INCREASED RHOA/RHO-KINASE SIGNALING MEDIATES SPONTANEOUS TONE IN AORTA FROM ANGIOTENSIN II-INDUCED HYPERTENSIVE RATS

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Abbreviations used in the paper:
Ang II, Angiotensin II; AT1, Ang II type I receptor; MLC, myosin light chain; RhoGEF, Rho guanine nucleotide exchange factor; RhoGDI, Rho GDP disassociation inhibitor; GPCR, G protein coupled receptor; MYPT1, myosin light chain phosphatase target subunit; ROS, reactive oxygen species; NO, nitric oxide; PE, phenylephrine; ACh, acetylcholine; EC, endothelial cells; DPI, diphenylene iodonium; LARG, leukemia-associated RhoGEF; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RGS, regulator of G protein signaling; DOCA, deoxycorticosterone; PKC, protein kinase C; L-NAME, Nω- nitro-L-arginine methyl ester, CPI-17, PKC-potentiated inhibitor protein of 17 kDa

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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 is 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 to their normotensive counterparts (73 ± 5% versus 7 ± 3% of phenylephrine-induced maximum contraction, respectively). The Rho-kinase inhibitor Y-27632 (0.1 to 10 μM) concentration-dependently inhibited spontaneous tone in aortic rings from Ang 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 RhoGEFs 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 myosin light chain phosphatase target subunit, which were both blocked by the NADPH oxidase inhibitor diphenylene iodonium (10 μM). In conclusion, these data suggest that chronic treatment with angiotensin II leads to up-regulation of the RhoA/Rho-kinase pathway, contributing to the 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.
INTRODUCTION

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 (Kagiyama et al., 2002; Seshiah et al., 2002). It is well known that activation of Ang II type I receptor (AT₁) increases free intracellular Ca²⁺ 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 Ca²⁺ sensitization signaling pathway through which smooth muscle is able to contract when the intracellular Ca²⁺ concentration is low (Somlyo and Somlyo, 2003).

The activity of RhoA is regulated by Rho guanine nucleotide exchange factors (RhoGEF) and Rho GDP disassociation inhibitors (RhoGDI) (Somlyo and Somlyo, 2003). In its inactive state, RhoA binds with GDP and forms a complex with RhoGDI in the cytosol. When RhoGEF is activated by G protein coupled receptors (GPCR) such as AT₁ 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.

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 animal 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 is to determine whether the RhoA/Rho-kinase pathway is involved in the spontaneous tone development and whether 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, un-coupled eNOS, xanthine oxidase and mitochondrial respiratory chain in various vascular beds. Previously, we and others reported that ROS produced by xanthine oxidase or released from 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 is 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 Laboratories) were implanted subcutaneously with osmotic mini pumps (Alzet, Durect Corp) 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., 2004). Systolic blood pressure was measured by tail cuff plethysmography every 3 days in conscious rats to monitor the progression of hypertension. Basal level of systolic blood pressure was measured on two 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 a cold physiological saline solution of the following composition (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.18, CaCl₂·2H₂O 1.6, MgSO₄·7H₂O 1.6, NaHCO₃ 25, dextrose 5.5, EDTA 0.03. After removal of adventitial tissue, aortae were cut into 2 mm rings and mounted in myograph organ chambers (Danish Myo Technology A/S).
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% O₂/5% CO₂ at 37 °C. The rings were pre-contracted by phenylephrine (PE, 1 μM) and cumulative relaxation curves to acetylcholine (ACh, 0.001 to 10 μM) were obtained on each ring for assessment of endothelium function. Changes in isometric force were recorded by a PowerLab 8/SP™ data acquisition system (ADInstruments). ACh-induced relaxations were expressed as percentage of PE-induced maximum 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 μM) to reduce any spontaneous tone developed during the equilibration period and the passive tension was re-adjusted to 30 mN. After washing and re-equilibration for 30 min, the aortic preparations were subjected to 10 μM PE contraction. ACh (1 μM) was added to the plateau phase of maximum contraction to determine whether the endothelium was intact or completely denuded. Finally, after washing off PE and ACh, spontaneous tone was allowed to develop over the time. The maximum spontaneous tone was expressed as percentage of PE-induced maximum contraction.

In another set of experiments, a Rho-kinase inhibitor Y-27632 (from 0.1 to 10 μM), NADPH oxidase inhibitors (diphenylene iodonium, DPI and apocynin) or vehicle (DMSO, final concentration is 0.1%) was added at the peak of spontaneous contraction. Y-27632, DPI or
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 pre-incubated with DPI (10 µM), vehicle (DMSO, final concentration is 0.1%) or Y-27632 (1 µM) for 15 min before challenged by Ang II (100 nM). Ang II-induced contractions in the absence or presence of different inhibitors were expressed as percentage of PE-induced maximum contraction.

Western Blot

Protein expressions of RhoA and Rho-kinase were determined by Western blot as described previously (Jin et al., 2004). Briefly, aortae from control and Ang II-treated rats were excised, cleaned and snap frozen in liquid nitrogen. Tissue was homogenized in a cold RIPA buffer (Upstate) and protein concentration was determined by the BCA kit (Pierce). Equal amounts of protein were loaded and separated by SDS-PAGE and subsequently transferred to nitrocellulose membrane. Antibodies against RhoA (Santa Cruz Biotechnology) and Rho-kinase (BD Biosciences) 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 β-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). Membrane fraction of RhoA is normalized
by β-actin protein levels and p-MYPT1 is normalized by MYPT1 protein expression.

RT-PCR

The RhoGEF primers were as follows:

PDZ-RhoGEF primers:
forward, 5’-GGGACCCCTCTTCGAGAACCAGCCAAA-3’;
reverse, 5’-GGGCAGCCAACCTTGGTCCTTGCAGG-3’.

Leukemia-associated RhoGEF (LARG) primers:
forward, 5’-AGCCATGCGCGCTGGAGTACAAAC-3’;
reverse, 5’-GCTCCAGGGGAATGAGGGTGGTC-3’.

p115RhoGEF primers:
forward, 5’-TCCGGACCAAGAGTGGGGACAAGA-3’;
reverse, 5’-TACCCAGGCTTCCCTTCCGGTCTG-3’.

And Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were:
forward, 5’-TGCATCCTGCACCACAACTGTT-3’;
reverse, 5’-ACAGCCCTTGGGAGCACCAGTGGAT-3’.
Total RNA (4 µg per reaction) extracted from aortae of control or Ang II-treated rats with TRIzol reagent (Invitrogen) was used for the first strand cDNA synthesis with superscript II kit (Invitrogen). cDNA equal to 0.04 µg total RNA was used for each PCR reaction under the following conditions: 94ºC for 2 min and 22 (for GAPDH) or 30 (for RhoGEFs) cycles at 94ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s, followed by 72ºC 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 mean ± SEM. Statistical significance between groups was evaluated with unpaired 2-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 mmHg in Ang II-treated rats compared to 120 ± 2 mmHg in control rats (n = 8, P < 0.01). There were no significant changes of the systolic blood pressure in control rats during the 14 day treatment.

Endothelial function was examined after aortic rings were pre-contracted by PE. Endothelium-dependent relaxation to ACh was mildly impaired in the aortic rings from Ang II-
treated hypertensive rats when compared to that from normotensive rats. The maximum relaxation in response to ACh was decreased in Ang II-treated hypertensive rats by 30% from normotensive rats (54 ± 5% from PE-induced maximum contraction in hypertensive rats vs. 77 ± 4% in normotensive rats, n=6, p < 0.01). However, pEC$_{50}$ of ACh is similar in both hypertensive and normotensive rats (7.0 ± 0.1 in hypertensive rats vs. 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 (Figure 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 maximum contraction). However, the increase was not significantly different from 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 PE-induced maximum 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 Figure 2, Y-27632 reduced the spontaneous tone in a concentration-dependent fashion with EC$_{50}$ equal to 0.5 μM. At 10 μM of Y-27632, the relaxation was 91 ± 5% from maximum 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 Ca\(^{2+}\) sensitization through activation of AT\(_1\) 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 (Figure 3A); whereas the protein expression of Rho-kinase was not significantly different between control and Ang II-treated rats (Figure 3B). These data suggest that increased RhoA protein expression may partly account for the increased Ca\(^{2+}\) sensitization and generation of spontaneous tone in Ang II rats.

mRNA expressions of RhoGEFs in Ang II-treated rats

Activation of RhoGEFs is a critical step to initiate the RhoA/Rho-kinase signaling cascade. LARG, p115RhoGEF and PDZ-RhoGEF have a regulator of G protein signaling (RGS) domain enabling them to directly interact with G\(_{\alpha}\) 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 semi-quantitative RT-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 when compared to those from control rats (Figure 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. Similarly, 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 maximum spontaneous tone by apocynin and 92 ± 2% by DPI, n=5-8, Figure 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 normotensive rats. DPI (10 µM) significantly reduced aortic smooth muscle contraction in the response to 100 nM of Ang II (Figure 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 (Figure 6B). This migration of RhoA was blocked by pre-incubation with DPI. Additionally, 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 (Figure 6C).
DISCUSSION

Development of spontaneous tone has been studied in blood vessels from several hypertensive animal models including spontaneous 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). While Ca\(^{2+}\)-dependent mechanisms are ascribed for the increased tone of aortae from Ang II-induced hypertensive rats, the Ca\(^{2+}\)-sensitization pathways are yet to be explored. In this study we demonstrate that the RhoA/Rho-kinase mediated Ca\(^{2+}\)-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 have 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 the spontaneous tone generation in DOCA-salt rats while protein expressions of RhoA and Rho-kinase is not altered in DOCA-salt rats when compared to those in the 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 those most studied RhoGEFs containing RGS domain as a direct link between GPCR and RhoA (Gohla et al., 1999; Gohla et al., 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, suggesting 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 demonstrated that NO destabilized the RhoA·GTP membrane binding through protein kinase G (Sauzeau et al., 2000).
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 $\text{N}^\omega$-nitro-L-arginine methyl ester (L-NAME) increased the generation of spontaneous tone in EC-intact aortic rings from Ang II-treated rats (data not shown). However, Wang and colleagues reported that spontaneous tone was developed in both EC-intact and EC-denuded aortae from Ang II-induced hypertensive rats (Di Wang et al., 1999). In addition, L-NAME 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 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 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 the 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 link between ROS and RhoA can be receptor tyrosine kinases which has been to shown to increases 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-potentiated inhibitor protein of 17 kDa (CPI-17) in spontaneous tone development in hypertensive rats. PKC and its down stream target CPI-17 are other potential mediators of Ca$^{2+}$ sensitization. Activation of CPI-17 through phosphorylation by
PKC inhibits MLC phosphatase catalytic subunit PP1C. 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; Somlyo and Somlyo, 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.
REFERENCE


FOOTNOTES

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FIGURE LEGEND

Figure 1. Spontaneous tone development in aortic rings from normotensive control and Ang II-induced hypertensive rats. **A.** On the left is a representative tracing of an EC-denuded aortic ring from a control rat. On the right is a tracing of an EC-denuded aortic ring from an Ang II-treated rat. ACh was added to verify the complete removal of endothelial cell layer. **B.** Summary of spontaneous tone generation in EC-intact or EC-denuded aortic rings from control and Ang II-induced hypertensive rats. Maximum spontaneous tone was expressed as % of PE-induced maximum contraction (White bar: EC-intact from control; grey bar: EC-intact from Ang II-treated; hatched bar: EC-denuded from control; black bar: EC-denuded from Ang II-treated rats). n = 5-8, **P < 0.01 vs. EC-intact aortic rings from control rats.

Figure 2. The Rho-kinase inhibitor (Y-27632) decreased spontaneous tone in EC-denuded aortic rings from Ang II-induced hypertensive rats. **A.** A representative tracing showing the relaxation effect of Y-27632 on spontaneous tone. **B.** A bar graph summarized the inhibitory effects of Y-27632 on spontaneous tone at different concentrations. The data were expressed as % relaxation from maximum spontaneous tone (n = 6).

Figure 3. Protein expressions of RhoA and Rho-kinase in aortae from normotensive control and Ang II-induced hypertensive rats. **A.** Top panel shows a representative immunoblot of RhoA (21 kDa) protein expression in two control and two Ang II-induced hypertensive rats. Bottom panel shows densitometry analysis of RhoA expression in control (white bar) and Ang II-treated rats (black bar) normalized by β-tubulin (n = 7; **P < 0.01 vs. control). **B.** Top panel shows a
representative immunoblot of Rho-kinase (160 kDa) protein expression in two control and two Ang II-induced hypertensive rats. Bottom panel shows densitometry analysis of Rho-kinase expression normalized by β-tubulin (n=7).

Figure 4. mRNA expressions of RhoGEFs in aortae from normotensive control and Ang II-induced hypertensive rats. A. Electrophoretic visualization of the amplicons of LARG, PDZ-RhoGEF and p115RhoGEF. GAPHD is used as an internal control. B. GAPDH normalized quantification of the PCR products for control (white bars) and Ang II-induced hypertensive rats (black bars, n=4).

Figure 5. Effects of NADPH oxidase inhibitors on the spontaneous tone in EC-denuded aortic rings from Ang II-induced hypertensive rats. A. Representative tracings showing the relaxation effects of apocynin (left) and DPI (right) on spontaneous tone. B. A bar graph summarized the inhibitory effects of apocynin and DPI. The data were expressed as % relaxation from maximum spontaneous tone (n = 4-8).

Figure 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 vs. Ang II-treated only. B. Top panel shows a representative immunoblot of 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 =
C. Top panel shows a representative immunoblot of MYPT1 phosphorylation at Thr696. Ang II inhibited MLC phosphatase activity by increased MYPT1 phosphorylation and pre-incubation of 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 vs. control; † P < 0.05 vs. control; ‡ P < 0.05 vs. Ang II-treated only).
**Figure 1**

A. 

An EC-denuded ring from a control rat. 

An EC-denuded ring from an Ang II-induced hypertensive rat.

B. 

Bar graph showing the % of PE-induced maximum contraction in EC-intact and EC-denuded conditions for control (Con) and Ang II (Ang II) treated tissue.

** **
Figure 2

A

EC-denuded aortic ring (Ang II-treated rat)

Rho-kinase inhibitor Y-27632 (in \(-\log M\) )

5 mN

wash

B

Percentage Relaxation from Maximum Tone

\[
\begin{array}{cccccc}
& -7 & -6.5 & -6 & -5.5 & -5 \\
Y-27632 (\log M) & & & & & \\
\end{array}
\]
Figure 3
Figure 4
Figure 5
Figure 6