Increased RhoA/Rho-Kinase Signaling Mediates Spontaneous Tone in Aorta from Angiotensin II-Induced Hypertensive Rats

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ABSTRACT
Spontaneous tone in large arteries may contribute to the pathogenesis of hypertension. Reactive oxygen species and Ca\(^{2+}\) influx have been shown to stimulate the development of spontaneous tone in isolated aortic rings in several models of hypertensive rats. The aim of this study was to investigate the role of the RhoA/Rho-kinase signaling pathway in the development of spontaneous tone in angiotensin II-induced hypertension and to explore the underlying mechanisms of RhoA/Rho-kinase activation. Our results showed that spontaneous tone was greatly enhanced in endothelium-denuded aortic rings from angiotensin II-induced hypertensive rats compared with their normotensive counterparts (73 ± 5 versus 7 ± 3% of phenylephrine-induced maximal contraction, respectively). The Rho-kinase inhibitor \((\mathcal{R})(-\mathcal{R})\)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) (0.1–10 \(\mu\)M) concentration dependently inhibited spontaneous tone in aortic rings from angiotensin II-treated rats. NADPH oxidase inhibitors diphenylene iodonium and apocynin also significantly reduced spontaneous tone. Chronic angiotensin II treatment markedly increased RhoA protein expression (57%) but had no effect on Rho guanine nucleotide exchange factor mRNA or Rho-kinase protein expression levels. In endothelium-denuded rings from normotensive rats, angiotensin II (100 nM) increased RhoA membrane translocation and phosphorylation of the myosin light chain phosphatase target subunit, which were both blocked by the NADPH oxidase inhibitor diphenylene iodonium (10 \(\mu\)M). In conclusion, these data suggest that chronic treatment with angiotensin II leads to up-regulation of the RhoA/Rho-kinase pathway, contributing to spontaneous tone development in rat aorta. Increased NADPH oxidase-dependent reactive oxygen species may be one of the mechanisms mediating the RhoA/Rho-kinase activation.

Angiotensin II (Ang II) produced by the renin-angiotensin system is an important hormone in the homeostasis of cardiovascular and renal function. Increased circulating Ang II promotes inflammation and cell growth and increases vascular reactivity (Kagiyma et al., 2002; Seshiah et al., 2002). It is well known that activation of Ang II type I receptor (AT\(_1\)) increases free intracellular Ca\(^{2+}\) concentration and myosin light chain (MLC) kinase activity, leading to MLC phosphorylation and subsequent smooth muscle contraction. Recent studies suggest that the phosphorylation state of MLC is also modulated by RhoA/Rho-kinase, a Ca\(^{2+}\) sensitization signaling pathway through which smooth muscle is able to contract when the intracellular Ca\(^{2+}\) concentration is low (Somlyo and Somlyo, 2003).

The activity of RhoA is regulated by Rho guanine nucleotide exchange factors (RhoGEFs) and Rho GDP disassociation inhibitors (Somlyo and Somlyo, 2003). In its inactive state, RhoA binds with GDP and forms a complex with GDP disassociation inhibitors in the cytosol. When RhoGEF is activated by G protein-coupled receptors such as AT\(_1\) or receptor tyrosine kinase, it facilitates the exchange of GTP for GDP on RhoA. RhoA-GTP migrates to the plasma membrane and consequently increases Rho-kinase activity. Rho-kinase phosphorylates MLC phosphatase target subunit (MYPT1) and thereby inhibits MLC phosphatase and prolongs MLC phosphorylation.

Abbreviations: Ang II, angiotensin II; AT\(_1\), Ang II type I receptor; MLC, myosin light chain; RhoGEF, Rho guanine nucleotide exchange factor; MYPT1, myosin light chain phosphatase target subunit; ROS, reactive oxygen species; NO, nitric oxide; PE, phenylephrine; ACh, acetylcholine; EC, endothelial cell, Y-27632, \((\mathcal{R})(-\mathcal{R})\)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide; DPI, diphenylene iodonium; DMSO, dimethyl sulfoxide; p-MYPT1, phosphorylated MYPT1; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LARG, leukemia-associated RhoGEF; RGS, regulator of G protein signaling; DOCA, deoxycorticosterone; PKC, protein kinase C; CPI-17, PKC-potentiated inhibitor protein of 17 kDa.
Hypertension is known to increase arterial tone in both small resistance arteries (myogenic tone) and large conduit arteries (spontaneous tone) that increase total peripheral resistance. Spontaneous tone is an increased contraction of isolated arteries without agonist stimulation. It has been observed in several types of hypertension including Ang II-induced hypertension, which is mediated by reactive oxygen species (ROS) and Ca\(^{2+}\) influx (Di Wang et al., 1999; Northcott et al., 2002; Ghosh et al., 2004). However, the role of the RhoA/Rho-kinase signaling pathway in the development of spontaneous tone in Ang II-induced hypertensive rats has not been investigated. In addition, whether the RhoA/Rho-kinase signaling pathway is up-regulated in Ang II-induced hypertensive animals is not known although studies have shown increased RhoA/Rho-kinase activity by Ang II in cell cultures (Yamakawa et al., 2000; Seko et al., 2003). The first aim of this study was to determine whether the RhoA/Rho-kinase pathway is involved in the spontaneous tone development and whether the RhoA/Rho-kinase pathway is up-regulated in blood vessels from Ang II-induced hypertensive rats.

Growing evidence suggests that ROS play an important role in the pathogenesis of hypertension. ROS not only reduce nitric oxide (NO) bioavailability and impair endothelium-dependent relaxation but also act as second-messenger molecules to modulate the responses of a cell to extracellular stimuli through activation of signaling pathways (Ohtsu et al., 2005). Sources of ROS include NADPH oxidase, uncoupled endothelial nitric-oxide synthase, xanthine oxidase, and the mitochondrial respiratory chain in various vascular beds. Previously, we and others reported that ROS produced by mitochondria increased Rho-kinase activity in arteries (Jin et al., 2004; Bailey et al., 2005). Studies have shown that Ang II-stimulated ROS production is mainly dependent on NADPH oxidase activity (Griendling et al., 2000). It is not clear whether Ang II-induced RhoA/Rho-kinase activation is dependent on the activation of NADPH oxidase. Therefore, the second aim of this study was to investigate the role of ROS stimulated by Ang II in activation of RhoA/Rho-kinase.

Materials and Methods

Animal Preparation and Blood Pressure Measurement

Sprague-Dawley rats (275–299 g; Harlan, Indianapolis, IN) were implanted s.c. with osmotic minipumps (Alzet; DURECT Corporation, Cupertino, CA) after they were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). The rats were divided into two groups: a control group infused with saline only and an Ang II-treated group infused with Ang II (40 ng/kg/min) as described previously (Zhao et al., 2005). Systolic blood pressure was measured by tail cuff plethysmography every 3 days in conscious rats to monitor the progression of hypertension. The basal level of systolic blood pressure was measured on 2 consecutive days before the implantation of minipumps. All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals at the Medical College of Georgia.

Measurement of Isometric Tension.

The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Aortae were rapidly excised and placed in an ice-cold physiological saline solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.18 mM KH\(_2\)PO\(_4\), 1.6 mM CaCl\(_2\), 1.6 mM MgSO\(_4\), 7H\(_2\)O, 25 mM NaHCO\(_3\), 5.5 mM dextrose, and 0.03 mM EDTA. After removal of adventitial tissue, aortae were cut into 2-mm rings and mounted in myograph organ chambers (DMT A/S, Aarhus, Denmark).

Assessment of Endothelium Function.

The aortic rings were allowed to equilibrate for 60 min under a passive tension of 30 mN in physiological saline solution gassed with 95% O\(_2\)/5% CO\(_2\) at 37°C. The rings were precontracted by phenylephrine (PE, 1 \(\mu\)M) and cumulative relaxation curves to acetylcholine (ACh, 0.001–10 \(\mu\)M) were obtained on each ring for assessment of endothelium function. Changes in isometric force were recorded by a PowerLab 8/35 data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia). ACh-induced relaxations were expressed as percentage of PE-induced maximal contraction.

Spontaneous Tone Development.

In some of the aortic rings, the endothelium layer was gently removed. After equilibration for 60 min, the endothelial cells (EC)-intact or EC-denuded aortic rings were then relaxed with sodium nitroprusside (10 \(\mu\)M) to reduce any spontaneous tone developed during the equilibration period and the passive tension was readjusted to 30 mN. After washing and re-equilibration for 30 min, the aortic preparations were subjected to 10 \(\mu\)M PE contraction. ACh (1 \(\mu\)M) was added to the plateau phase of maximal contraction to determine whether the endothelium was intact or completely denuded. Finally, after PE and ACh were washed off, spontaneous tone was allowed to develop over time. The maximal spontaneous tone was expressed as percentage of PE-induced maximal contraction.

Effect of NADPH Oxidase and Rho-Kinase Inhibitors on Ang II-Induced Contraction.

To determine the involvement of NADPH oxidase and Rho-kinase in Ang II-induced contraction of aortic rings from normotensive rats, the rings were preincubated with DPI (10 \(\mu\)M), vehicle (DMSO, final concentration is 0.1%), or Y-27632 (1 \(\mu\)M) to reduce any spontaneous tone. Finally, after PE and ACh were washed off, spontaneous tone was allowed to develop over time. The maximal spontaneous tone was expressed as percentage of PE-induced maximal contraction.

Western Blot

Protein expressions of RhoA and Rho-kinase were determined by Western blot as described previously (Jin et al., 2004). In brief, aortae from control and Ang II-treated rats were excised, cleaned, and snap-frozen in liquid nitrogen. Tissue was homogenized in an ice-cold radiimmunoprecipitation assay buffer (Upstate Biotechnology, Lake Placid, NY), and the protein concentration was determined by the BCA kit (Pierce Chemical, Rockford, IL). Equal amounts of protein were loaded and separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membrane. Antibodies against RhoA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Rho-kinase (BD Biosciences, San Jose, CA) were used to detect protein expressions. The RhoA and Rho-kinase protein expressions in normotensive controls and Ang II-induced hypertensive rats were normalized by \(\beta\)-tubulin.

To examine the effects of Ang II and NADPH oxidase-dependent ROS on RhoA/Rho-kinase activity, aortic rings were isolated from normotensive rats and mounted in the organ chambers after removal of endothelium. The rings were contracted with Ang II (100 nM) in the presence of DPI or vehicle (DMSO, final concentration is 0.1%). The rings were then immediately snap-frozen when the contraction reached to maximum. Ang II-induced RhoA translocation and phosphorylation of MYPT1 (p-MYPT1; Santa Cruz Biotechnology) was determined as described previously (Jin et al., 2004). The membrane fraction of RhoA was normalized by \(\beta\)-actin protein levels, and p-MYPT1 was normalized by MYPT1 protein expression.
Reverse Transcription-PCR

The PDZ-RhoGEF primers were as follows: forward, 5'-GGGACCTCCTTCCAGAGGAAA-3', and reverse, 5'-GGGCAGCAGCTTGTCCTGCAGG-3'. Leukemia-associated RhoGEF (LARG) primers were as follows: forward, 5'-AGCCAGTCCGCTGGAGTACAAC-3', and reverse, 5'-GGGCAGCAGCTTGTCCTGCAGG-3'.

p115RhoGEF primers were as follows: forward, 5'-TCGGAGTGGGGCAAGCAG-3', and reverse, 5'-TACCCAGGTCCTCTCGGCTTG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were as follows: forward, 5'-ACAGCCTTGGGACAGCAGTGGGAT-3', and reverse, 5'-ACAGCCTTGGGACAGCAGTGGGAT-3'.

Total RNA (4 μg/reaction) extracted from aortae of control or Ang II-treated rats with TRIzol reagent (Invitrogen, Carlsbad, CA) was used for the first-strand cDNA synthesis with superscript II kit (Invitrogen). cDNA equal to 0.04 μg of total RNA was used for each PCR reaction under the following conditions: 94°C for 2 min and 22 (for GAPDH) or 30 (for RhoGEFs) cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. The reaction products were analyzed by electrophoresis on agarose gel, and the expected product was extracted and verified by direct DNA sequencing. Results were expressed as the densitometry ratio of RhoGEF/GAPDH.

Statistical Analysis

Data are expressed as means ± S.E.M. Statistical significance between groups was evaluated with the unpaired two-tailed t test and one-way analysis of variance. A P value of <0.05 was considered significant.

Results

Blood Pressure and Aortic Endothelial Function.

Systolic blood pressure was elevated in Ang II-treated rats. The systolic blood pressure after 14 days of treatment was 191 ± 6 mm Hg in Ang II-treated rats compared with 120 ± 2 mm Hg in control rats (n = 8, P < 0.01). There were no significant changes in systolic blood pressure in control rats during the 14-day treatment.

Endothelial function was examined after aortic rings were
precontracted by PE. Endothelium-dependent relaxation to ACh was mildly impaired in the aortic rings from Ang II-treated hypertensive rats compared with that from normotensive rats. The maximal relaxation in response to ACh was decreased in Ang II-treated hypertensive rats by 30% from normotensive rats (54 ± 5% from PE-induced maximal contraction in hypertensive rats versus 77 ± 4% in normotensive rats, n = 6, p < 0.01). However, pEC50 of ACh was similar in both hypertensive and normotensive rats (7.0 ± 0.1 in hypertensive rats versus 7.2 ± 0.1 in normotensive rats).

Spontaneous Tone Development in Ang II-Induced Hypertensive Rats. Minimal spontaneous tone was observed in the aortic rings from normotensive control rats with intact endothelium (Fig. 1). There was a small development of tone in the EC-intact aortic rings from Ang II-treated rats and in the EC-denuded aortic rings from control rats (7 ± 2 and 7 ± 3% of PE-induced maximal contraction). However, the increase was not significantly different from that in the EC-intact aortic rings from control rats. The spontaneous tone generation in the EC-denuded aortic rings from Ang II-treated rats reached 73 ± 5% of the PE-induced maximal contraction.

Involvement of RhoA/Rho-Kinase in the Development of Spontaneous Tone. A selective Rho-kinase inhibitor, Y-27632, has been widely used for pharmacological analysis of Rho-kinase activity (Shimokawa, 2002). A cumulative concentration-relaxation curve of Y-27632 was constructed upon maximal spontaneous tone in EC-denuded aortic rings from Ang II-treated rats. As shown in Fig. 2, Y-27632 reduced the spontaneous tone in a concentration-dependent fashion with EC50 equal to 0.5 μM. At 10 μM Y-27632, the relaxation was 91 ± 5% from maximal spontaneous tone. Y-27632 had no significant effect on EC-denuded aortic rings from control rats (data not shown). These data suggest that Rho-kinase activity is increased in aorta from Ang II-induced hypertensive rats.

RhoA and Rho-Kinase Protein Expressions in Ang II-Treated Rats. Recent studies have suggested that Ang II increases RhoA/Rho-kinase-mediated Ca2+ sensitization through activation of AT1 receptor in cultured vascular smooth muscle cells (Yamakawa et al., 2000). The long-term effects of Ang II in vivo with respect to the regulation of RhoA and Rho-kinase protein expressions are not known. Therefore, we examined the protein expressions of RhoA and Rho-kinase in aortae from control and Ang II-treated rats using Western blot analysis. The results showed that RhoA protein expression was markedly increased by 57% in Ang II-treated rats (Fig. 3A); whereas the protein expression of Rho-kinase was not significantly different between control and Ang II-treated rats (Fig. 3B). These data suggest that increased RhoA protein expression may partly account for the increased Ca2+ sensitization and generation of spontaneous tone in Ang II-treated rats.

mRNA Expressions of RhoGEFs in Ang II-Treated Rats. Activation of RhoGEFs is a critical step in initiating the RhoA/Rho-kinase signaling cascade. LARG, p115RhoGEF, and PDZ-RhoGEF have a regulator of the G protein signaling (RGS) domain enabling them to interact directly with the Go subunit or receptor tyrosine kinase (Somlyo and Somlyo, 2000). Previously we reported up-regulation of these RhoGEFs in aortae from stroke-prone spontaneously hypertensive rats (Ying et al., 2004). Here we assessed the relative mRNA expressions of LARG, p115RhoGEF, and PDZ-RhoGEF in control and Ang II-treated rats by semiquantitative reverse transcription-PCR. The results showed that the mRNA expressions of these RGS domain-containing RhoGEFs were not significantly different in aortae from Ang II-treated rats compared with those from control rats (Fig. 4).

Effect of NADPH Oxidase Activation on Spontaneous Tone. Evidence has shown that increased NADPH oxidase-dependent ROS production plays a major role in the induction of spontaneous tone in aortae from Ang II-treated rats. Likewise, our data also demonstrate that two different NADPH oxidase inhibitors, apocynin (100 and 300 μM) and DPI (10 μM), attenuated the spontaneous tone in EC-denuded aortic rings from Ang II-treated rats (73 ± 8% relaxation from maximal spontaneous tone by apocynin and 92 ± 2% by DPI, n = 5–8, Fig. 5). DMSO as a vehicle control did not have a significant effect on the spontaneous tone (data not shown).

Effect of the NADPH Oxidase Inhibitor on RhoA/Rho-Kinase Activity. Next, we determined whether increased NADPH oxidase activity by Ang II results in an activation of Rhoa/Rho-kinase in vitro using EC-denuded aortic rings from Ang II-induced hypertensive rats. As shown in Fig. 6, the concentration-relaxation curve of Y-27632 was constructed upon maximal spontaneous tone in EC-denuded aortic rings from Ang II-induced hypertensive rats. At 10 μM Y-27632, the relaxation was 91 ± 5% from maximal spontaneous tone. Y-27632 had no significant effect on EC-denuded aortic rings from control rats (data not shown). These data suggest that Rho-kinase activity is increased in aorta from Ang II-induced hypertensive rats.
normotensive rats. DPI (10 μM) significantly reduced aortic smooth muscle contraction in the response to 100 nM Ang II (Fig. 6A). Ang II-induced contraction was also mediated by Rho-kinase because the contraction was significantly decreased by Y-27632 (1 μM). Ang II increased the migration of RhoA to the membrane fraction, an indication of RhoA activation (Fig. 6B). This migration of RhoA was blocked by preincubation with DPI. In addition, in the presence of DPI, Ang II-induced MYPT1 phosphorylation was markedly decreased, suggesting that increased Rho-kinase activity is partly dependent on NADPH oxidase activation (Fig. 6C).
Discussion

Development of spontaneous tone has been studied in blood vessels from several hypertensive animal models including spontaneously hypertensive rats, deoxycorticosterone (DOCA)-salt hypertensive rats and Ang II-induced hypertensive rats (Sekiguchi et al., 1998; Di Wang et al., 1999; Ghosh et al., 2004). Whereas Ca²⁺-dependent mechanisms are ascribed for the increased tone of aortae from Ang II-induced hypertensive rats, the Ca²⁺ sensitization pathways are yet to be explored. In this study we demonstrate that the RhoA/Rho-kinase mediated Ca²⁺ sensitization is up-regulated in aortae from Ang II-induced hypertensive rats, and it plays a significant role in the development of spontaneous tone. In addition, our results indicate that increased NADPH oxidase-dependent ROS by Ang II contribute to enhanced RhoA/Rho-kinase activity.

Increased activity of RhoA/Rho-kinase has been associated with hypertension since administration of Rho-kinase inhibitors normalizes blood pressure in hypertensive subjects but has no effects on normotensive subject (Masumoto et al., 2001). Studies have suggested that spontaneous tone may have a role in the pathogenesis of hypertension (Di Wang et al., 1999; Northcott et al., 2004). It has been reported that the RhoA/Rho-kinase pathway is involved in spontaneous tone generation in DOCA-salt rats whereas protein expressions of RhoA and Rho-kinase is not altered in DOCA-salt rats compared with those in sham-operated control rats (Northcott et al., 2002). In Ang II-induced hypertensive rats, it is not known whether RhoA/Rho-kinase contributes to the development of spontaneous tone. Our observation of Y-27632 concentration dependently decreasing spontaneous tone suggests that increased Rho-kinase activity is a major factor for the development of spontaneous tone. In vitro studies have shown that RhoA/Rho-kinase mediates Ang II-induced vasoconstriction, cellular hypertrophy, and protein synthesis in vascular smooth muscle cells (Yamakawa et al., 2000). Whether the RhoA/Rho-kinase pathway is up-regulated in Ang II-induced hypertensive rats is not known. Our results suggest that the protein expression of RhoA but not Rho-kinase is increased, which may partly lead to enhanced Rho-kinase activity and thereby contribute to the development of spontaneous tone.

RhoGEFs promote the cycling of GDP-bound inactive RhoA toward GTP-bound active RhoA. LARG, PDZ-RhoGEF, and p115 RhoGEF are the most studied RhoGEFs containing the RGS domain as a direct link between G protein-coupled receptors and RhoA (Gohla et al., 1999, 2000). Previously, a study from our laboratory demonstrated that mRNA expressions of RGS-containing RhoGEFs were increased in aortae from stroke-prone spontaneously hypertensive rats, suggest-

Fig. 6. NADPH oxidase activation mediated Ang II-stimulated RhoA/Rho-kinase activity in the aortic rings from normotensive rats. A, aortic smooth muscle contractions were increased in response to Ang II (100 nM, white bar). Ang II-induced contraction was decreased by DPI (10 μM, black bar) or Y-27632 (1 μM, gray bar). n = 6; **, P < 0.01 versus Ang II-treated only. B, top panel shows a representative immunoblot of the membrane fraction of RhoA. Ang II (100 nM) increased RhoA translocation to the membrane, which was blocked by DPI (10 μM). Bottom panel shows the densitometry analysis of membrane fraction of RhoA normalized by β-actin (n = 3; *, P < 0.05 versus control (Con); †, P < 0.05 versus Ang II-treated only). C, top panel shows a representative immunoblot of MYPT1 Thr696 phosphorylation at Thr696. Ang II inhibited MLC phosphatase activity by increased MYPT1 phosphorylation, and preincubation with DPI reduced the effect of Ang II on MYPT1. Bottom panel shows the densitometry analysis of MYPT1 phosphorylation, which is normalized by total MYPT1 protein expression in aorta (n = 3; **, P < 0.01 versus control; †, P < 0.05 versus control; ‡, P < 0.05 versus Ang II-treated only).
ing their potential role in increased vasoconstriction in hypertension (Ying et al., 2004). In vascular smooth cells, Ang II increases LARG mRNA expression but not PDZ-RhoGEF or p115RhoGEF via AT1 receptor activation (Ying et al., 2005). However, in Ang II-induced hypertensive rats, Ang II up-regulated RhoA/Rho-kinase signaling may be independent of the alteration of RhoGEF transcription since there were no significant changes in RhoGEF mRNA expressions in Ang II-treated rats.

Increased RhoA/Rho-kinase activity may be caused by reduced bioavailability of the upstream inhibitory regulator of RhoA/Rho-kinase NO. Sauzeau et al. (2000) demonstrated that NO destabilized RhoA-GTP membrane binding through protein kinase G. We noticed the development of spontaneous tone only in EC-denuded but not in EC-intact aortic rings, which may be explained by the observation of mild endothelial dysfunction in aortic rings isolated from Ang II-induced hypertensive rats. There is still basal release of NO from the endothelium that prevents the activation of RhoA/Rho-kinase, since administration of NO synthase inhibitor N\textit{}-nitro-L-arginine methyl ester increased the generation of spontaneous tone in EC-intact aortic rings from Ang II-treated rats (data not shown). However, Di Wang et al. (1999) reported that spontaneous tone was developed in both EC-intact and EC-denuded aortae from Ang II-induced hypertensive rats. In addition, N\textit{}-nitro-L-arginine methyl ester, did not further increase the magnitude of spontaneous tone in Ang II-induced hypertensive rats, suggesting that the endothelium dysfunction may be severe. The inconsistency is probably due to different rat strains as well as the infusion rate of Ang II, which is 10 to 20 times higher than the rate we used in the present study.

In hypertension, impaired endothelium-dependent vasodilation is associated with increased ROS production. It has been reported that NADPH oxidase is the major source of ROS, which is activated by Ang II in smooth muscle cells, fibroblasts, and endothelial cells (Griendling et al., 1994; Touyz et al., 2002). Inhibition of NADPH oxidase or treatment with antioxidants lowers blood pressure in hypertensive animals (Swei et al., 1999; Somers et al., 2000; Wu et al., 2001). ROS generated by NADPH oxidase has been shown to be important in the modulation of spontaneous tone in DOCA-salt hypertensive rats and Ang II-treated hypertensive rats, consistent with our results that NADPH oxidase inhibitors decrease spontaneous tone (Di Wang et al., 1999; Ghosh et al., 2004). Previously we reported that generation of ROS by xanthine oxidase and its substrate xanthine increased RhoA/Rho-kinase-mediated aortic smooth muscle contraction (Jin et al., 2004). However, xanthine oxidase inhibitors appear to be ineffective in blocking of spontaneous tone in both DOCA-salt hypertensive rats and Ang II-treated rats, suggesting that increased ROS is independent of xanthine oxidase (Di Wang et al., 1999; Ghosh et al., 2004).

Studies have shown that Ang II not only has long-term effects on NADPH oxidase but also can acutely increase the activity of NADPH oxidase (Seshiah et al., 2002). Our results show that the NADPH oxidase inhibitor DPI reduces aortic smooth muscle contraction in response to Ang II, indicating the involvement of NADPH oxidase-dependent ROS. Moreover, DPI prevents RhoA translocation and MYPT1 phosphorylation induced by Ang II. These data suggest that Ang II-stimulated RhoA/Rho-kinase activity is mediated by increased NADPH oxidase-dependent ROS. In cases where NO production is significantly decreased and/or ROS levels are increased, the activation of RhoA/Rho-kinase may contribute to increased vascular tone. Additional experiments are required to determine whether NADPH oxidase activation contributes to the increase in RhoA/Rho-kinase activity in vivo and to elucidate how ROS stimulate RhoA/Rho-kinase activity. Putative molecules linking between ROS and RhoA can be receptor tyrosine kinase, which has been shown to increase RhoGEF activity through phosphorylation (Somlyo and Somlyo, 2003).

A limitation of this study is that we have not investigated the possible involvement of protein kinase C (PKC) and PKC-activated inhibitor protein of 17 kDa (CPI-17) in spontaneous tone development in hypertensive rats. PKC and its downstream target CPI-17 are other potential mediators of Ca\textsuperscript{2+} sensitization. Activation of CPI-17 through phosphorylation by PKC inhibits the MLC phosphatase catalytic subunit PP\textsubscript{1}C. However, studies also indicate that Rho-kinase may have a direct role on activation of CPI-17 because Y-27632 decreases agonist-induced CPI-17 phosphorylation (Somlyo and Somlyo, 1994, 2000).

In conclusion, our data demonstrate that the up-regulation of the RhoA/Rho-kinase pathway plays an important role in the development of spontaneous tone in aortae from Ang II-induced hypertensive rats. Enhanced levels of NADPH-dependent ROS and reduced NO bioavailability might activate RhoA/Rho-kinase and increase MYPT1 phosphorylation, which lead to augmented arterial smooth muscle contractility. Overall these data aid in understanding the molecular signaling events involved in the onset of spontaneous tone and the progression of hypertension.

References
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