Acta Biochim Biophys Sin, 2015, 47(7), 539–547
doi: 10.1093/abbs/gmv047
Advance Access Publication Date: 11 June 2015
Original Article

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Angiotensin II induces an increase in MMP-2 expression in idiopathic ascending aortic aneurysm via AT1 receptor and JNK pathway

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Received 8 January 2015; Accepted 16 March 2015

Abstract

The cellular and molecular mechanisms responsible for human idiopathic ascending aortic aneurysm (IAAA) remain unknown. Matrix metalloproteinase-2 (MMP-2) is a key enzyme for the degradation of extracellular matrix in aneurysmal walls. The aim of this study was to elucidate the role of the angiotensin II (Ang II) pathway in MMP-2 induction in IAAA aortic walls. Quantitative polymerase chain reaction and western blot analysis were used to compare the MMP-2 mRNA and protein levels in ascending aortic specimens with those in IAAA patients ($n = 10$) and heart transplant donors ($n = 5$) without any aortopathy. It was found that MMP-2 expression was significantly increased, which was associated with elastic lamellae disruption in IAAA walls. Additionally, the expression levels of angiotensinogen (AGT) and Ang II in the ascending aortic tissues from individuals with and without IAAAs were detected by western blot analysis and radioimmunoassay, respectively. The results demonstrated that the expressions of AGT and Ang II protein were significantly increased in the ascending aortic tissues of IAAA patients. Furthermore, whether Ang II induces MMP-2 expression was investigated using human IAAA walls *ex vivo* culture. It was found that exogenous Ang II increased the MMP-2 expression in a dose-dependent manner, which was completely inhibited by the Ang II type 1 receptor (AT1R) inhibitor candesartan and was mediated by c-Jun N-terminal kinase (JNK) activation. Taken together, these results indicate that Ang II can induce an increase of MMP-2 expression via AT1R and JNK in *ex vivo* cultured IAAA aortic walls, and suggest that angiotensin receptor blocker (ARB) drugs and JNK inhibitors have the potential in the prevention or treatment of IAAAs.

Key words: idiopathic ascending aortic aneurysm, angiotensin II, matrix metalloproteinase-2, angiotensin II type 1 receptor, c-Jun NH2-terminal kinase

Introduction

The majority of ascending aortic aneurysms is not related to any specific etiology and should be regarded as idiopathic ascending aortic aneurysms (IAAAs) [1]. IAAA is a severe life-threatening disease. The global burden of disease 2010 project demonstrated that the overall global death rate from aortic aneurysms increased from 2.49 per 100,000 individuals to 2.78 per 100,000 individuals from 1990 to 2010 [2]. In addition, IAAA patients are most often asymptomatic until dissection or rupture occurs, resulting in 94%–100% mortality, particularly in the absence of surgery [3,4]. Sixty percent of thoracic

aortic aneurysms involve the ascending aorta [5]. However, the cellular and molecular mechanisms involved in the formation and progression of IAAAs remain unclear.

Recent studies have demonstrated that the expression of matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, is significantly increased in the walls of IAAAs [6–8]. Increased MMP-2 expression can degrade the extracellular matrix of the aortic tunica media, leading to the loss of the normal structure of the aortic wall and to the progressive expansion of the aorta lumen. Among the MMPs, MMP-9 has been shown to play a major role in the formation and progression of IAAAs. In contrast, the role of MMP-2 in this process remains controversial. Recent studies demonstrated that MMP-2 levels did not significantly increase in aorta specimens of IAAA patients [9,10].

Angiotensin II (Ang II) plays important physiological roles in the modulation of blood pressure, cell migration, cell growth, and extracellular matrix deposition [11]. Recent reports demonstrated that Ang II infusion promoted ascending aortic aneurysms and abdominal aortic aneurysms in apolipoprotein E−/− (ApoE−/−) and low-density lipoprotein receptor−/− (LDLR−/−) knockout mice [12–15]. Losartan reduced the aortic growth rate and prevented progressive aortic wall architecture deterioration in Marfan syndrome mice [16], whereas it attenuated aortic root dilation in Marfan syndrome patients [17]. These findings demonstrate that Ang II is involved in the development of abdominal and thoracic aortic aneurysms in both human and animal models [18,19]. Nishimoto *et al.* [20] found that the concentrations of Ang II were increased in abdominal aortic aneurysm tissues compared with in control specimens. Additionally, Marfan syndrome aortic root aneurysms had significantly high Ang II concentrations [21]. However, thus far, local Ang II concentrations in IAAAs have not been reported.

Several reports demonstrated that c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 1/2 participated in abdominal aortic aneurysm formation and development, because phosphorylated JNK and ERK were highly expressed in human abdominal aortic aneurysm tissues [22,23]. However, whether JNK and ERK are involved in IAAA pathogenesis remains unknown. Exogenous Ang II could induce human IAAAs to produce MMP-9 via ERK activation *in vitro* [24]. But whether Ang II contributes to MMP-2 production in IAAAs remains unknown. Some studies showed that Ang II type 2 receptor (AT2R) expression was inhibited in vascular smooth muscle cells (VSMCs) from rat thoracic aortas when cultured in high-serum medium [25,26]. Conversely, Brogelli *et al.* [25] demonstrated the expression of *AT2R* mRNA in *ex vivo* aorta organ culture model at high serum concentration. Additionally, serum deprivation induced the cultured VSMCs from adult internal thoracic to switch from the synthetic to the contractile phenotype [27]. Organ cultures can be performed to examine the effect of Ang II on the hemodynamic effects or neurohumoral adaptations of intact IAAA walls in the absence of Ang II [25]. Furthermore, organ cultures can also be used to analyze the potential signaling pathways involved in MMP-9 expression modulated by Ang II [23,24]. In the present study, we used *ex vivo* cultured adult IAAAs as the experiment model to investigate the effect of Ang II on MMP-2 up-regulation and explore whether the Ang II receptors are involved in its signaling pathways.

Materials and Methods

Regents and antibodies

Ang II was purchased from Sigma (St Louis, USA). Mitogen-activated protein kinases (MAPKs) inhibitors SB203580, PD98059, and SP600125, and Ang II receptors inhibitors candesartan and PD123319 were purchased from Selleck Chemicals (Houston, USA). Fetal bovine serum, cell culture media, and TRIzol Reagent were purchased from Life Technologies (Grand Island, USA). Bicinchoninic acid (BCA) protein assay reagents were purchased from Pierce (Rockford, USA). Anti-angiotensinogen (AGT) and anti-Ang II antibody were from Abbiotec (San Diego, USA). Anti-MMP-2 antibody was purchased from Bioworld Technology (St Louis Park, USA). Anti-alpha smooth muscle actin (α-SMA) antibody was from Abcam (Cambridge, UK). Rabbit anti-JNK, anti-phospho-JNK1/2, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, and anti-phospho-p38 antibodies were from Cell Signaling Technology (Beverly, USA). Anti-GAPDH antibody was from Santa Cruz (Santa Cruz, USA).

Patients' information and collection of plasma and aortic tissues

Ascending aortic specimens were obtained from 10 patients (8 men and 2 women) ranging from 33 to 66 years old at the time of elective operative ascending aortic aneurysm repair at Fuwai Hospital from October to December 2013. The clinical characteristics of these patients are presented in **Table 1**. Among our IAAA patients (aneurysm size <55 mm), five patients received open surgery because of severe regurgitation, and three patients received open surgery because of ascending aorta overgrowth (>5 mm/6 months). As a control, normal aortic specimens (n = 5) were obtained from five heart transplant donors died from traffic accidences without any diagnosis of aortopathy. Informed consent was obtained from all the study subjects. None of the subjects received angiotensin-converting enzyme inhibitor or angiotensin receptor blocker (ARB) drugs. No patients with IAAAs had aortic dissection, inflammatory aortic disease, or known connective tissue disorder such as Marfan syndrome and Loeys–Dietz syndrome. The obtained aortic walls were placed in 10% formalin for histological and immunohistochemical analysis or immediately snap-frozen in liquid nitrogen for RNA and protein extraction. All of the experimental protocols using human specimens were approved by the Institutional Ethics Committee of the Fuwai Hospital of the Peking Union Medical College.

Ex vivo organ culture of IAAA walls

Ex vivo organ culture was performed as described previously [23]. In brief, IAAA ascending aortic walls (n = 5) were obtained when ascending aortic aneurysm repair surgery was performed. After the aortic intima and adventitia were dissected, the aortic tunica media were cut into ~1 mm^2 pieces. Then, equal wet weight tissue samples in the minced medium were placed into each well of 6-well plates and cultured

Table 1. Profile of thoracic aortic aneurysm patients

No.	Age	Gender	Max AO (mm)	Smoking	AR	HT	HL	DM
1	51	F	43	No	Moderate	No	No	No
2	33	M	53	Yes	Severe	No	No	No
3	51	M	47	Yes	Moderate	No	No	No
4	54	M	54	Yes	No	Yes	No	No
5	66	M	81	Yes	Severe	Yes	No	No
6	62	M	64	No	Severe	Yes	No	No
7	43	M	53	No	Severe	No	No	No
8	48	M	44	No	Mild	No	No	No
9	64	F	47	No	Severe	No	No	No
10	49	M	45	No	Severe	Yes	No	No

Max AO, maximum diameter of aorta; AR, aortic valve regurgitation; HT, hypertension; HL, hyperlipidemia; DM, diabetes mellitus.

with serum-free Dulbecco's modified Eagle's medium (DMEM) for 24 h. Then, the culture medium was replaced with fresh serum-free DMEM. IAAA aortic pieces in culture were incubated with or without 1 μM Ang II type 1 receptor (AT1R) inhibitor candesartan, 10 μM AT2R antagonists PD123319, or 20 μM JNK inhibitor SP600125. After 48 h, IAAA aortic pieces in culture were treated with 1 μM Ang II for 48 h to detect the expression of MMP-2 protein, and or treated for 30 min to detect the level of pJNK in response to Ang II with or without SP600125 pre-treatment.

Radioimmunoassay

Aortic tissue samples (200–400 mg, IAAAs group $n = 10$; Control group $n = 5$) were homogenized in 1 ml of 50 mM Tris–HCl buffer (1.0 M NaCl, 2.0 M urea, 0.2 mM phenylmethanesulfonyl fluoride, 0.1% Brij-35, 0.1% EDTA, pH 7.4) at 4°C, centrifuged at 10,000 *g* for 10 min, and the supernatant was collected. The pellets were re-homogenized in 1 ml of 50 mM Tris–HCl and centrifuged at 10,000 *g* for 10 min at 4°C. The combined supernatants were centrifuged at 10,000 *g* for 15 min at 4°C, and the final supernatant was collected for analysis. Total protein concentrations were determined by the BCA protein assay kit (Pierce). The Ang II concentration in the supernatants was measured by a radioimmunoassay (RIA) kit (North Institute of Biological Technology, Beijing, China) according to the manufacturer's instructions. All the measurements were repeated in duplicate. The results were normalized to total protein and expressed as picograms Ang II per milligrams total aortic protein.

Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated using TRIzol reagent from each aortic wall according to the manufacturer's instruction. RNA was quantified using a spectrophotometer (NanoDrop ND-3300, Thermo Scientific, Waltham, USA) at the absorbance of 260 nm/280 nm. Total RNA (1 μg) was reverse transcribed to the cDNA using a RevertAid first strand cDNA synthesis kit (Thermo Scientific). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was perform in a 20 μl reaction volume with 0.2 μM each primer and 2.5 mM $MgCl_2$, using KAPA SYBR® FAST qPCR Kits (KAPA, Wilmington, USA) in the ABI PRISM®7900HT (Applied Biosystems, Carlsbad, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control. The primer sequences used for PCR and the sizes of the predicted PCR products are presented in Table 2. The amplification program was 95°C for 3 min, and then 40 cycles consisting of 95°C for 30 s, 60°C for 20 s, and 62°C for 20 s. The amplification products were analyzed by a melting curve, which confirmed the presence of a single PCR product in all reactions apart from the negative controls. The specificity of the PCR products was confirmed by drawing dissolution curves. The results of the qPCR were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Table 2. Primer sequences and product sizes for *MMP-2* and *GAPDH*

Gene	Primer	Sequence (5′–3′)	PCR product length (bp)
MMP-2	Forward	CCAAGTCTGGAGCGATGTG	125
	Reverse	GTCCGTCCTTACCGTCAAAG	
GAPDH	Forward	CAGGGCTGCTTTTAACTCTGG	180
	Reverse	TCCTGGAAGATGGTGATGGG	

Western blot analysis

The aortic tissue proteins were extracted using a RIPA lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1.2% Triton X-114, 1 mM NaF, and 200 mM $NaVO_4$) containing 1 tablet/10 ml of protease inhibiter cocktail tablets (Roche, Mannheim, Germany). Protein concentrations were determined by the BCA protein assay kit. For western blot analysis, 100 μg of tissue proteins was separated on 10% sodium dodecyl sulfate–polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA). Non-specific binding sites were blocked by incubating the membranes in TBS-0.1% Tween-20 (TBST) with 5% skimmed milk for 1 h at room temperature. After that, the membranes were incubated with primary antibodies (1:1000 dilution for MMP-2, 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 three times with TBST. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) for 1 h at room temperature, and washed three times with TBST. The blots were developed according to chemiluminescence kit protocol (Millipore), and analyzed using Quantity One 4.6.2 Software (Bio-Rad, Hercules, USA). Human GAPDH was used as the internal control.

Histological analysis

Ascending aortic samples of the control group ($n = 3$) and the IAAA group ($n = 5$) were fixed with 4% paraformaldehyde for 24 h at room temperature and cryosectioned at 3 μm thickness. Hematoxylin and eosin (HE) staining was used to assess aortic wall morphology and inflammatory changes. Elastin Verhoeff-van Gieson (EVG) stain was used to examine elastin present in the aortic wall. To quantify the staining of elastin and collagen, three images were taken randomly from high-power fields of each sample slide (magnification, ×400). The average positive staining of these three fields for each slide was calculated by Image-Pro Plus software 5.0 (Media Cybernetics, Rockville, USA).

Immunofluorescence assay

Ascending aortic preparations from the IAAA patients ($n = 5$) and the control group ($n = 3$) were fixed with 4% paraformaldehyde for 24 h. Tissue samples were embedded with paraffin, cut into 3 μm sections, mounted on gelatin-coated slides, then dewaxed and rehydrated with graded alcohols. The slides were microwaved in boiling 0.01 M sodium citrate buffer for 2 min, and then blocked in 5% goat serum solution for 20 min at room temperature. Then, the slides were incubated with primary antibodies against Ang II (1:100 dilutions in PBS), α-SMA (1:200 dilutions in PBS), and MMP-2 (1:200 dilutions in PBS) overnight at 4°C. Finally, the slides were incubated with Alexa Fluor 488-conjugated AffiniPure goat anti-Rabbit IgG secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) at 1:300 dilution for 30 min at 37°C. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindol (DAPI, Beyotime, Jiangsu, China) for 5 min. For negative controls, PBS solution was used instead of primary antibody. Slides were mounted with anti-fading reagent (Beyotime) and examined with a Leica TCS SP8 confocal spectral microscope (Wetzlar, Germany).

Statistical analysis

Data are presented as means ± SEM from at least three individual experiments. Group differences were analyzed by analysis of variance, and individual group differences were tested by the *post hoc* Fisher's protected least significant difference test. A P-value of <0.05 was considered statistically significant. All data were analyzed with SPSS 21.0 software.

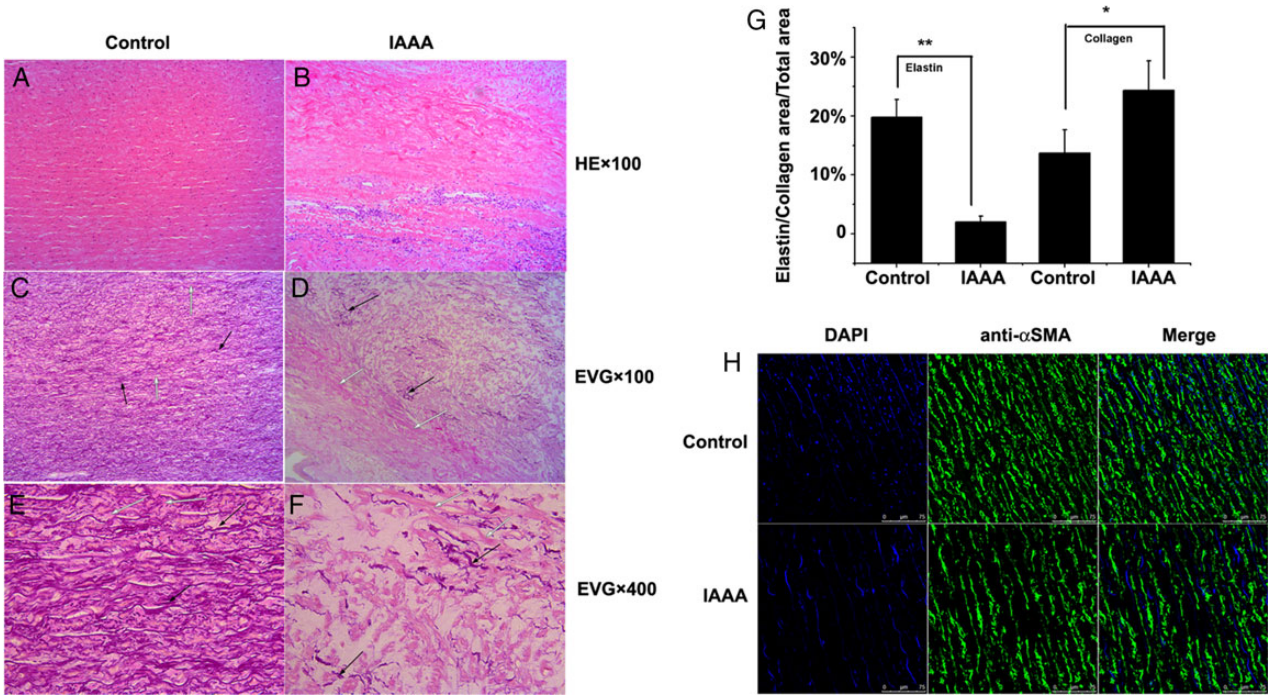

Figure 1. Histology of IAAAs and normal aortas Representative HE stained sections of ascending aorta from the control (A) and IAAAs (B) groups and EVG-stained sections from the control (C, E) and IAAAs (D, F) groups. White arrows indicate collagen; black arrows indicate the elastin (original magnification, A, B, C, D, ×100; E, F, ×400). (G) Comparison of ascending aortic expression of elastin and collagen as measured by quantitative histology in patients IAAAs and controls. $*P < 0.05$, $**P < 0.01$ vs. control. (H) SMCs are labeled with anti-αSMA as determined by immunofluorescence. Only the media of ascending aorta were provided. The nuclei were counterstained with DAPI. Sections were analyzed using confocal microscopy. The scale bars represent 75 μm.

Results

Histology of IAAAs and normal aortas

Representative images stained with HE (**Fig. 1A,B**) and EVG (**Fig. 1C–G**) demonstrated that IAAA patients exhibited severe elastin fragmentation, and remarkable elastin reduction and more collagen deposition. Aortas from the control group displayed intact and regularly arrayed elastic fibers in the intima and media and extremely few collagen fibers. In the control group, smooth muscle cells (SMCs) were distributed evenly and abundantly throughout the media layer. However, in the IAAA group, many SMCs were lost in the media layer (**Fig. 1H**). IAAAs had extensive inflammatory cell infiltration, while no significant inflammatory cell infiltration was observed in the control patient group.

Increased MMP-2 expression in IAAAs

First, we examined MMP-2 mRNA and protein expression levels in human IAAA aortic walls and normal aortic walls by qPCR, western blot analysis, and immunofluorescence assay. MMP-2 expression in IAAA patients was increased to 2.5 folds at the mRNA level and to 1.9 folds at the protein level compared with the control patients ($P = 0.013$ and $P = 0.025$, respectively, **Fig. 2A,B**). Immunofluorescence staining also confirmed that MMP-2 protein expression in aortic tunica media samples was higher in the IAAA patients than that in the control group (**Fig. 2C**).

Elevated AGT and Ang II expression in IAAAs

The expressions of AGT and Ang II protein were examined in human IAAA aortic walls and normal aortic walls by western blot analysis, RIA, and immunofluorescence assay. Local AGT protein expression in IAAA patients was increased to 2.3 folds as determined by western blot analysis ($P = 0.001$, **Fig. 3A**), indicating that Ang II expression may be increased in IAAAs. Furthermore, the RIA results confirmed that the aortic local Ang II concentrations in IAAA patients had an ~60% increase compared with the control patients ($P = 0.03$, **Fig. 3B**). Additionally, the immunofluorescence assay also showed that Ang II expression was increased in IAAA patients (**Fig. 3C**).

p38 MAPK, ERK1/2, and JNK expression and phosphorylation in IAAAs

Western blot analysis was performed to measure the phosphorylated and total amount of p38 MAPK, ERK1/2, and JNK in aortic wall samples from individuals with or without IAAAs. Compared with in normal aortas, total p38 (t-p38) expression was elevated in IAAA aortas ($P < 0.001$, **Fig. 4B**), while phosphorylated p38 (p-p38) and the ratio of p-p38/t-p38 was not elevated ($P = 0.198$ and $P = 0.124$, **Fig. 4B**, respectively). Additionally, the levels of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated JNK (p-JNK) were markedly increased in human IAAAs ($P = 0.001$, **Fig. 4C**; $P = 0.004$, **Fig. 4D**, respectively), whereas the levels of total ERK1/2 (t-ERK1/2) and total JNK (t-JNK) were not changed ($P = 0.625$, **Fig. 4C**; $P = 0.365$, **Fig. 4D**, respectively). Furthermore, the ratios of p-ERK/t-ERK and p-JNK/t-JNK were significantly elevated ($P = 0.001$, **Fig. 4C**; $P = 0.042$, **Fig. 4D**, respectively).

Ang II-induced up-regulation of MMP-2 protein in *ex vivo* cultured aortic walls from IAAA patients

To evaluate the Ang II-induced MMP-2 up-regulation in IAAAs, aortic walls from IAAA patients ($n = 5$) were cut into small pieces and

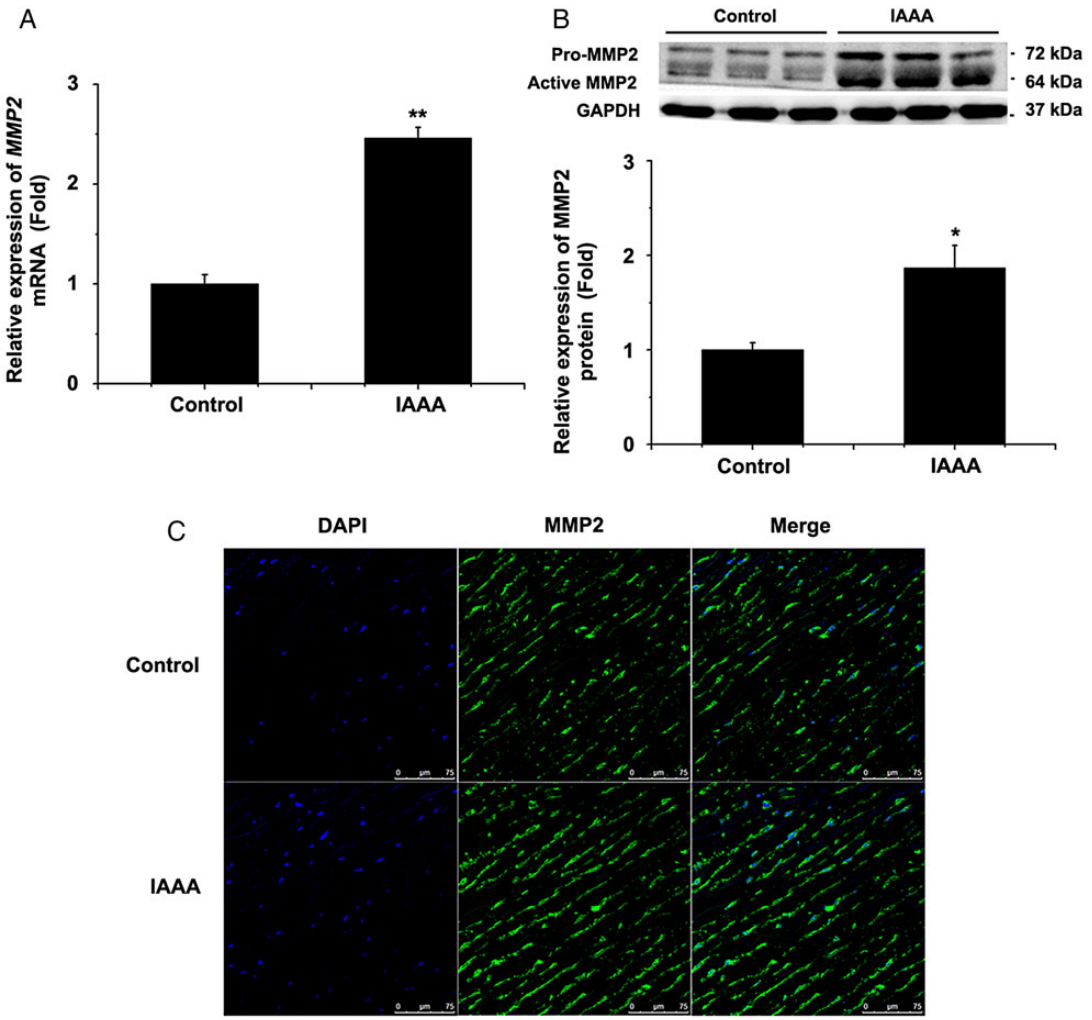

Figure 2. Increased MMP-2 expression in IAAAs The MMP2 expression at mRNA level (A) and protein level (B) in the ascending aorta of patients with IAAAs was detected by qPCR and western blot analysis, respectively. $*P < 0.05$, $**P < 0.01$ vs. control. (C) Representative immunofluorescence photographs of paraffin ascending aorta sections stained with anti-MMP-2 antibodies from three or more experiments. The nuclei were counterstained with DAPI. The scale bars represent 75 μm.

incubated *in vitro* under the different concentrations of exogenous Ang II for 48 h in 6-well plates. Western blot analysis showed that 0.1 μM Ang II significantly increased the MMP-2 protein level compared with the group without Ang II stimulation ($P = 0.005$, **Fig. 5A**), and more increase for MMP-2 protein levels under the treatment with 1 and 10 μM Ang II ($P < 0.001$, **Fig. 5A**) was observed. These results demonstrated that Ang II up-regulates the MMP-2 expression in a dose-dependent manner in *ex vivo* cultured human IAAA aorta pieces.

Ang II stimulated the expression of MMP-2 via the AT1 receptor in *ex vivo* cultured aortic walls from IAAA patients

Human aortic smooth muscles cells express both AT1R and AT2R. Therefore, we examined which receptor was involved in Ang II-induced MMP-2 up-regulation. *In vitro* cultured IAAA aortic walls were pretreated with candesartan (1 μM for 48 h) or PD123319 (10 μM for 48 h), and the tissues were subsequently treated with Ang II (1 μM for 48 h). Candesartan treatment completely inhibited the Ang II-induced increase in MMP-2 protein expression ($P = 0.003$, **Fig. 5B**), but PD123319 treatment did not ($P = 0.651$, **Fig. 5B**). Therefore, these results indicated that Ang II might induce MMP-2 up-regulation via AT1R in *ex vivo* cultured aortic walls.

Ang II induced the expression of MMP-2 via the JNK pathway, but not via the ERK1/2 or p38 MAPK pathway in *ex vivo* cultured aortic walls from IAAA patients

After the regulatory effect of Ang II on the MMP-2 expression was confirmed, we further explored the possible signal transduction pathway. *Ex vivo* cultured IAAA aortic walls were incubated with 1 μM exogenous Ang II for 30 min, then the activation of p38 MAPK, ERK1/2, and JNK was detected by western blot analysis. The results showed that exogenous Ang II-induced significant JNK phosphorylation in human IAAA aortic walls compared with no Ang II stimulation ($P < 0.001$, **Fig. 6A**). The Ang II-induced JNK activation was abrogated by treatment with the selective JNK inhibitor SP600125 (20 μM) ($P = 0.001$, **Fig. 6A**). In contrast, p38 MAPKs and ERK1/2 were not activated in IAAA *ex vivo* cultured aortic walls (data not shown).

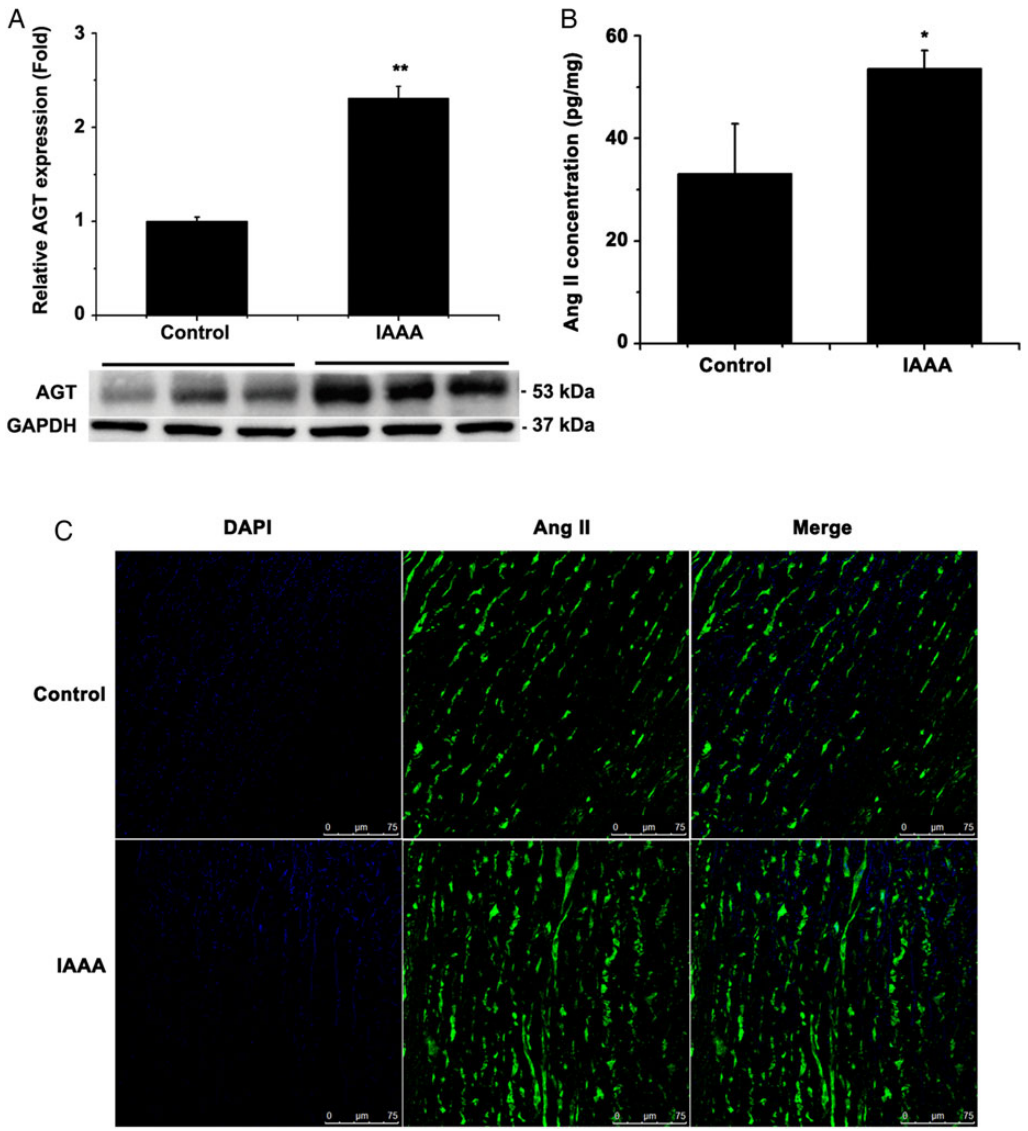

Figure 3. Elevated AGT and Ang II expression in IAAAs (A) The relative AGT protein levels in the ascending aorta of patients with IAAAs and controls. $^{**}P < 0.01$ vs. control. (B) The Ang II concentration in the aorta of patients with IAAAs was determined using RIA. $^{*}P < 0.05$ vs. control. (C) Representative photographs of immunofluorescence staining for Ang II on paraffin sections of ascending aortas from the control and IAAA groups. The nuclei were counterstained with DAPI. These photomicrographs are representative of similar results from three or more experiments. Bars represent 75 μm.

In addition, the potential role of JNK in the Ang II-induced MMP-2 expression was explored in *ex vivo* cultured human IAAA aorta pieces. The IAAA aortic walls were stimulated with Ang II (1 μM) for 48 h after pre-treatment with or without 20 μM SP600125 for 48 h. It was found that MMP-2 expression was markedly increased in IAAA aortic walls in response to Ang II compared with control aortic walls ($P = 0.008$, **Fig. 6B**). SP600125 pre-treatment suppressed the Ang II-induced MMP-2 expression, indicating that this effect is mediated by JNK in *ex vivo* cultured human IAAA aortic walls ($P = 0.016$, **Fig. 6B**).

Discussion

Histologically, IAAAs are characterized by elastic fiber fragmentation, collagen fiber accumulation, SMC loss, and degenerate tissue replacement with interstitial pools of basophilic mucopolysaccharide materials, termed cystic medial necrosis or cystic medial degeneration. No significant differences were observed between specimens from patients with IAAAs and those with Marfan syndrome [28,29]. In this study, we confirmed that MMP-2 expression was significantly increased at both transcriptional and translational levels in aortic specimens of IAAA patients compared with in normal aortas, which is consistent with previous studies [8,9,30]. In addition, the expressions of MMP-1, MMP-3, MMP-9, and MMP-14 were markedly increased in IAAAs [6,9]. Previous studies and our results demonstrated that an increased level of MMPs results in increased proteolysis of the aortic walls, which is strongly associated with the initiation and development of IAAAs. To our knowledge, this is the first report which demonstrates an increase of local Ang II concentrations in the aneurysmal walls of patients with IAAAs. Nagashima *et al.* [21] demonstrated that the Ang II concentration in aorta specimens from Marfan syndrome patients was significantly higher than that in the normal

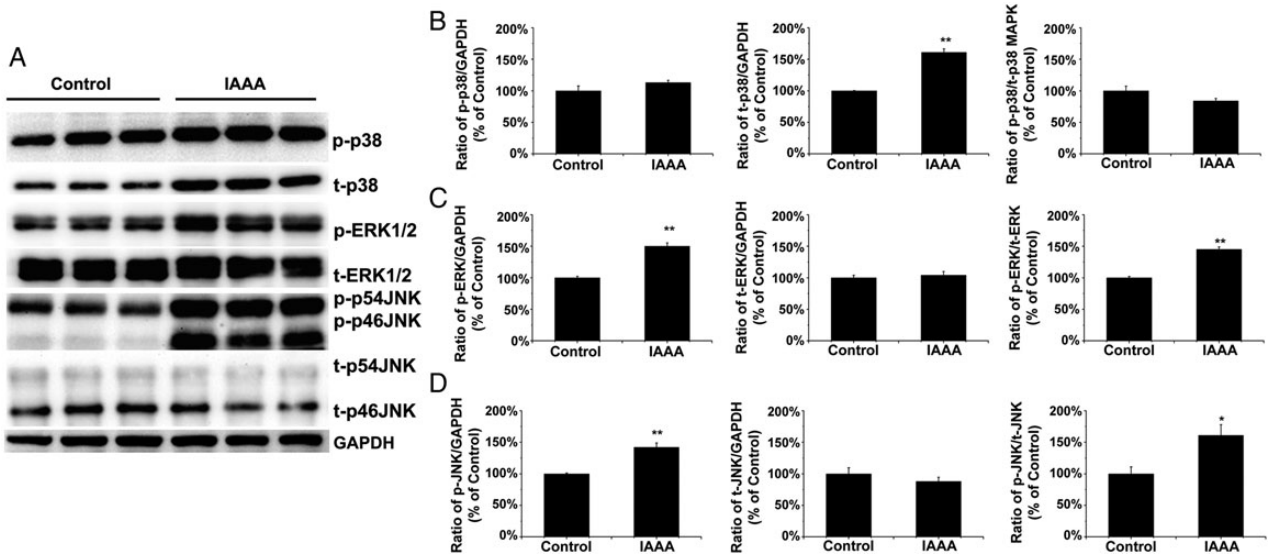

Figure 4. p38 MAPK, ERK1/2, and JNK expression and phosphorylation in IAAAs (A) The phosphorylated and total amount of p38 MAPK, ERK1/2, and JNK in aortic wall samples from individuals with or without IAAAs were detected by western blotting. (B–D) The quantification of the western blots. GAPDH served as the internal control of protein loading. $^{*}P<0.05$, $^{**}P<0.01$ vs. control.

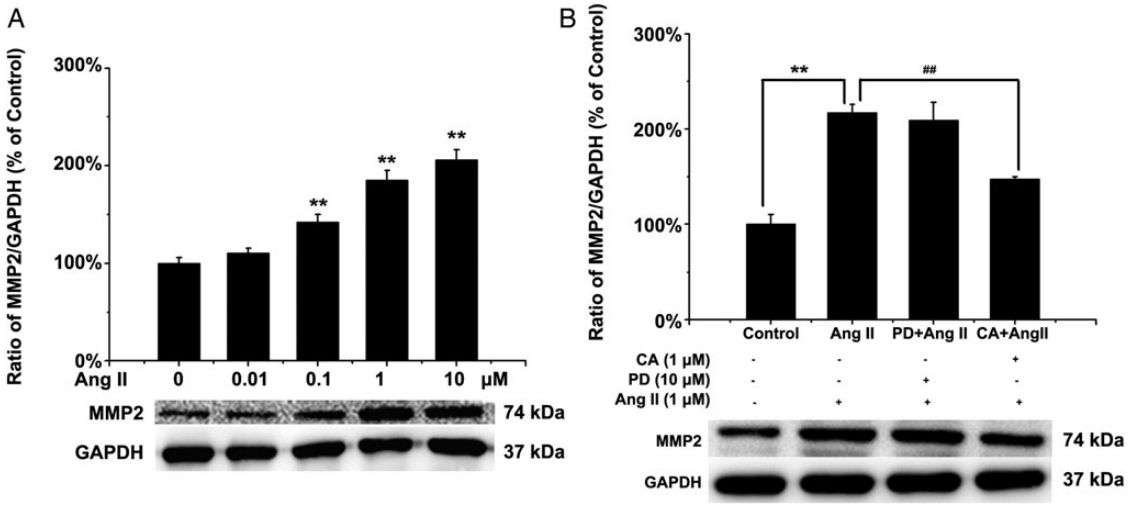

Figure 5. Ang II-induced MMP-2 protein expression through AT1 receptor in *ex vivo* cultured IAAA aortic walls (A) *Ex vivo* cultured aortic pieces from IAAA patients ($n=5$) were incubated in the presence of different concentrations of Ang II for 48 h. (B) *Ex vivo* cultured aneurysmal aorta walls were pre-treated with either candesartan (10 μM, AT1R inhibitor) or PD123319 (10 μM, AT2R antagonist) for 48 h, followed by 1 μM Ang II treatment for 48 h. The group without Ang II incubation was used as control. $^{**}P<0.01$ vs. untreated control group; $^{\#\#}P<0.01$ vs. treated with Ang II alone. CA, candesartan; PD, PD123319.

aorta. In addition, Nishimoto *et al.* [20] found that increased local Ang II might play a crucial role in the development of abdominal aortic aneurysms. These data demonstrated that Ang II participates not only in the pathogenesis of Marfan syndrome aortic root aneurysms and abdominal aortic aneurysms but also in the formation of IAAAs. Moreover, Ang II infusion promotes ascending aortic aneurysms and abdominal aortic aneurysms in ApoE and LDL knockout mice [12–14]. Ang II also induces MMP-2 expression and increases its activity in *ex vivo* cultured rat aortic SMCs [31]. All these studies have provided evidence for an Ang II-based mechanism underlying the ascending aortic aneurysm.

MAPK pathways have been identified as an important signaling pathway involved in controlling cell growth, proliferation, differentiation, and adaptation [32]. Three parallel MAPK intracellular signaling pathways have been described: JNK, ERK1/2, and p38 MAPK [32]. Yoshimura *et al.* [23] showed that phosphorylated JNK was highly expressed in human abdominal aortic aneurysm tissues. Moreover, the inhibition of JNK in abdominal aneurysm mouse models induced by Ang II and $CaCl_2$ resulted in the prevention of aortic dilatation and medial thinning as well as the preservation of the aortic wall architecture. In addition, human abdominal aortic aneurysm tissue showed significantly elevated levels of phosphorylated ERK compared with control aorta tissues, and ERK knockout mice failed to form elastase-induced abdominal aortic aneurysms [22]. These data demonstrated that JNK and ERK are involved in the pathogenesis of abdominal aortic aneurysms. Our results demonstrated that

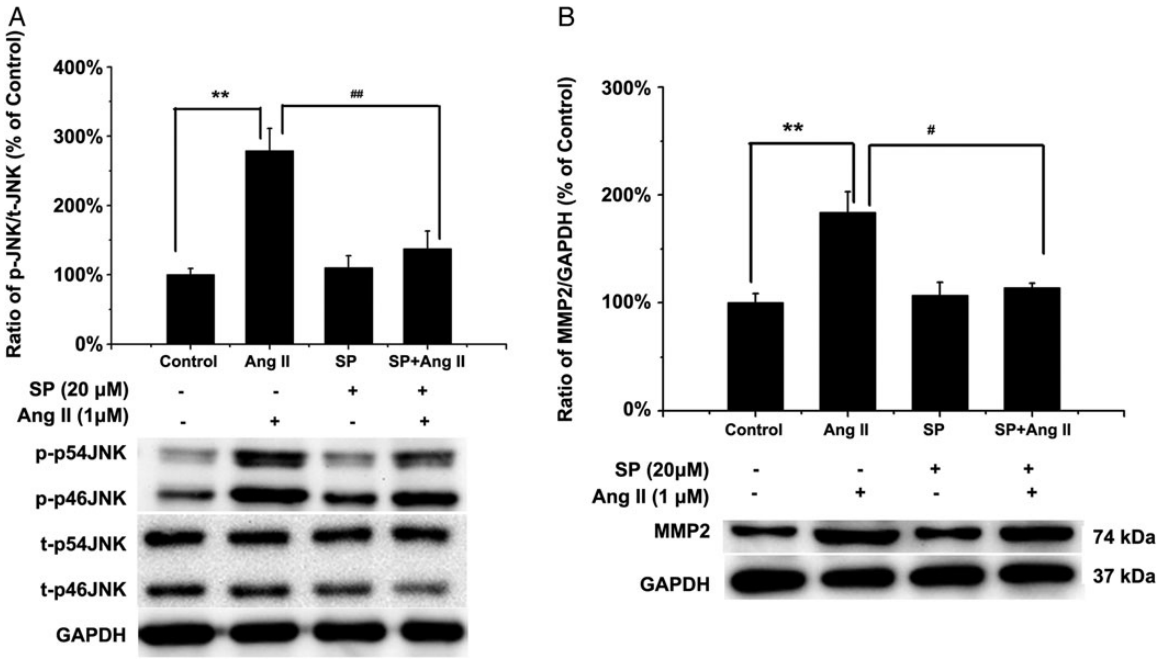

Figure 6. Ang II-induced MMP-2 up-regulation is blocked by JNK signaling pathway inhibition in *ex vivo* cultured IAAA aortic walls Aortic walls from IAAA patients ($n=5$) were cut into small pieces and stimulated with 1 μM Ang II with or without pre-treatment of JNK inhibitor SP600125 (SP), and then aortic pieces were lysed and analyzed for the expression levels of p-JNK, t-JNK (A) and MMP-2 (B) by western blotting. Data are expressed as percentages of control. $^{**}P<0.01$ vs. untreated control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Ang II alone.

phosphorylated JNK and ERK were significantly higher in IAAAs than in normal aorta. Moreover, p-JNK/t-JNK and p-ERK/t-ERK were also markedly increased in IAAAs. However, p-p38 was not elevated in IAAAs. These results suggested that p-JNK and p-ERK might be involved not only in abdominal aortic aneurysm formation but also in IAAA initiation.

In our experiments, the expressions of Ang II and MMP-2 were found to be significantly increased in IAAAs. However, the relationship between Ang II and MMP-2 in IAAAs remains unclear. Whether Ang II participates in MMP-2 production by VSMCs in aortic tunica media remains to be elucidated. This is the first study which demonstrates that Ang II up-regulates MMP-2 protein expression in a dose-dependent manner in *ex vivo* cultured human IAAA walls. The increase in MMP-2 protein level was observed under the treatment of Ang II ranging from 0.1 to 10 μM. Nagasawa *et al.* [24] reported that Ang II could induce MMP-9 up-regulation in *ex vivo* cultures from IAAA tissues. Wang *et al.* [33] demonstrated that total MMP-2 protein expression and activity in the carotid artery were significantly increased in young rats infused with Ang II and that Ang II increased the MMP-2 activity in *ex vivo* cultured young rat carotid arterial rings. Additionally, MMP-2 expression and activity were increased in Ang II-induced abdominal aortic aneurysms in LDLR −/− or ApoE −/− mice [34,35].

Furthermore, we also demonstrated that the Ang II-induced increase in MMP-2 expression was specifically mediated by the AT1R, because the AT1R inhibitor candesartan prevented Ang II-induced MMP-2 expression in an *ex vivo* culture model obtained from IAAAs. However, the AT2R antagonist PD123319 had no effect on MMP-2 expression. These findings were consistent with a previous study which demonstrated that the increased MMP-2 activity was completely inhibited by the AT1R antagonist losartan in Ang II-treated *ex vivo* cultured carotid arterial rings from young rats [33].

Moreover, Ang II-induced MMP-2 expression in IAAAs is dependent on the JNK signaling pathway. Our results showed that the JNK inhibitor SP600125 could significantly attenuate Ang II-induced MMP-2 up-regulation, while the p38 MAPK inhibitor SB203580 and the ERK antagonist PD98059 showed no effect on MMP-2 up-regulation (**Supplementary Fig. S1**). JNK was markedly activated in aortas of Ang II-induced abdominal aortic aneurysm mice [34]. Although p-ERK was markedly elevated in IAAAs, this protein may not be involved Ang II-elicited MMP-2 up-regulation in *ex vivo* cultured IAAA walls. Because our IAAA sample size was rather small, this enhanced ERK1/2 phosphorylation in IAAAs needs to be confirmed by further investigation in large sample size. In the present study, it was found that exogenous SP600125 alone did not lead to a decrease in endogenous MMP-2 expression in cultured IAAA walls. However, Yoshimura *et al.* [23] found that SP600125 alone could lower the basal and TNF-α-induced secretion of MMP-9 in *ex vivo* human abdominal aortic aneurysm tissue. Taken together, Ang II-induced MMP-2 expression may play a critical role in the pathogenesis of IAAAs. Therefore, understanding the mechanisms underlying Ang II-induced MMP-2 expression in IAAAs may be important for the development of new therapeutic strategies.

In summary, our results demonstrated that MMP-2 expression and Ang II concentrations were significantly increased in IAAAs. Ang II stimulation caused an increase in MMP-2 expression via the JNK signaling pathway in *ex vivo* cultured IAAAs, suggesting that the JNK signaling pathway is an important modulator of MMP-2 during IAAA formation. Inhibiting the expression or phosphorylation of JNK isoforms may be a potential therapy to prevent IAAA formation.

Supplementary Data

Supplementary data is available at *ABBS* online.

Acknowledgements

The authors would like to thank Dr Wang H and Dr Li L for their technical assistance.

Funding

This work was supported by a grant from State Key Laboratory of Cardiovascular Disease, Chinese National Center for Cardiovascular Diseases (No. 2014-11).

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