttrell et al29 have demonstrated that overexpression of WT c-Src or a constitutively active mutant c-Src increases tyrosine kinase activity in Shc immunoprecipitates, resulting in increased Shc tyrosine phosphorylation and greater Shc-Grb2 complex formation. Because Ang II has been shown to increase tyrosine phosphorylation of Shc and because Shc-Grb2 can lead to ERK1/2 stimulation, one possibility is that Ang II activation of ERK1/2 by c-Src is via Gqβ,γ. However, Ang II–mediated ERK1/2 activation is likely via a mechanism different from that activated by Gq-coupled receptor agonists, because it has been shown that Gq-coupled receptors stimulate the ERK1/2 pathway independent of Gqβ,γ.26

The role of Src family kinases in Ang II–mediated signal transduction appears to be tissue specific.29,30 Sadoshima and Ishida et al11 reported that Src family kinases have both specific and overlapping functions and that phosphorylation of cortactin and tensin is completely c-Src dependent, whereas focal adhesion kinase and paxillin phosphorylation are dependent on both c-Src and Fyn. Finally, it should be noted that other tyrosine kinases, such as Pyk2 (also termed CADTK or RAFTK), may be involved in Ang II–mediated ERK1/2 activation.21,22

The major finding of the present study is that c-Src activity is required for Ang II stimulation of ERK1/2 in VSMCs. This conclusion is supported by several results. First, in both rVSMCs and mVSMCs, AT1 receptor stimulation rapidly activated c-Src and ERK1/2. Second, inhibition of c-Src function by the specific inhibitor CP-188,556, by use of c-Src–independent pathways was intact in Src knockout VSMCs (Src−/− mVSMCs), or by use of retroviral transduction of dominant-negative c-Src into rVSMCs compared with control rVSMCs (Fig 9). The inhibition was greatest at 10 nmol/L Ang II (68.0±8.3% inhibition, P<.05, n=3). Several other rVSMC clones transduced with exogenous chicken c-Src or dominant-negative c-Src showed similar results.

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Our data support the concept that Src family kinases have both specific and overlapping functions. In Src−/− mVSMCs, ERK1/2 activation by Ang II was decreased dramatically but not completely. However, the Src family kinase inhibitor CP-188,556 completely abolished ERK1/2 activation. These results might be explained by our finding that Fyn and Lyn are more highly expressed in Src−/− mVSMCs. From these data, we infer that Src family kinases may have redundant roles in signal transduction. In addition, there is specificity in Src family kinase function, because increased Fyn and Lyn expression in Src−/− mVSMCs could not fully compensate for the lack of c-Src as measured by ERK1/2 activation. Recently, it has been reported30 that Src family kinases have both specific and overlapping functions with respect to phosphorylation of cytoskeletal proteins: cortactin and tensin phosphorylation are completely c-Src dependent, whereas focal adhesion kinase and paxillin phosphorylation are dependent on both c-Src and Fyn. Finally, it should be noted that other tyrosine kinases, such as Pyk2 (also termed CADTK or RAFTK), may be involved in Ang II–mediated ERK1/2 activation.21,22

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Izumo et al. have demonstrated that in cardiac myocytes Ang II activates Fyn and stimulates the association of Shc with Fyn and the formation of an SHC–Grb–Sos complex. These authors have suggested that the nonreceptor tyrosine kinase, Fyn, activates Ras through this complex formation. A more recent report by Zou et al. has shown in liver epithelial cells that Ang II stimulation of p70<sup>S6K</sup> is dependent on CADTK, whereas activation of p90<sup>RSK</sup> (an ERK1/2 substrate) is independent of CADTK. We have shown in the present study that in rVSMCs ERK1/2 activation by Ang II is dependent on c-Src. Finally, Schieffer et al. have shown in rVSMCs that Ras activation by Ang II is dependent on c-Src, supporting our present results. Further studies will be required to define the tissue-specific mechanisms for c-Src activation by the AT<sub>1</sub> receptor and for c-Src activation of ERK1/2.

Acknowledgments
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References
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