Involvement of Rho-Kinase and the Actin Filament Network in Angiotensin II–Induced Contraction and Extracellular Signal–Regulated Kinase Activity in Intact Rat Mesenteric Resistance Arteries


Abstract—We have previously shown that angiotensin II (Ang II) and pressure increase extracellular signal–regulated kinase (ERK) 1/2 activity synergistically in intact, pressurized resistance arteries in vitro. However, the mechanisms by which pressure and Ang II activate ERK1/2 in intact resistance arteries remain to be determined. The purpose of the present study was to investigate the involvement of Rho-kinase and the actin filament network in Ang II– and pressure-induced ERK1/2 activation, as well as in the contractile response induced by Ang II. Mesenteric resistance arteries (200 to 300 μm) were isolated, mounted in an arteriograph, and stimulated by pressure, Ang II, or both. Activation of ERK1/2 was then measured by an in-gel assay. In mesenteric resistance arteries maintained at 70 mm Hg, Ang II (0.1 μmol/L) induced contraction (29±1.4% of phenylephrine, 10 μmol/L–induced contraction) and significantly increased ERK1/2 activity. Selective inhibition of Rho-kinase by Y-27632 (10 μmol/L) and selective disruption of the actin filament network by cytochalasin B (10 μmol/L) both decreased the Ang II–induced contraction by 78±1.2% and 87±1.9%, respectively, and significantly diminished ERK1/2 activity. In the absence of Ang II, increasing intraluminal pressure from 0 to 70 or 120 mm Hg increased ERK1/2 activity. ERK1/2 activity at 120 mm Hg was similar to that observed at 70 mm Hg in the presence of Ang II. Pressure-induced ERK1/2 activation was markedly attenuated by cytochalasin B but not by Y-27632. These results indicate that whereas pressure-induced ERK1/2 activation requires an intact actin filament network, but not Rho-kinase, the activation of ERK1/2 and the contraction induced by Ang II require both Rho-kinase and an intact actin filament network in isolated, intact mesenteric resistance arteries. (Arterioscler Thromb Vasc Biol. 2001;21:1288-1293.)

Key Words: Ang II ■ MAP-kinase ■ Rho-kinase ■ actin filament network ■ resistance arteries

High luminal pressure and angiotensin II (Ang II) are both involved in the pathogenesis of cardiovascular diseases, such as hypertension. Hypertension is characterized by increased vascular resistance associated with changes in arterial wall structure and function in resistance arteries. Ang II receptors are coupled to a wide variety of signal transduction elements, including protein kinase C, c-Src tyrosine kinase, protein tyrosine phosphorylation, and extracellular signal–related kinase (ERK) 1/2. These pathways certainly play an important role in the regulation of vascular tone. We and others have previously shown that mechanical strain in the vessel wall induced by pressure activates ERK1/2 in vascular smooth muscle cells (VSMCs) both in vivo and in vitro. However, scarce information is available on the cellular signaling pathways mediating Ang II–induced contraction and on its possible interaction with pressure in the activation of ERK1/2, especially in intact resistance arteries.

Recent findings suggest that the small GTPase Rho and its target Rho-kinase play a crucial role in the regulation of blood pressure in vivo. In accordance with this concept, several in vitro studies have shown that the activated form of Rho-kinase plays a role in smooth muscle contraction, myosin light-chain phosphorylation, sensitization of contractile proteins to Ca2+, and stress fiber formation. Our recent study showed that Ang II and pressure both activate ERK1/2 via signaling pathways involving protein kinase C and tyrosine kinase. Thus, we hypothesized that Rho-kinase could be involved in Ang II–induced contraction and/or ERK1/2 activation in intact resistance arteries. In addition, Rho-kinase may also be regulated by cytoskeletal structures, and cytoskeletal elements play an important role in the mechanotransduction of flow and pressure in blood vessels. Indeed, disruption of cytoskeletal elements by cytochalasin B alters the generation...
of second messengers in various types of cells. Cytochalasin B also alters stretch-induced ERK1/2 activation in cultured VSMCs. Thus, the actin filament network could play a role in pressure-induced ERK1/2 activation in intact resistance arteries and possibly be involved in contraction, ERK1/2 activation, or both induced by Ang II.

Thus, the purpose of the present study was to investigate the signaling pathways of Ang II and intraluminal pressure in intact, pressurized, mesenteric resistance arteries by using Y-27632 (a specific Rho-kinase inhibitor) and cytochalasin B (an actin filament network disruptor).

**Methods**

Mesenteric resistance arteries (diameter 200 to 300 μm) from 12-week-old Wistar rats were dissected and mounted in an arteriograph, as described previously. The cannulated arterial segments were submerged in 6 mL of sterile, serum-free Dulbecco’s modified Eagle’s medium and exposed to an intraluminal pressure of 70 mm Hg for 1 hour while kept in an incubator containing 5% CO2 at 37°C. The functional integrity of the endothelial cell layer was assessed by testing the endothelium-dependent vasodilating effect of acetylcholine (10 μmol/L) after preconstriction with phenylephrine (10 μmol/L). Vessels not responding by full relaxation to acetylcholine were discarded.

**Functional Investigations**

Under continuous monitoring of vessel diameter by a video image analyzer (Living System), the arteries were challenged with Ang II (0.1 μmol/L), phenylephrine (10 μmol/L), or KCl (60 mmol/L) at 70 mm Hg of intraluminal pressure. No vessel was exposed to >1 drug. These experiments were performed in the absence or continuous presence of Y-27632 (10 μmol/L), PD98059 (10 μmol/L), or cytochalasin B (10 μmol/L).

**ERK1/2 Activity**

In arterial segments in which mitogen-activated protein kinase activation was measured, phenylephrine and acetylcholine were not used. After a 1-hour equilibration period under an intraluminal pressure of 70 mm Hg, arterial segments were exposed to the following conditions for 5 minutes: (1) 70 mm Hg, (2) 120 mm Hg, (3) 70 mm Hg and Ang II (0.1 μmol/L), or (4) 120 mm Hg and Ang II (0.1 μmol/L). This procedure was done in the absence or presence of Y-27632 (a selective inhibitor of Rho-kinase; 10 μmol/L), cytochalasin B (a selective disrupter of the actin filament network; 10 μmol/L), candesartan (an Ang II receptor type 1 inhibitor; 10 μmol/L), or PD98059 (2-[2’-amino-3’-methoxyphenyl]-ox-anaphthalen-4-one, a mitogen-activated protein kinase/ERK kinase inhibitor; 10 μmol/L). These inhibitors were added to the arterial preparation during the whole experiment (1-hour equilibration period and 5 minutes of stimulation).

In a preliminary series of experiments, we had measured the activation of ERK1/2 after 2, 5, 10, and 15 minutes of stimulation with pressure or with pressure plus Ang II. The ERK1/2 activity induced by pressure was 277±38%, 289±28%, 239±6%, and 265±13% (n=3) after 2, 5, 10, and 15 minutes, respectively, and after pressure plus Ang II, was 541±58%, 599±31%, 635±28%, and 578±62% (n=3) after 2, 5, 10, and 15 minutes, respectively.

The arterial segments were then immediately frozen in LN4 and stored at −80°C. In each set of experiments, an unmounted arterial segment that remained unexposed to pressure and drugs for 65 minutes was used as a “control preparation” whose ERK1/2 activity was considered 100%. Frozen arterial segments were pulverized and resuspended in ice-cold lysing buffer (40 μL), as described earlier for large arteries and adapted to resistance arteries. Suspensions were incubated on ice for 15 minutes and then centrifuged (12 000 g, 15 minutes, 4°C). The detergent-soluble supernatant fractions were retained, and protein content in each sample was determined by Lowry’s method.

ERK1/2 activity was assessed by an in-gel assay in gels containing myelin basic protein, a substrate for ERK1/2. A 12-μL aliquot of arterial extract and 8 μL of the sample were loaded on a 0.5 mg/mL myelin basic protein–containing 10% sodium dodecyl sulfate–polyacrylamide gel. After electrophoresis, SDS was removed from the gel. Phosphorylation of myelin basic protein was carried out by incubating the gels with 50 μCi of [32P]ATP, 100 mmol/L dithiothreitol, and 10 mmol/L ATP. Repeated washing of the gel terminated the reaction. Then the gels were placed in a 45-mL bath containing 10% propanol, 10% acetic acid, and Coomassie Blue. Excess dye was washed away by repeated rinsing of the gel. Subsequently, the gels were dried and analyzed for determination of ERK1/2 activity by using a Bio Imaging Analyzer (BAS1000, MacBAS).

**Drugs**

PD123319 was supplied by Research Biochemical International; PD98059 by New England Biolabs; Ang II, phenylephrine, cytochalasin B, and acetylcholine by Sigma Chemical Co; Dulbecco’s modified Eagle’s medium by Life Technology; and candesartan by Astra. Y-27632 was a kind gift of Yoshimoto Pharmaceutical Industries (Japan).

**Statistical Analysis**

Results are expressed as mean±SEM. Differences between means were evaluated by 1-way ANOVA. Differences were considered significant when P<0.05.

**Results**

Effect of PD98059 on Contraction

Phenylephrine (10 μmol/L, n=3), KCl (60 mmol/L, n=3), and Ang II (0.1 μmol/L) induced contraction in mesenteric resistance arteries (Figure 1). The contractions induced with phenylephrine and KCl were not affected by PD98059. However, the Ang II–induced contraction was significantly decreased by PD98059.

KCl- and Phenylephrine-Induced Contraction and ERK1/2 Activation

Phenylephrine (10 μmol/L, n=5) induced contraction in mesenteric resistance arteries, which was significantly attenuated by Y-27632 but not by cytochalasin B (Figure 2A). Similarly, KCl (60 mmol/L, n=5) induced contraction in mesenteric resistance arteries. This contraction was affected by neither Y-27632 nor cytochalasin B (Figure 2B). Neither
phenylephrine (10 μmol/L, n=3) nor a high potassium level (60 mmol/L KCl, n=3) induced an increase ERK1/2 activity (Figure 2C).

Ang II–Induced Contraction and ERK1/2 Activation

In arteries subjected to zero pressure for 65 minutes, addition of Ang II (0.1 μmol/L) for the final 5 minutes caused an increase in ERK1/2 activity (121% compared with control, n=3). Pressure (70 mm Hg) by itself increased ERK1/2 activity (290±13% compared with control, n=3). In addition, in pressurized arteries, Ang II further increased ERK1/2 activity (620±40% compared with control, n=3).

In isolated and pressurized mesenteric resistance arteries, Ang II (0.1 μmol/L, n=5) induced contraction (29±1.4% of phenylephrine-induced contraction) (Figure 3A). The magnitude of this response was significantly decreased in the presence of Y-27632 (a selective inhibitor of Rho-kinase, 10 μmol/L; n=5) or cytochalasin B (a selective disrupter of the actin filament network, 10 μmol/L; n=5) (Figure 3A). Candesartan (a selective Ang II type 1 receptor antagonist, 10 μmol/L) abolished the Ang II–induced contractile response (n=5, data not shown).

Ang II (0.1 μmol/L, n=9) added to arteries maintained under a pressure of 70 mm Hg increased ERK1/2 activity by 106% compared with arteries exposed to 70 mm Hg alone (Figure 3B). This additional ERK1/2 activation by Ang II, however, was not observed when intraluminal...
pressure was increased to 120 mm Hg (n=6, Figure 3B). Both Y-27632 and cytochalasin B (Figure 4) significantly decreased the activation of ERK1/2 by Ang II. Ang II–stimulated ERK1/2 activation was inhibited by Ang II type 1 receptor antagonism with 10 μmol/L candesartan, whereas the Ang II type 2 receptor antagonist PD123319 had no effect (n=5, data not shown).

### Pressure-Induced ERK1/2 Activation

Increasing intraluminal pressure in mesenteric resistance arteries from 0 to 70 mm Hg increased ERK1/2 activity (320±63%, n=8) (Figures 3B and 5A). A further increase in pressure from 70 to 120 mm Hg (n=6) resulted in an additional rise in ERK1/2 activity (Figure 3B). Pressure-induced ERK1/2 activation was not altered by Rho-kinase inhibition (Y-27632, 10 μmol/L; n=5) (Figures 5A and 5B). In contrast, cytochalasin B (10 μmol/L, n=7) decreased significantly the ERK1/2 activation induced by pressure (Figures 5A and 5B). Manipulation of the arteries during the mounting procedure did not activate ERK1/2 (346±63% in arteries subjected to 70 mm Hg and 92±4% in arteries mounted in the arteriograph but not subjected to pressure or drugs). In the presence of PD98059 (10 μmol/L, n=7), ERK1/2 activity in pressurized arteries was abolished (Figures 5A and 5B).

### Discussion

The main findings of this study are as follows: (1) In intact, rat mesenteric resistance arteries, the signaling pathways of Ang II–induced contraction and ERK1/2 activation involve both Rho-kinase activity and the intact actin filament network and (2) the signaling pathway of stretch (pressure)-induced ERK1/2 activation involves the intact actin filament network but not Rho-kinase.

Rho-kinase, the target of small G-protein Rho, is known to be involved in various functional activities of VSMCs, including contraction\(^{11,21}\) and the regulation of calcium sensitivity.\(^{22}\) The finding of the present study showing that Rho-kinase is involved in Ang II–induced contraction is in agreement with a previous work by Uehata et al\(^{11}\) showing that Rho-kinase is involved in the phenylephrine- but not in the K\(^+\)-induced contractile response of mesenteric resistance arteries. The effect of mitogen-activated protein kinase/ERK kinase inhibition on Ang II–induced contraction was considerably smaller compared with that obtained by Rho-kinase inhibition by Y-27632.\(^{3}\) Thus, these findings suggest that the signaling pathways linking Rho-kinase to ERK1/2 and the contractile apparatus are different in intact mesenteric resistance arteries subjected to physiological conditions of pressure and flow. Further investigations are required to clarify these differences in signaling pathways.

We found that in isolated, pressurized mesenteric resistance arteries, increases in intraluminal pressure enhanced ERK1/2 activity. This response was strongly amplified by Ang II type 1 receptor stimulation with Ang II. The first question arising from this observation is whether the increase in ERK1/2 activity due to Ang II was caused by Ang II per se or whether it was nonspecifically triggered by the contraction. In fact, pressure induces distension of the cannulated artery and generates mechanical strain on the smooth muscle cells, resulting in increased tension and stress in the vessel wall. However, when an arterial segment is contracted, its diameter decreases and this produces a decrease in strain, tension, and stress in the arterial wall. Thus, it is unlikely that an increase in ERK1/2 activity after isobaric contraction induced by Ang II could have been due to the change in diameter. Furthermore, if the contraction due to Ang II and not Ang II itself were responsible for the activation of ERK1/2, then phenylephrine and potassium, which produced an equivalent contraction, also should have activated ERK1/2. This was not the case. In addition (and by contrast with nonpressurized arteries),\(^{3}\) the effect of Ang II was much
higher in pressurized arteries, suggesting that ERK1/2 activation, which is implicated in VSMC hypertrophy and hyperplasia during remodeling of resistance arteries in response to frequent and/or sustained elevations in blood pressure, might be much more effective when circulating Ang II is higher. A similar synergism between pressure and Ang II was previously observed in the induction of fibronectin expression in isolated and pressurized rabbit aortas. We suggest that the synergistic effect of Ang II (probably together with other factors) and pressure on ERK1/2 activation might be important in the pathogenesis of vascular remodeling and increased peripheral resistance in hypertension.

In intact mesenteric resistance arteries (present study), pressure-induced ERK1/2 activation required an intact actin filament network but did not require Rho-kinase activation. Numaguchi et al have shown that mechanotransduction of stretch in cultured VSMCs isolated from the rat thoracic aorta involves both Rho-kinase and the intact actin filament network. The discrepancy between these 2 findings could reflect differences in vessel type (aorta vs resistance artery) and/or in experimental approaches (cultured cells vs intact arterial segment). Indeed, as pointed out by Hill et al, several reasons can explain the considerable differences in responses to stretch in cultured cells compared with intact vessel segments. For example, smooth muscle cells exhibit a synthetic phenotype rather than a contractile phenotype as seen in intact vessels. Cells in culture lose their spindle shape, whereas in situ cells are arranged circumferentially and are almost perpendicular to the long axis of the vessel. Another difference between the 2 types of preparation is that intact vessels contain a smooth muscle and an endothelial layer. Nevertheless, it is reasonable to state that endothelial cells, when also subjected to stretch when pressure is applied in intact arteries, cannot account for a large proportion of the response (ERK1/2 activation), because in 200- to 300-μm mesenteric arteries, they represent <10% of the cell population. Nevertheless, such an issue requires further investigations.

In a previous study, we have shown that in intact, pressurized resistance arteries, Ang II–induced ERK1/2 activation involves protein kinase C and c-Src–dependent tyrosine kinase. In the present study, we found that Rho-kinase inhibition markedly decreased Ang II–induced ERK1/2 activation under the same conditions. Furthermore, the attenuation of Ang II–dependent ERK1/2 activation by cytochalasin B indicated that in intact resistance arteries, signaling pathways involve not only Rho-kinase but also the actin filament network. Yamakawa et al have shown that Rho-kinase is not involved in Ang II–induced ERK1/2 activation in cultured VSMCs. It is also probable that the difference reflects the different experimental conditions (cultured cells vs whole, perfused arteries). The difference observed in ERK1/2 activation by Ang II and pressure cannot be attributed to a difference in time course of the activation process, because a large number of studies have previously reported that ERK1/2 activation induced by mechanical and pharmacological stimulation reaches a maximum between 2 and 10 minutes of stimulation.

To better compare whole arteries and cell cultures, it would be useful to investigate how stretch and Ang II influence ERK1/2 activity in fresh, cultured VSMCs from resistance arteries.

In summary, we found that in intact, pressurized resistance arteries, (1) Ang II–induced contraction and ERK1/2 activation (chemical transduction) involved both Rho-kinase and an intact actin filament network and (2) pressure-induced ERK1/2 activation (mechanotransduction) required an intact actin filament network but not Rho-kinase. These results support the concept that Rho-kinase and actin filaments may have a different role in signal transduction due to pressure and Ang II in resistance arteries, although pressure and Ang II act synergistically in the control of blood pressure.

References


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_Arterioscler Thromb Vasc Biol._ 2001;21:1288-1293
doi: 10.1161/hq0801.093653

_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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