Angiotensin II Induces an Increase in Matrix Metalloproteinase 2 Expression in Aortic Smooth Muscle Cells of Ascending Thoracic Aortic Aneurysms Through JNK, ERK1/2, and p38 MAPK Activation

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Abstract: In this study, we hypothesized that angiotensin II (Ang II) induces matrix metalloproteinase 2 (MMP-2) upregulation in aneurysmal smooth muscle cells (ASMCs) derived from ascending thoracic aortic aneurysms (ATAAs). We compared MMP-2 protein levels in ascending aortic specimens using Western blot and plasma concentrations by enzyme-linked immunosorbent assay between ATAA (n = 40) and coronary heart disease patients (n = 40). Additionally, the protein level of angiotensinogen (AGT) in the ascending aorta and the plasma concentration of Ang II were detected by Western blot and radioimmunoassay, respectively, in ATAA and coronary heart disease patients. In ATAA patients, Ang II and MMP-2 plasma levels were significantly increased ($P < 0.05$). Additionally, AGT and MMP-2 protein levels in the aorta of ATAA patients were higher ($P < 0.01$). Enhanced AGT suggested that the amount of Ang II in aneurysmal aorta specimens may be also increased, which was confirmed by immunofluorescent staining for Ang II. Moreover, we investigated the effect of Ang II on MMP-2 upregulation by ASMCs and determined the Ang II receptors and intracellular signaling pathways that are involved. Our results showed that treatment with Ang II significantly increased the expression of MMP-2 through the Ang II type 1 receptor (AT1R) and activated the 3 major mitogen-activated protein kinases (MAPKs), JNK, ERK1/2, and p38 MAPK. In conclusion, these results indicate that Ang II can induce MMP-2 expression elevation through AT1R and MAPK pathways in ASMCs and suggest that there is therapeutic potential for angiotensin receptor blocker drugs and MAPK inhibitors in the prevention and treatment of ATAAs.

Key Words: angiotensin II, aortic aneurysm, thoracic, matrix metalloproteinase 2, p38 MAPK, ERK1/2, JNK

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INTRODUCTION

Ascending thoracic aortic aneurysm (ATAA) is a severe life-threatening disease characterized by progressive dilatation and rupture of the ascending thoracic aorta.[1] Once ATAAs rupture, mortality is exceedingly high, approximately 94%–100%.[2] The diagnosis of ATAA has increased because of improvement in imaging. As there are no validated medical therapies for ATAAs, treatment is confined to surgical repair. Thus, there is an urgent need to determine the pathogenic mechanisms of this disease for development of effective pharmacological therapies.

Several studies have demonstrated that matrix metalloproteinase expression, particularly matrix metalloproteinase 2 (MMP-2), was significantly elevated in the walls of ATAAs.[3–6] Furthermore, enhanced MMP-2 expression was mainly produced by aortic smooth muscle cells (SMCs) derived from the tunica media of aortic aneurysms.[7,8] Increased MMP-2 can degrade components of the extracellular matrix, such as elastin and collagen fibers, leading to loss of the normal structure of the aortic wall and progressive expansion of the aortic lumen, which is thought to be an important event in ATAA formation.[9,10] However, the possible cellular and molecular mechanisms of MMP-2 upregulation in ATAAs were unknown; additionally, whether the MMP-2 expression increases in ATAAs remains controversial. Although most previous studies have indicated that MMP-2 expression increases in aortic aneurysms,[3–6] some studies have reported that MMP-2 expression levels in ATAAs are not significantly different from those observed in normal aorta specimens.[11,12]

Angiotensin II (Ang II) is a multifunctional octapeptide with diverse actions that modulates vasomotor tone, cell migration, cell growth, apoptosis, and extracellular matrix deposition.[13] Recently, accumulating evidence has demonstrated that Ang II is strongly associated with the formation

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and progression of thoracic aortic aneurysms in humans and mouse models.[14–17] For example, Nishimoto et al[18] demonstrated that Ang II–forming enzymes, including chymase and angiotensin-converting enzyme (ACE), were significantly increased in abdominal aortic aneurysms. This finding suggests that the increase in aorta Ang II formation through activation of ACE and chymase may play a crucial role in developing abdominal aortic aneurysmal lesions. Nagashima et al[19] showed that Ang II concentration was significantly higher in Marfan syndrome aortas than in control patients without aortic diseases. Daugherty et al[20] demonstrated that infusion of Ang II led to development of aneurysmal aortas in apolipoprotein E (ApoE)–deficient mice. To date, however, circulating and local Ang II concentrations in nonsyndrome ATAA have not been reported.

It has become evident that Ang II in vitro can stimulate rat vascular smooth muscle cells (VSMCs) and human umbilical vein endothelial cells to produce, secrete, and activate MMP-2.[21–23] Recent studies have indicated that the mitogen-activated protein kinase (MAPK) cascades are involved in Ang II–mediated regulation of MMP-2 protein expression in human retinal pigment epithelium cells,[24] human umbilical vein endothelial cells,[25] and rat aortic SMCs.[26] In addition, Wang et al[23] showed that in vivo and ex vivo Ang II significantly increased MMP-2 expression and activity in rat carotid arteries. However, it is not yet known whether Ang II contributes to MMP-2 production and intracellular signaling pathways involved in aneurysmal smooth muscle cells (ASMCs) derived from ATAAs. Therefore, we hypothesised that Ang II stimulates MMP-2 upregulation by ASMCs through activation of MAPKs cascades. Therefore, in this study, we investigated the following issues: (1) whether Ang II and MMP-2 increase in the plasma and aneurysmal walls of ATAAs; (2) whether Ang II receptors are expressed in ASMCs; (3) whether Ang II stimulates ASMCs to upregulate MMP-2 expression; (4) which Ang II receptors participate in Ang II-induced MMP-2 expression by ASMCs; (5) whether activation of p38 MAPK, extracellular signal-regulated kinase (ERK) 1/2, and c-Jun N-terminal kinase (JNK) mediate Ang II-induced MMP-2 expression upregulation.

MATERIALS AND METHODS

Reagents and Antibodies

The following reagents and antibodies were used in this study: anti-MMP-2 from Bioworld Technology (St. Louis Park, MN), rabbit anti-c-Jun amino kinase terminal kinase (JNK), anti-phospho-JNK1/2, anti-extracellular signal-regulated kinase (ERK)1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38 from Cell Signaling Technology (Beverly, MA); anti-GAPDH from Santa Cruz Biotechnology (Dallas, TX); angiotensinogen (AGT) and angiotensin II antibodies from Abbiotec (San Diego, CA); and anti–smooth muscle α-actin, anti–angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) from Abcam (Cambridge, United Kingdom). Angiotensin II was purchased from Sigma (St. Louis, MO). MAPK inhibitors SB203580, PD98059, and SP600125 and Ang II receptor inhibitors candesartan and PD123319 were purchased from Selleck Chemicals (Houston, TX). Fetal bovine serum, cell culture media, and TRIzol Reagent were purchased from Life Technologies (Grand Island, NY). BCA protein assay reagents were purchased from Pierce (Rockford, IL).

Patients and Collection of Plasma and Aortic Tissues

Aneurysmal ascending aortic specimens and plasma specimens (n = 40) (21 men and 19 women) were obtained at the time of elective operative ascending thoracic aortic aneurysm repair from patients ranging in age from 43 to 74 years at Fuwai Hospital between February 2013 and December 2013. As a control, normal plasma and aortic specimens (n = 40) were obtained from patients with coronary heart diseases (CHD, 24 men and 16 women, 45–75 years) without any diagnosis of aortopathy who underwent coronary artery bypass surgery at Fuwai Hospital between March 2013 and October 2013. In addition, the aorta samples of both ATAA and CHD patients were taken from the anterior wall of the ascending aorta adjacent to the sinotubular ridge. None of the study subjects received angiotensin-converting enzyme inhibitors or angiotensin receptor blockers before surgery. None of the ATAA patients had aortic dissection, inflammatory aortic disease, or known connective tissue disorders, such as Marfan syndrome or Loeys–Dietz syndrome. The blood sample was centrifuged at 1000*g* for 10 minutes at 4°C, and supernatants were stored at −80°C until use. The obtained ATAA walls were immediately placed in liquid nitrogen for RNA and protein extraction. Informed consent was obtained from all study subjects. All of the experimental protocols using human specimens were approved by the Institutional Ethics Committee of Fuwai Hospital of Peking Union Medical College.

Human Aortic Smooth Muscle Cell Culture

Primary culture of ASMCs was initiated by explant outgrowth from ascending aortic aneurysmal specimens obtained from 8 ATAA patients during surgery repair. Because it is very difficult to culture SMCs from ascending aorta of CHD patients, we only cultured normal SMCs using explants outgrowth of aorta obtained from the heart transplant donors without aortopathy. The identity of aortic SMCs was confirmed morphologically and by positive immunostaining for smooth muscle α-actin. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin G, 100 mg/mL streptomycin, and 10% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator. The purity of the human aortic SMCs was greater than 97%, as confirmed by immunocytochemical staining of α-smooth muscle actin (α-SMA, Abcam). All experiments were performed using human SMCs in 2–8 subcultures.

Reverse Transcription Polymerase Chain Reaction

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was quantified using a spectrophotometer

(NanoDrop ND-3300; Thermo Scientific, Waltham, MA) at an absorbance of 260 nm/280 nm. Total RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) using a RevertAid First Strand cDNA Synthesis Kit (K1622 Thermo Scientific, Waltham, MA). The expression of AT1R and AT2R mRNA was assessed by reverse transcription polymerase chain reaction (RT-PCR). Single-stranded cDNA was amplified with 35 cycles of PCR. PCR conditions were 15 seconds of denaturation at 94°C, 20 seconds of annealing at 58°C, and 40 seconds of extension at 72°C. The number of cycles was 35, and the sequences of the oligonucleotide primers used for PCR and the sizes of the predicted PCR products are shown in Table 1. PCR products (10 μL) were electrophoresed on 2.0% agarose, stained with ethidium bromide, and visualized by ultraviolet absorption. Densitometric signals were quantified using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA).

Western Blot Analysis

The aortic SMCs and tissues were collected by centrifugation for protein extraction using RIPA lysis buffer (50 mM TrisHCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1.2% Triton-X-114, 1 mM NaF, 200 mM NaVO4) and 1 tablet per 10 mL of protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA Protein Assay Kit. For Western blotting, 20 μg of total cellular proteins (100 μg of tissue protein) were separated on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Nonspecific binding sites were blocked by incubating the membranes in TBS-0.1% Tween-20 with 5% skimmed milk for 1 hour at room temperature, and the membranes were incubated with primary antibodies (1:1000 dilution for MMP-2, AT1R, AT2R, t-p38, p-p38, t-ERK1/2, p-ERK1/2, t-JNK1/2, p-JNK1/2, GAPDH; 1:500 dilution for AGT) overnight at 4°C and then washed 3 times with TBS-T. The membranes were then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:5000 dilution) for 1 hour at room temperature and washed 3 times with TBS-T. The blots were developed according to the chemiluminescence kit protocol (Millipore, Billerica, MA) and analyzed using Quantity One 4.6.2 software. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize protein loading.

TABLE 1. Primer Sequences and Product Sizes for AT1R and AT2R

Gene	Primer	Sequence (5′ to 3′)	PCR Product Length
AT1R	Forward	GATTGTCCCAAAGCTGGAAG	104 bp
	Reverse	ATCACCACCAAGCTGTTTCC	
AT2R	Forward	TTCCCTTCCATGTTCTGACC	191 bp
	Reverse	AAACACACTGCGGAGCTTCT	

AT1R and AT2R indicate angiotensin II type 1 and type 2 receptors.

Immunofluorescence and Confocal Laser Scanning Microscopy

Ascending aortic preparations from ATAAs patients (n = 5) and CHD group (n = 5) were fixed in 4% paraformaldehyde for 24 hours. Tissue samples were embedded with paraffin, cut into 3 μm sections, mounted on gelatin-coated slides, and then dewaxed and rehydrated with graded alcohols. The slides were microwaved in boiling 0.01 M sodium citrate buffer for 2 minutes, and then blocked in 5% goat serum solution for 20 minutes at room temperature. Next, the slides were incubated with primary antibody for Ang II (1:100 dilutions in PBS) overnight at 4°C. Finally, the slides were incubated with Alexa Fluor 488-conjugated AffiniPure Goat Anti-Rabbit IgG secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) at a 1:300 dilution for 30 minutes at 37°C. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, Jiangsu, China) for 5 minutes. For negative controls, PBS solution was used instead of primary antibody. Slides were mounted with antifading reagent (Beyotime, Jiangsu, China) and examined with a Leica TCS SP8 confocal spectral microscope (Wetzlar, German).

Radioimmunoassay and Enzyme-linked Immunosorbent Assay

Plasma from 40 ATAA and 40 CHD patients was centrifuged at 1000*g* for 15 minutes at 4°C, and supernatants were stored at −80°C until use. Clinical characteristics of all patients participating in this study are shown in Table 2. According to the manufacturer's instructions, the Ang II concentration in the plasma of patients with ATAA and CHD was measured using an RIA kit (North Institute of Biological Technology, Beijing, China); concentrations of MMP-2 in plasma were determined using an enzyme-linked immunosorbent assay test (ELH-MMP-2-001; RayBiotech, Norcross, GA).

TABLE 2. Demographic Characteristics of the Study Groups in Plasma Analysis Study

	ATAAs (n = 40), n (%)	CHD (n = 40), n (%)	*P*
Age, median, (min, max), yrs	54 (43–74)	56 (45–75)	0.433
Male:female	21:19	24:16	0.652
Smoking	24 (60.0)	27 (67.5)	0.642
Hypertension	22 (55.0)	21 (52.5)	1.000
Hyperlipidemia	4 (10.0)	13 (32.5)	0.027
Diabetes mellitus	3 (7.5)	8 (20.0)	0.193
ACEI	0	0	1.000
ARB	0	0	1.000
Beta-blocker	18 (45.0)	19 (47.5)	1.000
Calcium channel blocker	20 (50.0)	15 (37.5)	0.367
Antilipid medication	2 (5.0)	10 (25.0)	0.025
Antihyperglycemic medication	3 (7.5)	7 (17.5)	0.311

ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

All measurements were performed with sufficiently diluted plasma samples, and every sample was measured in duplicate. Only samples with coefficients of variation of 10% or less were used. The mean level of Ang II and MMP-2 was calculated for data analysis. All Ang II concentration values are presented as picograms per milliliter, and all MMP-2 concentration values are presented as nanograms per milliliter.

Statistical Analysis

Data are presented as the mean ± SEMs. Group differences were analyzed by analysis of variance, and individual group differences were tested using the post hoc Fisher's protected least significant difference test. P-values <0.05 were considered statistically significant. All data were analyzed with SPSS 21.0 software.

RESULTS

Patient Characteristics

A total of 80 patients were enrolled in the study. All patients underwent preoperative transthoracic echocardiography. The median diameter of aortic aneurysms was 5.1 cm and ranged from 4.5 to 6.6 cm. No significant differences were observed in age, gender, smoking, hypertension, or diabetes mellitus between patients with ATAA compared with CHD. There was a significant difference in the presence of hypercholesterolemia between the 2 groups (Table 2).

Ang II and MMP-2 Concentrations are Increased in the Plasma of Patients With ATAAs

To elucidate whether Ang II and MMP-2 concentrations in the plasma of ATAA patients increased compared with control plasma, we enrolled 40 ATAA patients and 40 CHD patients as controls to detect plasma levels of Ang II and MMP-2. Demographic characteristics of these patients are described in Table 2. The results showed that Ang II plasma concentrations in the ATAA patients were significantly higher than in CHD patients ($P = 0.021$; Fig. 1A). MMP-2 plasma concentrations in the ATAA patients were also increased compared with CHD patients ($P = 0.016$; Fig. 1E).

AGT, Ang II, and MMP-2 Protein Expression are Elevated in Aneurysmal Wall Specimens of ATAAs

Next, we examined AGT and MMP-2 protein expression levels in aneurysmal and control aorta walls by Western blot. The results demonstrated that local AGT protein expression in aneurysmal walls was increased by approximately 100% compared with the CHD group ($P < 0.001$; Fig. 1B). This finding indicates that the amount of Ang II in the ascending aorta of ATAAs may be augmented. Furthermore, we confirmed that Ang II amounts in ATAA tunica media of aorta of ATAA were higher than that in CHD using immunofluorescent staining (Figs. 1C1–C3, D1–D3). In addition, the expression level of MMP-2 protein in the aneurysmal walls of ATAA patients was significantly higher than in the CHD group ($P = 0.003$; Fig. 1F).

Ang II Type 1 and 2 Receptors are Expressed in ATAA-Derived SMCs

Given that aortic SMCs express AT1R and AT2R, we determined AT1R and AT2R expression in aneurysm-derived SMCs by RT-PCR. RT-PCR (Figs. 2A–C) identified the presence of both AT1 and AT2 receptors in ASMCs.

Ang II Induces MMP-2 Protein Expression in ASMCs in a Dose- and Time-Dependant Manner

To elucidate the effect of Ang II on MMP-2 protein expression in ASMCs, we incubated different Ang II concentrations with ASMCs for different amounts of time. The quantification of MMP-2 protein induced by different Ang II concentrations was performed at 48 hours by Western blotting (Fig. 3A). Incubation with 0.01 μM Ang II significantly elevated the MMP-2 protein level compared with the group without Ang II stimulation ($P = 0.014$; Fig. 3A), and there were greater increases in MMP-2 protein with 1 and 10 μM Ang II (Fig. 3A; $P = 0.008$ and $P = 0.004$, respectively). Stimulation of ASMCs with 0.1 μM Ang II also caused a significant time-dependent increase in MMP-2 protein. Incubation with 0.1 μM Ang II for 18 hours elevated the MMP-2 protein level ($P = 0.062$; Fig. 3B), and greater increases in MMP-2 protein levels were observed with 0.1 μM Ang II for 24 hours and 48 hours (Fig. 3B; $P = 0.002$ and $P = 0.001$, respectively). These results demonstrate that Ang II upregulates MMP-2 protein expression in a dose- and time-dependant manner in cultured ASMCs. Similar results were also obtained when normal SMCs were incubated with Ang II at different doses for different amounts of time (data not shown).

Ang II Induces Upregulation of MMP-2 Expression and ERK1/2 Phosphorylation Through AT1R

Because ASMCs both express AT1R and AT2R, we examined which receptor was involved in Ang II–induced MMP-2 upregulation. ASMCs were incubated with the AT1R antagonist candesartan (0.1 μM for 60 minutes) and AT2R antagonist PD123319 (10 μM for 60 minutes) followed by 0.1 μM Ang II for 48 hours. Upregulation of MMP-2 protein expression was completely inhibited by candesartan at 0.1 μM ($P = 0.004$; Fig. 4A). In contrast, PD123319 had no effect on Ang II–induced MMP-2 protein expression at 10 μM ($P = 0.795$; Fig. 4B). In addition, ASMCs were pretreated with candesartan (0.1 μM for 30 minutes) and PD123319 (10 μM for 30 minutes) and subsequently stimulated with Ang II (0.1 μM for 5 minutes). Phosphorylation of ERK1/2 was completely inhibited by candesartan at 0.1 μM ($P = 0.031$; Fig. 4C). In contrast, PD123319 had no effect on Ang II–induced ERK1/2 phosphorylation at 10 μM ($P = 0.334$; Fig. 4D). Additionally, both candesartan and PD123319 had no effect on increased expression of MMP-2 in the absence of Ang II stimulation ($P = 0.629$ and $P = 0.834$; Figs. 4A, B). These results indicated that Ang II induced MMP-2 expression upregulation and ERK1/2 phosphorylation through AT1R in ASMCs.

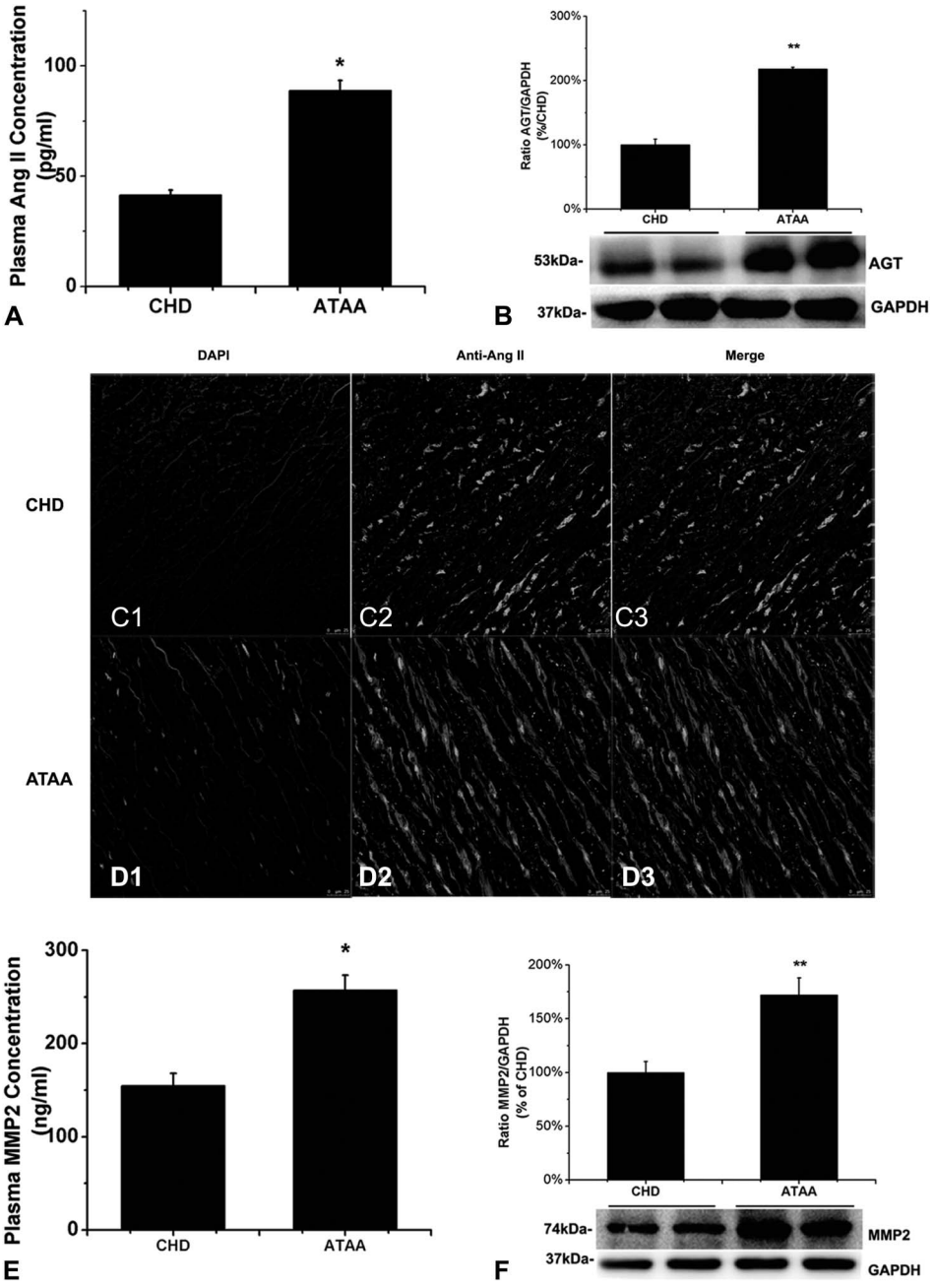

FIGURE 1. Analysis of plasma concentrations of Ang II and MMP-2 and tissue protein levels of Ang II, AGT, and MMP-2 in ATAA patients. A, The graph shows that Ang II plasma concentrations determined using radioimmunoassay were increased in the plasma of patients with ATAAs. $*P < 0.05$ versus CHD. ATAA (n = 40); CHD (n = 40). B, Bottom panel: A representative Western blot analysis. AGT protein expression by Western blot was elevated in aneurysmal walls of patients with ATAAs. Top panel: The graph shows relative AGT protein levels normalized to GAPDH relative to control. $**P < 0.01$ versus CHD. ATAA (n = 40); CHD (n = 40). C1–C3 and D1–D3, Representative photographs of immunofluorescent staining for Ang II (green color) on paraffin sections of ascending aortas from CHD and ATAA group. The nuclei were counterstained with DAPI (blue). These photomicrographs are representative of similar results obtained from at least 3 separate experiments. Sections were analyzed using confocal microscopy. The scale bar refers to 25 μm. E, The graph shows that MMP-2 plasma concentrations by enzyme-linked immunosorbent assay were increased in the plasma of patients with ATAAs. $*P < 0.05$ versus CHD. ATAA (n = 40); CHD (n = 40). F, Typical Western blot analysis (bottom panel). The graph shows MMP-2 protein expression by Western blot was elevated in aneurysmal walls of patients with ATAAs (top panel). $**P < 0.01$ versus CHD. ATAA (n = 40); CHD (n = 40).

Ang II Induces Activation of p38 MAPK, ERK1/2, and JNK in ASMCs

Several studies have demonstrated that Ang II induces a number of cellular responses through p38 MAPK, ERK1/2, and JNK in various cell types, such as endothelial and myocardium cells. Therefore, we examined whether Ang II stimulation activates these kinases in ASMCs. ASMCs were stimulated with 0.1 μM Ang II for 0, 2, 5, 10, 30, and 60 minutes. As shown in Figure 5, Ang II induced rapid phosphorylation of p38 MAPK, ERK1/2, and JNK, which reached a peak within several minutes and then gradually decreased (2.2-fold at 5 minutes vs. control, $P = 0.037$, Figs. 5A, D; 2.8-fold at 5 minutes and 2.7-fold at 10 minutes vs. control, $P < 0.01$, Figs. 5B, D; 1.9-fold at 5 minutes vs. control, $P = 0.01$, Figs. 5C, D, respectively). Total p38 MAPK, ERK1/2, and JNK were not statistically different at any of the 5 time points.

Ang II-induced Increase in MMP-2 Expression is Mediated by JNK, ERK1/2, and p38 MAPK in ASMCs

We explored the potential role of JNK, ERK, and p38 MAPK in the Ang II–induced increase in MMP-2 expression in cultured ASMC cells. ASMC cells were stimulated with

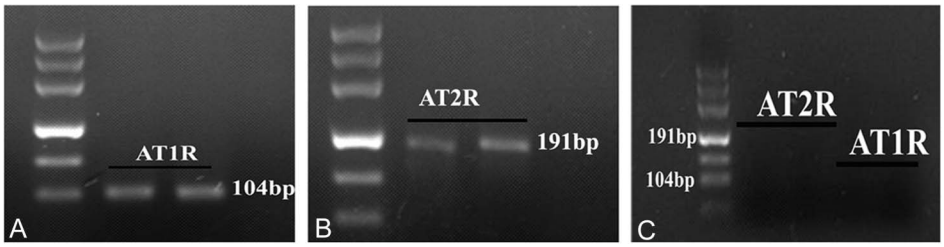

FIGURE 2. Ang II type 1 and 2 receptors are expressed in human ASMCs. RT-PCR (A–C) was used to detect AT1R and AT2R mRNA in ASMCs. Non–reverse transcribed RNA was used as the negative control. The AT1R and AT2R PCR product lengths are shown in the image margin.

Ang II (0.1 μM) for 48 hours with or without pretreatment with selective inhibitors of JNK, ERK, and p38 MAPKs (10 μM SP600125, 10 μM PD98059, and 20 μM SB203580 for 60 minutes, respectively). Pretreatment with SP600125, PD98059, or SB203580 suppressed Ang II–induced increases in MMP-2 expression (Fig. 6; $P = 0.02$, $P = 0.039$ and $P = 0.018$, respectively), which indicates that these effects are mediated by JNK, ERK, and p38 MAPKs in ASMCs.

DISCUSSION

This study confirmed that MMP-2 was significantly elevated in the plasma and aneurysmal aortas of ATAA patients compared with CHD patients, which is consistent with previous studies.[27] In addition, MMP-1, MMP-3, MMP-9, and MMP-14 were also found to be markedly increased in ATAAs.[4,11] Our study results as well as others that increased levels of active MMPs result in increased proteolysis of the aortic wall have been strongly associated with the initiation and development of ATAAs. To our knowledge, this study was the first to demonstrate that Ang II in the plasma of patients with non-Marfan syndrome ATAAs was increased compared with CHD patients. Additionally, Western blot for the first time showed that AGT was augmented in aorta specimens from ATAAs, indicating that the local amount of Ang II may be increased. The elevation of Ang II amounts in ATAA wall was also confirmed by immunofluorescence. Moreover, Nagashima et al[19] showed that Ang II concentrations in Marfan syndrome aortas were significantly higher than in normal aortas using the enzyme-linked immunosorbent assay method. In addition, Ang II infusion promotes ascending aortic aneurysms in ApoE or low-density lipoprotein receptor knockout hypercholesterolemic mice.[16,28] Losartan, an AT1R antagonist, reduced aortic growth rates and prevented progressive deterioration of aortic wall architecture in Marfan syndrome mice[27] and attenuated aortic root dilation of Marfan syndrome patients.[28] All these studies have provided evidences that Ang II–based mechanisms are an underlying cause of aneurysms localized to the ascending aorta. Ang II through AT1R enhanced NADPH oxidase activity and consequent reactive oxygen species generation in cultured VSMCs[29]; reactive oxygen species generated by NADH/NADPH oxidase may activate aortic MMPs and promote SMC apoptosis, which contributes to the formation of aneurysms.[30] In our present investigation, Ang II through AT1R induced MMP-2 upregulation in cultured VSMCs, causing elastic fiber degradation followed by aortic dilation. In addition, the mechanism by which Ang II through AT1R increases expression of cyclooxygenase 2 in VSMCs may contribute to the pathogenesis of thoracic aortic aneurysms.[31–34]

Our results for the first time demonstrated that Ang II upregulated MMP-2 protein expression in a dose- and time-dependent manner in cultured SMCs derived from human ATAAs. MMP-2 protein elevation was observed with Ang II concentrations ranging from 0.01 to 10 μM and time intervals from 18 to 48 hours with 0.1 μM Ang II. In addition, we demonstrated that this response was specifically mediated by the AT1 receptor. This was because the AT1R blocker candesartan inhibited Ang II–induced expression of MMP-2 in aneurysm-derived VSMCs, but the AT2R antagonist PD123319 had no effect. These findings were consistent with a previous study in cultured human retinal pigment epithelium cells in which Ang II induced upregulation of MMP-2 through the AT1 receptor.[24] Furthermore, the effect of Ang II on ERK1/2 phosphorylation was abolished by the AT1R antagonist candesartan, whereas the AT2R blocker PD123319 had no effect. JNK and p38 MAPK also showed similar results (data not shown). Moreover, these data for the

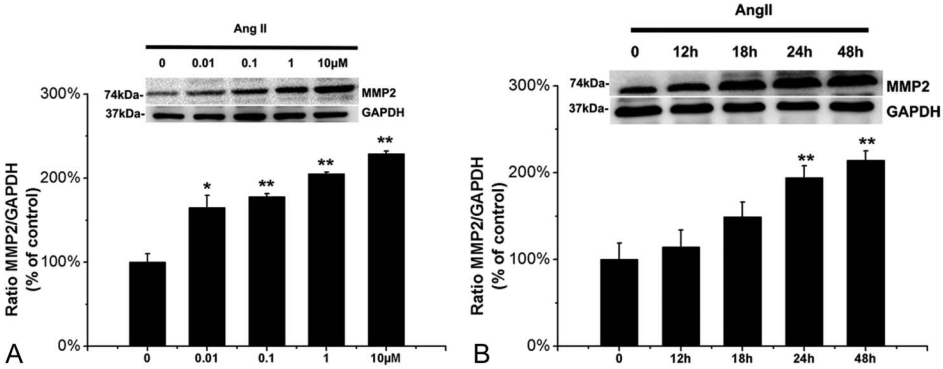

FIGURE 3. Ang II induces MMP-2 protein expression in ASMCs. A, ASMCs were incubated for 48 hours in the presence of various concentrations of Ang II. B, ASMCs were incubated in the absence or presence of 0.1 μM Ang II for the indicated time intervals. The cell lysates were then collected. Levels of MMP-2 protein in cell lysates were measured by Western blot. The group without Ang II incubation was used as a control. The graph shows the relative MMP-2 protein levels normalized to GAPDH relative to control. Values are means ± SEMs of 3 individual experiments. *$P < 0.05$, **$P < 0.01$ versus control.

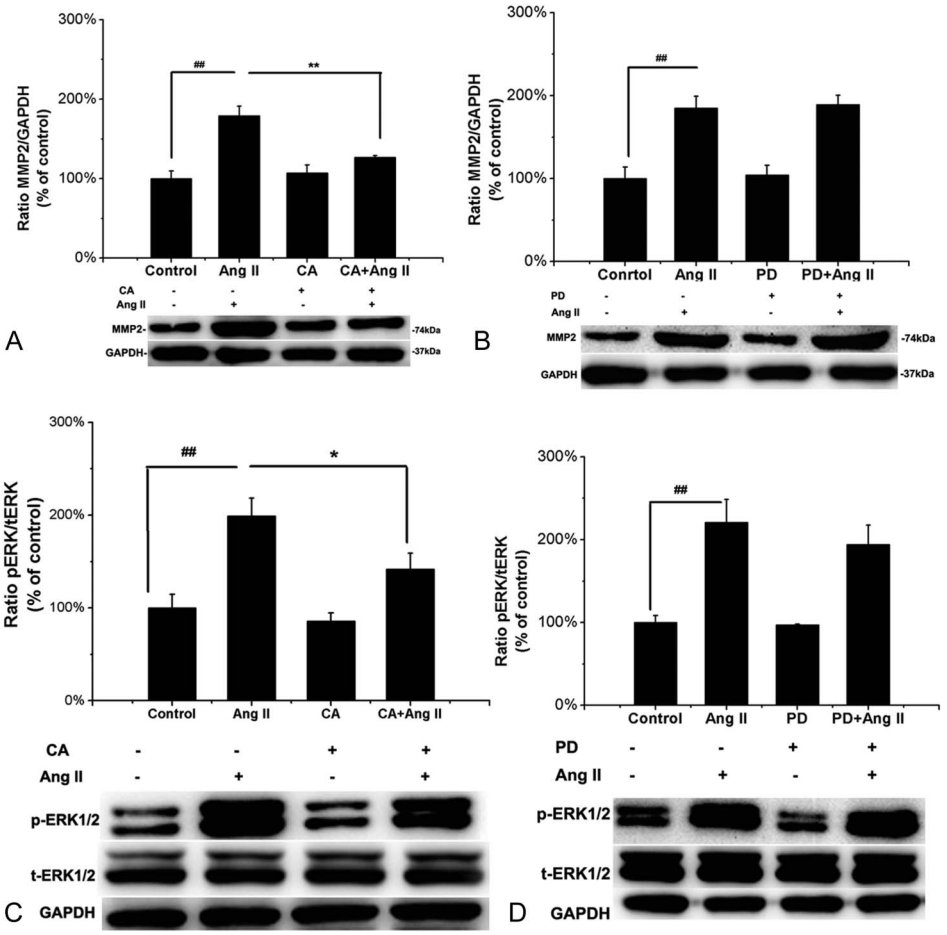

FIGURE 4. Ang II induces MMP-2 protein expression and ERK phosphorylation in ASMCs through AT1R. A and B, Ratio of MMP-2/GAPDH (top panel). C and D, The graph shows relative p-ERK1/2 protein levels normalized to total ERK1/2 relative to control (top panel). A–D, Western blot from a representative experiment (bottom panel). The means ± SEMs of 3 individual experiments are shown. $^{\#\#}P < 0.01$ versus untreated control cells; $^{*}P < 0.05$ versus cells treated with Ang II.

first time demonstrated that exposure to Ang II induced AT1R-mediated activation of ERK, JNK, and p38 MAPK signaling pathways in SMCs originating in aneurysms. These data are compatible with previous in vitro results obtained with the use of cultured rat VSMCs.[35] JNK, ERK1/2, and p38 MAPK are reported to mediate various cell responses

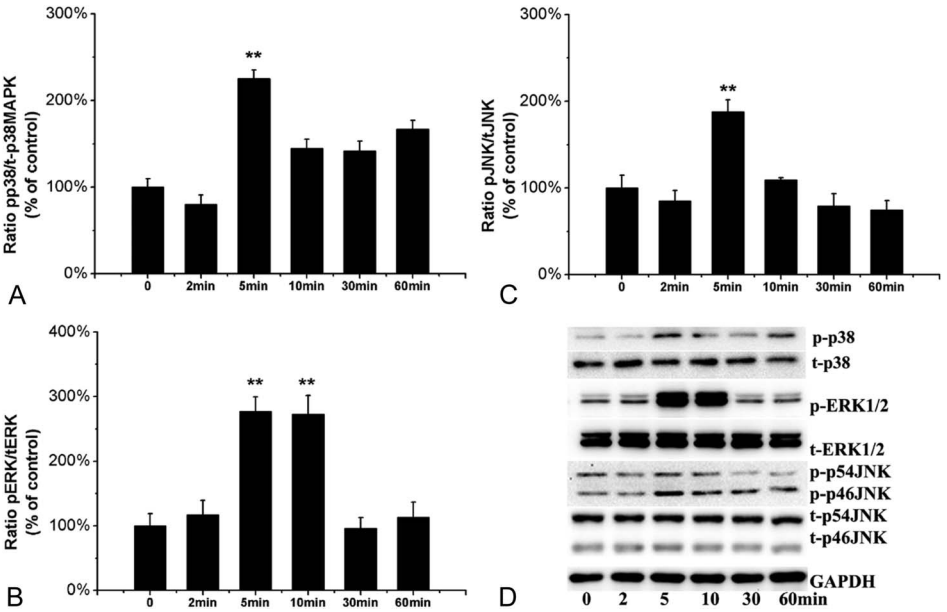

FIGURE 5. Ang II activates p38 MAPK, ERK1/2, and JNK in ASMCs. ASMCs were stimulated with 0.1 μM Ang II for the time course indicated, and then cells were lysed and analyzed by Western blotting for phosphorylated and total p38 MAPK (p-p38 and t-p38), phosphorylated and total ERK1/2 (p-ERK1/2 and t-ERK1/2), and phosphorylated and total JNK (p-JNK and t-JNK). A–C, Bar graphs corresponding to mean results of 3 independent experiments normalized to their corresponding loading controls. D, Representative Western blots for evaluation of phosphorylated protein in cell lysates. The results are expressed as the means ± SEMs of 3 individual experiments. $^{**}P < 0.01$ compared with untreated control cells.

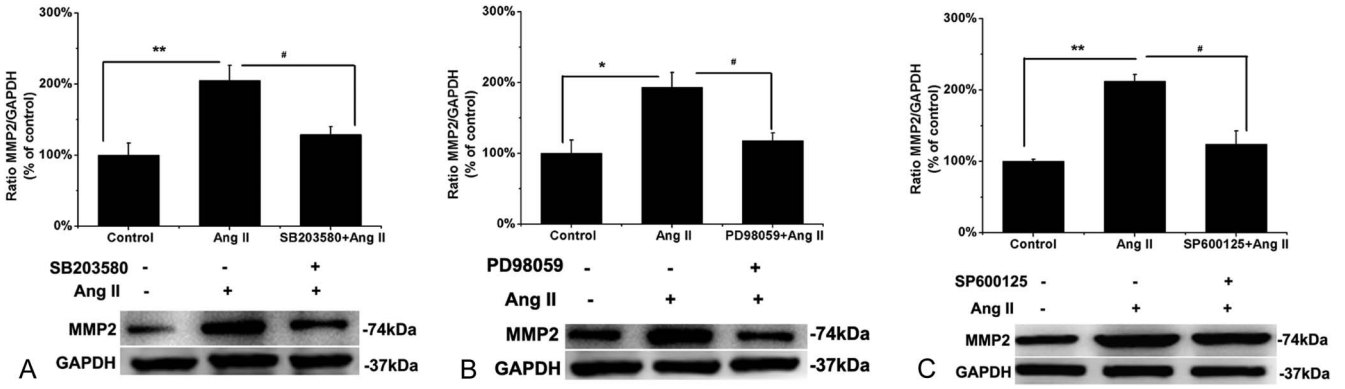

FIGURE 6. Inhibition of JNK, ERK1/2, and p38 MAPK blocks Ang II–induced MMP-2 protein expression in ASMCs by the selective inhibition of JNK SP600125, ERK1/2 PD98059, and p38 MAPK SB203580. A–C, Ratio of MMP-2/GAPDH (top panel). A–C, Representative Western blot for MMP-2 (bottom panel). Data are expressed as percentages of control and are the means ± SEMs of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus untreated control cells; $^{\#}P < 0.05$ versus Ang II alone.

of VSMCs.[36–38] However, there have been no reports regarding the role of MAPK signaling pathways activated by Ang II in aneurysm-derived SMCs. This study first showed that Ang II–induced MMP-2 expression in aneurysm-derived SMCs was dependent on signaling through JNK, ERK1/2, and p38 MAPK signaling pathways. Ang II markedly elicited JNK, ERK1/2, and p38 MAPK phosphorylation at 5 or 10 minutes, and JNK inhibitor SP600125, ERK inhibitor PD98059, and p38 MAPK inhibitor SB203580 significantly suppressed Ang II–induced MMP-2 upregulation in ASMCs. In addition, inactivation of the JNK pathway attenuated Ang II–induced abdominal aortic aneurysm initiation in low-density lipoprotein receptor–deficient mice.[39] Nagasawa et al[40] demonstrated that Ang II enhanced MMP-9 production independent of Smad2 activation through the ERK signaling pathway in human nonsyndromic thoracic aortic aneurysm walls. Furthermore, inhibition of ERK activation suppressed Ang II–induced abdominal aortic aneurysm formation in ApoE knockout mice.[41]

Our study has several important limitations that warrant discussion. First, there is an absence of parallel experimental investigation in SMCs from patients with CHD. We did not examine the role of Ang II in detail in SMCs from patients with CHD. Because the ascending aortic tissues from CHD patients who underwent coronary artery bypass surgery were very small and broken, it was difficult to excise the adventitial layer and scrape the endothelium of aorta for the culture of the SMCs. Additionally, the cultured SMCs were not pure because they were from different CHD individuals. In this study, we used explants outgrowth from ascending aortas obtained from heart transplant donors to culture SMCs as parallel experimental investigation. Our results showed that Ang II induced MMP-2 expression in these cells in a dose- and time-dependant way through AT1 receptor. We did not present these data in our manuscript because we thought that we may not need to as these results were similar with those obtained in ASMCs. Second, we did not examine the levels of renin in plasma and tissues for ATAA and CHD patients studied in this study. Finally, our data do not provide insight as to the potential cause and effect relationship between the elevation of Ang II and MMP-2 expression and ATAAs.

Despite these limitations, this study serves as a foundation for future investigation by demonstrating that ATAAs exhibit increased MMP-2 expression and Ang II amount. Furthermore, these data suggested that the mechanism of Ang II–induced MMP-2 expression may participate in the pathogenesis of ATAAs. Therefore, understanding the mechanisms underlying Ang II–induced MMP-2 expression in aneurysm-derived SMCs is important for developing new therapeutic strategies. Further studies into the functional significance of individual cytokines that are altered in aortic aneurysms will play a pivotal role in increasing our understanding of the pathogenesis of ascending thoracic aortic aneurysms and contribute to clinical improvements.

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REFERENCES

1. Elefteriades JA, Farkas EA. Thoracic aortic aneurysm clinically pertinent controversies and uncertainties. *J Am Coll Cardiol.* 2010; 55:841–857.
2. Johansson G, Markstrom U, Swedenborg J. Ruptured thoracic aortic aneurysms: a study of incidence and mortality rates. *J Vasc Surg.* 1995;21:985–988.
3. Phillippi JA, Green BR, Eskay MA, et al. Mechanism of aortic medial matrix remodeling is distinct in patients with bicuspid aortic valve. *J Thorac Cardiovasc Surg.* 2014;147:1056–1064.
4. Ikonomidis JS, Jones JA, Barbour JR, et al. Expression of matrix metalloproteinases and endogenous inhibitors within ascending aortic aneurysms of patients with bicuspid or tricuspid aortic valves. *J Thorac Cardiovasc Surg.* 2007;133:1028–1036.
5. Lesauskaite V, Tanganelli P. Morphogenesis of thoracic aorta aneurysms: investigation of matrix metalloproteinases and their tissue inhibitors [in Lithuanian]. *Medicina (Kaunas).* 2002;38:31–35.
6. Segura AM, Luna RE, Horiba K, et al. Immunohistochemistry of matrix metalloproteinases and their inhibitors in thoracic aortic aneurysms and aortic valves of patients with Marfan's syndrome. *Circulation*. 1998;98 (suppl 19):II331–II337.

7. Patel MI, Melrose J, Ghosh P, et al. Increased synthesis of matrix metalloproteinases by aortic smooth muscle cells is implicated in the etiopathogenesis of abdominal aortic aneurysms. *J Vasc Surg.* 1996;24:82–92.
8. Crowther M, Goodall S, Jones JL, et al. Increased matrix metalloproteinase 2 expression in vascular smooth muscle cells cultured from abdominal aortic aneurysms. *J Vasc Surg.* 2000;32:575–583.
9. El-Hamamsy I, Yacoub MH. Cellular and molecular mechanisms of thoracic aortic aneurysms. *Nat Rev Cardiol.* 2009;6:771–786.
10. Booher AM, Eagle KA. Diagnosis and management issues in thoracic aortic aneurysm. *Am Heart J.* 2011;162:38–46.e1.
11. Koullias GJ, Ravichandran P, Korkolis DP, et al. Increased tissue microarray matrix metalloproteinase expression favors proteolysis in thoracic aortic aneurysms and dissections. *Ann Thorac Surg.* 2004;78:2106–2110.
12. Ikonomidis JS, Jones JA, Barbour JR, et al. Expression of matrix metalloproteinases and endogenous inhibitors within ascending aortic aneurysms of patients with Marfan syndrome. *Circulation*. 2006;114(suppl 1): I365–I370.
13. Touyz RM. Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. *Exp Physiol.* 2005;90:449–455.
14. Lu H, Rateri DL, Bruemmer D, et al. Involvement of the renin-angiotensin system in abdominal and thoracic aortic aneurysms. *Clin Sci (Lond).* 2012;123:531–543.
15. Moltzer E, Essers J, van Esch JH, et al. The role of the renin-angiotensin system in thoracic aortic aneurysms: clinical implications. *Pharmacol Ther.* 2011;131:50–60.
16. Daugherty A, Rateri DL, Charo IF, et al. Angiotensin II infusion promotes ascending aortic aneurysms: attenuation by CCR2 deficiency in apoE-/- mice. *Clin Sci (Lond).* 2010;118:681–689.
17. Bruemmer D, Daugherty A, Lu H, et al. Relevance of angiotensin II-induced aortic pathologies in mice to human aortic aneurysms. *Ann N Y Acad Sci.* 2011;1245:7–10.
18. Nishimoto M, Takai S, Fukumoto H, et al. Increased local angiotensin II formation in aneurysmal aorta. *Life Sci.* 2002;71:2195–2205.
19. Nagashima H, Sakomura Y, Aoka Y, et al. Angiotensin II type 2 receptor mediates vascular smooth muscle cell apoptosis in cystic medial degeneration associated with Marfan's syndrome. *Circulation*. 2001;104(12 suppl 1):I282–I287.
20. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest.* 2000;105:1605–1612.
21. Luchtefeld M, Grote K, Grothusen C, et al. Angiotensin II induces MMP-2 in a p47phox-dependent manner. *Biochem Biophys Res Commun.* 2005;328:183–188.
22. Ham SA, Lee H, Hwang JS, et al. Activation of peroxisome proliferator-activated receptor delta inhibits angiotensin II-induced activation of matrix metalloproteinase-2 in vascular smooth muscle cells. *J Vasc Res.* 2014;51:221–230.
23. Wang M, Zhang J, Spinetti G, et al. Angiotensin II activates matrix metalloproteinase type II and mimics age-associated carotid arterial remodeling in young rats. *Am J Pathol.* 2005;167:1429–1442.
24. Pons M, Cousins SW, Alcazar O, et al. Angiotensin II induced MMP-2 activity and MMP-14 and basigin protein expression are mediated via the angiotensin II receptor type 1 mitogen-activated protein kinase 1 pathway in retinal pigment epithelium. *Am J Pathol.* 2011;178:2665–2681.
25. Jimenez E, Perez de la Blanca E, Urso L, et al. Angiotensin II induces MMP 2 activity via FAK/JNK pathway in human endothelial cells. *Biochem Biophys Res Commun.* 2009;380:769–774.
26. Ehrlichman LK, Ford JW, Roelofs KJ, et al. Gender-dependent differential phosphorylation in the ERK signaling pathway is associated with increased MMP2 activity in rat aortic smooth muscle cells. *J Surg Res.* 2010;160:18–24.
27. Huusko T, Salonurmi T, Taskinen P, et al. Elevated messenger RNA expression and plasma protein levels of osteopontin and matrix metalloproteinase types 2 and 9 in patients with ascending aortic aneurysms. *J Thorac Cardiovasc Surg.* 2013;145:1117–1123.
28. Rateri DL, Moorleghen JJ, Balakrishnan A, et al. Endothelial cell-specific deficiency of Ang II type 1a receptors attenuates Ang II-induced ascending aortic aneurysms in LDL receptor-/- mice. *Circ Res.* 2011;108:574–581.
29. Griendling KK, Minieri CA, Ollerenshaw JD, et al. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res.* 1994;74:1141–1148.
30. Ejiri J, Inoue N, Tsukube T, et al. Oxidative stress in the pathogenesis of thoracic aortic aneurysm: protective role of statin and angiotensin II type 1 receptor blocker. *Cardiovasc Res.* 2003;59:988–996.
31. He R, Guo DC, Sun W, et al. Characterization of the inflammatory cells in ascending thoracic aortic aneurysms in patients with Marfan syndrome, familial thoracic aortic aneurysms, and sporadic aneurysms. *J Thorac Cardiovasc Surg.* 2008;136:922–929.e1.
32. Hu ZW, Kerb R, Shi XY, et al. Angiotensin II increases expression of cyclooxygenase-2: implications for the function of vascular smooth muscle cells. *J Pharmacol Exp Ther.* 2002;303:563–573.
33. Gitlin JM, Trivedi DB, Langenbach R, et al. Genetic deficiency of cyclooxygenase-2 attenuates abdominal aortic aneurysm formation in mice. *Cardiovasc Res.* 2007;73:227–236.
34. Chapple KS, Parry DJ, McKenzie S, et al. Cyclooxygenase-2 expression and its association with increased angiogenesis in human abdominal aortic aneurysms. *Ann Vasc Surg.* 2007;21:61–66.
35. Eguchi S, Dempsey PJ, Frank GD, et al. Activation of MAPKs by angiotensin II in vascular smooth muscle cells—metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *J Biol Chem.* 2001;276:7957–7962.
36. Deguchi J, Makuuchi M, Nakaoka T, et al. Angiotensin II stimulates platelet-derived growth factor-B chain expression in newborn rat vascular smooth muscle cells and neointimal cells through Ras, extracellular signal-regulated protein kinase, and c-Jun N-terminal protein kinase mechanisms. *Circ Res.* 1999;85:565–574.
37. Lee HM, Lee CK, Lee SH, et al. p38 mitogen-activated protein kinase contributes to angiotensin II-stimulated migration of rat aortic smooth muscle cells. *J Pharmacol Sci.* 2007;105:74–81.
38. Touyz RM, El Mabrouk M, He G, et al. Mitogen-activated protein extracellular signal-regulated kinase inhibition attenuates angiotensin II mediated signaling and contraction in spontaneously hypertensive rat vascular smooth muscle cells. *Circ Res.* 1999;84:505–515.
39. Tsai SH, Huang PH, Peng YJ, et al. Zoledronate attenuates angiotensin II-induced abdominal aortic aneurysm through inactivation of Rho/ROCK-dependent JNK and NF-kappaB pathway. *Cardiovasc Res.* 2013;100:501–510.
40. Nagasawa A, Yoshimura K, Suzuki R, et al. Important role of the angiotensin II pathway in producing matrix metalloproteinase-9 in human thoracic aortic aneurysms. *J Surg Res.* 2013;183:472–477.
41. Zhang Y, Naggar JC, Welzig CM, et al. Simvastatin inhibits angiotensin II-induced abdominal aortic aneurysm formation in apolipoprotein E-knockout mice: possible role of ERK. *Arterioscler Thromb Vasc Biol.* 2009;29:1764–1771.