0026-895X/06/6903-932–940$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:932–940, 2006

Vol. 69, No. 3
17830/3090185
Printed in U.S.A.

Angiotensin II Up-Regulates the Leukemia-Associated Rho Guanine Nucleotide Exchange Factor (RhoGEF), a Regulator of G Protein Signaling Domain-Containing RhoGEF, in Vascular Smooth Muscle Cells

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Received August 10, 2005; accepted December 13, 2005

ABSTRACT

In vascular smooth muscle, stimulation of heterotrimeric G protein-coupled receptors (GPCRs) by various contractile agonists activates intracellular signaling molecules to result in an increase in cytosolic Ca^{2+} and the subsequent phosphorylation of myosin light chain (MLC) by Ca^{2+}/calmodulin-dependent MLC kinase. In addition, a portion of agonist-induced contraction is partially mediated by the Ca^{2+}-independent activation of the small G protein RhoA and a downstream target, Rho-kinase. The activation of RhoA is controlled by several regulatory proteins, including guanine nucleotide exchange factors (GEFs). GEFs activate RhoA by promoting the release of GDP and then facilitating the binding of GTP. There are three Rho-specific GEFs (RhoGEFs) in vascular smooth muscle that contain a binding domain [regulator of G protein signaling (RGS) domain] capable of linking GPCRs to RhoA activation: PDZ-RhoGEF, leukemia-associated RhoGEF (LARG), and p115RhoGEF. We hypothesized that RGS domain-containing RhoGEFs, especially LARG, participate in linking GPCR to RhoA activation in vascular smooth muscle. We observed that angiotensin II up-regulates LARG via the AT1 receptor, and this up-regulation is signaled via the phosphatidylinositol 3-kinase pathway. Furthermore, angiotensin II treatment caused a small, but significant, increase in the component of contractile responses sensitive to Rho-kinase antagonism. These observations support the hypothesis that RhoGEFs, particularly LARG, participate in linking GPCR to RhoA activation in vascular smooth muscle.

Angiotensin II, one of the most powerful vasoconstrictor substances, plays a key role in blood pressure regulation (Kim and Iwao, 2000; Touyz and Schiffrin, 2000; Touyz, 2003). In addition to its short-term vasoconstrictor action, angiotensin II has long-term actions on the structure and function of blood vessels by regulating the expression of particular genes. The octapeptide also stimulates the secretion of aldosterone from adrenal glomerulosa cells, and it has trophic effects on the heart.

In vascular smooth muscle cells (VSMCs), there is growing evidence indicating that some of the cellular effects of angiotensin II are mediated through the RhoA/Rho-kinase signaling pathway (Touyz and Schiffrin, 2000). In vitro studies have demonstrated the vasoconstrictor effects of RhoA/Rho-kinase to be the result of Rho-kinase-mediated inhibition of myosin light chain (MLC) phosphatase through phosphorylation of the myosin binding subunit (Somlyo and Somlyo, 2003). MLC phosphatase inhibition results in the maintenance of phosphorylated MLC promoting the binding of actin and myosin for force generation. Activation of this Ca^{2+}-

This study was supported by National Institutes of Health grants HL74167 and HL71138 and by American Heart Association grant 0130364N.

A preliminary report of this investigation was presented at Experimental Biology, April 17–21, 2004, Washington DC.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.017830.

ABBREVIATIONS: VSMC, vascular smooth muscle cell; MLC, myosin light chain; LARG, leukemia-associated Rho guanine nucleotide exchange factor; RGS, regulators of G protein signaling; RhoGEF, Rho-specific guanine nucleotide exchange factor; PI3-kinase, phosphatidylinositol 3-kinase; GPCR, G protein-coupled receptor; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; AG-490, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD123319, *S*-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo(4,5-*c*)pyridine-6-carboxylic acid; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; GAPDH, glyceraldehye-3-phosphate dehydrogenase; RT-PCR, real-time-polymerase chain reaction; RACE, rapid amplification of cDNA ends; PE, phenylephrine; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PDTC, pyrrolidinedithiocarbamate; kb, kilobase(s); SP600125, 1,9-pyrazoloanthrone anthra(1,9-*cd*)pyrazol-6(2*H*)-one; Y-27632, *N*-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride

sensitizing pathway is regulated by the binding state of RhoA. Similar to other small GTPases from the Rho family, RhoA is activated upon binding of GTP. In the inactive state, RhoA is primarily a cytosolic component and is bound to GDP and a guanine nucleotide dissociation inhibitor. Post-translational modification (geranylgeranylation) enables the necessary membrane binding and subsequent activation of RhoA. Activation also requires a guanine nucleotide exchange factor (GEF), which promotes the exchange of GTP for GDP. Leukemia-associated RhoGEF (LARG) and its homologs PDZ-RhoGEF and p115RhoGEF make up a subgroup of RhoGEFs, known as regulators of G protein signaling (RGS) domain-containing RhoGEFs. The RGS domain of these proteins mediates their binding to and activation by $G_{12/13}$ in response to extracellular stimuli (Hart et al., 1998; Tanabe et al., 2004). LARG has also been shown to interact with G_q, providing a mechanism to directly link G_q with RhoA activation (Booden et al., 2002). We previously demonstrated that the expression of these RhoGEFs was significantly increased in arteries from stroke-prone spontaneous hypertensive rats (Ying et al., 2004). Therefore, we hypothesized that RGS domain-containing RhoGEFs, especially LARG, may play a role in angiotensin II-induced Ca^{2+} sensitization.

In addition to its vasoconstrictor action, angiotensin II plays a key role in the remodeling of the vasculature in several disease states, such as hypertension and atherosclerosis (Kim and Iwao, 2000; Touyz and Schiffrin, 2000; Touyz, 2003). Recent studies have demonstrated that this mitogenic activity of angiotensin II is dependent on the phosphatidylinositol 3-kinase (PI3-kinase) signaling pathway in VSMCs (Dugourd et al., 2003; Yamakawa et al., 2003). Because the proliferative responses to angiotensin II in VSMCs involves the AT1 receptor, we hypothesized that inhibition of PI3-kinase signaling would block the ability of angiotensin II to up-regulate the RhoGEFs, particularly, LARG.

In the present study, we observed that angiotensin II up-regulates LARG via the AT1 receptor, and this up-regulation is signaled via the PI3-kinase pathway. Furthermore, angiotensin II treatment caused a small, but significant, increase in the component of contractile responses sensitive to Rho-kinase antagonism. These observations support the hypothesis that RhoGEFs, particularly LARG, participate in linking G protein-coupled receptors (GPCRs) to RhoA activation in vascular smooth muscle.

Materials and Methods

Materials. Collagenase, elastase, Y-27632, PD98059, phenylephrine, ammonium pyrrolidinecarbodithioate, SB203580, wortmannin, AG490, SP600125, LY294002, and PD123319 were purchased from Sigma-Aldrich (St. Louis, MO). Losartan was a gift from Dr. Michael W. Brands (Medical College of Georgia, Augusta, GA).

Preparation and Culture of Rat VSMCs. Primary cultures of rat VSMCs were prepared as described previously (Jing et al., 1999). In brief, the thoracic aortas of Sprague-Dawley rats (Harlan, Indianapolis, IN) were isolated aseptically, and the adventitia and outer media were stripped off and discarded. Then, the vessels were minced, placed in collagenase solution at 37°C for 30 min, and washed. The remaining media were incubated in collagenase and elastase with gentle agitation until the cells were dispersed. The resulting cell suspension was centrifuged, and the cells were seeded onto a plastic dish after resuspension. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C under a 95% air, 5% CO_2 atmosphere.

Preparation of cDNA. Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Four micrograms of RNA was subjected to digestion by DNase I (Invitrogen) and then used for reverse transcription reaction. The first strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions. After dilution by adding 40 μl of water, cDNA was used for polymerase chain reaction (PCR) or stored at −20°C.

Polymerase Chain Reaction. As described previously (Ying et al., 2004), primers were designed using the Primer3 program based on the known mRNA sequences of each gene. To prevent possible contamination with genomic DNA, care was taken to ensure that primer binding sites for each gene were located in different exons. PDZ-RhoGEF primers were forward, 5′-gggaccct cttcgagaacgc-caaa-3′ and reverse, 5′-gggcagccacttgtccttgtcagg-3′. LARG primers were forward, 5′-agccatgcgcgctggagtacaaac-3′ and reverse, 5′-gctc-caggggaatgaggggatgtc-3′. p115RhoGEF primers were forward, 5′-tccggaccaagagtggggacaaga-3′ and reverse, 5′-tacccaggcttcccttccggtc tg-3′. Glyceraldehye-3-phosphate dehydrogenase (GAPDH) primers were forward, 5′-tgcatcctgcaccaccaactgctt-3′ and reverse, 5′-acagcct-tgg cagcaccagtggat-3′. One microliter of cDNA was first used to amplify the house-keeping gene, GAPDH. Amplification products from this reaction were compared by scanning densitometry, and the calculated ratios of products were used to dilute the original cDNA samples so as to obtain equivalent GAPDH cDNA levels between samples. RhoGEF transcripts were subsequently amplified from the adjusted cDNA preparations. PCR was performed in a mixture of 25 μl containing 12.5 μl of *Taq*PCR master mix (QIAGEN, Valencia, CA), 0.1 μM forward primer, 0.1 μM reverse primer, and 1 μl of cDNA. The reaction cycles were as follows: 94°C for 2 min and 22 (GAPDH) or 30 (RhoGEFs) cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. Products were analyzed by electrophoresis on 2% agarose gels. Gel images were recorded and analyzed with Kodak image station 440CF (Kodak IBI, New Haven, CT), and the results were expressed as the densitometric ratio of RhoGEF/GAPDH (percentage).

Real-Time Quantitative PCR. The QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) was used to quantify the LARG and GAPDH mRNA levels in samples according to manufacturer's instructions. In brief, mixtures (one reaction contains 10 μl of 2× master mix, 1 μl of 10 nM forward primer, 1 μl of 10 nM reverse primer, and 7 μl of water) were prepared and added to each PCR tube, and then 1 μl of cDNA was added. Reactions were performed on the Smart Cycler system (Cepheid, Sunnyvale, CA) with the following thermal cycle conditions: 15 min at 95°C to activate the hotStartaq DNA polymerase; then 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 30 s; and finally a melting curve analysis of PCR product. The fluorescent signal at each cycle was plotted versus the cycle number. The threshold cycle, the cycle number at which an increase above background fluorescence could be reliably detected, was determined for each sample. The results were expressed as the difference between the threshold cycle of GAPDH and that of LARG. The specificity and identity of the PCR products were verified by the melting curve analysis. Primers were designed by PrimerquestSM (Integrated DNA Technologies, Coralville, IA), and the sequences were as follows: 5′-TCACCACCATGG AGAAGGC-3′ and 5′-GCTA-AGCAGTTGGTGGTGCA-3′ for GAPDH with a 178-base pair amplicon, and 5′-AAGCAGACGAACTCCAAGGAGACT-3′ and 5′-CAGGT-GCTGAAACAATGCGGAGAA-3′ for LARG with a 117-base pair amplicon.

5′ Rapid Amplification of cDNA Ends RT-PCR. 5′ RACE RT-PCR was performed on rat aortic RNA using RNA ligase mediated-RACE reagents following the manufacturer's instructions (Ambion, Austin, TX). The primary PCR was performed using the Outer

Adapter Primer (Ambion) and the following gene-specific primer: 5′-gctccaggggaatgaggggatgtc-3′. The secondary PCR was performed on 4 μl of a 1:100 dilution of the primary PCR reaction using the Inner Adapter Primer and the following nested gene-specific primer: 5′-atgtcataatgggagacatatgtc-3′. For both PCR reactions, the following reaction conditions were used with AmpliTaq (Applied Biosystems, Foster City, CA): 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. A pool of the different RACE products was ligated into pCR2.1 (Invitrogen). Fifteen plasmids were isolated from recombinant clones and sequenced.

Tissue Preparation and Isometric Force Measurement. All animal procedures were performed in accordance with the *Guiding Principles for Research Involving Animals and Human Beings* of the American Physiological Society (http://www.the-aps.org/publications/journals/guide.htm). Male Sprague-Dawley rats (275–300 g) were anesthetized with pentobarbital sodium (50 mg/kg i.p.), and the thoracic aorta was quickly removed and cleaned in physiological salt solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.6 mM $CaCl_2{\cdot}2H_2O$, 1.6 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 5.5 mM dextrose, and 0.03 mM EDTA. The aorta was cut into 2-mm rings, and the endothelium was gently removed. Rings were incubated with DMEM (Invitrogen) containing either 100 nM angiotensin II (100 nM) or vehicle for 3 h at 37°C. After incubation, the aortic rings were mounted in a muscle bath containing physiological salt solution at 37°C and bubbled with 95% O_2, 5% CO_2. Isometric force generation was recorded with a Multi Myograph system (Danish Myo Technology A/S, Aarhus, Denmark). A resting tension of 3 g was imposed on each ring, and the rings were allowed to equilibrate for 1 h. The rings were contracted with phenylephrine (PE; 10 nM), and acetylcholine (1 μM) was added during the plateau phase of the contraction to verify efficient removal of endothelium (no relaxation). Then, the drugs were washed out until the contractile response returned to baseline levels. Thereafter, a concentration-dependent contractile response curve to PE or a concentration-dependent relaxation response curve to Y-27632 was recorded (these relaxation responses were recorded in 10 nM PE precontracted rings).

Detection of LARG Protein by Western Blot Analysis. Aortic rings were incubated in DMEM containing either 100 nM angiotensin II or vehicle at 37°C for the designated time and immediately snap frozen in liquid nitrogen. The rings were homogenized and solubilized in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride, with 0.25% sodium-deoxycholate and 1.0% Nonidet P-40) and centrifuged at 10,000*g* and 4°C for 30 min. The supernatant was collected and subjected to Western blot analysis. In brief, 80 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membrane. The membrane was then incubated with LARG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or β-actin (Sigma-Aldrich) antibody. Finally, the membranes were incubated with a horseradish peroxidase-linked secondary antibody and visualized with an enhanced chemiluminescence kit (Amersham Biosciences Inc., Piscataway, NJ). Band density was quantified by densitometric analysis using a Kodak Image Station 440CF (Kodak Scientific Imaging Systems, Rochester, NY).

Data Analysis. Data are presented as mean ± S.E.M., and treatment effects were compared by unpaired *t* test adjusted with the Bonferroni correction. $p < 0.05$ was taken as the level of significance.

Results

Angiotensin II Transiently Up-Regulates LARG mRNA and Protein in Rat Vascular Smooth Muscle Cells. To determine whether the RGS domain-containing RhoGEFs were regulated by angiotensin II, rat aortic rings were prepared and stimulated with 100 nM angiotensin II in vitro, and RhoGEF mRNA transcripts were examined by RT-PCR. Results demonstrated that LARG mRNA was significantly increased after 30 min of stimulation and reached a peak after 1 h of stimulation (Fig. 1A). It is noteworthy that PDZ-RhoGEF and p115RhoGEF did not significantly change at any interval after stimulation with angiotensin II (Fig. 1B). When stimulated by vehicle for 1, 2, 8, and 24 h, no alteration of mRNA expression level of these RhoGEFs was observed (data not shown). LARG protein levels were consistently elevated after angiotensin II stimulation, with a significant increase after 3 h and peaking at 6 h of stimulation (Fig. 1, C and D).

Because the major cell type in the aorta is the VSMC, we hypothesized that the induction of LARG by angiotensin II occurred in these cells. To test this hypothesis, a RT-PCR analysis of LARG mRNA was established (its specificity and efficiency are demonstrated in Fig. 2, A and B) and was used to examine whether angiotensin II can induce LARG in cultured VSMCs. Results showed that angiotensin II transiently up-regulated LARG mRNA in cultured VSMCs (Fig. 2C) with a similar time course to that observed in aortic rings, and the induction of LARG by angiotensin II occurred in a concentration-dependent manner. Concentrations as low as 1 nM angiotensin II significantly increased the expression of LARG mRNA, and maximal induction was observed at an angiotensin II concentration of 1 μM (Fig. 2D).

The AT1 Receptor Mediates the Induction of LARG mRNA by Angiotensin II. To date, two high-affinity plasma membrane receptors for angiotensin II, AT1 and AT2, have been cloned and pharmacologically characterized. Both receptors can be detected in vascular smooth muscle (Zhuo et al., 1995) and have been shown to mediate the modulation of gene expression by angiotensin II. Therefore, specific inhibitors were used to identify which type of receptor was in-

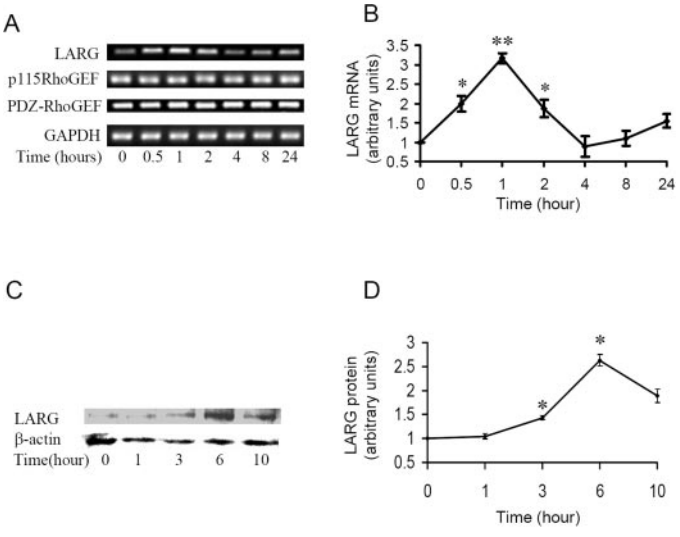

Fig. 1. Angiotensin II increased the expression of LARG in aortic rings. Aortic rings were prepared and cultured in DMEM containing 100 nM angiotensin II or vehicle for the indicated time. mRNA and protein expression levels of LARG were measured by RT-PCR and Western blot, respectively. A, a representative gel image of RT-PCR products. B, after normalization to GAPDH, LARG mRNA expression relative to nonstimulated control (0) was calculated and is presented as mean ± S.E.M. of three separate experiments. C, representative image of Western blot analysis. D, LARG protein expression relative to nonstimulated control is presented as mean ± S.E.M. of four separate experiments. *, $p < 0.05$; **, $p < 0.01$.

volved in the induction of LARG by angiotensin II. Results showed that the AT1 blocker losartan but not the AT2 receptor blocker PD123319 abolished the induction of LARG mRNA by angiotensin II (Fig. 3), suggesting that the induction of LARG by angiotensin II was mediated by the AT1 receptor.

The Induction of LARG mRNA by Angiotensin II Is via the PI3-Kinase Signaling Pathway. Because a variety of signaling pathways can be activated by angiotensin II binding to the AT1 receptor, a set of inhibitors were used to identify which signaling pathway(s) was involved in the induction of LARG by angiotensin II. We observed that a PI3-kinase inhibitor (wortmannin) completely blocked the induction of LARG by angiotensin II, and a mitogen-activated protein kinase kinase inhibitor (PD98059) and an MAPK inhibitor (SB203580) partially blocked the induction of LARG, whereas a Janus tyrosine kinase 2 inhibitor (AG490) and a c-Jun NH_2-terminal kinase inhibitor (SP600125) did not significantly affect the induction of LARG mRNA by angiotensin II (Fig. 4A). Consistent with the results from wortmannin, another PI3-kinase inhibitor, LY294002, significantly attenuated the induction of LARG by angiotensin II (Fig. 4B).

NF-κB is a common downstream transcription factor of PI3-kinase. To test whether NF-κB mediates the induction of LARG by angiotensin II, VSMCs were pretreated with ammonium pyrrolidinedithiocarbamate (PDTC), a compound shown to specifically inhibit the mobilization of NF-κB but not other transcription factors in response to endotoxin (Ziegler-Heitbrock et al., 1993). As shown in Fig. 4B, PDTC dramatically attenuated the induction of LARG by angiotensin II. To identify potential NF-κB binding sites in the promoter region of LARG, 5′ RACE RT-PCR was used to determine the transcription start sites of LARG. Two prominent amplicons were observed in the secondary PCR reaction (Fig. 5A), and after sequencing, two major transcription start sites were identified (Fig. 5B). The LARG promoter region was then scanned using an online program, TRES (http://bioportal.bic.nus.edu.sg/tres/). In the analyzed region, two potential NF-κB binding sites were identified (Fig. 5B). Similar analyses were performed on the promoter regions of human p115 RhoGEF, human PDZ-RhoGEF, and mouse LARG as well, because the sequences of their 5′ noncoding region were available via GenBank. It is noteworthy that the potential NF-κB binding site was identified only in the promoter region of mouse LARG but not in other genes examined (Table 1).

Pretreatment with Angiotensin II Increases Rho-Kinase Antagonist-Induced Vasodilation. Because LARG can link heterotrimeric G proteins to RhoA and may thus be involved in Ca^{2+} sensitization of vascular smooth muscle, we predicted that pretreatment with angiotensin II would increase the Ca^{2+} sensitization of rat aortic rings induced by other agonists. A Rho-kinase antagonist, Y-27632, has commonly been used to test RhoA/Rho-kinase-mediated Ca^{2+} sensitization in blood vessels (Uehata et al., 1997; Weber and Webb, 2001). Therefore, we investigated how pretreatment with angiotensin II affected the vasodilator response to Y-27632 in PE-precontracted rat aortic rings. Results demonstrated that pretreatment with angiotensin II significantly increased the vasodilator response to Y-27632 at 100 and 300 nM (Fig. 6B). However, pretreatment with angiotensin II did not change contractile responses to PE as demonstrated by the lack of shift in the curve before and after treatment (Fig. 6A). To rule out the possibility that increased RhoA or Rho-kinase contributes to the increased vasodilator response to Y-27632, we also used Western blot to measure RhoA and Rho-kinase, and RT-PCR to measure two subunits of myosin light chain phosphatase in aortic rings after stimulation with angiotensin II (100 nM) for 4 h. No significant differences were observed at the mRNA expression levels of these components of the RhoA/Rho-kinase signaling pathway before or after treatment with angiotensin II (data not shown).

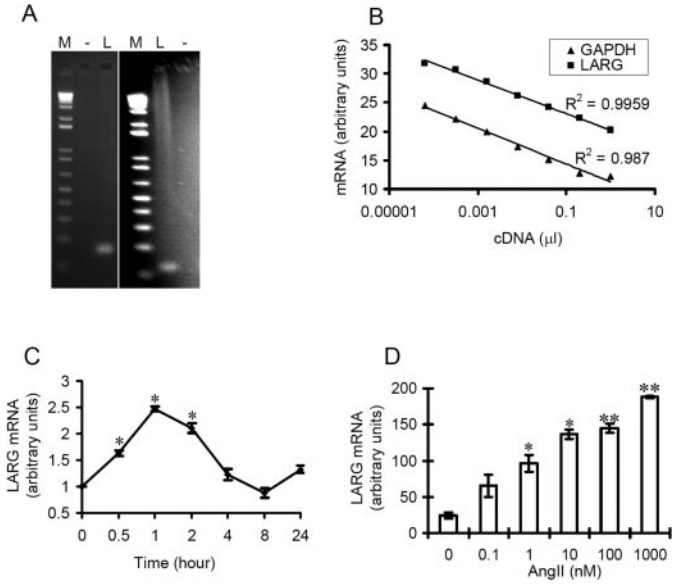

Fig. 2. Concentration-dependent induction of LARG mRNA by angiotensin II in cultured VSMCs. A, to establish the LARG mRNA assay based on real-time PCR, primers for GAPDH and LARG were designed, and their specificity was verified by visualization of RT-PCR products with 2% agarose gel. A representative gel image is presented, demonstrating that only the expected band was evident. M, 1-kb Plus DNA ladder (Invitrogen); −, minus control; L, LARG. B, efficiency and reliability of LARG mRNA assay was verified by measuring serially diluted (1:5) samples. C, rat VSMCs were stimulated with 100 nM angiotensin II for the indicated time. LARG mRNA assay was then performed. Results are presented as mean ± S.E.M. of three separate experiments. D, rat VSMCs were stimulated with the indicated concentration of angiotensin II for 1 h, and then LARG mRNA assay was performed. Results are presented as mean ± S.E.M. of six separate experiments. *, $p < 0.05$; **, $p < 0.01$.

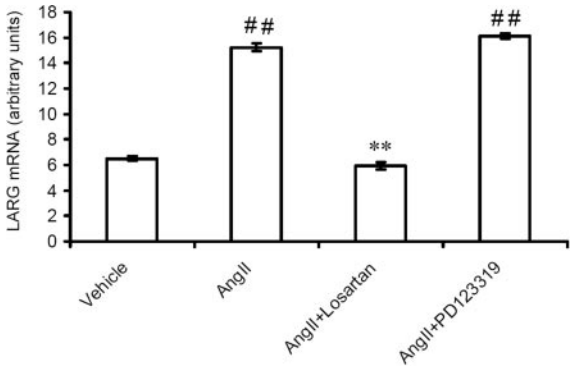

Fig. 3. Induction of LARG by angiotensin II is mediated by the AT1 receptor in VSMCs. Rat VSMCs were pretreated with 1 μM AT1 blocker losartan, 1 μM AT2 blocker PD123319, or vehicle for 15 min and then stimulated with 100 nM angiotensin II or treated with vehicle for 1 h. LARG mRNA expression level was measured by real-time PCR. Results are presented as mean ± S.E.M. of three separate experiments. **, $p < 0.01$; ##, $p < 0.01$, in comparison with vehicle.

Discussion

Here, we demonstrate that angiotensin II up-regulates LARG via the AT1 receptor, and this up-regulation is signaled via the PI3-kinase pathway. Furthermore, angiotensin II treatment caused a small, but significant, increase in the component of contractile responses sensitive to Rho-kinase antagonism.

Two angiotensin II receptors, AT1 and AT2, are detectable in the vasculature (Zhuo et al., 1995; Touyz, 2003). Using specific inhibitors, we observed that the induction of LARG by angiotensin II was mediated by the AT1 receptor. The AT1 receptor can activate multiple downstream intracellular signal transduction pathways (Brasier et al., 2000; Kim and Iwao, 2000; Touyz and Schiffrin, 2000; Touyz, 2003). Of these, the PI3-kinase/NF-κB pathway is noteworthy because of the key role played by angiotensin II in inflammation. This pathway has been shown to be responsible for the induction of interleukin-6 and interleukin-8 mRNA by angiotensin II in adipocytes (Touyz and Schiffrin, 2000). The present study demonstrated that a PI3-kinase inhibitor, wortmannin, abolished the induction of LARG by angiotensin II. Another PI3-kinase inhibitor, LY294002, dramatically reduced the induction of LARG by angiotensin II. Because the underlying mechanisms for these two inhibitors are different, the results seemed to be reliable.

NF-κB is a transcription factor downstream from PI3-ki-

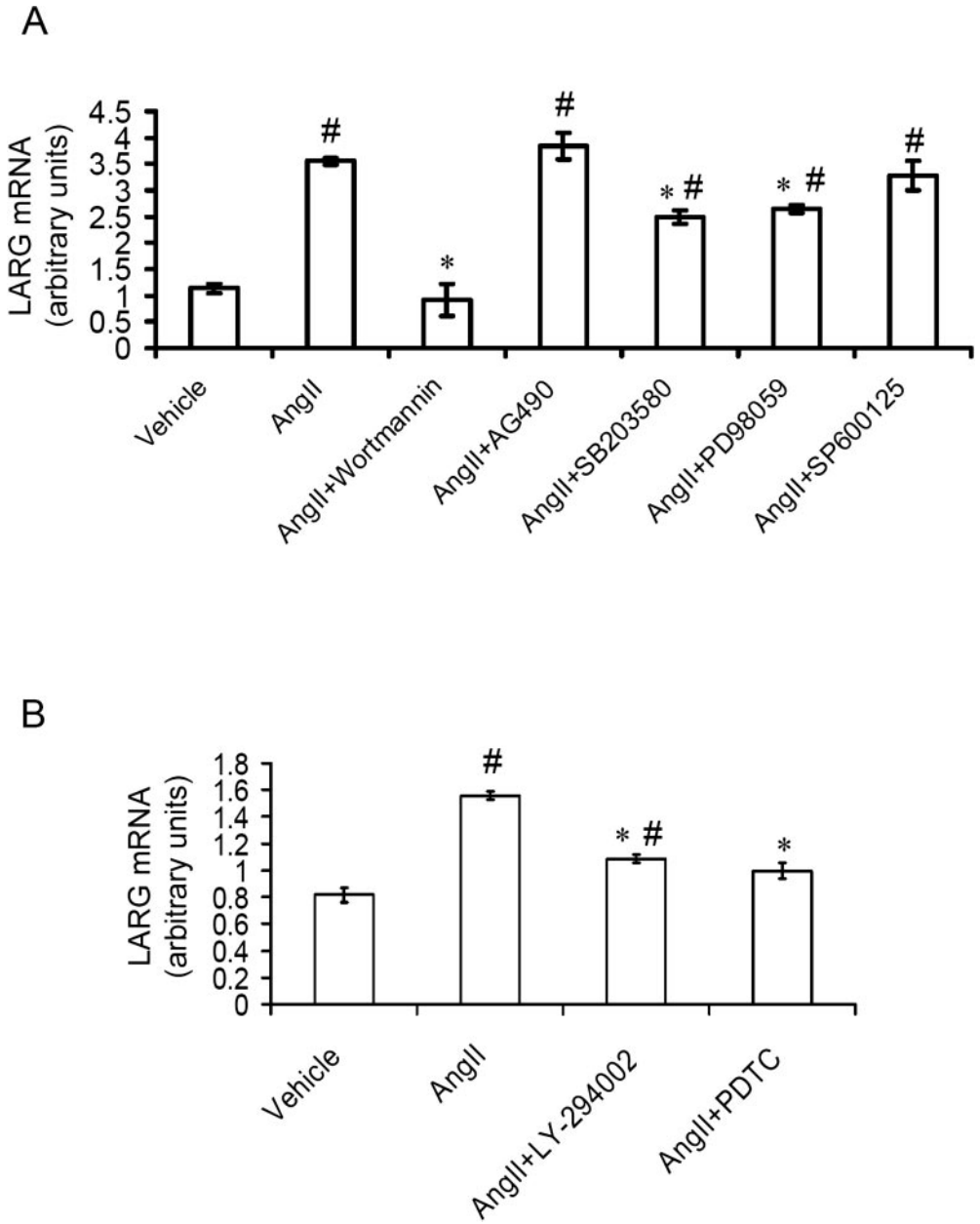

Fig. 4. The PI3-kinase pathway was required for the induction of LARG by angiotensin II in VSMCs. A, rat VSMCs were pretreated with 0.1 μM wortmannin, 10 μM SB203580, 10 μM SP600125, 25 μM AG490, 50 μM PD98059, or vehicle and treated with 100 nM angiotensin II or vehicle for 1 h. LARG mRNA expression level was then measured by real-time PCR. Results are presented as mean ± S.E.M. of three separate experiments. B, effect of another PI3-kinase inhibitor (LY294002; 20 μM) and a NF-κB inhibitor (PDTC; 300 μM) on the induction of LARG by angiotensin II. Results are presented as mean ± S.E.M. of four separate experiments. *, $p < 0.05$, in comparison with angiotensin II; #, $p < 0.05$, in comparison with vehicle.

nase. A NF-κB inhibitor, PDTC (Ziegler-Heitbrock et al., 1993), abolished the induction of LARG by angiotensin II. Consistent with this observation, two potential NF-κB binding sites were identified in the promoter region of rat LARG. It is noteworthy that potential NF-κB binding sites were also present in the promoter region of mouse LARG but not human p115 RhoGEF or human PDZ-RhoGEF, suggesting the induction of LARG by angiotensin II may not be limited to the rat. Our results showed that the induction of LARG by angiotensin II was partially blocked by either the mitogen-activated protein kinase kinase inhibitor PD98059 or the MAPK inhibitor SB203580, indicating the involvement of the MAPK pathway. However, no signal transducer and activator of transcription 3 binding site was identified within the analyzed region. There are two possibilities for this discrepancy: 1) the analyzed sequence is not long enough, because functional *cis*-elements can be located up to 10 kb from the transcription start site (Swallow, 2003); and 2) there is cross-

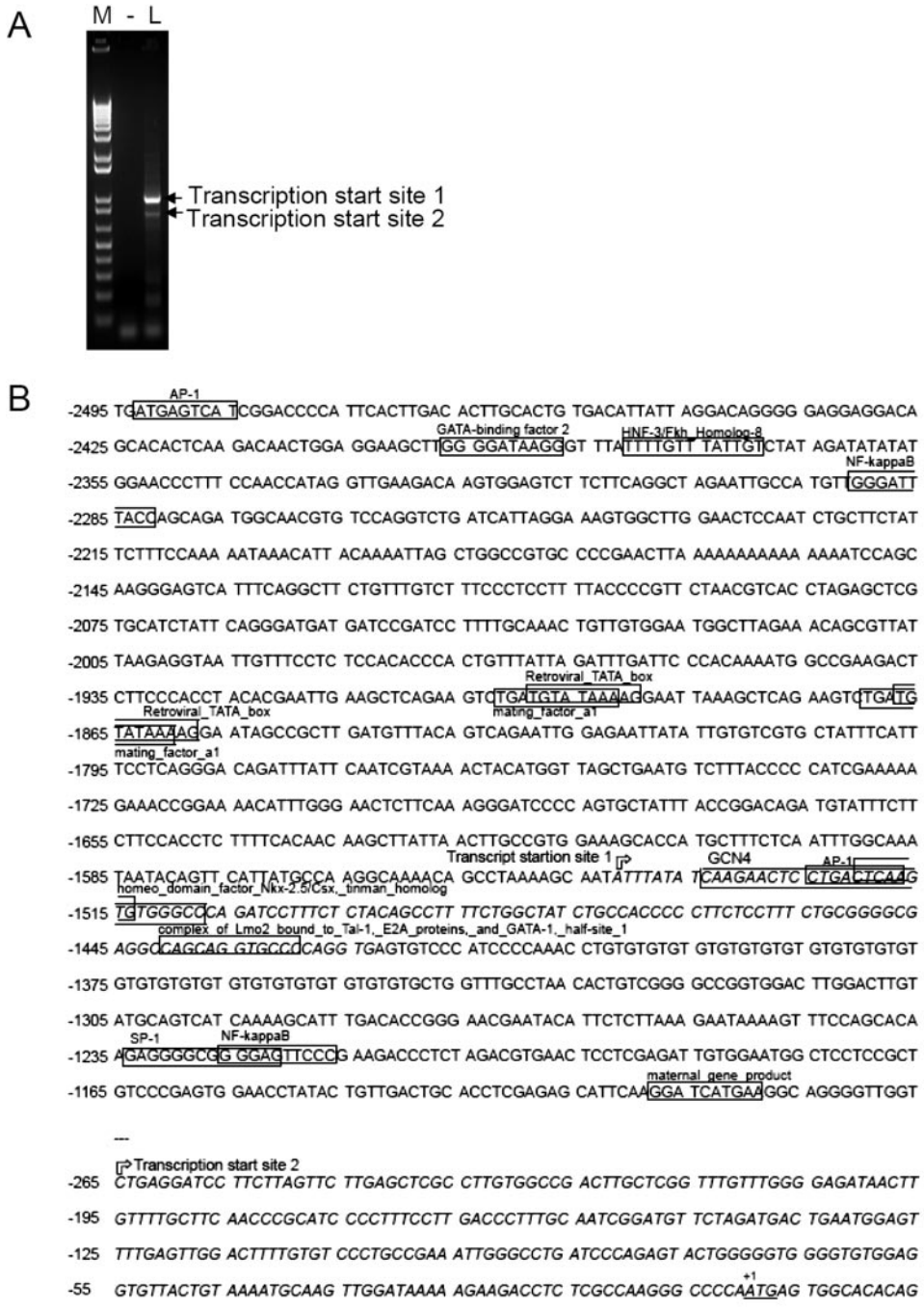

Fig. 5. Determination of transcript start sites of rat LARG gene. A, total RNA was extracted from rat VSMCs or aorta, and then 5′ RACE RT-PCR was performed according to manufacturer's instructions. A, gel image of PCR products (aorta) was presented. M, 1-kb Plus DNA ladder (Invitrogen); −, minus control; L, LARG. B, online program TRES was used to search transcription factor binding sites using TRANSFAC weight matrices within 3000 base pairs upstream from the start codon. Predicted transcription binding sites are shown in frames. The arrow indicates the position of the transcription start.

TABLE 1

Transcription factor binding sites in the promoter region of RhoGEF genes

Transcription factor binding sites in the promoter region of RhoGEF genes were scanned using an online program, TRES (http://bioportal.bic.nus.edu.sg/tres/), with TRANSFAC weight matrices. The similarity score was in the range of 0 to 100, and the cut-off value was set at 95. Except for rat LARG, the transcript start sites of other genes were obtained by searching the GenBank database (human p115RhoGEF, accession no.NM_198977; human PDZ-RhoGEF, accession no. NM_014784; and mouse LARG, accession no. NM_027144). The first nucleotide upstream from the full-length cDNA was set as −1.

Position	Transcription Factor	Similarity
		%
Rat LARG		
−834	Maternal gene product	96.5
−940	NF-κB	97.6
−948	Stimulating protein 1	97.1
−1146	Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1	97.2
−1155	Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1	99.1
−1156	Snail	95.2
−1234	Homeodomain factor Nkx-2.5/Csx, tinman homolog	100
−1239	AP-1 binding site	97.4
−1248	GCN4	95.1
−1581	Retroviral TATA box	95.1
−1584	Mating factor a1	96
−1613	Retroviral TATA box	95.1
−1616	Mating factor a1	96
−1921	*Xenopus* forkhead domain factor 2	95.4
−2005	NF-κB	95.4
−2096	HNF-3/Fkh homolog-8	95.9
−2111	GATA-binding factor 2	95.3
−2207	AP-1 binding site	98.8
−2881	Tal-1β/E47 heterodimer	97.2
Mouse LARG		
−463	Adf-1	96.6
−466	Adf-1	98.3
−1132	Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1	97.4
−1141	Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1	99.1
−1142	Snail	95.2
−1224	Homeodomain factor Nkx-2.5/Csx, tinman homolog	98.6
−1239	Sox-5	99.8
−1421	Ikaros 1	95.5
−1575	Retroviral TATA box	95.1
−1890	*Xenopus* forkhead domain factor 2	95.4
−1974	NF-κB	95.4
−2458	Bicoid	96.1
−2793	Homeodomain factor Nkx-2.5/Csx, tinman homolog	100
Human p115RhoGEF		
−45	Stimulating protein 1	97.7
−58	GC box elements	95.5
−176	Stimulating protein 1	97.8
−578	Activator protein 4	98.5
−704	Ikaros 1	96.7
−992	Bicoid	99
−1029	Homeodomain factor Nkx-2.5/Csx, tinman homolog	100
−1289	Bicoid	99
−1424	Bicoid	99
−1675	Ikaros 1	96.2
−1853	GATA-binding factor 2	96.2
−2021	GCR1	95.6
−2138	Activator protein 4	95.7
−2279	Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1	98.4
−2364	Zeste transvection gene product	95
−2563	PAR-type chicken vitellogenin promoter-binding protein	97.7
−2649	Heat shock factor (yeast)	97.1
−2654	Heat shock factor (yeast)	97.1
Human PDZ-RhoGEF		
−100	NF-κB (p65)	97.6
−169	Tramtrack 69K	95.7
−798	Chorion factor 1	96.9
−886	Dorsal	97.6
−1439	Bicoid	99
−1588	Broad-complex Z4	95.1
−1728	Sox-5	99.1
−1763	GATA-binding factor 2	96.2
−1795	Viral homolog of thyroid hormone receptor α1	97
−1983	Myoblast determining factor	96.5
−2033	Fushi tarazu	95.9
−2103	Retroviral poly A downstream element	95.2
−2146	Myoblast-determining factor	99.4
−2167	Bicoid	99
−2303	Bicoid	95.1
−2484	*Xenopus* forkhead domain factor 1	95.4
−2781	Yeast factor complex HAP2/3/5, homolog to vertebrate NF-Y/CP1/CBF	95

AP-1, adaptor protein.

talk between elements of the MAPK pathway and NF-κB (Karin, 2004).

Studies have shown that LARG can activate RhoA after exposure to extracellular stimuli (Vogt et al., 2003; Wang et al., 2004). Because LARG has been suggested to play a role in RhoA/Rho-kinase-mediated Ca^{2+} sensitization, we predicted that the induction of LARG by angiotensin II would affect Ca^{2+}-sensitization of vascular smooth muscle. Supporting this hypothesis, our results revealed that pretreatment with angiotensin II significantly increased the vasodilator response to Y-27632, a Rho-kinase inhibitor. This interpretation is consistent with that drawn from studies of arteries from hypertensive animals. In several animal models of hypertension, it has been reported that expression levels for various components of the RhoA/Rho-kinase signaling pathway are increased in hypertensive arteries compared with normotensive values (Weber and Webb, 2001; Seko et al., 2003; Yoneda et al., 2003; Lee et al., 2004; Ying et al., 2004; Hu and Lee, 2005). In addition, contractile responses to various agonists in arteries from hypertensive animals are more sensitive to the inhibitory properties of Rho-kinase antagonists than arteries from normotensive animals. In hypertensive patients, dilator responses to Rho-kinase antagonists are increased in the forearm vasculature compared with normotensive values (Masumoto et al., 2001). The interpretation of these various studies has been that the contractile re-

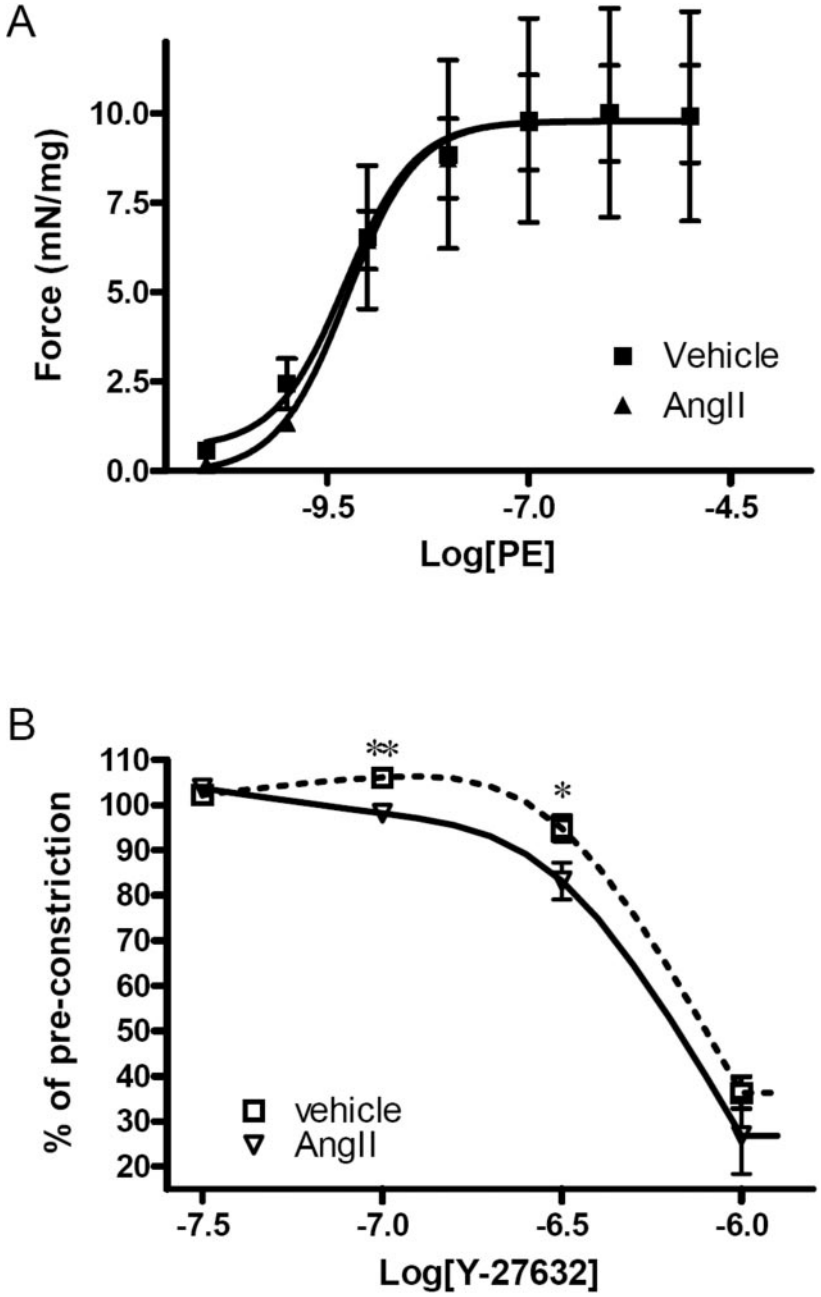

Fig. 6. Angiotensin II increased Rho-kinase mediated Ca^{2+} sensitization in rat aortic rings. Aortic rings were prepared and incubated in DMEM containing either 100 nM angiotensin II or vehicle for 3 h and then were mounted in a muscle bath. After 1 h of equilibration, the aortic rings were contracted by 0.01 μM PE, and the concentration-dependent relaxation-response curve to Y-27632 was recorded. A, concentration-dependent contractile response induced by PE was recorded. B, concentration-dependent relaxation-response curve to Y-27632. *, $p < 0.05$; **, $p < 0.01$.

sponse of the smooth muscle cell is dependent on both a Ca^{2+}-dependent pathway (phospholipase C/inositol 1,4,5-trisphosphate, protein kinase C) and a Ca^{2+}-sensitizing pathway (RhoA/Rho-kinase). The change in sensitivity or responsiveness to the Rho-kinase antagonist reflects that a greater proportion of the contractile response is maintained by the RhoA/Rho-kinase signaling pathway in the arteries from the hypertensive animals than in arteries from normotensive animals. Our experiments seem consistent with these previous studies in arteries from hypertensive animals where there is an up-regulation of the RhoA/Rho-kinase pathway. When the LARG was increased, there was an associated increase in the sensitivity of the system to inhibition by the Rho-kinase antagonist. Thus, it may be that a greater proportion of the contractile response to PE has shifted to the Ca^{2+}-sensitizing pathway in these arteries where LARG expression is increased.

Although pretreatment with angiotensin II significantly increased the vasodilator response to Y-27632, it did not change the concentration-dependent contractile response curve to PE. As LARG may act as a GTPase activating protein for heterotrimeric G proteins (Kozasa et al., 1998; Hakoshima et al., 2003), one possible explanation is that LARG activates the RhoA/Rho-kinase pathway at the expense of another signaling pathway, for example Ca^{2+}-dependent signaling. However, this remains to be tested. It has been shown that the maximal contraction of aorta by PE did not change in mineralocorticoid hypertensive rats, but the vasodilator response to Y-27632 increased in these vascular segments compared with normotensive values (Weber and Webb, 2001). RhoA/Rho-kinase-mediated Ca^{2+} sensitization is increased in hypertension (Uehata et al., 1997; Somlyo and Somlyo, 2003; Lee et al., 2004), and the mechanism remains poorly understood. We previously demonstrated that the expression of LARG increased in arteries from stroke-prone spontaneous hypertensive rats (Ying et al., 2004), and the current study provides a possible mechanism for increased RhoA/Rho-kinase-mediated Ca^{2+} sensitization in hypertension.

In conclusion, our results demonstrate that angiotensin II up-regulates LARG via the AT1 receptor, and this up-regulation causes a small, but significant, increase in the component of contractile responses sensitive to Rho-kinase antagonism, suggesting that RhoGEFs, particularly LARG, may participate in linking GPCR to RhoA activation in vascular smooth muscle.

Acknowledgments

We are grateful to Dr. Chin-Chen Wu for helpful suggestions and comments during the preparation of the manuscript.

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