Molecular Pharmacology Fast Forward. Published on December 14, 2005 as doi:10.1124/mol.105.017830


MOL 17830

MOLPHARM/2005/017830

# Angiotensin II Up-regulates Leukemia-associated Rho Guanine Nucleotide Exchange Factor (LARG), a RGS Domain Containing RhoGEF, in Vascular Smooth Muscle Cells

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Molecular Pharmacology Fast Forward. Published on December 14, 2005 as DOI: 10.1124/mol.105.017830
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MOL 17830

Running title: Induction of LARG by angiotensin II

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Text pages: 30

Tables: 1

Figures: 6

References: 28

Words in abstract: 207

Words in introduction: 526

Words in discussion: 918

Abbreviations used: GPCR, G-protein-coupled receptors; MLC, myosin light chain; LARG, leukemia-associated Rho guanine nucleotide exchange factor; RGS, regulators of G-protein signaling; RhoGEFs, Rho specific guanine nucleotide exchange factors; RhoGAPs, Rho specific GTPase activating proteins; GDIs, guanine nucleotide dissociation inhibitors; VSMCs, vascular smooth muscle cells.

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## Abstract

In vascular smooth muscle, stimulation of heterotrimeric G-protein-coupled receptors (GPCR) 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 (MLCK). Additionally, 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, LARG (leukemia-associated RhoGEF), 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 (PI3-kinase) pathway. Further, angiotensin II treatment caused a small, but significant, increase in the component of contractile responses sensitive to Rho-kinase antagonism. These observations support the

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hypothesis that RhoGEFs, particularly LARG, participate in linking GPCR to RhoA activation in vascular smooth muscle.

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## Introduction

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 $Ca^{2+}$-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 guanosine triphosphate (GTP). In the inactive state, RhoA is primarily a cytosolic component, and is bound to guanosine diphosphate (GDP) and a

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guanine nucleotide dissociation inhibitor (GDI). 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, comprise 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 (Tanabe et al., 2004; Hart et al., 1998). 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.,

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2003; Yamakawa et al., 2003). Since 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. Further, 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 (GPCR) to RhoA activation in vascular smooth muscle.

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## Materials and Methods

**Materials:** Collagenase, elastase, Y-27632, PD98059, phenylephrine, ammonium pyrrolidinecarbodithioate, SB203580, wortmannin, AG490, SP600125, LY-294002 and PD123319 were purchased from Sigma (St. Louis, MO). Losartan was a gift from Dr. Michael W. Brands (Medical College of Georgia, Augusta, GA).

**Preparation and culture of rat vascular smooth muscle cells (VSMCs):** Primary cultures of rat VSMCs were prepared as previously described (Jing et al., 1999). Briefly, the thoracic aortas of Sprague-Dawley rats (Harlan, Indianapolis, Indiana) 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 minutes, and washed. The remaining media was incubated in collagenase and elastase with gentle agitation until the cells were dispersed. The resulting cell suspension was centrifuged, and the cells 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

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TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Four µg RNA was subjected to digestion by Dnase I (Invitrogen, Carlsbad, CA) and then used for reverse transcription reaction. The first strand cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After dilution by adding 40µl of water, cDNA was used for polymerase chain reaction (PCR) or stored at -20°C.

**PCR:** As previously described (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 cttcgagaacgccaaa-3′; reverse, 5′-gggcagccacttgtccttgtcagg-3′. LARG primers were: forward, 5′-agccatgcgcgctggagtacaaac-3′; reverse, 5′-gctccaggggaatgaggggatgtc-3′. p115RhoGEF primers were: forward, 5′-tccggaccaagagtggggacaaga-3′; reverse, 5′-tacccaggcttcccttccggtc tg-3′. GAPDH primers were: forward, 5′-tgcatcctgcaccaccaactgctt-3′; reverse, 5′-acagccttgg cagcaccagtggat-3′. One µl cDNA was first used to amplify the house-keeping gene, glyceraldehye-3-phosphate dehydrogenase (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

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levels between samples. RhoGEF transcripts were subsequently amplified from the adjusted cDNA preparations. PCR was performed in a mixture of 25µl containing 12.5µl Taq PCR master mix (Qiagen, Valencia, CA), 0.1µM forward primer, 0.1µM reverse primer, and 1µl cDNA. The reaction cycles were as follows: 94°C for 2 min and 22 (GAPDH) or 30 (RhoGEFs) cycles of 94°C for 30s, 60°C for 30s and 72°C for 30s, 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, New Haven, CT), and the results were expressed as the densitometric ratio of RhoGEF/GAPDH (%).

**Real-time (RT) 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. Briefly, mixtures (one reaction contains 10µl 2× master mix, 1µl 10nM forward primer, 1µl 10nM reverse primer, and 7µl water) were prepared and added to each PCR tube, then 1µl 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 15s, 60°C for 30s, 72°C for 30s, and finally a melting curve analysis of PCR product. The fluorescent signal at each cycle was plotted versus the cycle number. The threshold cycle C(t), the cycle number at which an increase above background fluorescence could be reliably detected, was determined for each sample.

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The results were expressed as the difference between the C(t) 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′-GCTAAGCAGTTGGTGGTGCA -3′ for GAPDH with a 178 bp amplicon; 5′-AAGCAGACGAACTCCAAGGAGACT-3′ and 5′-CAGGTGCTGAAACAATGCGGAGAA-3′ for LARG with a 117 bp amplicon.

**5' RACE RT-PCR:** 5' RACE RT-PCR was performed on rat aortic RNA using RNA ligase mediated (RLM)-RACE reagents following the manufacturer's instructions (Ambion, Austin, TX). The primary PCR was performed using the Outer Adapter Primer (Ambion, Austin, TX) 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 (Perkin-Elmer, Shelton, CT): 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min. A pool of the different RACE products was ligated into pCR2.1 (Invitrogen, Carlsbad, CA). Fifteen plasmids were isolated from recombinant clones and sequenced.

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**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. Male Sprague-Dawley rats (275–300 g) were anesthetized with pentobarbital sodium (50 mg/kg ip), and the thoracic aorta was quickly removed and cleaned in physiological salt solution (PSS) of the following composition (mM): 118 NaCl, 4.7 KCl, 1.18 $KH_2PO_4$, 1.6 $CaCl_2 \cdot 2H_2O$, 1.6 $MgSO_4 \cdot 7H_2O$, 25 $NaHCO_3$, 5.5 dextrose, and 0.03 EDTA. The aorta was cut into 2-mm rings, and the endothelium was gently removed. Rings were incubated with DMEM (Invitrogen, Carlsbad, CA) containing either 100nM angiotensin II (100nM) or vehicle for 3 hours at 37°C. After incubation, the aortic rings were mounted in a muscle bath containing PSS 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). A resting tension of 3 g was imposed on each ring, and the rings were allowed to equilibrate for 1 hour. 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. Subsequently, 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 pre-contracted rings).

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**Detection of LARG protein by Western blot analysis.** Aortic rings were incubated in DMEM containing either 100nM 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 RIPA buffer (mM): 50 Tris·HCl (pH 7.4), 150 NaCl, 1 EDTA, 1 NaF, 1 $Na_3VO_4$, and 1 PMSF, with 0.25% Na-deoxycholate and 1.0% NP-40, and centrifuged at 10,000 g and 4°C for 30 min. The supernatant was collected, and subjected to Western blot analysis. Briefly, 80μg of protein were separated by SDS-PAGE and subsequently transferred to nitrocellulose membrane. The membrane was then incubated with LARG (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Sigma, St. Louis, MO) antibody. Finally, the membranes were incubated with a horseradish peroxidase-linked secondary antibody and visualized with an enhanced chemiluminescence kit (Amersham, Piscataway, NJ). Band density was quantified by densitometric analysis using a Kodak image station 440CF (Kodak, New Haven, CT).

**Data Analysis.** Data are presented as mean ± standard error of the 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.

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## Results

**Angiotensin II transiently up-regulates LARG mRNA and protein in rat vascular smooth muscle cells:** To determine if 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 minutes of stimulation, and reached a peak after one hour of stimulation (Figure 1A). It is noted that PDZ-RhoGEF and p115RhoGEF did not significantly change at any interval after stimulation with angiotensin II (Figure 1B). When stimulated by vehicle for 1 hour, 2 hours, 8 hours, and 24 hours, no alteration of mRNA expression level of these RhoGEFs was observed (data not shown). LARG protein levels were consistently elevated following angiotensin II stimulation, with a significant increase after 3 hours and peaking at 6 hours of stimulation (Figure 1C and D).

Since the major cell type in the aorta is the vascular smooth muscle cell (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 Figure 2A and B), and was used to examine if angiotensin II can induce LARG in cultured VSMCs. Results showed that angiotensin II

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transiently up-regulated LARG mRNA in cultured VSMCs (Figure 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 of angiotensin II significantly increased the expression of LARG mRNA, and maximal induction was observed at an angiotensin II concentration of 1 μM (Figure 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 (Zhou 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 involved 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 (Figure 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:** Since 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

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pathway(s) was involved in the induction of LARG by angiotensin II. It was observed that a PI3-kinase inhibitor (wortmannin) completely blocked the induction of LARG by angiotensin II, and a MEK inhibitor (PD98059) and a MAPK inhibitor (SB203580) partially blocked the induction of LARG, whereas a JAK2 inhibitor (AG490) and a JNK inhibitor (SP600125) did not significantly affect the induction of LARG mRNA by angiotensin II (Figure 4A). Consistent with the results from wortmannin, another PI3-kinase inhibitor, LY-294002, significantly attenuated the induction of LARG by angiotensin II (Figure 4B).

NF-κB is a common downstream transcription factor of PI3-kinase. To test if NF-κB mediates the induction of LARG by angiotensin II, VSMCs were pre-treated with ammonium pyrrolidinecarbodithioate (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 Figure 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 (Figure 5A), and after sequencing, two major transcription start sites were identified (Figure 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

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potential NF-κB binding sites were identified (Figure 5B). Similar analyses were performed on the promoter regions of human p115 RhoGEF, human PDZ-RhoGEF, and mouse LARG as well, since the sequences of their 5' non-coding region were available via GenBank. Interestingly, the potential NF-κB binding site was identified only in the promoter region of mouse LARG, but not in other genes examined (Table 1).

**Pre-treatment with angiotensin II increases Rho-kinase antagonist-induced vasodilation:** Since LARG can link heterotrimeric G proteins to RhoA and may thus be involved in $Ca^{2+}$-sensitization of vascular smooth muscle, it was predicted that pre-treatment 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, it was investigated how pre-treatment with angiotensin II affected the vasodilator response to Y-27632 in PE-pre-contracted rat aortic rings. Results demonstrated that pre-treatment with angiotensin II significantly increased the vasodilator response to Y-27632 at 100 nM and 300 nM (Figure 6B). However, pre-treatment with angiotensin II did not change contractile responses to PE as demonstrated by the lack of shift in the curve before and after treatment (Figure 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

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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 hours. 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).

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## 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. Further, 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 (Touyz, 2003; Zhuo et al., 1995). 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 (Touyz and Schiffrin, 2000; Touyz, 2003; Brasier et al., 2000; Kim and Iwao, 2000). 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 (IL-6) and interleukin-8 (IL-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, LY-294002, dramatically reduced the induction of LARG by angiotensin II. Since the underlying mechanisms for these two inhibitors are different, the results appeared to be reliable.

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NF-κB is a transcription factor downstream from PI3-kinase. 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. Notably, 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 MEK inhibitor, PD98059, or the MAPK inhibitor, SB203580, indicating the involvement of the MAPK pathway. However, no STAT3 binding site was identified within the analyzed region. There are two possibilities for this discrepancy: 1) the analyzed sequence is not long enough, since functional cis-elements can be located up to 10 kb from the transcription start site (Swallow, 2003); and/ or 2) there is cross-talk between elements of the MAPK pathway and NF-κB (Karin, 2004).

Studies have shown that LARG can activate RhoA following exposure to extracellular stimuli (Wang et al., 2004; Vogt et al., 2003). Since LARG has been suggested to play a role in RhoA/Rho-kinase-mediated $Ca^{2+}$-sensitization, it was 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 pre-treatment with

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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 to normotensive values (Hu and Lee, 2005; Lee et al., 2004; Nakano, 2003; Seko et al, 2003; Weber and Webb, 2001; Ying et al, 2004; Yoneda et al, 2003). Additionally, 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 to normotensive values (Masumoto et al, 2001). The interpretation of these various studies has been that the contractile response of the smooth muscle cell is dependent on both a $Ca^{2+}$-dependent pathway (phospholipase C/$IP_3$, 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 appear consistent with these earlier 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

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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 pre-treatment 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 (GAP) for hetereotrimeric G proteins (Hakoshima et al, 2003; Kozasa et al., 1998), 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. Interestingly, 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 to normotensive values (Weber and Webb, 2001). RhoA/Rho-kinase-mediated $Ca^{2+}$-sensitization is increased in hypertension (Uehata et al., 1997; Lee et al., 2004; Somlyo and Somlyo, 2003), and the mechanism remains poorly understood. We previously demonstrated that the expression of LARG increased in arteries from SHRSP (Ying et al. 2004), and the current study provides a possible mechanism for increased RhoA/Rho-kinase-mediated $Ca^{2+}$-sensitization in hypertension.

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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.

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## Acknowledgements

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

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## Footnotes

This study was supported by grants from the National Institutes of Health (HL-74167, HL-71138) and the American Heart Association (0130364N).

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

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## Figure legends

**Figure 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 non-stimulated control (0) was calculated and was presented as mean ± S.E.M. of three separate experiments; C, a representative image of western blot analysis; D, LARG protein expression relative to non-stimulated control was presented as mean ± S.E.M. of four separate experiments. *$p < 0.05$, **$p < 0.01$.

**Figure 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, Carlsbad, CA); -, minus control; L, LARG. B, the efficiency and reliability of LARG mRNA assay was verified by measuring serially diluted (1:5) samples.

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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 ± SEM of three separate experiments. D, rat VSMCs were stimulated with the indicated concentration of angiotensin II for 1 hour, and then LARG mRNA assay was performed. Results are presented as mean ± S.E.M. of six separate experiments. *$p < 0.05$, **$p < 0.01$.

**Figure 3. The induction of LARG by angiotensin II is mediated by the AT1 receptor in VSMCs.** Rat VSMCs were pretreated with 1μM AT1 blocker, losartan, 1μM AT2 blocker, PD123319, or vehicle for 15 minutes, and then stimulated with 100 nM angiotensin II or treated with vehicle for 1 hour. 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.

**Figure 4. The PI3-kinase pathway was required for the induction of LARG by angiotensin II in VSMCs.** A, Rat VSMCs were pretreated with 0.1μM wortmannin, 10μM SB203580, 10μM SP600125, 25μM AG490, 50μM PD98059, or vehicle, and treated with 100 nM angiotensin II or vehicle for 1 hour. LARG mRNA expression level was then measured by real-time PCR. Results are presented as mean ± S.E.M. of three separate experiments. B, the effect of another PI3-kinase inhibitor (LY-294002, 20μM) and a NF-κB inhibitor (PDTC, 300μM) on the induction of LARG by angiotensin II.

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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.

**Figure 5. The 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, a gel image of PCR products (aorta) was presented. M, 1 kb Plus DNA ladder (Invitrogen, Carlsbad, CA); -, minus control; L, LARG. B, An online program, TRES (http://bioportal.bic.nus.edu.sg/tres/), was used to search transcription factor binding sites using TRANSFAC Weight Matrices within 3000 basepairs upstream from the start codon. Predicted transcription binding sites are shown in frames. The arrow indicates the position of the transcription start.

**Figure 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 hours, and then were mounted in a muscle bath. After 1 hour 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, The concentration-dependent contractile response induced by PE was recorded; B, The concentration-dependent relaxation response curve to Y-27632. *p < 0.05, **p < 0.01.

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**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 data base of GenBank (Access number: human p115RhoGEF, NM_198977; human PDZ-RhoGEF, NM_014784; mouse LARG, NM_027144). The first nucleotide upstream from the full-length cDNA was set as -1.

rat LARG

| Position | Transcription factor | similarity |
|---|---|---|
| -834 | maternal_gene_product | 96.5 |
| -940 | ⇨NF-kappaB | 97.6 |
| -948 | stimulating_protein_1 | 97.1 |
| -1146 | complex_of_Lmo2_bound_to_Tal- | 97.2 |
| -1155 | complex_of_Lmo2_bound_to_Tal- | 99.1 |
| -1156 | Snail | 95.2 |
| -1234 | homeo_domain_factor_Nkx- | 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_fork_head_domain_factor_2 | 95.4 |
| -2005 | ⇨NF-kappaB | 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-1beta/E47_heterodimer | 97.2 |

mouse LARG

| Position | Transcription factor | similarity |
|---|---|---|
| -463 | Adf-1 | 96.6 |
| -466 | Adf-1 | 98.3 |
| -1132 | complex_of_Lmo2_bound_to_Tal- | 97.4 |
| -1141 | complex_of_Lmo2_bound_to_Tal- | 99.1 |
| -1142 | Snail | 95.2 |
| -1224 | homeo_domain_factor_Nkx- | 98.6 |
| -1239 | Sox-5 | 99.8 |
| -1421 | Ikaros_1 | 95.5 |
| -1575 | Retroviral_TATA_box | 95.1 |
| -1890 | Xenopus_fork_head_domain_factor_2 | 95.4 |
| -1974 | ⇨NF-kappaB | 95.4 |
| -2458 | Bicoid | 96.1 |
| -2793 | homeo_domain_factor_Nkx- | 100 |

human p115RhoGEF

| Position | Transcription factor | similarity |
|---|---|---|
| -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 | homeo_domain_factor_Nkx- | 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- | 98.4 |
| -2364 | Zeste_transvection_gene_product | 95 |
| -2563 | PAR- | 97.7 |
| -2649 | heat_shock_factor_(yeast) | 97.1 |
| -2654 | heat_shock_factor_(yeast) | 97.1 |

human PDZ-RhoGEF

| Position | Transcription factor | similarity |
|---|---|---|
| -100 | NF-kappaB_(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_rec | 97 |
| -1983 | myoblast_determining_factor | 96.5 |
| -2033 | fushi_tarazu | 95.9 |
| -2103 | Retroviral_Poly_A_downstream_eleme | 95.2 |
| -2146 | myoblast_determining_factor | 99.4 |
| -2167 | Bicoid | 99 |
| -2303 | Bicoid | 95.1 |
| -2484 | Xenopus_fork_head_domain_factor_1 | 95.4 |
| -2781 | yeast_factor_complex_HAP2/3/5,_hom | 95 |

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# Figure 1

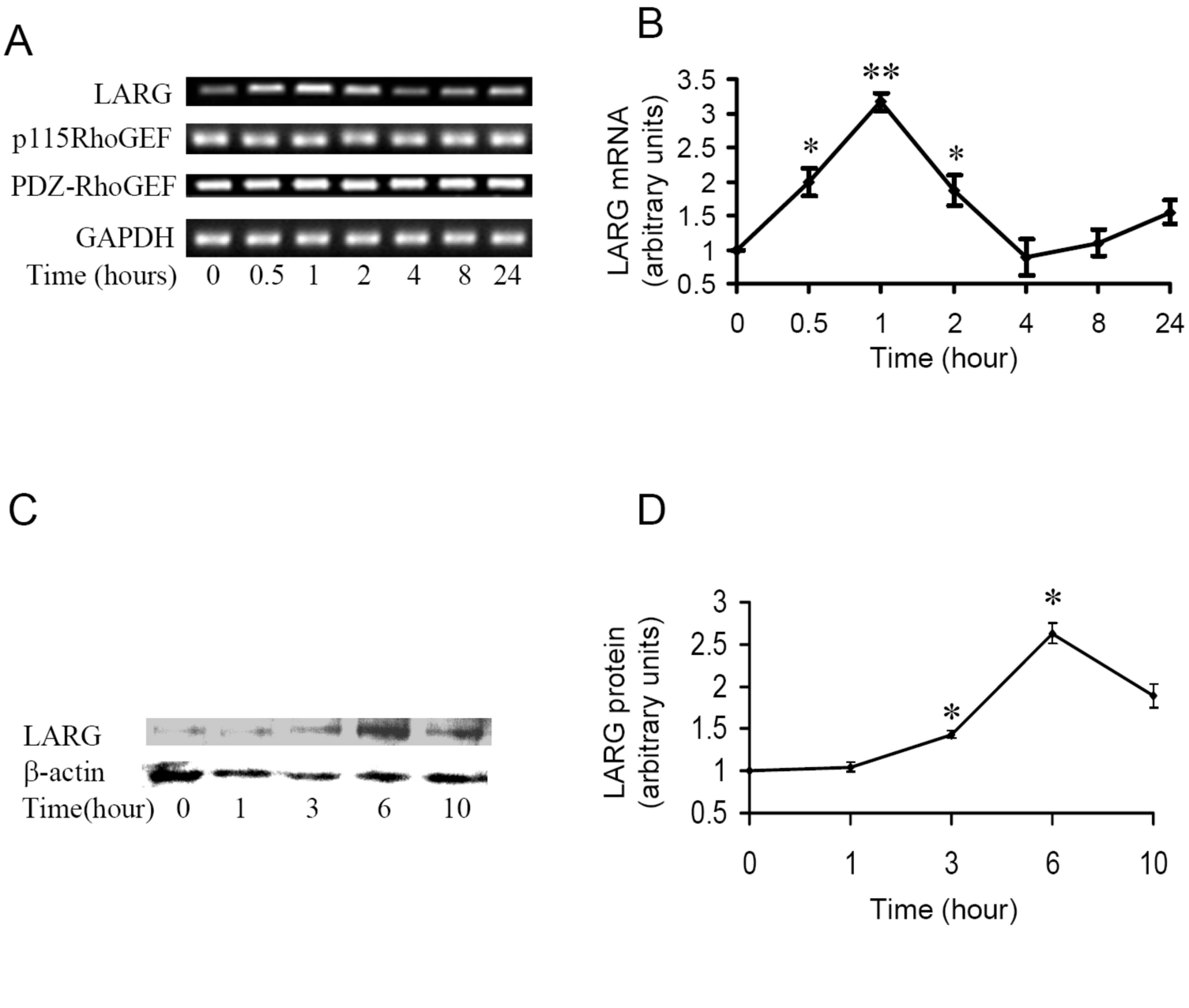

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Figure 2

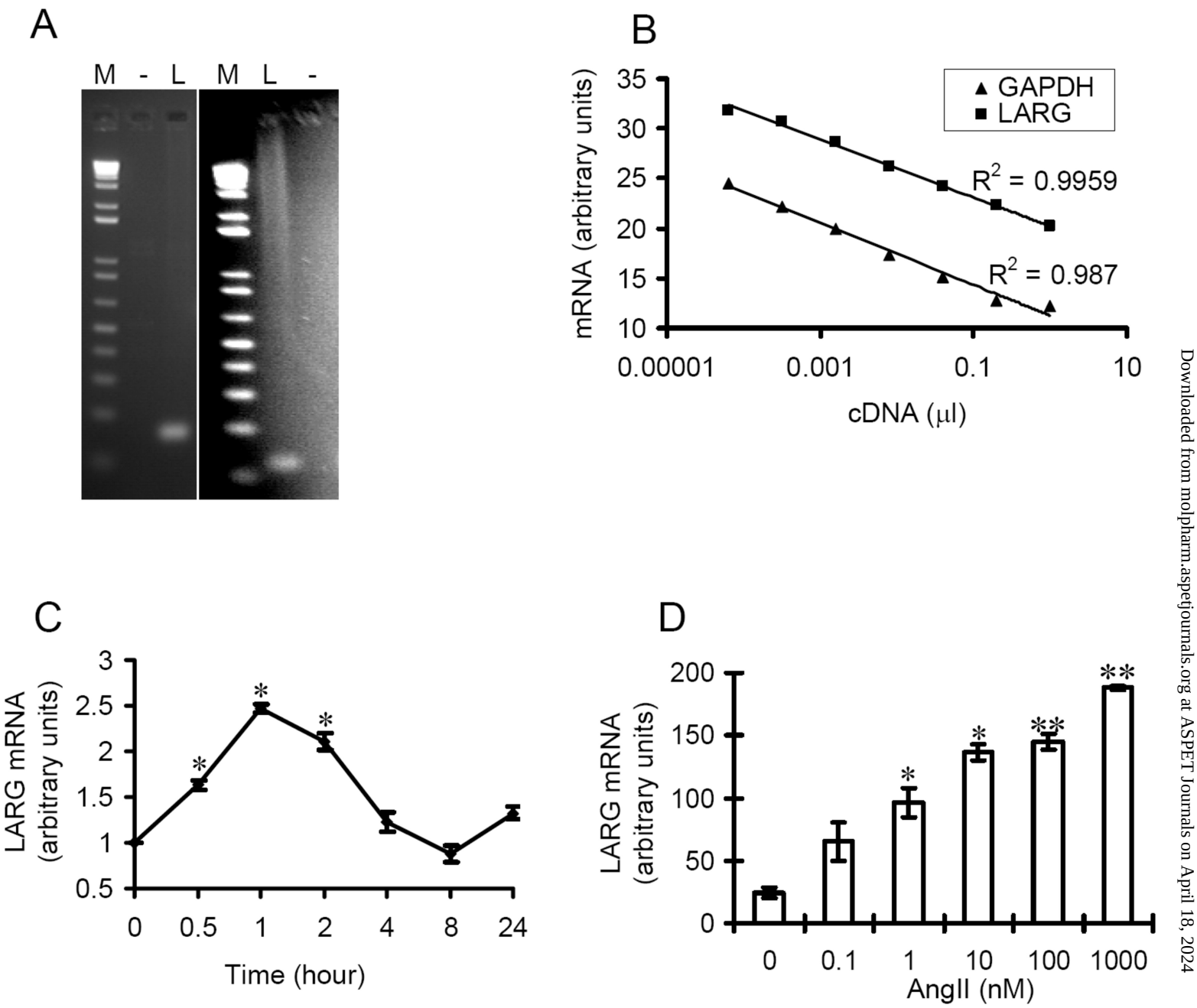

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Figure 3

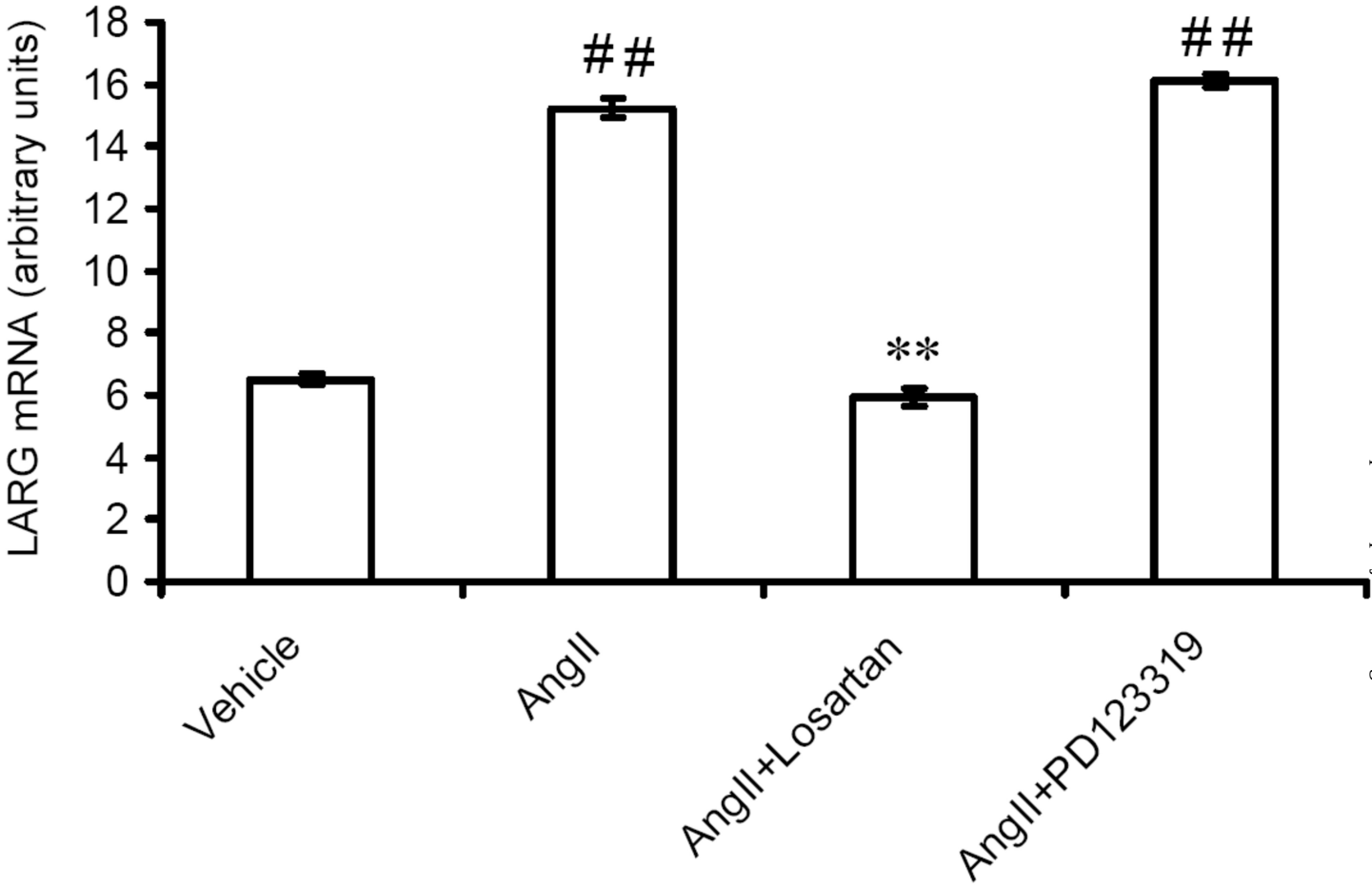

Figure 4

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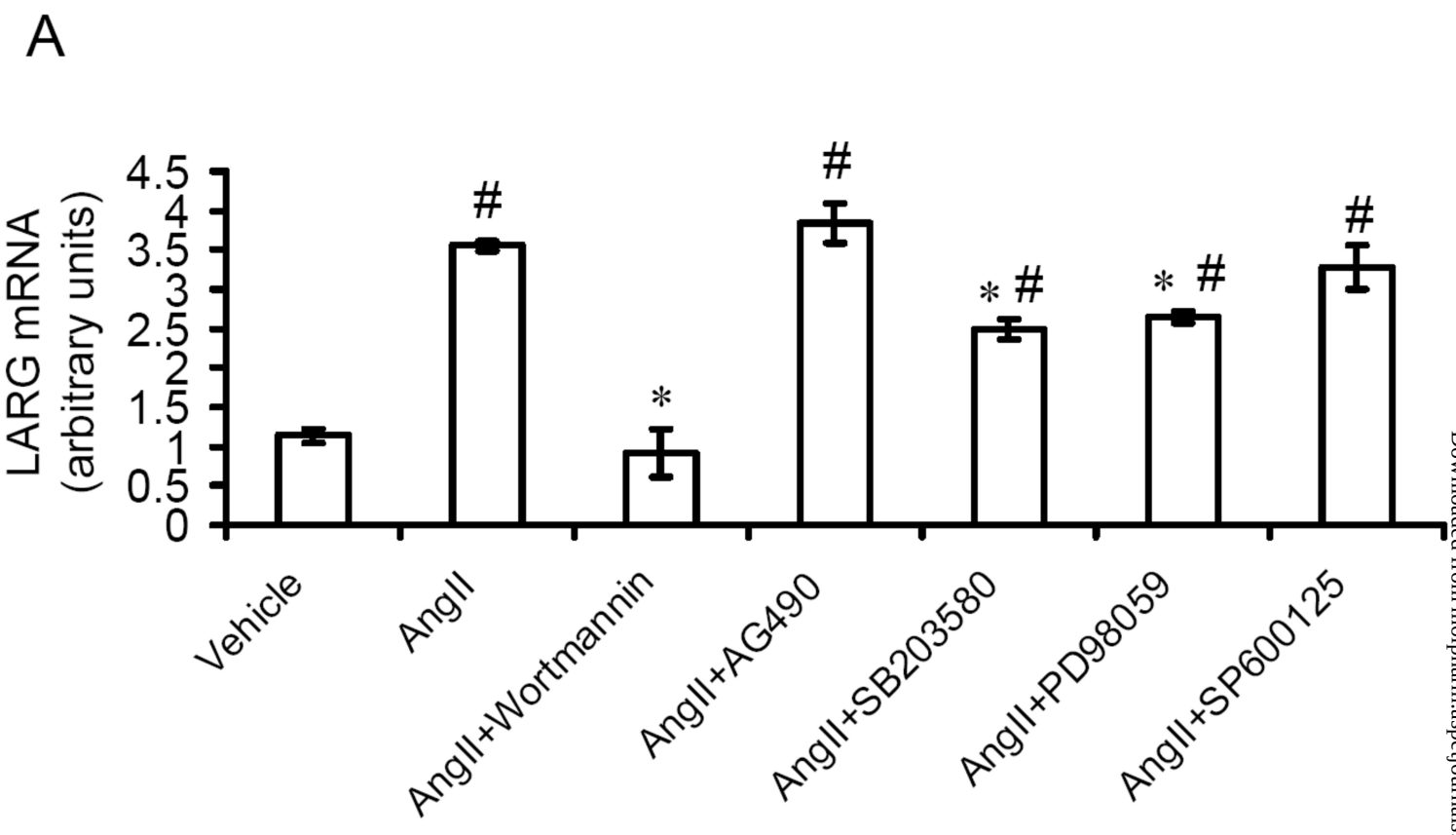


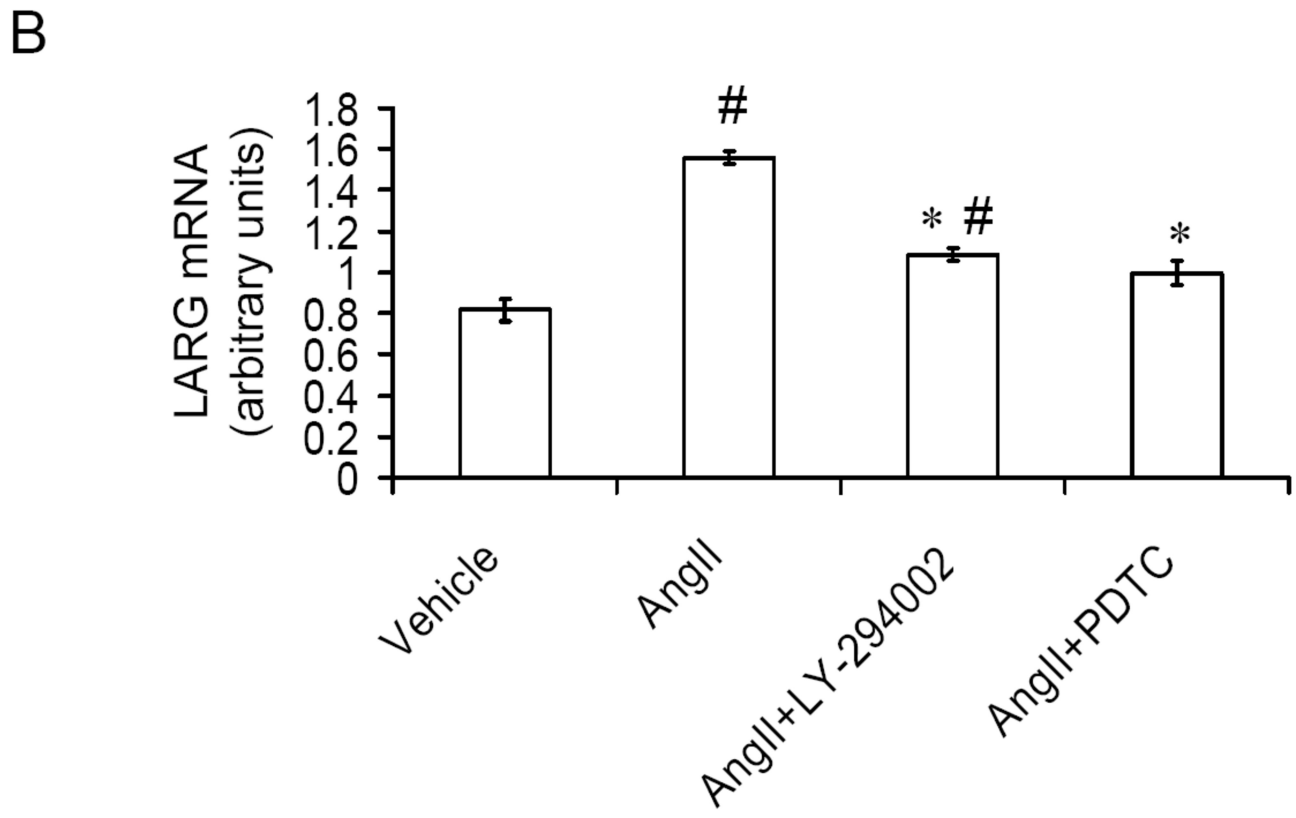

# Figure 5

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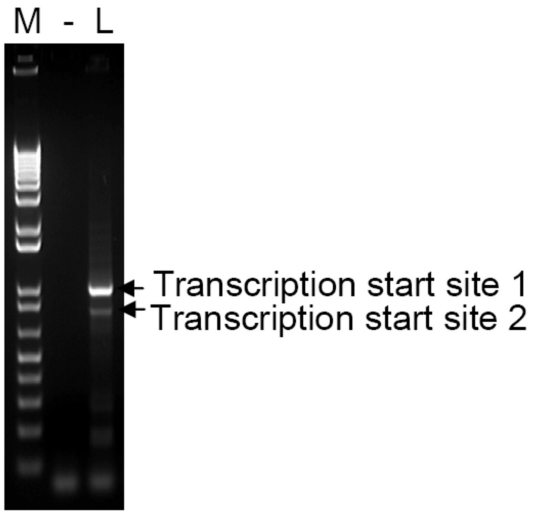
A




B

AP-1
-2495 TGATGAGTCA TCGGACCCCA TTCACTTGAC ACTTGCACTG TGACATTATT AGGACAGGGG GAGGAGGACA

GATA-binding factor 2
HNF-3/Fkh_Homolog-8
-2425 GCACACTCAA GACAACTGGA GGAAGCTTGG GGATAAGGGT TTATTTTGTT TATTGTCTAT AGATATATAT

NF-kappaB
-2355 GGAACCCTTT CCAACCATAG GTTGAAGACA AGTGGAGTCT TCTTCAGGCT AGAATTGCCA TGTTGGGATT

-2285 TACCAGCAGA TGGCAACGTG TCCAGGTCTG ATCATTAGGA AAGTGGCTTG GAACTCCAAT CTGCTTCTAT

-2215 TCTTTCCAAA AATAAACATT ACAAAATTAG CTGGCCGTGC CCCGAACTTA AAAAAAAAAA AAAATCCAGC

-2145 AAGGGAGTCA TTTCAGGCTT CTGTTTGTCT TTCCCTCCTT TTACCCCGTT CTAACGTCAC CTAGAGCTCG

-2075 TGCATCTATT CAGGGATGAT GATCCGATCC TTTTGCAAAC TGTTGTGGAA TGGCTTAGAA ACAGCGTTAT

-2005 TAAGAGGTAA TTGTTTCCTC TCCACACCCA CTGTTTATTA GATTTGATTC CCACAAAATG GCCGAAGACT

Retroviral_TATA_box
-1935 CTTCCCACCT ACACGAATTG AAGCTCAGAA GTCTGATGTA TAAAAGGAAT TAAAGCTCAG AAGTCTGATG

Retroviral_TATA_box
mating_factor_a1
-1865 TATAAAAGGA ATAGCCGCTT GATGTTTACA GTCAGAATTG GAGAATTATA TTGTGTCGTG CTATTTCATT

mating_factor_a1
-1795 TCCTCAGGGA CAGATTTATT CAATCGTAAA ACTACATGGT TAGCTGAATG TCTTTACCCC CATCGAAAAA

-1725 GAAACCGGAA AACATTTGGG AACTCTTCAA AGGGATCCCC AGTGCTATTT ACCGGACAGA TGTATTTCTT

-1655 CTTCCACCTC TTTTCACAAC AAGCTTATTA ACTTGCCGTG GAAAGCACCA TGCTTTCTCA ATTTGGCAAA

Transcript startion site 1
GCN4
AP-1
homeo_domain_factor_Nkx-2.5/Csx,_tinman_homolog
-1585 TAATACAGTT CATTATGCCA AGGCAAAACA GCCTAAAAGC AATATTTATA TCAAGAACTC CTGACTCAAG

-1515 TGTGGGCCCA GATCCTTTCT CTACAGCCTT TTCTGGCTAT CTGCCACCCC CTTCTCCTTT CTGCGGGGCG

complex_of_Lmo2_bound_to_Tal-1,_E2A_proteins,_and_GATA-1,_half-site_1
-1445 AGGCCAGCAG GTGCCCCAGG TGAGTGTCCC ATCCCCAAAC CTGTGTGTGT GTGTGTGTGT GTGTGTGTGT

-1375 GTGTGTGTGT GTGTGTGTGT GTGTGTGCTG GTTTGCCTAA CACTGTCGGG GCCGGTGGAC TTGGACTTGT

-1305 ATGCAGTCAT CAAAAGCATT TGACACCGGG AACGAATACA TTCTCTTAAA GAATAAAAGT TTCCAGCACA

SP-1
NF-kappaB
-1235 AGAGGGGCGG GGAGTTCCCG AAGACCCTCT AGACGTGAAC TCCTCGAGAT TGTGGAATGG CTCCTCCGCT

maternal_gene_product
-1165 GTCCCGAGTG GAACCTATAC TGTTGACTGC ACCTCGAGAG CATTCAAGGA TCATGAAGGC AGGGGTTGGT

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Transcription start site 2
-265 CTGAGGATCC TTCTTAGTTC TTGAGCTCGC CTTGTGGCCG ACTTGCTCGG TTTGTTTGGG GAGATAACTT

-195 GTTTTGCTTC AACCCGCATC CCCTTTCCTT GACCCTTTGC AATCGGATGT TCTAGATGAC TGAATGGAGT

-125 TTTGAGTTGG ACTTTTGTGT CCCTGCCGAA ATTGGGCCTG ATCCCAGAGT ACTGGGGGTG GGGTGTGGAG

+1
-55 GTGTTACTGT AAAATGCAAG TTGGATAAAA AGAAGACCTC TCGCCAAGGG CCCCAATGAG TGGCACACAG

Figure 6

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