Angiotensin II Stimulates Extracellular Signal–Regulated Kinase Activity in Intact Pressurized Rat Mesenteric Resistance Arteries

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Abstract—The activation of extracellular signal–regulated kinases 1/2 (ERK1/2) was assessed in isolated rat mesenteric resistance arteries (200-μm diameter) in a pressure myograph and stimulated for 5 minutes by angiotensin II (Ang II, 0.1 μmol/L) with a pressure of 70 mm Hg. ERK1/2 activity was measured by using an in-gel assay, and ERK1/2 phosphorylation was measured by Western blot analysis with use of a phospho-specific ERK1/2 antibody. Ang II (0.1 μmol/L) induced contraction (28% of phenylephrine contraction, 10 μmol/L). ERK kinase inhibitor PD98059 (10 μmol/L) attenuated this contraction by 36% but not that to phenylephrine or K^+ (60 mmol/L). In unpressurized arteries, Ang II increased ERK1/2 activity by 26%, and pressure (70 mm Hg) itself increased ERK1/2 activity by 72%. Ang II and pressure together acted synergistically, increasing ERK1/2 activity by 264%. Thus, in pressurized vessels, Ang II (0.1 μmol/L) increased ERK1/2 activity by 112%, calculated as $[(364/172)-1]\times100$, which was confirmed by a measured 72% increase in ERK1/2 phosphorylation. Ang II type 1 receptor blockade by candesartan (10 μmol/L) abolished the Ang II–induced increase in ERK1/2 activity, but Ang II type 2 receptor blockade (PD123319, 10 μmol/L) did not. The Ang II–induced increase in ERK1/2 activity was inhibited by protein kinase C inhibitors Ro-31-8220 (1 μmol/L) and Go-6976 (300 nmol/L) and tyrosine kinase inhibitors genistein (1 μmol/L, general) and herbimycin A (1 μmol/L, c-Src family). The present findings show for the first time in intact resistance arteries that ERK1/2 activation is rapidly regulated by Ang II, is synergistic with pressure, and is involved in contraction. The ERK1/2 signaling pathway apparently includes upstream protein kinase C and c-Src. **(*Hypertension*. 2000;36:617-621.)**

Key Words: angiotensin II ■ receptors, angiotensin II ■ protein kinases ■ mesenteric arteries ■ rats

Angiotensin II (Ang II), through either the Ang II type 1 receptor (AT_1R) or the Ang II type 2 receptor (AT_2R), is coupled to a wide variety of signal transduction enzymes in vascular smooth muscle. These include protein kinase C (PKC), tyrosine kinases, and extracellular signal–regulated protein kinase 1/2 (ERK1/2),[1,2] a cascade that is also involved in mechanical signal transduction.[3] Furthermore, Ang II has been identified as a potent regulator that induces hypertrophy, hyperplasia, or both in smooth muscle cells[4] and resistance arteries[5,6] and plays a critical role in the pathogenesis of hypertension.[7] Moreover, acute elevation of blood pressure in vivo caused by Ang II increases ERK1/2 activity.[8,9] Therefore, Ang II has been suggested to be an activator of ERK1/2, and this activation could play an important role in the morphological changes of resistance arteries and, thus, could possibly be associated with hypertension.[1]

However, evidence indicating a role for ERK1/2 in hypertension-associated changes in resistance artery morphology is, at present, circumstantial in that the evidence is based on cultured vascular smooth muscle cells (VSMCs) either from larger arteries[10] or resistance arteries.[1] Such culture preparations have the following difficulties: phenotypes may change; connections between VSMCs, endothelial cells, and the extracellular matrix are lost; and the normal wall stress is absent.

Therefore, given this situation, we have developed methods that allow for the study of ERK1/2 activation in intact resistance arteries under intraluminal pressure. We used a pressure myograph in which small arteries (diameter $\approx$200 μm) were kept under constant pressure and activated with Ang II for a given period while their diameter could be controlled. Thereafter, vessels were snap-frozen for biochemical analysis. This method has enabled us to clarify the degree to which Ang II can bring about ERK1/2 activation to Ang II and the role of such activation in the Ang II–induced contraction. We have also taken the opportunity to clarify the receptor subtype of Ang II (AT_1R or AT_2R) involved in

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ERK1/2 activation and to obtain evidence concerning the intracellular transduction pathways of Ang II–induced ERK1/2 activation with special regard to PKC and tyrosine kinase. The results provide the novel information that Ang II causes activation of ERK1/2 in intact resistance arteries, thus providing direct evidence that this pathway is potentially of importance in the control of peripheral resistance.

Methods

Contractile Effect of Ang II

Mesenteric resistance arteries (200-μm diameter) were dissected out from 12-week-old Wistar rats and mounted in a pressure myograph as described previously.[11,12] The pressure myograph used in the present study contained 2 chambers and allowed vessel diameter to be measured online by use of a video-microscope arrangement. Each resistance artery was connected to a perfusion circuit consisting of a glass reservoir and a pressure chamber, allowing the control of hydrostatic pressure in the intraluminal compartment. The arterial segments were then allowed to equilibrate in 6 mL of sterile serum-free DMEM in an incubator containing 5% CO_2 at 37°C, with an internal pressure of 70 mm Hg for 1 hour. The integrity of the endothelium was assessed by testing the vasodilator effect of acetylcholine (10 μmol/L) after precontraction of the arteries with phenylephrine (10 μmol/L). Vessels in which acetylcholine was not able to induce full relaxation were discarded.

Under continuous monitoring of vessel diameter, the arteries (at 70 mm Hg) were challenged with Ang II (0.1 μmol/L), phenylephrine (10 μmol/L), or K^+ (60 mmol/L, replacement of 50% DMEM with K^+ saline[13]). Experiments were performed either with no other drugs present or with 2-(2′-amino-3′-methoxyphenyl)-oxanaphtalen-4-one (PD98059, an ERK kinase [MEK] inhibitor, 10 μmol/L) or with candesartan (an AT_1R inhibitor, 10 μmol/L), present throughout the equilibration and Ang II stimulation periods. Each vessel was exposed only once to a drug.

ERK1/2 Activity Protocols

Vessels were mounted on the pressure myograph and equilibrated at 70 mm Hg as described above for 1 hour. Subsequently, with intraluminal pressure maintained at 70 mm Hg, arteries were exposed to Ang II (0.1 μmol/L) or not for 5 minutes. Furthermore, for each set of experiments, a "control vessel" that remained unmounted (zero pressure) and unexposed to drugs for 65 minutes was prepared. The effect of Ang II (0.1 μmol/L) on such an unmounted vessel for the final 5 minutes was also investigated. At the end of each experiment, arteries were immediately frozen in liquid nitrogen and stored at −80°C.

Nine types of experiments were performed in which the following were included during both the equilibration period and the Ang II stimulation period: (1) no drugs, (2) candesartan (10 μmol/L), (3) PD123319 (AT_2R inhibitor, 10 μmol/L), (4) candesartan plus PD123319, (5) Go-6976 (calcium-dependent PKC inhibitor, 300 nmol/L), (6) Ro-31-8220 (nonselective PKC inhibitor, 1 μmol/L), (7) genistein (general tyrosine kinase inhibitor, 1 μmol/L), (8) herbimycin A (c-Src–dependent tyrosine kinase inhibitor, 10 μmol/L), and (9) PD98059 (10 μmol/L). In a 10th group, vessels were exposed only to pressure. To ensure that only the effects of AT_1R were observed for protocols 5 through 8, PD123319 (10 μmol/L) was included in these experiments.

Tissue Extraction

Frozen vessel segments were pulverized and resuspended in ice-cold lysis buffer (40 μL).[14] Artery extracts were incubated for 15 minutes on ice and then centrifuged (12 000*g* for 15 minutes at 4°C). The detergent-soluble supernatant fractions were retained, and protein contents in samples (≈340 ng, in-gel assay; 15 to 20 μg, Western blot assay) were determined by the Lowry method.

In-Gel ERK1/2 Assay

ERK1/2 activity was assessed by using an in-gel assay. In the present study, gels containing a substrate for ERK1/2, myelin basic protein, were used as described previously.[14] To 12-μL aliquots of artery extracts, we added 8 μL of sample buffer (11.2 mmol/L glycerol, 1.29 g SDS, 12 mL Tris-KCl, 5.1 mL β-mercaptoethanol, and 0.2% bromophenol blue), which was loaded on a 10% SDS-polyacrylamide gel containing 0.5 mg/mL myelin basic protein. After electrophoresis, SDS was removed from the gel and treated.[14]

Phosphorylation of myelin basic protein was carried out by incubating the gels with 50 μCi of [^{32}P]ATP, 100 mmol/L dithiothreitol, and 10 mmol/L ATP. The reaction was stopped by washing twice for 10 minutes in 10% propanol and 10% acetic acid. Subsequently, the gels were washed (3 times) for 10 minutes with 10% acetic acid and 10% sodium pyrophosphate. Then, the gels were incubated in 45 mL of 10% propanol, 10% acetic acid, and Coomassie blue. The coloration was washed away by washing the gel (3 times) for 15 minutes with 10% acetic acid and 10% sodium pyrophosphate. After these procedures, the gels were dried and passed for assay to determine ERK1/2 activity. After correction for protein content, ERK1/2 activity in the various conditions was expressed as a percentage of the activity measured in parallel-processed control vessels (unpressurized in the absence of Ang II).

Western Blotting of ERK1/2

Phosphorylated (activated) ERK1/2 (p-ERK1/2) was measured in arteries by using the immunoblotting method. Arteries were processed, homogenized, and rapidly frozen (−80°C) as described above. To 20-μL aliquots, 14 μL of sample buffer (as used in the in-gel assay) was added, and samples were heated (95°C, 3 minutes) and cooled before loading on gel. Proteins were separated (Mini Gel Protean II System, Bio-Rad; 200 V, 35 minutes, with 300 mL of 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS) on a 10% resolving gel and 4% stacking gel (Tris-HCl Ready Gel, Bio-Rad) and then transferred (35 V, overnight, 4°C, with 800 mL of 25 mmol/L Tris, 192 mmol/L glycine, and 10% methanol) to polyvinylidene difluoride blotting membranes (Immobilon-P, Millipore). Membranes were then washed in TBS-T (10 mmol/L Tris/base pH 7.5, 0.1 mol/L NaCl, 1 mmol/L EDTA, and 0.1% Tween 20) and blocked for 1 hour (5% fat-free dry milk in TBS-T). After the membranes were washed, they were incubated (overnight, 4°C) with primary antibody against p-ERK1/2 (1:500, Santa Cruz) in fresh blocking solution, washed again, and incubated (1 hour at room temperature) with horseradish peroxidase–conjugated secondary antibody (1:1000, Santa Cruz). Membranes were washed, and p-ERK1/2 bands (44 and 42 kDa) were visualized by using the ECL Plus Chemiluminescence Kit (Amersham). The Storm860 (Molecular Dynamics) Imaging System and Image Quant software were used for quantification. After correction for protein content, phosphorylated ERK1/2 of Ang II–stimulated vessels was expressed relative to parallel-processed vessels, which had been pressurized but not stimulated.

Suppliers

PD123319 was supplied by Research Biochemicals International; PD98059, by New England Biolabs; Go-6976 and Ro-31-8220, by Calbiochem; genistein, herbimycin A, Ang II, phenylephrine, and acetylcholine, by Sigma; and DMEM, by Life Technology. Candesartan was a kind gift of AstraZeneca (Mölndal, Sweden).

Statistical Analysis

Results are expressed as mean±SEM. Differences between the 10 groups mentioned above were tested by 1-way ANOVA, and because this was significant ($P<0.001$), subsequent *t* tests were used (Figures 2A and 3) to compare groups 2 through 10 with group 1 (pressure plus Ang II). Values of $P<0.05$ were considered significant.

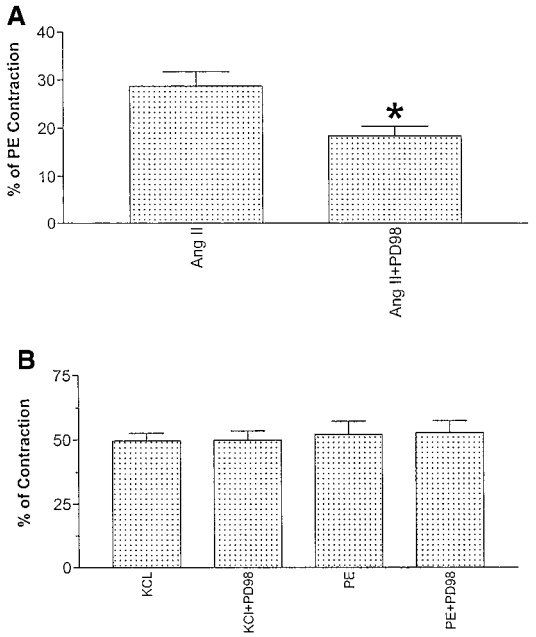

Figure 1. A, Effect of MEK inhibitor PD98059 (PD98, 10 μmol/L) on Ang II–induced contraction (0.1 μmol/L) on mesenteric resistance arteries expressed with respect to contraction to phenylephrine (PE, 10 μmol/L; n=7). B, Effect of MEK inhibitor PD98 (10 μmol/L) on K^+ (60 mmol/L, n=5)– and PE (10 μ 77, n=6)–induced contraction on mesenteric resistance arteries. Data are mean±SEM. *$P<0.05$ vs without PD98.

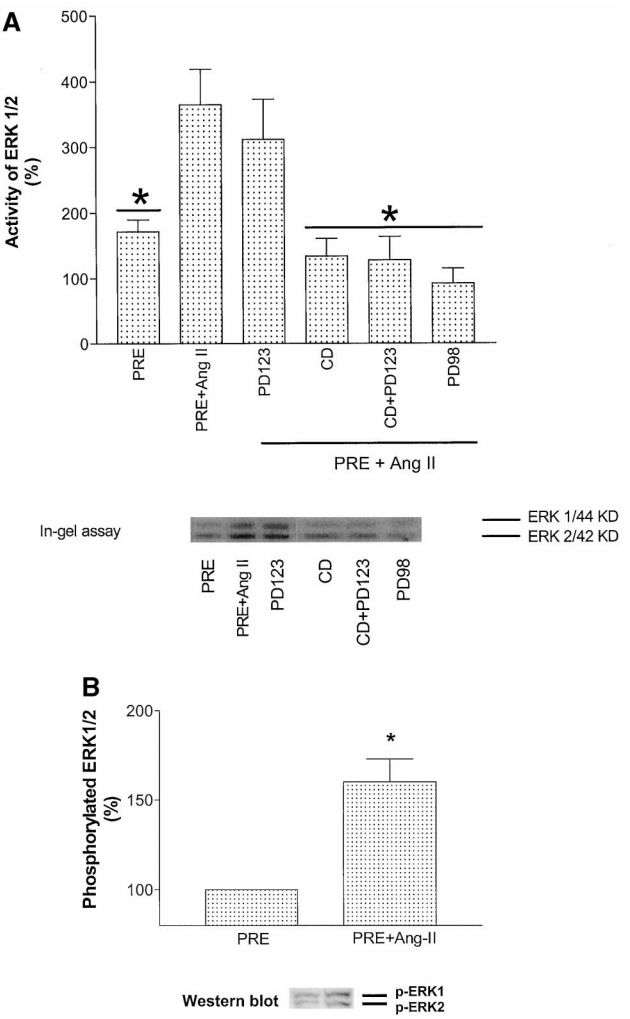

Figure 2. A, Graph at top shows effect of pressure and Ang II with and without drugs on ERK1/2 activity measured by in-gel assay. Beneath the graph is a typical autoradiogram of in-gel assay of ERK1 and ERK2 activity (44 and 42kDa, respectively). Activity is expressed with respect to activity in control vessels unpressurized for 65 minutes without drugs. PRE indicates vessel exposed to 70 mm Hg intravascular pressure for 65 minutes (n=7); PRE+Ang II, Ang II added to pressurized vessel for final 5 minutes (n=10). ERK1/2 activity in pressurized vessels exposed to Ang II for final 5 minutes is shown; AT_2R blocker PD123319 (PD123, 10 μmol/L; n=5), AT_1R blocker candesartan (CD, 10 μmol/L; n=5), candesartan plus PD123319, or MEK inhibitor PD98059 (PD98, 10 μmol/L; n=5) was present for entire 65-minute period. Data are mean±SEM. *$P<0.05$ vs PRE+Ang II. B, Conditions were same as in panel A, but ERK1/2 phosphorylation was measured by Western blot analysis (graph at top). Typical chemifluorescence blot is shown in bottom of panel. ERK1/2 phosphorylation for PRE+Ang II is expressed relative to phosphorylation in pressurized vessels in absence of Ang II (PRE). Data are mean±SEM. *$P<0.05$ vs PRE.

Results

Effects of PD098059 on Ang II–Induced Contraction

Ang II (0.1 μmol/L) induced contractions of arteries (Figure 1). If the MEK inhibitor PD98059 (10 μmol/L) was included in the equilibration and activation solutions, the contractile response to Ang II was attenuated (Figure 1A). Candesartan (10 μmol/L) abolished the contractile response to Ang II (n=5, data not shown). Contractions to phenylephrine (10 μmol/L) or K^+ (60 mmol/L) were not inhibited by the MEK inhibitor (PD98059, 10 μmol/L) (Figure 1B).

Effects of Ang II on ERK1/2 Activity

In arteries submitted to zero pressure for 65 minutes, the addition of Ang II (0.1 mmol/L) for the final 5 minutes caused a slight increase in ERK1/2 activity by 26±2% (n=5, $P<0.01$). Pressure (70 mm Hg) itself increased ERK1/2 activity by 72% compared with arteries without pressure (Figure 2A). However, in such pressurized arteries, Ang II had a synergistic effect with pressure, inasmuch as the combination gave an increase in ERK1/2 activity of 264% compared with arteries without pressure (Figure 2A). This indicates that in pressurized vessels, Ang II causes a 112%, calculated as $[(364/172)-1]\times100$, increase in ERK1/2 activity, and this result was confirmed by Western blot analysis; Ang II increased ERK1/2 phosphorylation by 72% (Figure 2B).

In the presence of PD98059 (10 μmol/L), ERK1/2 activity of the pressurized vessels was not increased by Ang II stimulation (Figure 2A). AT_1R antagonism with candesartan (10 μmol/L) inhibited Ang II–stimulated ERK1/2 activity (Figure 2A), whereas the AT_2R antagonist PD123319 (10 μmol/L) had no effect (Figure 2A). Candesartan and PD123319 together caused the same inhibition of Ang II–induced ERK1/2 activity as did candesartan alone (Figure 2A).

The presence of the PKC inhibitor Ro-31-8220 (1 μmol/L) or Go-6976 (300 nmol/L) prevented Ang II–induced ERK1/2 activity (Figure 3). Likewise, the presence of the tyrosine kinase inhibitor genistein (1 μmol/L) or herbimycin A

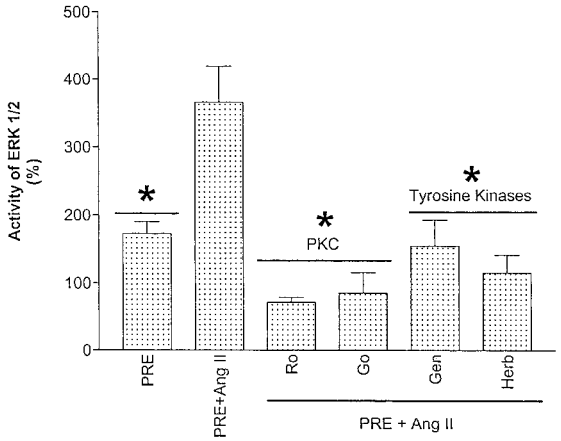

Figure 3. ERK1/2 activity in pressurized vessels exposed to Ang II for 5 minutes, with PKC inhibitors Ro-31-8220 (Ro, 10 μmol/L; n=5) or Go-6967 (Go, 0.3 μmol/L; n=5) or tyrosine kinase inhibitors genistein (Gen, 1 μmol/L; n=5) or herbimycin A (Herb, 1 μmol/L; n=5) present for entire 65-minute period. PRE indicates vessel exposed to 70 mm Hg intravascular pressure for 65 minutes (n=7); PRE+Ang II, Ang II added to pressurized vessel for final 5 minutes (n=10). Activity is expressed with respect to activity in control vessels unpressurized for 65 minutes without drugs. Data are mean±SEM. *$P<0.05$ vs PRE+Ang II.

(1 μmol/L) prevented Ang II–induced ERK1/2 activity (Figure 3).

Discussion

The major finding of the present study is that Ang II is able to cause rapid activation of ERK1/2 in intact pressurized resistance arteries, providing for the first time direct evidence that the ERK1/2 pathway is of potential importance in the control of peripheral resistance.

The ability of Ang II to activate the ERK1/2 pathway through AT_1Rs is well established in vascular VSMCs cultured either from conduit arteries[10] or from resistance arteries.[1,2] This pathway is thought to play a key role in the ability of Ang II to cause VSMC hypertrophy and hyperplasia.[8,9,15,16] Activation of ERK1/2 also contributes to smooth muscle cell hypertrophy/hyperplasia during arterial remodeling induced by frequent and/or sustained elevations in blood pressure,[17] probably associated with the ability of stretch to activate ERK1/2.[18,19] Other activators of ERK1/2 include cellular contacts with matrix.[20,21] Thus, there is good circumstantial evidence that ERK1/2 activation is involved in the ability of Ang II to cause vascular remodeling. The present investigation provides important further support for this concept by showing that Ang II can activate ERK1/2 in intact resistance arteries.

The ≈2-fold Ang II–induced increase in ERK1/2 activity and ERK1/2 phosphorylation seen in the present study with pressurized resistance arteries is similar to that observed by Touyz et al[1] in VSMCs derived from spontaneously hypertensive rat (SHR) resistance vessels. Similar to our findings, the Ang II–induced increase was inhibited by the MEK inhibitor PD98059. However, it is of interest that the Ang II–induced increase in ERK1/2 activity that we saw in nonpressurized vessels was only 26%, similar to the ≈30% increase reported by Touyz et al in the VSMCs of normotensive Wistar-Kyoto rats. Moreover, as indicated in Results, there is synergy between the effects of Ang II and pressure, with the combination greatly exceeding the sum of the individual effects. This suggests that subjection of vessels to pressure is important for an accurate estimation of the effects of Ang II on ERK1/2 activity. Furthermore, compared with its effect on WKY VSMCs, Ang II has a greater effect on ERK1/2 activity in VSMCs from SHR resistance arteries[1] but not in VSMCs from SHR aorta.[10] This fact indicates that it is now important that the effects of Ang II on ERK1/2 activity in intact resistance arteries of SHR be investigated.

Our finding that MEK inhibition with PD98059 attenuated the contractile response to Ang II is also a novel observation regarding intact resistance arteries, although it has previously been seen in VSMCs from rat resistance arteries.[1] The ERK1/2 dependence of vasoconstrictors has also been observed in VSMCs from rat aorta,[22] rat tail artery,[23] and human resistance arteries.[2] However, there have been discrepancies regarding the specificity of PD98059, especially in high concentrations (40 μmol/L), at which this drug also caused inhibition of responses to potassium in rat middle cerebral arteries.[24] In our experiments, at a lower concentration of PD98059 (10 μmol/L), the inhibition was specific for Ang II and did not have any effect on phenylephrine or K^+ contractions. The pathways involved remain to be determined, but the results support the concept of cross talk between the intracellular growth and contractile signaling mechanisms.[25]

The ability of candesartan (AT_1R antagonist) to block completely the Ang II–induced ERK1/2 activity and the lack of a further effect of PD123319 (AT_2R antagonist) show that Ang II–induced ERK1/2 activity is mediated by AT_1R in mesenteric resistance arteries and is not modified by AT_2R activation. These findings are supported by those of Touyz et al,[1] who also showed that the Ang II–induced ERK1/2 activation in VSMCs of human subcutaneous resistance arteries is mediated by AT_1R. In contrast, in cultured neuronal cells from the neonatal rat hypothalamus and brain stem, as well as in VSMCs from fetal and postnatal mice, activation of AT_2Rs reduces ERK1/2 activity.[26,27] These observations show the importance of not extrapolating from one experimental situation to another and suggest that ERK1/2 regulation may depend on the developmental stages and type of cell.

Our finding that incubation of arteries with Ro-31-8220 (calcium-dependent and -independent PKC inhibitor) or Go-6976 (calcium-dependent PKC inhibitor) inhibited Ang II–induced ERK1/2 activity suggests that calcium-dependent PKC is an upstream mediator of ERK1/2 activation. This finding is in agreement with previous studies in aortic VSMCs[28] and in cardiac myocytes,[29] showing that ERK1/2 signaling pathways are PKC dependent. In cardiac fibroblasts, however, Ang II–induced ERK1/2 activity was insensitive to the PKC inhibitor.[30] Furthermore, in the rabbit aorta, pressure-induced ERK1/2 activity was not suppressed by high concentrations of PKC inhibitors.[14] Therefore, present evidence suggests that the involvement of PKC in ERK1/2 activation differs between Ang II and pressure induction.

The present results also indicate that c-Src tyrosine kinase is located upstream from the ERK1/activation cascade, given that both the general tyrosine kinase inhibitor (genistein) and

the c-Src family tyrosine kinase inhibitor (herbimycin A) decreased Ang II–induced ERK1/2 activity. The results are consistent with the finding that VSMCs from c-Src–deficient transgenic mice also show a decrease in Ang II–induced ERK1/2 activity.[31] These results, in accordance with several other studies, emphasize the importance of c-Src in Ang II–stimulated ERK1/2 activity in VSMCs,[32,33] as in cardiac cells.[34]

In summary, the findings of the present study indicate for the first time that Ang II induces a substantial and rapid increase in ERK1/2 activity in intact pressurized resistance arteries, which we studied in the rat mesentery. Furthermore, ERK1/2 activity is involved in the contraction induced by Ang II, and this activity appears to involve the activation of AT_1R, calcium-dependent PKC, and c-Src. The results suggest that ERK1/2 activity mediates both the structural and functional effects of Ang II in these resistance arteries. Therefore, the results support a potential role for ERK1/2 activation in the control of peripheral resistance and, indeed, of hypertension, thus providing a basis for further work to test this possibility.

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