Activation of RhoA and Inhibition of Myosin Phosphatase as Important Components in Hypertension in Vascular Smooth Muscle

Tetsuya Seko, Masaaki Ito, Yasuko Kureishi, Ryuji Okamoto, Nobuyuki Moriki, Katsuya Onishi, Naoki Isaka, David J. Hartshorne, Takeshi Nakano

Abstract—Two mechanisms are proposed to account for the inhibition of myosin phosphatase (MP) involved in Ca\(^{2+}\)-sensitization of vascular muscle, ie, phosphorylation of either MYPT1, a target subunit of MP or CPI-17, an inhibitory phosphoprotein. In cultured vascular aorta smooth muscle cells (VSMCs), stimulation with angiotensin II activated RhoA, and this was blocked by pretreatment with 8-bromo-cGMP. VSMCs stimulated by angiotensin II, endothelin-1, or U-46619 significantly increased the phosphorylation levels of both MYPT1 (at Thr96) and CPI-17 (at Thr38). The angiotensin II–induced phosphorylation of MYPT1 was completely blocked by 8-bromo-cGMP or Y-27632 (a Rho-kinase inhibitor), but not by GF109203X (a PKC inhibitor). In contrast, phosphorylation of CPI-17 was inhibited only by GF109203X. Y-27632 dramatically corrected the hypertension in N\(^{\text{nitro}}\)-nitro-L-arginine methyl ester (L-NAME)–treated rats, and this hypertension also was sensitive to isosorbide mononitrate. The level of the active form of RhoA was significantly higher in aortas from L-NAME–treated rats. Expression of RhoA, Rho-kinase, MYPT1, CPI-17, and myosin light chain kinase were not significantly different in aortas from L-NAME–treated and control rats. Activation of RhoA without changes in levels of other signaling molecules were observed in three other rat models of hypertension, ie, stroke-prone spontaneously hypertensive rats, renal hypertensive rats, and DOCA-salt rats. These results suggest that independent of the cause of hypertension, a common point in downstream signaling and a critical component of hypertension is activation of RhoA and subsequent activation of Rho-kinase. (Circ Res. 2003;92:411-418.)

Key Words: hypertension ■ RhoA ■ Rho-kinase ■ myosin phosphatase ■ Ca\(^{2+}\) sensitization

Although the mechanisms underlying development of hypertension are not established, one critical feature observed in most cases of hypertension is increased peripheral resistance, implying enhanced constriction of the relevant vessels. A major determinant of vascular tone is the level of myosin light chain (MLC) phosphorylation that is controlled by the Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase (MLCK) and by myosin phosphatase (MP). However, there is no fixed relationship between cytosolic Ca\(^{2+}\) and MLC phosphorylation and this can vary depending on conditions. An increased phosphorylation and tension can occur at suboptimal Ca\(^{2+}\) levels and is referred to as Ca\(^{2+}\) sensitization and occurs frequently after stimulation by many agonists. A major contributor to this effect is inhibition of MP and over the last decade this was shown to be operating via a small GTPase RhoA-linked pathway.

There is a strong evidence to implicate Rho-kinase as a downstream target in the RhoA-linked pathway, and it has been shown that Rho-kinase inhibitors, such as Y-27632, block the agonist-induced Ca\(^{2+}\) sensitization in smooth muscle. At a molecular level, one mechanism responsible for inhibition of MP is that phosphorylation by Rho-kinase of the target subunit of MP, MYPT1, at Thr96 (for the human isoform) inhibits activity of the catalytic subunit. Another mechanism involves CPI-17, a smooth muscle–specific inhibitory protein for MP. Phosphorylation at Thr38, by several kinases including protein kinase C (PKC), increases the inhibitory potency of CPI-17. Activation of MP also is thought to occur via the NO/cyclic GMP (cGMP) pathway, and this decreases MLC phosphorylation at a given Ca\(^{2+}\) concentration, ie, Ca\(^{2+}\) desensitization. Molecular basis for activation of MP is not established, but may involve the prevention of RhoA activation to form GTP-RhoA after phosphorylation of RhoA by cGMP-dependent protein kinase I (cGKI).

Rho-kinase inhibitors have been widely used to illustrate the participation of Rho-kinase in several pathological conditions. In the cardiovascular field, these include hypertension, coronary artery spasm, effort angina, vascular inflammation and remodeling, myocardial cell hypertro-
The marked effect of Y-27632 (a selective Rho-kinase inhibitor) on various rat models of hypertension, namely, spontaneously hypertensive rats (SHR), 2-kidney 1-clip renal hypertensive rats, and DOCA-salt rats, indicate an important role for Rho-kinase in various types of hypertension. The possible involvement of Rho-kinase in human hypertension also was reported recently. Thus, several lines of evidence indicate a central role for Rho-kinase in hypertension, but the molecular mechanism controlled by Rho-kinase phosphorylation is not established.

To explore this topic in more detail, we investigated RhoA/Rho-kinase signaling in VSMCs with respect to their role in hypertension. It was found that agonist stimulation induced phosphorylation of MYPT1 at Thr696 via the RhoA pathway. In addition, it was shown that cGMP signaling inhibited phosphorylation of MYPT1 by blocking the activation of RhoA and that hypertension induced by chronic inhibition of NO synthesis could be reduced by the Rho-kinase inhibitor. In 4 different rat models of hypertension, the activation of RhoA/Rho-kinase signaling in VSMCs with respect to their role in hypertension. It was found that agonist stimulation induced phosphorylation of MYPT1 at Thr696 via the RhoA pathway. In addition, it was shown that cGMP signaling inhibited phosphorylation of MYPT1 by blocking the activation of RhoA and that hypertension induced by chronic inhibition of NO synthesis could be reduced by the Rho-kinase inhibitor. In 4 different rat models of hypertension, the activation of RhoA in the aorta was detected, suggesting that a common mechanism in hypertension involves initial activation of RhoA and subsequent activation of Rho-kinase.

Materials and Methods

Materials

Y-27632 (R[R]trans-N-[4-pyridyl]-4-[l-amino ethyl]-cyclohexane carboxamide) and isosorbide mononitrate (ISMN) were generously provided by Mitsubishi Pharma Corp (Osaka, Japan) and Toaeryo Corp (Tokyo, Japan), respectively. The following chemicals were used: N' nitro-L-arginine methyl ester (L-NAME; Sigma), 8-bromocGMP (Calbiochem), U-46619 (Cayman Chemical), GF109203X (BIOMOL Research Laboratories), angiotensin II, and endothelin-1 (Peptide Institute).

Animal Preparation

The experimental procedures were approved by the Animal Investigation Committee of the Pie University School of Medicine. All hypertensive rat models except stroke-prone spontaneously hypertensive rats (SHRSP) were prepared from male Sprague-Dawley (SD) rats (Japan SLC, Hamamatsu, Japan). L-NAME–treated hypertensive rats were prepared by the addition of L-NAME (1 mg/mL) to the drinking water for more than 3 weeks. The control group received untreated drinking water. The preparations of 2-kidney, 1-clip renal hypertensive rats (2K1C) with the minor modification of using an acril clip (0.2 mm slit) and DOCA-salt hypertensive rats were prepared as previously described. SHRSP and aged-matched Wister Kyoto rats (WKY) were obtained from Diseases Model Cooperative Research Association (Kyoto, Japan). The angiotensin II–induced hypertensive rat model was prepared by continuous infusion of angiotensin II. Angiotensin II (dissolved in 0.01 N acetic acid) was infused subcutaneously using on osmotic pump (model 2002, Alza Corporation) at a dose of 200 ng/kg per minute for 7 days. Rats from all groups were used when 10 to 12 weeks old. The systolic blood pressure was measured by the tail cuff method (Softron Co Ltd). Y-27632 and ISMN, dissolved in MilliQ water at a concentration of 2 mg/mL, were administrated orally using metal tube to L-NAME–treated and control rats. In the previous study, using the same condition, the plasma level of Y-27632 was elevated near to that required for the relaxation of isolated blood vessel stimulated by agonists (M. Uehata, personal communication).

Culture of Vascular Smooth Muscle Cells

Vascular smooth cell cultures were prepared by enzymatic digestion of aortas from 10-week-old SD rats. The cells were propagated in DMEN containing 10% FBS in 5% CO₂ at 37°C and were used between passage 0 and 3. High levels of β-caldesmon and calponin expression were observed in these VSMCs and time-lapse microscopy revealed contraction of about half of the VSMCs on angiotensin II stimulation, indicating a differentiated phenotype for these cells.

Tissue Preparation

Aortas were removed from the hypertensive rats, immediately frozen in liquid nitrogen, and homogenized using a Cryo-Press (Microtec Co Ltd) in a modified buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 150 mmol/L NaCl, 0.25% Na-deoxycholate, 1 mmol/L EDTA, 0.1% SDS) with protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μmol/L p-amidinophenylmethanesulfonyl fluoride hydrochloride). After centrifugation at 10,000g for 30 minutes at 4°C, the supernatant was applied to SDS-PAGE and subsequent Western blotting.

Western Blot Analysis

Protein-matched samples were separated by SDS-PAGE and transferred to PVDF (Millipore) membrane. The primary antibodies used in this study were mouse monoclonal anti-ROKα and anti-ROKβ (Transduction Laboratories), rabbit polyclonal anti-RhoA (Santa Cruz Biotechnology Inc), anti-MYPT1, anti-CPI-17, and anti-MLCK antibodies. Immunodetection was accomplished using appropriate horseradish peroxidase-linked secondary antibodies (Amersham) and the enhanced chemiluminescence (ECL) kit.
Activated RhoA for the pull-down assay. Detection of RhoA was performed by Western blot using anti-RhoA antibody.

**Determination of the Phosphorylation Levels of MYPT1 and CPI-17**

Subconfluent cells were incubated in serum-free medium for 24 hours, then various agonists (angiotensin II, endothelin-1, U-46619) were added to the medium. In some experiments, serum-starved cells were pretreated with 8-bromo-cGMP, Y-27632, or GF109203X before stimulation with angiotensin II. At the times indicated, reactions were terminated by addition of ice-cold trichloroacetic acid (final 10% wt/vol) and the samples were processed as described previously.

Phosphorylation of MYPT1 at Thr696 and of CPI-17 at Thr38 were analyzed by Western blot using anti-phospho pMYPT1T696 and anti-MYPT1 antibody, respectively. Bottom, Densitometrical data are summarized. Mean density of phosphorylated MYPT1 (top) versus the total MYPT1 density (middle) for the controls was expressed as 1 arbitrary unit. Control, VSMCs without stimulation; Ang II, VSMCs pretreated with angiotensin II for 10 minutes; U-46619 (2 μmol/L) for 10 minutes; Ang II + Y-27632, VSMCs pretreated with Y-27632 (10 μmol/L) for 30 minutes, followed by the stimulation with angiotensin II for 10 minutes; Ang II + Y-27632, VSMCs pretreated with Y-27632 (10 μmol/L) for 30 minutes, followed by the stimulation with angiotensin II (1 μmol/L) for 10 minutes; ET-1, VSMCs stimulated with endothelin-1 (0.1 μmol/L) for 10 minutes; U-46619, VSMCs stimulated with U-46619 (2 μmol/L) for 2 minutes. n=5 to 8. *P<0.01.

**Results**

**Activation of RhoA in VSMCs by Angiotsensin II**

RhoA activation was determined by affinity precipitation of the active GTP-bound RhoA using a glutathione S-transferase (GST)-fusion protein of the Rhobinding domain of the Rho effector rhotekin (GST-RBD) as previously described. The plasmid for GST-RBD was a generous gift from Dr M.A. Schwartz (The Scripps Research Institute, La Jolla, Calif). In brief, frozen aortas from hypertensive and control rats or VSMCs stimulated by agonists were lysed with RhoA-RBD buffer. Then GST-RBD bound to glutathione Sepharose 4B was added to the lysate to selectively bind activated RhoA for the pull-down assay. Detection of RhoA was performed by Western blot using anti-RhoA antibody.

**Phosphorylation of MYPT1 at Thr696 in VSMCs**

Levels of MYPT1 phosphorylation at the inhibitory site (Thr696) were examined in VSMCs. As shown in Figure 2A, stimulation with angiotensin II (1 μmol/L) significantly increased the level of MYPT1 phosphorylation. A lower dose of angiotensin II (0.1 μmol/L) enhanced MYPT1 phosphorylation to a similar extent (data not shown). Pretreatment with 8-bromo-cGMP (100 μmol/L) and Y-27632 (10 μmol/L) markedly suppressed angiotensin II–induced phosphorylation of MYPT1. Both values were significantly (P<0.01) lower than control (non-angiotsensin II–treated) cells. However, angiotensin II–induced MYPT1 phosphorylation was insensitive to pretreatment with either 8-bromo-cGMP or Y-27632 (Figure 2A).

**Phosphorylation of CPI-17 in VSMCs**

Phosphorylation of CPI-17 in VSMCs after agonist stimulation also was monitored. As shown in Figure 3A, stimulation with angiotensin II (1 μmol/L) significantly increased the level of CPI-17 phosphorylation at Thr38. The phosphorylation of CPI-17 by angiotensin II was insensitive to pretreatment with either 8-bromo-cGMP or Y-27632 (Figure 3A), but was significantly inhibited by GF109203X (5 μmol/L) (Figure 4B). Endothelin-1 (0.1 μmol/L) and U-46619 (2 μmol/L), as shown in Figure 3B, significantly increased the level of CPI-17 phosphorylation at Thr38.

**Effect of Y-27632 on L-NAME–Induced Hypertension**

The antagonistic effect of cGMP on RhoA signaling indicated the involvement of Rho-kinase in hypertension induced by inhibition of NO synthesis. Chronic inhibition
of NO synthesis by oral administration of L-NAME increased blood pressure. As shown in Figure 5, after 3 weeks the systolic blood pressure in L-NAME–treated groups was about 40 mm Hg higher than control. The effects of Y-27632 (30 mg/kg) on systolic blood pressures of the L-NAME–treated and control rats are shown as time courses in Figure 5 (top panel). The changes in blood pressure also are plotted (Figure 5, bottom panel). Oral administration of Y-27632 significantly decreased the blood pressure in the L-NAME–treated rats but had little effect on controls. The antihypertensive effect of Y-27632 was observed at 1 hour, peaked at 5 hours and lasted for 7 hours. Administration of lower doses (5 mg/kg) of Y-27632 caused no significant change in blood pressure in treated and control groups (data not shown). As expected, the administration of ISMN (30 mg/kg) to the L-NAME–treated rats caused a significant and persistent fall in blood pressure (data not shown). The same dose of ISMN had no effects on SHRSP (data not shown).

Expression of Molecules Related to Rho-Kinase Signaling in Rat Hypertensive Models

The expression levels of the following proteins were evaluated in thoracic aortas of hypertensive and control rats: RhoA, Rho-kinase (ROKα and ROKβ isoforms), MYPT1, CPI-17, and MLCK. Western blot analyses were carried out using specific antibodies. As summarized in the Table, there were no significant changes in expression of these proteins in the L-NAME–treated hypertensive group and the control normotensive group. Similarly, no significant changes in expression of these molecules were detected in the other three rat hypertensive models, namely DOCA-salt rats, renal hypertensive rats, and SHRSP. Hypertension in the two former models was reported to be sensitive to Y-27632.8

Activation of RhoA in Various Rat Hypertensive Models

The levels of GTP-bound active form of RhoA in aortas from hypertensive rat models were compared with those in controls.
RhoA activation were monitored in the angiotensin II development of chronic hypertension in vivo, changes in acute effect of angiotensin II on RhoA activity in VSMCs to significantly higher in all rat hypertensive models. To compare the shown in Figure 6, the levels of GTP-RhoA were significantly higher in all rat hypertensive models vs those in the normotensive counterparts. The sensitivity of phosphorylation of MYPT1 site (Thr696 in human MYPT1) was phosphorylated via a RhoA/Rho-kinase pathway after agonist stimulation of VSMCs; (2) MYPT1 phosphorylation at Thr696 was inhibited by cGMP-signaling as a result of inhibition of RhoA activation; (3) CPI-17 was phosphorylated at Thr38 in VSMCs on agonist stimulation by a PKC-linked pathway; (4) the Rho-kinase inhibitor, Y-27632, normalized L-NAME--induced hypertension; and (5) increased activation of RhoA and the subsequent increase in Rho-kinase activity are common pathobiological phenomena observed in hypertension.

It is established that Ca2+ sensitization of vascular smooth muscle involves a G-protein-coupled mechanism(s) linked to activation of RhoA, subsequent activation of Rho-kinase and inhibition of MP.5,6 Many agonists can initiate this process, including U-46619 and endothelin-1. Earlier data suggested that angiotensin II was not effective,28 although our results demonstrated that angiotensin II caused RhoA activation in VSMCs. This is consistent with the proposal that in VSMCs, the receptors for various vasoconstrictors, including angiotensin II, are coupled to both Gq/G11 and G12/G13. The latter are thought to be linked to RhoA/Rho-kinase activation.29 Although there are other cellular targets for RhoA, including PIP5-kinase and protein kinase N, the most likely downstream partner for RhoA in this aspect of smooth muscle function is Rho-kinase.

Rho-kinase has several substrates but those implicated in MP inhibition are MYPT1 and CPI-17.12 The latter also is regulated by phosphorylation with PKC.11 Both proteins were phosphorylated at the inhibitory sites, Thr696 for MYPT1 and Thr38 for CPI-17, after treatment with the three agonists (angiotensin II, endothelin-1, and U-46619). However, phosphorylation of the two proteins was sensitive to different protein kinase inhibitors, MYPT1 phosphorylation was blocked by the Rho-kinase inhibitor, Y-27632, and CPI-17 phosphorylation was blocked by the PKC inhibitor, GF109203X. Previously it was found that CPI-17 phosphorylation was insensitive to another Rho-kinase inhibitor, hydroxyfasudil, in serotonin-stimulated human thoracic artery.30 The sensitivity of phosphorylation of MYPT1 to 8-bromo-cGMP, but not CPI-17, is another important point of difference. It was suggested that the antagonistic role of cGMP and

Expression of Rho-Kinase Signal-Related Molecules in Various Types of Hypertensive Rat Models

<table>
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<tr>
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<th>RhoA</th>
<th>ROKα</th>
<th>ROKβ</th>
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<th>CPI-17</th>
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<td>ND</td>
<td>100±7</td>
<td>104±8</td>
<td>98±10</td>
</tr>
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Values are expressed as percent relative expression levels of the molecules in the hypertensive models vs those in the normotensive counterparts. Data are mean±SEM. n=3 to 4. ND indicates not determined.
cAMP to RhoA signaling reflected phosphorylation of RhoA by cGKIα or cAMP-dependent protein kinase and a resulting block in signaling function. It should be noted that the proposed block of RhoA function would not activate MP above the non-phosphorylated control level but would only prevent inhibition. The effect of 8-bromo-cGMP on the angiotensin II–induced phosphorylation of MYPT1 would be consistent with the participation of RhoA/Rho-kinase. Thus, of the two candidates involved phosphorylation of MYPT1 seems to be more important in the hypertensive response. The ineffectiveness of a PKC inhibitor on chronically hypertensive rats supports this hypothesis. This is the first report to demonstrate phosphorylation at the inhibitory site (Thr696) of MYPT1 in VSMCs by vasoconstrictor agonists. Although the above evidence favors a role for Rho-kinase, phosphorylation of MYPT1 by other kinases may occur via different signaling pathways. Within the limitations imposed by our experiments, it is suggested that PKC plays a major role in agonist-induced CPI-17 phosphorylation. Recently it was reported that CPI-17 was phosphorylated by PKCa and δ isoforms in histamine-induced vascular constriction. Although CPI-17 is phosphorylated by Rho-kinase in vitro, the corresponding in vivo phosphorylation was not observed in our experiments. Also, it is interesting that Etter et al showed that CPI-17 was dephosphorylated during relaxation of carotid artery induced by NO donors. In contrast, the above results indicate that increased cGMP levels did not inhibit CPI-17 phosphorylation. The reason for this discrepancy is not known, although the difference between normal and hypertensive tissues may be a factor.

The increased level of GTP-RhoA (activated) could be due to either a shift in the balance of GDP-RhoA/GTP-RhoA, or to increased expression of RhoA in the hypertensive state. Examples of the latter include increased expression of RhoA in hypertensive rat models and an upregulation of Rho-kinase RNA in SHR carotid arteries. In a different system, Wang et al showed a considerably higher level of RhoA in corpus cavernosum, compared with ileum, that was reflected by an increased Ca2+ sensitivity. However, it is shown above that expression of RhoA and several other signaling molecules in the hypertensive rat models remained at normotensive levels. The molecular basis for development of hypertension in the rat models is not known. However, it is evidently upstream of the activation of RhoA and may involve receptors and ion channels at the membrane level and/or subsequent signaling via trimeric G proteins and various RhoA partners, such as RhoA guanine nucleotide exchange factors (RhoA-GEFs). In this scenario, the activation of RhoA/Rho-kinase would be secondary to a causative upstream event. As suggested earlier the elevated Rho-kinase activity in the vasculature may represent a positive feedback mechanism that would further increase vascular resistance above normotensive levels.

Other factors that may contribute to hypertension have been cited. These include enhancement of the renin angiotensin system in 2K1C renal hypertensive rats, increased tissue angiotensin II in SHR and elevated endothelin-1 in DOCA-salt rats and SHRSP. Endothelial dysfunction and lowered NO production was observed in L-NAME–treated rats and DOCA-salt rats, but these changes in SHRSP and renal hypertensive models are controversial. In addition, mechanical stress was reported to activate RhoA in VSMCs. Each of these factors associated with hypertension are translated to merge at a common point, namely the activation of RhoA and subsequent activation of Rho-kinase.

The normal process of Ca2+ sensitization in vascular tissue and hypertension both involve activation of RhoA. In addition, it was shown recently that Rho-kinase contributes to arterial tone in both normal and hypertensive vessels. Yet, a puzzling feature, shown originally by Uehata et al and confirmed above, is that inhibition of Rho-kinase does not lower blood pressure in normotensive rats. In addition, it was reported that no significant increase in Ca2+ sensitivity was found in vessels from SHR. One obvious difference between the acute effect of Ca2+ sensitization and the more chronic hypertensive state are the time periods involved. The
acute phase is relatively rapid and in our results, this is mimicked by the changes in RhoA observed in VSMCs after 10 minutes incubation with angiotensin II. The development of angiotensin II–induced hypertension takes several days. Under the conditions used in our study, the systolic blood pressure was only slightly increased after 1 day and reached a steady maximum at 5 days. The longer time period may be required to cause a sustained molecular change rather than the cyclic and relatively rapid changes found in Ca$^{2+}$ sensitization. Another suggestion is that any effect of Y-27632 on the sensitization of smooth muscle mediated by a Rho-kinase inhibitor plays a key role for coronary artery spasms in a porcine model with interleukin-1β.

These studies suggest that RhoA functions as a molecular switch in hypertension and thus the signaling pathway(s) leading to and including RhoA/Rho-kinase would be an appropriate target for therapeutic intervention. Further studies are required to define the molecular changes that occur upstream of RhoA activation that are responsible for development of hypertension. For practical reasons the above studies were carried out with rat aorta, but it is necessary to analyze other parts of the vasculature, particularly the resistance vessels.

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