

Inhibition of angiotensin II-induced contraction of human airway smooth muscle cells by angiotensin-(1-7) via downregulation of the RhoA/ROCK2 signaling pathway

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Abstract. Sustained renin-angiotensin system (RAS) activation in asthmatic patients plays a crucial role in airway hyperresponsiveness and airflow limitation. Angiotensin II (Ang II), as a key peptide of RAS, contributes to the contraction of human airway smooth muscle by activating the RhoA/Rho-associated coiled-coil containing protein kinase 2 (ROCK2) signaling pathway. Angiotensin-(1-7) [Ang-(1-7)], is a component of the angiotensin I converting enzyme 2 (ACE2)-Ang-(1-7)-Mas axis which counteracts the detrimental effects of the ACE-Ang II-angiotensin type 1 receptor (AT1R) axis *in vivo*; however, whether Ang-(1-7) can inhibit the effect of Ang II in the contraction of human airway smooth muscle cells (HASMCS) is unknown. In our study, collagen gel lattices and immunofluorescence were used to evaluate the contraction of HASMCS induced by Ang II. Real-time PCR and western blot analysis were performed to confirm the regulatory mechanism and the participating signaling pathway. Ang II caused the contraction of HASMCS; this effect was reversed by Ang-(1-7). In addition, irbesartan and A779, which are inhibitors of AT1R and Mas, respectively, attenuated the effect of Ang II and Ang-(1-7). Furthermore, Y-27632, an inhibitor of ROCK2, attenuated the Ang II-induced contraction of HASMCS by blocking the RhoA/ROCK2 signaling pathway which is involved in this contraction, and thus may be a major regulator involved in the basal maintenance of contractility in HASMCS. These data demonstrate that Ang II induces the contraction of HASMCS and that this effect can be reversed by Ang-(1-7), partially through the downregulation of the RhoA/ROCK2 signaling pathway.

Introduction

Airway narrowing is the final common pathway leading to symptoms and physiological changes in asthma. Airway smooth muscle contraction in response to multiple bronchoconstrictor mediators and neurotransmitters is the predominant mechanism of airway narrowing. The lung has the capacity to locally generate angiotensin II (Ang II) (1,2). Under the pathological conditions of asthma, with antigen sensitization and challenge, the airway renin-angiotensin system (RAS) is triggered, thereby elevating the Ang II concentration, which then contributes to bronchoconstriction and bronchial hyperactivity (3,4). New constituents of RAS, angiotensin-(1-7) [Ang-(1-7)] and its main inducer, angiotensin I converting enzyme 2 (ACE2), have been discovered. As opposed to Ang II, Ang-(1-7) has been shown to suppress heart or vascular muscle remodeling, proliferation, migration, inflammation and fibrosis (5-7). Ang-(1-7) acting via its receptor, Mas (8,9), is considered a protective factor, with beneficial effects as opposed to the Ang II-angiotensin type 1 receptor (AT1R), particularly in acute lung injury induced by severe acute respiratory syndrome (SARS) (10,11). As previously reported, AT1R expression increased in patients with asthma associated with airway remodeling and dysfunction; however, valsartan, an AT1R antagonist, inhibits AT1R expression and partially inhibits structural airway changes in chronic ovalbumin-exposed rats (12).

Ang II activates the small guanosine triphosphatase (GTPase), RhoA, and its downstream effector, Rho-associated coiled-coil containing protein kinase 2 (ROCK2), which plays a main role in the intensity and persistence of vascular smooth muscle cell contraction and vasoconstriction (13,14). According to recent research, Ang II acts via AT1R in vascular smooth muscle cells (15,16), hepatic stellate cells (17) and airway smooth muscle cells (18,19). The RhoA/ROCK2 signaling pathway also plays a crucial role in biological functions, including contraction, migration and immunoregulation. Rho kinase activation is associated with the maintenance of the contractile phenotype of human airway smooth muscle cells (HASMCS) (18).

In the present study, we investigated the possible effects of Ang-(1-7) on HASMC contraction. We hypothesized that

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Ang-(1-7) may inhibit Ang II-induced airway smooth muscle cell contraction via the Mas receptor. We further hypothesized that Ang-(1-7) treatment may suppress the Ang II-induced activation of the RhoA/ROCK signaling pathway, a possible mechanism for its inhibitory effect on smooth muscle cell contraction.

Materials and methods

Ethics statement. All experimental procedures and protocols in this study were approved by the Ethics Committee of Nanfang Hospital, Southern Medical University (NFEC-201109-K1). Prior to the experiments, the patients were informed of the objectives and provided written informed consent to participate in the study.

Cell isolation and culture. HASMCs were isolated from the lobar or main bronchus obtained from lung resection donors, approved by the Division of Thoracic Surgery. The cells were maintained as primary culture in Dulbecco's modified Eagle's medium (DMEM; Hyclone) with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin and 100 U/ml streptomycin. The morphology and phenotype of the cells was identified, with a purity of $\geq 95\%$. Cells from passages 3-8 were grown to confluence and were harvested by trypsin digestion and used for the experiments. To evaluate whether Ang II affects HASMCs in a time-dependent manner, Ang II was used to stimulate the cells for 5, 15, 30 or 60 min, at a concentration of 10^{-7} M under serum-free conditions. In all other experiments, 10^{-7} mol/l Ang II with or without Ang-(1-7) was added to the cell cultures followed by incubation for 15 min. Y-27632 (a ROCK-2 inhibitor) and irbesartan (IRB, an AT1R inhibitor) were used at a concentration of 10^{-5} M, 0.5 h prior to the addition of Ang II (10^{-7} M).

Gel contraction assay. HASMCs were treated with IRB (10^{-5} M) or Y-27632 (10^{-5} M) for 30 min, then Ang II (10^{-7} M) with or without Ang-(1-7) (10^{-7} M) was added to the culture medium and the cells were then added to the collagen suspension. Buffer without Ang II was used as the control. Type 1 rat-tail collagen suspension (5 mg/ml; Shengyou Biotechnology Co., Ltd.) was prepared according to the manufacturer's instructions. The final collagen suspension (1 mg/ml) containing the HASMCs in 0.76 ml (1×10^5 cells) with or without pre-treatment, was cast in 35-mm culture plates and allowed to polymerize (20 min, 37°C). Once polymerized, the gels were carefully detached from the culture plates and filled with serum-free medium. The gels were equilibrated overnight after detachment to avoid the initial contraction. The surface area of the collagen gels was measured using ImageJ analysis software. All experiments were performed in triplicate. The relative maximum was expressed by the formula: [(gel surface area of control - gel surface area of test substance)/gel surface area of control] $\times 100\%$.

Immunofluorescence. HASMCs were cultured on slides at a density of 5×10^4 cells/cm² and incubated for 24 h to permit cell attachment. Under serum-free conditions, the cells were treated with various reagents as described in the section 'Cell isolation and culture'. Subsequently, the cells were

fixed with 3.7% paraformaldehyde for 10 min. After washing with phosphate-buffered saline (PBS), the cells were treated with 0.1% Triton X-100 for 5 min to permeabilize them. To visualize F-actin stress fibers, the cells were stained with 2 $\mu\text{g/ml}$ of TRITC-conjugated phalloidin (Sigma) for 20 min at room temperature and then restained with 4'-6-diamidino-2-phenylindole (DAPI, BioTime) for the visualization of the nuclei. After mounting, images were obtained using an inverted fluorescence microscope (Olympus-FL 500; Tokyo, Japan).

Real-time PCR. Total RNA was isolated from the HASMCs by using the RNAiso Plus kit. The cDNA was generated by the PrimeScript[®] RT reagent kit (from Takara Biotechnology Co., Ltd.). In order to analyze messenger RNA (mRNA), we synthesized the template cDNA for the subsequent PCR analyses. Real-time PCR analysis of the rat genes was performed by the use of SYBR-Green-based assays with the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using SYBR[®] Premix Ex Taq[™] II (from Takara Biotechnology Co., Ltd.). The human RhoGEF (NM-198997), RhoAGTP (NM-001664.2) and ROCK2 (NM-021804.2) primers were designed by Primer 5.0 software (Table I). The primers were used at a concentration of 0.4 μM in each reaction. Cycling conditions were as follows: step 1, 30 sec at 95°C ; step 2, 5 sec at 95°C and 34 sec at 60°C ; step 3, 15 sec at 95°C , 1 min at 60°C and 15 sec at 95°C , with repetition of step 2 for 35 times. Data from the reaction were collected and analyzed by the 7500 software v2.0.4, using a standard curve. The relative quantification of gene expression was normalized to GAPDH.

Western blot analysis. Total protein was extracted using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotech, Haimen, China), containing protease inhibitors (Roche, Basel, Switzerland) or phosphatase inhibitors (Sigma). Protein samples (20 μg) were heated at 100°C for 7 min before loading and separation on 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Subsequently, the membranes were blocked with 5% bovine serum albumin in TBST buffer (20 mM Tris, 500 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. The membranes were then incubated at 4°C overnight with various primary antibodies at a 1:1,000 dilution in 5% bovine serum albumin buffer. The following primary antibodies were used: rabbit anti-phosphorylated myosin light chain (P-MLC), rabbit anti-phospho-moesin, rabbit anti-MLC and rabbit anti-moesin (all from Cell Signaling Technology, Danvers, MA, USA). To visualize the positive protein bands, horseradish peroxidase-linked secondary antibodies and the ECL system (Thermo, USA) were used. The density of the individual bands developed on radiographic film was then quantified using a densitometric scanner with Gel-Pro Analyzer 4 software.

Statistical analyses. All data are presented as the means \pm SEM, based on experiments repeated in triplicate, unless otherwise specified. Multiple comparisons were analyzed using one-way analysis of variance (ANOVA) with the Statistical Package for

Table I. Primers used in the real-time PCR analyses.

Gene name	Accession no.	Primers	Size (bp)
ARHGEF1	NM_198997.1	5'-AACCAAGCCGTGCGTGAC-3' 5'-TGAAGCTCGCTCAGCATAGGG-3'	109
RhoAGTP	NM_001664.2	5'-AGATATGGCAAACAGGATT-3' 5'-TTTCACAAGACAAGGCAC-3'	150
ROCK2	NM_021804.2	5'-TATGCGGATTCACCTTGTAG-3' 5'-AGTCGTACCTCCCTATCTG-3'	137

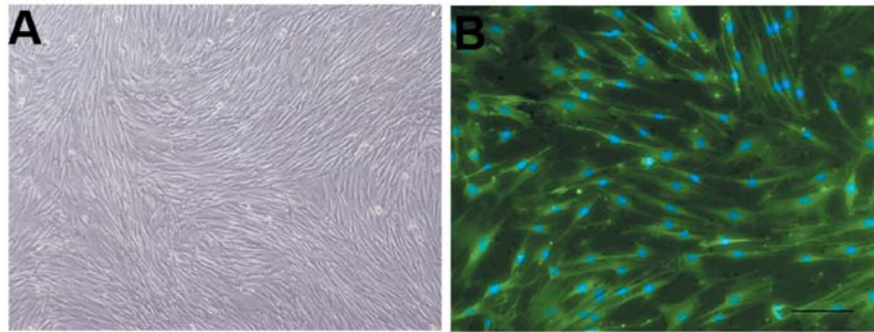


Figure 1. Primarily cultured HASMCs were identified by their typical morphology and phenotype. (A) The confluent cells displayed the typical 'hill and valley' appearance. (B) HASMCs immunostained with anti- α -actin antibody revealed the positive expression of α -actin. (scale bar, 200 μ m).

Social Sciences (SPSS) 13.0 (Statistical Software for Social Sciences, Chicago, IL). Differences were considered to be statistically significant when $P < 0.05$.

Results

Cell culture. Primary cultured HASMCs displayed the typical 'hill and valley' appearance when confluent under an inverted light microscope. HASMCs were initially characterized by positive immunostaining for α -smooth muscle actin (α -SMA). There they were identified by their typical morphology and phenotype, degree of purity $\geq 95\%$ (Fig. 1).

Ang II induction of collagen gel contraction. In order to validate whether Ang II induces the contraction of HASMCs embedded in collagen gel, a hydrated collagen gel assay was utilized. Fig. 2 displays a representative experiment with collagen lattice contraction. As shown in Fig. 2 (upper panel), the control group exposed to gel buffer only, demonstrated a minimal decrease in the collagen surface area. Compared with the control cells, Ang II at 10^{-7} M caused an obvious decrease in surface gel area. The cells co-treated with Ang II and Ang-(1-7) demonstrated a slight decrease in the collagen surface area compared with Ang II, and A779 reversed the effect of Ang-(1-7) by blocking the Mas receptor. The addition of the inhibitors, Y-27632 and IRB, to the HASMCs for 0.5 h prior to a 60-min exposure to Ang II, resulted in significant loss of gel contraction compared with Ang II alone (Fig. 2, upper panel). The quantification of percentage gel contraction is shown in Fig. 2 (bottom panel).

Ang II induction of increased actin stress fiber formation. Primary cultured HASMCs developed a fusiform shape, with some quantity and a regular arrangement of actin stress fibers. The Fig. 3 showed cytoskeletal reorganizations triggered by Ang II (10^{-7} M, 60 min) compared with the other groups in sparsely cultivated HASMCs typically stained with fluorophore-conjugated phalloidin with increased mass and stress fibers. Within 30 min of co-treatment with Ang II and Ang-(1-7), staining of the F-actin stress fibers showed a significant decrease in stress fibers in the HASMCs, compared with treatment with Ang II alone. A779 blocked the effect of Ang-(1-7), and the Ang II-induced increase in actin stress fiber formation was partially reversed by treatment with Y-27632 or IRB (Fig. 3).

Ang II activates the RhoA/ROCK-2 signaling pathway. Ang II induces various pathological effects through AT1R, such as contraction, migration, cell growth or hypertrophy, via different signaling pathways (20). However, the mechanisms behind the effect of Ang II on HASMCs and the role of the RhoA/ROCK2 signaling pathway remain unknown. Therefore, we performed real-time PCR in order to evaluate the expression of the genes, ARHGEF1, RhoAGTP and ROCK2, which are key molecules of the RhoA/ROCK2 signaling pathway. Following treatment with Ang II, the expression of ARHGEF1, RhoAGTP and ROCK2 in the HASMCs increased, indicating the activation of the RhoA/ROCK2 pathway (Fig. 4). Ang-(1-7) partially reversed the increase in ARHGEF1, RhoAGTP and ROCK2 expression, similar to IRB and Y-37632.

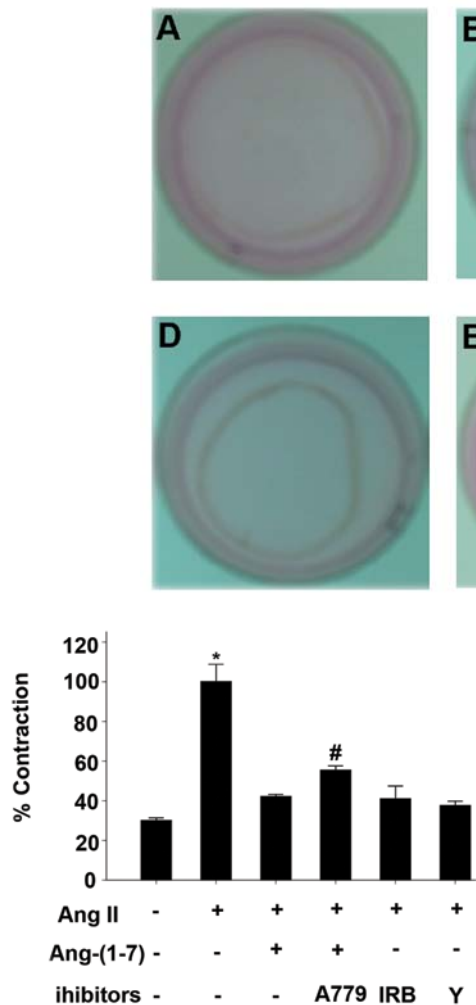


Figure 2. Mean maximal contraction of collagen gels with various stimuli, compared with the initial surface area. Upper panel: representative images of gel contraction in response to various stimuli. Lower panel: After incubation with buffer, the cells were treated with 10^{-7} M Ang II and Ang-(1-7) with or without pre-treatment with 10^{-5} M A779, 10^{-5} M irbesartan (IRB) or 10^{-5} M Y-27632 (Y, a ROCK-2 inhibitor) followed treatment with 10^{-7} M Ang II for 60 min. A, Control; B, Ang II; C, Ang II + Ang-(1-7); D, Ang II + Ang-(1-7) + A779; E, Ang II + IRB; F, Ang II + Y-27632 (* $P < 0.05$ vs. the other groups; # $P < 0.05$ vs. the other groups).

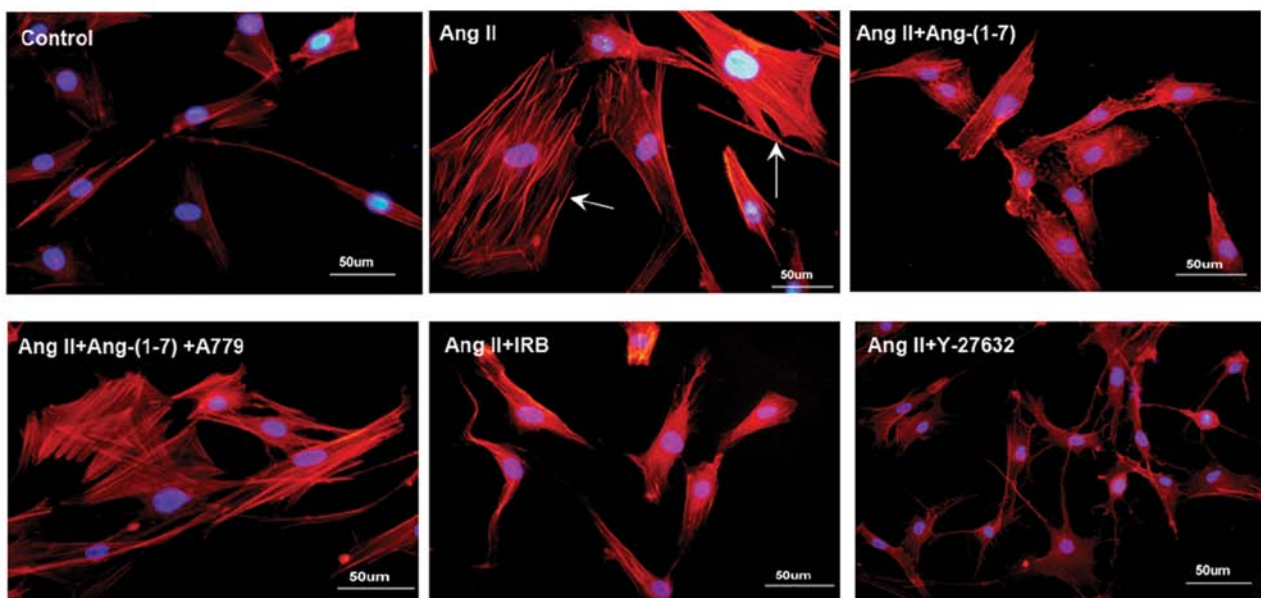


Figure 3. Cytoskeletal reorganizations triggered by the Ang II treatment (10^{-7} M, 60 min) of HASMCs. The staining HASMCs with TRITC-phalloidin showed cytoskeletal changes in the corresponding group F-actin (red) and DAPI (blue). After a 60-min exposure to 10^{-7} M Ang II, the cells had increased numbers of stress fibers and developed fiber-positive projections. Following costimulation with Ang II and Ang-(1-7), there were fewer stress fibers. Furthermore, the cells were pre-incubated for 30 min with A779, an inhibitor of Mas, irbesartan (IRB), or Y-27632 (Y) at a concentration of 10^{-5} M and then treated with Ang II or co-treated with Ang II and Ang-(1-7) for 60 min. The Ang II-induced increase in stress fiber formation was partially inhibited by Ang-(1-7), IRB and Y-27632 (Y). In addition, the effect of Ang-(1-7) was partially reversed by A779. All scale bars, 50 μ m.

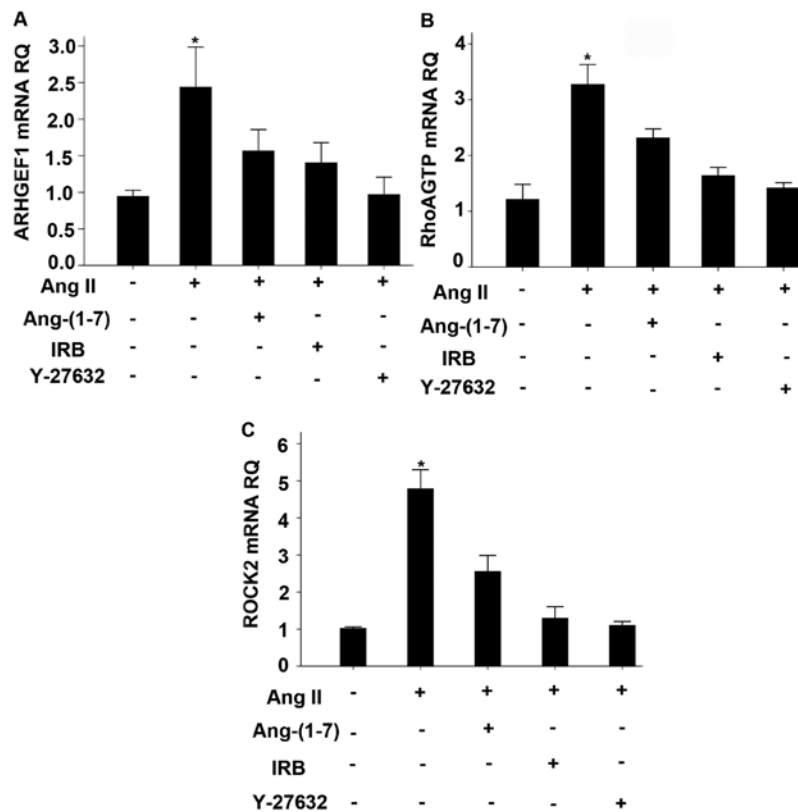


Figure 4. The RhoA/ROCK2 signaling pathway participates in the HASMC contraction induced by Ang II. Following exposure to 10^{-7} M Ang II for 30 min, the relative amounts of the genes, (A) ARHGEF1, (B) RhoAGTP and (C) ROCK2 increased significantly. After costimulation with Ang II and Ang-(1-7), or pre-incubation with irebesartan (IRB) and Y-27632 (Y), the relative gene expression (RQ) was attenuated significantly ($P < 0.05$ vs. the other groups).

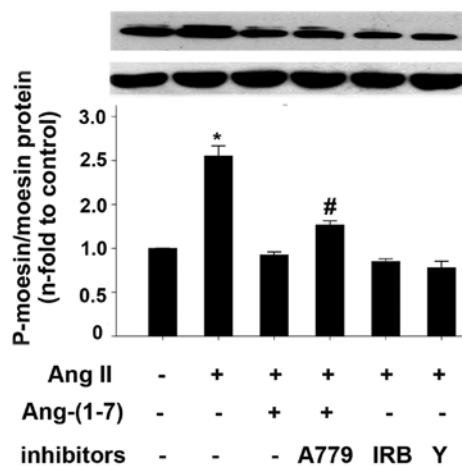


Figure 5. Representative immunoblotting and quantitative analysis of P-moesin and moesin protein expression in HASMCs. HASMCs were stimulated with Ang II with or without Ang-(1-7) and the inhibitors (Ang II and Ang-(1-7), 10^{-7} M, for 30 min); HASMCs were pre-treated with A779, irebesartan (IRB) and Y-27632 (Y), for 30 min at a concentration of 10^{-5} M ($P < 0.05$ vs. the other groups; # $P < 0.05$ vs. the other groups).

Ang II enhances the level of phosphorylated moesin (P-moesin) and P-MLC. In order to elucidate the mechanism by which Ang II induces HASMC contraction, we then determined how the Ang II-treated HASMCs respond to Ang-(1-7), IRB and Y-27632 co-treatment compared to the untreated HASMCs. Once the RhoA/ROCK pathway was activated, increasing

RhoAGTP levels activated the ROCK2 synthesis, which was reflected by the phosphorylation of moesin, as shown by western blot analysis in Fig. 5. Furthermore, moesin [a member of the ezrin/radixin/moesin (ERM) family of proteins], a substrate of ROCK2, provides a crucial link with the F-actin cytoskeleton and membrane proteins at the cell periphery (21). The P-moesin protein was assayed to elucidate how ERM proteins affect the cell cytoskeleton and contraction function.

Furthermore, to evaluate the effect of Ang II on MLC expression, we exposed the HASMCs to Ang II for different amounts of time, as described in Materials and methods, then measured P-MLC protein expression by western blot analyses. Fig. 6 shows the optical time of the Ang II stimulation of HASMCs, which is in accordance with a previous study (22). The Ang II stimulation of HASMCs occurred in a time-dependent manner. As shown, Ang II induced an increase in P-MLC expression as early as 5 min and this increase peaked at 15 min.

One of the major consequences of the activation of the RhoA/ROCK2 pathway is the phosphorylation of MLC, which is critically involved in the contraction of HASMCs. As demonstrated in Fig. 7, the exposure of the activated HASMCs to Ang II led to increased amounts of P-MLC. These effects were significantly inhibited by co-treatment with Ang-(1-7), IRB and Y-27632. Similarly, pre-treatment with A779 attenuated the inhibitory effect of Ang-(1-7) in response to Ang II stimulation. The above results suggest that Ang-(1-7) negatively regulates the contractile response of HASMCs to Ang II via the inhibition of the RhoA/ROCK2 pathway.

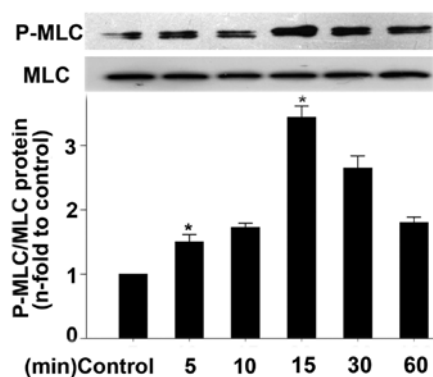


Figure 6. Time-dependence of Ang II-triggered P-MLC expression. HASMCs were treated with 10^{-7} M Ang II for different periods of time and then lysed. P-MLC and MLC expression was assessed by western blot analysis and quantified as described in Materials and methods.

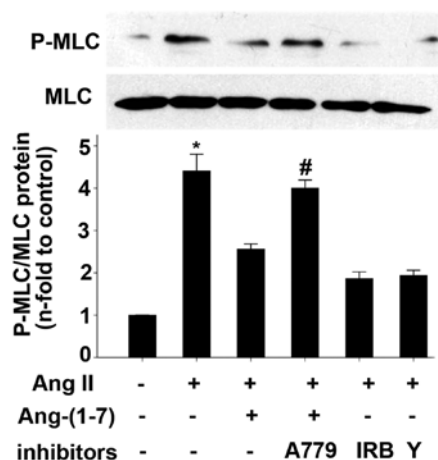


Figure 7. P-MLC/MLC expression in HASMCs treated with various stimuli. Ang II (10^{-7} M), with or without Ang-(1-7), was added to the cell cultures and the cells were incubated for 30 min. Y-27632 (Y), irbesartan (IRB) and A779 were used at a concentration of 10^{-5} M, 0.5 h prior to the addition of Ang II (10^{-7} M). * $P < 0.05$ vs. the other groups; # $P < 0.05$ vs. the other groups.

Discussion

The results from the present study demonstrated that Ang II (10^{-7} M) caused the significant contraction of primary cultured HASMCs in embedded in collagen gels. This effect was inhibited by IRB and Y-27632. Ang-(1-7) suppressed the contraction of HASMCs caused by Ang II and the inhibitory effects of Ang-(1-7) were partially reversed by the Ang-(1-7) receptor antagonist, A799. The difference in contraction among the groups was confirmed by immunofluorescence. Ang II, a key peptide fragment of RAS, was originally described as an important regulator of blood pressure and electrolytic balance, while Ang-(1-7) has demonstrated cardiovascular protection. Ang II is considered to induce the contraction of airway smooth muscle and bronchoconstriction directly and potentiates methacholine (Mch)-induced bronchoconstriction in mild asthmatic patients (23). A previous meta-analysis suggested that the insertion/deletion (I/D) polymorphism of the ACE gene, a key gene in Ang II synthesis, is a risk factor for asthma (24). In the current study, we demonstrate the role of

Ang II in HASMC contraction and investigated the potential mechanism involved.

Collagen gel contraction assay is a common method used to examine several constrictors, such as cysteinyl leukotrienes (cysLTD4), and has been used to measure Mch-induced contractile capacity in established human bronchial smooth muscle cells (25,26). It had been used to examine the contractile capacity of HASMCs between asthmatic and non-asthmatic patients (27). In our study, we found out that Ang II as a vasoconstrictor, induced an increase in the contractility of HASMCs. LTD4 and Mch had a similar effect.

Ang II and Ang-(1-7) are considered as the 'devil and angel' in RAS (6), one counteracting the effects of the other. Ang-(1-7) plays a protective role through the Mas receptor by inhibiting AT1R. However, it is not clear which intracellular signaling pathway is involved. Several factors, such as extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, ERK Akt phosphorylation and mitogen-activated protein (MAP) kinase inhibition seem to be involved in this signaling pathway (28-30). A previous study indicated that the chronic hypotensive effects of losartan in normal rats were mediated in part through the action of Ang-(1-7) (31). Another study showed that the Mas receptor is essential in mediating the endothelium-dependent relaxation response induced by perivascular adipose tissue, thus highlighting the important role of Ang-(1-7) (32). In the lung, Ang-(1-7) inhibits the apoptosis of alveolar epithelial cells (AECs) through Mas and ACE-2 regulates the AEC survival by balancing pro-apoptotic Ang II and its anti-apoptotic degradation product, Ang-(1-7) (33). Further studies are required to clarify the roles of Ang-(1-7) in vasodilatation, natriuresis, anti-proliferation and the increase in the bradykinin-nitric oxide (NO) system, counteracting the effects of Ang II. Therefore, we investigated whether Ang-(1-7) has an inhibitory effect on the Ang II-induced contraction of HASMCs.

In our study, the receptor inhibitors, IRB and Mas, were used to elucidate the potential mechanism of cell contraction, stress fiber formation and corresponding protein changes. Our results confirmed that the contraction of HASMCs in response to Ang II is mediated through the well-characterized RhoA/ROCK2 signal pathway.

As previously reported, in vascular cells, endogenous ACE2 counteracts the Ang II-mediated cellular response partly by the upregulation of Ang-(1-7) through Mas (30). However, in our study, we proved that Ang-(1-7) inhibits Ang II-mediated cellular contraction via the downregulation of the RhoA/ROCK2 signaling pathway. ROCK2, a cytosolic small GTPase Rho kinase, plays a predominant role in vascular smooth muscle cell contractility (14). The activation of RAS also leads to the phosphorylation of moesin downstream of Rho and MLC. Moreover, previous studies have proven that Ang II activates the RhoA/ROCK-2 signaling pathway in vascular smooth muscle cells (34,35).

However, to date, no information is available on the mechanisms causing the contraction induced by Ang II in HASMCs at the cellular level. In our study, the inhibition of the RhoA/ROCK2 signaling pathway was clearly demonstrated by the inhibition of the contraction of HASMCs in the group pre-treated with Y-27632, as the phosphorylation of MLC downstream of Rho was decreased significantly.

From the above results, we hypothesized that Ang-(1-7) may weaken the effects of Ang II by downregulating the RhoA/ROCK2 signaling pathway. The activation of key molecules, including RhoGEFs, RhoAGTP and ROCK2, was associated with the contraction of HASMCs and cytoskeletal rearrangement. Y-27632, as a ROCK2 inhibitor, demonstrated a significant inhibitory effect on the increased contraction by inhibiting the increase in stress fibers. This correlated with the dephosphorylation of moesin. Traction forces generated by the increase in stress fibers induced by the addition of Ang II leads to a significant increase in cell contractility (36,37).

In conclusion, our study reveals a function of RAS in regulating airway narrowing through its induction of intense HASMC contraction. Further studies are required to better understand the underlying mechanism; however, the RhoA/ROCK2 signaling pathway plays a key role in the Ang II/Ang-(1-7)-mediated regulation of HASMC contraction.

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