

Original Article

Kaempferol attenuates angiotensin II-induced vascular fibrosis involving the jnk and ERK1/2 pathways

Xia Chen, Li Zhang, Guolin Wu, Hongsong Li, Fangliang Zhang, Xiangdong Xu

Department of Cardiology, Shanghai Jiading District Central Hospital, Shanghai 201800, China

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Abstract: The objective of the study was to investigate the effect of kaempferol on angiotensin II (Ang II)-induced vascular fibrosis. In vascular smooth muscle cells (VSMCs), Ang II induced cell proliferation in dose- and time-dependent manners. Ang II up-regulated the expression of collagen I (Coll I) and fibronectin (FN), and increased intracellular ROS production and expression of gp^{91phox}. Kaempferol attenuated the effects of Ang II on VSMCs. Ang II significantly induced the JNK and ERK1/2 pathways in VSMCs, and the activation was inhibited by kaempferol treatment. In addition, Ang II induced high blood pressure in rats. Expression of Coll I and FN in aorta, and ROS and MDA levels in serum and aorta were also increased in the Ang II-infused rats. Treatment with kaempferol prevented vascular fibrosis of Ang II induced hypertension in rats. These findings demonstrated that kaempferol attenuated Ang II-induced vascular fibrosis. The JNK and ERK1/2 pathways were involved in the protective effect of kaempferol.

Keywords: Kaempferol, angiotensin II, vascular fibrosis, hypertension, JNK, ERK1/2

Introduction

Hypertension is a worldwide medical condition with increased morbidity and mortality. According to some statistics, approximately 7.1 million deaths per year can be directly attributed to hypertension [1]. It is estimated that 1.56 billion people will suffer from hypertension by 2025 [2]. Vascular fibrosis is a major complication of hypertension, which is characterized by excessive accumulation of extracellular matrix (ECM) in the vessel wall including structural proteins and adhesive proteins [3]. Fibrosis contributes to vascular remodeling and increases peripheral resistance and blood pressure [4]. Thus, regression of vascular fibrosis is valuable for alleviating hypertension.

Angiotensin II (Ang II) is an humoral factor that plays a major role in vascular remodeling and perivascular fibrosis [5]. Ang II has been proven to induce vascular fibrosis, and blockade of Ang II receptor inhibits vascular fibrosis in rats [6, 7]. Ang II binds to AT1 receptor and acts its biological functions through several signaling pathways [8]. Among the many pathways, MAPK family is one of the most important Ang II

relative pathways [9]. It has been reported that three of major MAPK, including JNK, p38, ERK1/2 can be induced by Ang II to influence cell survival, apoptosis, differentiation [10].

Kaempferol is a flavonoid compound, which is found in vegetables, fruits and herbs [11]. Numerous studies have shown that kaempferol possesses a wide range of pharmacological activities, such as anti-cancer, anti-oxidative, neuroprotective and cardioprotective [12, 13]. However, the molecular mechanism of kaempferol on hypertension is still unknown.

In the present study, the protective effect of kaempferol on Ang II-induced vascular fibrosis was evaluated. The results showed that kaempferol attenuated the hypertension induced by Ang II, and alleviated vascular fibrosis. Our finding indicated that kaempferol might be a promising candidate for preventing hypertension.

Material and methods

Cell isolation and culture

Rat primary vascular smooth muscle cells (VSMCs) were isolated from thoracic aorta of

10 weeks old Sprague-Dawley rats (Charles River Laboratories, Beijing, China) as described previously [14]. VSMCs were cultured in smooth muscle cell basal medium supplemented with VSMC growth kit (ATCC). For the evaluation of the dose- and time dependent effect of kaempferol on VSMCs, cells were pretreated with increasing concentrations of kaempferol (6.25, 12.5, 25, 50, 100, 200 $\mu\text{mol/L}$, Sigma, USA) for different time (0, 1, 2, 4, 6 h) and subsequently stimulated with Ang II (0.1 $\mu\text{mol/L}$, Sigma, USA) for 48 h. For other experiments, VSMCs were pretreated in the presence or absence of kaempferol (100 $\mu\text{mol/L}$) for 6 h and then stimulated with Ang II.

Cell proliferation assay

Cell proliferation was measured by BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions. Briefly, VSMCs were cultured in 96-well plates for 48 h, and BrdU was added to the medium. The cells were fixed by fixing solution followed by incubation with detection antibody solution. After adding the HRP-conjugated secondary antibody solution, tetramethylbenzidine (TMB) substrate was added. Finally, the plates were measured at 450 nm by a micro-plate reader (Bio-Rad, Hercules, CA).

RT-PCR

Total RNA of VSMCs was extracted using Trizol reagent (Invitrogen, CA, USA) and reverse transcribed to cDNA by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, IN, USA). The primers of fibronectin (FN), collagen (Coll I) and GAPDH were synthesized by Sangon (Shanghai, China). Primers used for amplification were as follows: FN forward 5'-GTGAA GAACG AGGAG GATGT G, reverse 5'-GTGAT GGCGG ATGAT GTAGC; Coll I forward 5'-TGCCG TGACC TCAAG ATGTG, reverse 5'-GGTCT GTTTC CAGGG TTG; GAPDH forward 5'-ACCAC AGTCC ATGCC ATCAC, reverse 5'-TCCAC CACCT GTTGC TGTA. SYBR Green Gene Expression Assay (Qiagen, Valencia, CA) was used for the quantitative PCR. The relative quantification of gene expression was calculated as fold changes according to the $2^{-\Delta\Delta C_t}$ method and was normalized by GAPDH in each sample.

Western blotting

Protein concentration was assayed using the micro-BCA protein assay kit (Pierce, Rockford, IL). Proteins were separated by 10% SDS-PAGE

and transferred to a nitrocellulose membrane (Amersham Pharmacia, Germany). Then, membrane was blocked with 5% nonfat milk in PBST buffer at room temperature for 1 h. The membrane was hybridized with 1:2000 dilutions of primary antibodies against FN, Coll I, JNK, p-JNK, p38, p-p38, ERK, p-ERK, gp91^{phox} and β -actin (Sigma, CA) at 4°C overnight. After washing for five times, membrane was incubated with goat anti-mouse immunoglobulin (1:5000, Sigma, CA) at room temperature for 1 h. Chemiluminescence detection was performed using an Bio-Rad ChemiDoc MP Imaging System. The densitometric analysis was carried out using Image J software.

Measurement of ROS in VSMCs

The intracellular ROS in VSMCs was assessed using the H2DCF-DA assay. Briefly, VSMCs with different treatments were washed twice with PBS, and then stained with 10 μM H2DCF-DA for 30 min at 37°C. The DCF fluorescence was detected by a fluorescence microscope (Olympus BX51, Japan) at 488 nm excitation and 525 nm emission wavelength. The mean fluorescence intensity of each group was calculated from five fields.

Animal models

Fifteen male Sprague-Dawley rats (210-250 g, Charles River Laboratories) were kept in a temperature-controlled room, with a 12-12 h light/dark cycle. Animals were fed with standard rat chow and water. All procedures in this study were followed the standard guidelines for animal care and protection. The rats were randomly separated into 3 groups: 1) control group (physiologic saline); 2) Ang II treatment group; 3) kaempferol + Ang II treatment group. Miniosmotic pumps (model 2002, Alza Corp., USA) were inserted subcutaneously for the infusion of Ang-II (100 ng/kg/min) or physiologic saline. Kaempferol (120 mg/kg/d) was treated by intraperitoneal injection. After 14 days, the rats were anesthetized with pentobarbital (50 mg/kg, Sigma, USA), and the blood was collected by cardiac puncture. Thorax was opened, and the descending aorta was collected for further use.

Detection of blood pressure

Systolic blood pressure (SBP) of the rats was detected by the photoelectric tail-cuff method.

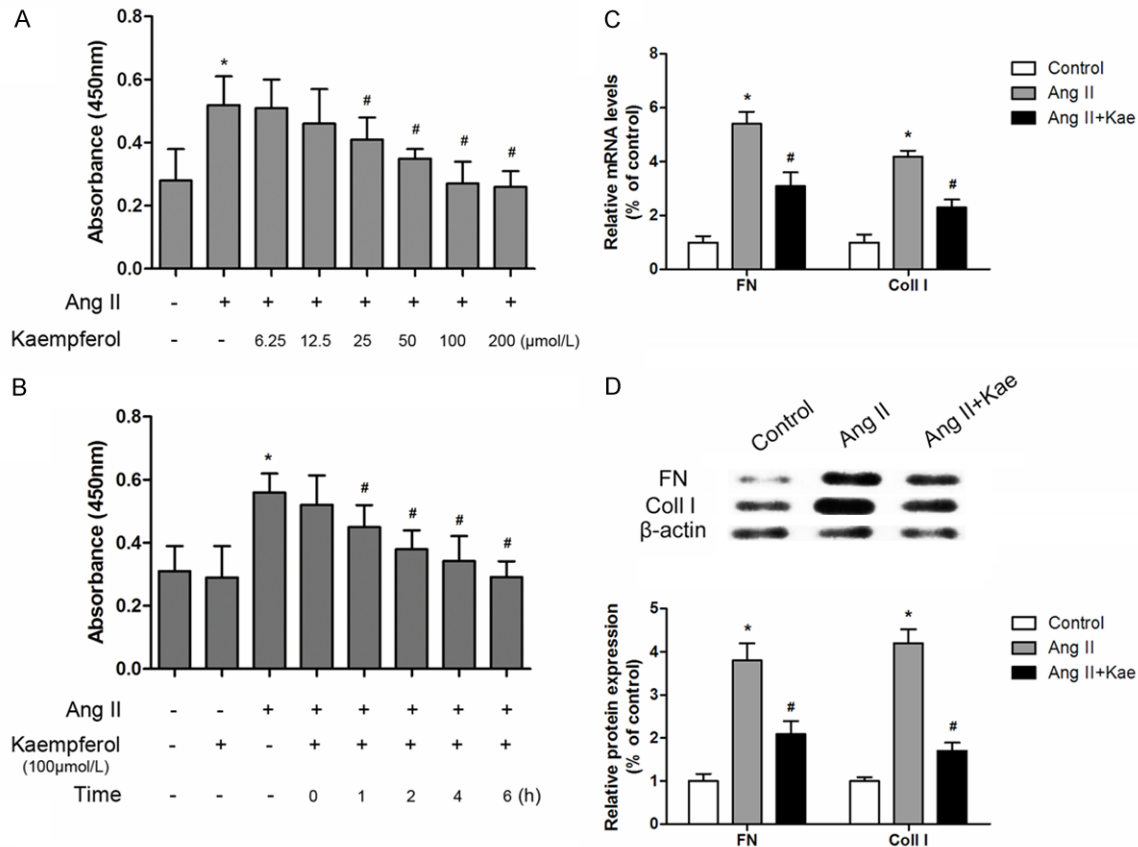


Figure 1. Kaempferol inhibited cell proliferation and fibrosis of VSMCs induced by Ang II. A. Dose-dependent effect of kaempferol on cell proliferation. VSMCs were pretreated with different doses of kaempferol (6.25, 12.5, 25, 50, 100, 200 μmol/L) for 4 h and were stimulated by Ang II. Cell proliferation was measured by BrdU assay. B. Time-dependent effect of kaempferol on cell proliferation. VSMCs were pretreated with kaempferol (100 μmol/L) for different time (0, 1, 2, 4, 6 h) and were stimulated by Ang II. C. mRNA levels of FN and Coll I. VSMCs were pretreated with kaempferol (100 μmol/L) for 6 h and were stimulated by Ang II. The mRNA levels of FN and Coll I were detected by RT-PCR. D. Protein levels of FN and Coll I. The protein levels of FN and Coll I were detected by western blotting. * $P < 0.05$ vs. control group, # $P < 0.05$ vs. Ang II group. Data are represented as mean \pm SD of three dependent experiments.

SBP was recorded at 0, 1, 2, 4, 6, 8, 10, 12 and 14 days after the materials administration.

Detection of MDA and ROS

The aorta was minced and homogenized in four volumes of ice-cold PBS buffer. The homogenate was centrifuged at 9000 g for 20 min at 4°C, and the supernatant was collected. Detection of MDA and ROS in serum and aorta homogenate was performed using commercial kits (Nanjing Jiancheng Bio, Nanjing, China) according to the manufacturer's protocol.

Statistical analysis

All values are expressed as means \pm SD of three independent experiments or five animals. Statistical analysis was performed with Graph-

Pad Prism 6 software (GraphPad Prism Software, Inc.). The significance of differences was evaluated by one-way ANOVA. $P < 0.05$ was considered significant.

Results

Kaempferol inhibited Ang II-induced cell proliferation and fibrosis of VSMCs

Ang II has been shown to play an important role in the development of vascular diseases [10]. As shown in **Figure 1**, Ang II induced cell proliferation of VSMCs and expression of Coll I and FN. Kaempferol, an active flavonoid compound, has been proved to possess protective effect of vascular diseases. The results of **Figure 1A** and **1B** showed that kaempferol inhibited cell proliferation of VSMCs in a dose- and time-

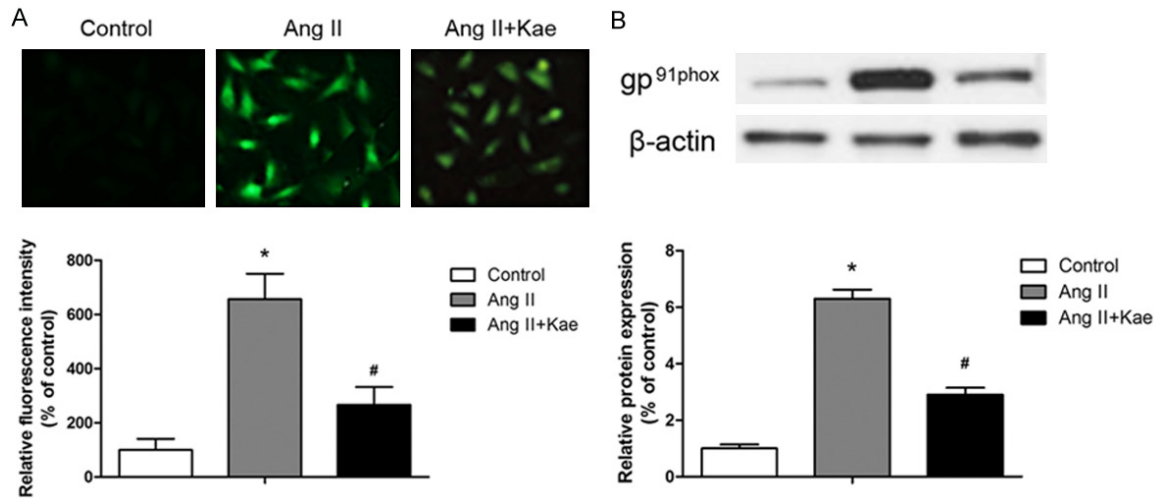


Figure 2. Kaempferol inhibited ROS production and gp^{91phox} expression of VSMCs induced by Ang II. VSMCs were pretreated with kaempferol (100 μmol/L) for 6 h and were stimulated by Ang II. A. The intracellular ROS production in VSMCs was assessed using the H2DCF-DA assay. B. The expression of gp^{91phox} was detected by western blotting. **P*<0.05 vs. control group, #*P*<0.05 vs. Ang II group. Data are represented as mean ± SD of three dependent experiments.

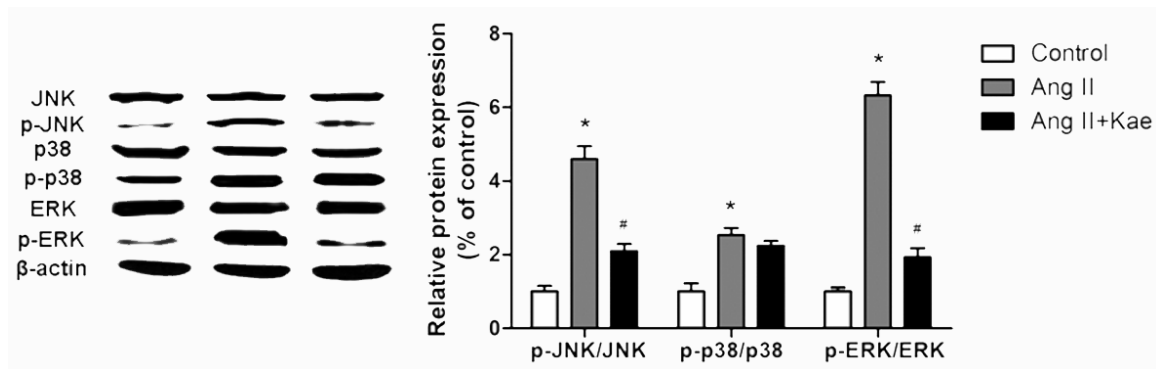


Figure 3. Kaempferol inhibited Ang II-induced activation of MAPK pathway. After cultured with the presence or absence of kaempferol/Ang II, expression of JNK, p-JNK, p38, p-p38, ERK, and p-ERK were detected by western blotting. **P*<0.05 vs. control group, #*P*<0.05 vs. Ang II group. Data are represented as mean ± SD of three dependent experiments.

dependent manner. After determination of dose and pretreated time, VSMCs were pretreated with kaempferol (100 μmol/L) for 6 h and were stimulated by Ang II. The mRNA and protein levels of FN and Coll I were detected. RT-PCR and western blotting assays showed that kaempferol also inhibited the induction of Coll I and FN expression, indicating kaempferol inhibited fibrosis of VSMCs induced by Ang II.

Kaempferol suppressed Ang II-induced oxidative stress in VSMCs

Since oxidative stress plays an essential role in VSMCs dysfunction, the effect of kaempferol

on oxidative stress in Ang II-induced VSMCs. The intracellular ROS in Ang II-treated VSMCs was increased by 6.57-fold, compared to the control VSMCs (**Figure 2A**). Kaempferol significantly inhibited the Ang II-induced ROS production. Expression of gp^{91phox}, a key subunit of NADPH oxidase, was induced by Ang II, and the induction was attenuated by kaempferol (**Figure 2B**).

Kaempferol inhibited Ang II-induced activation of ERK1/2 and JNK in VSMCs

MAPK pathway was reported to be involved in the effect of Ang II on VSMCs [15]. The path-

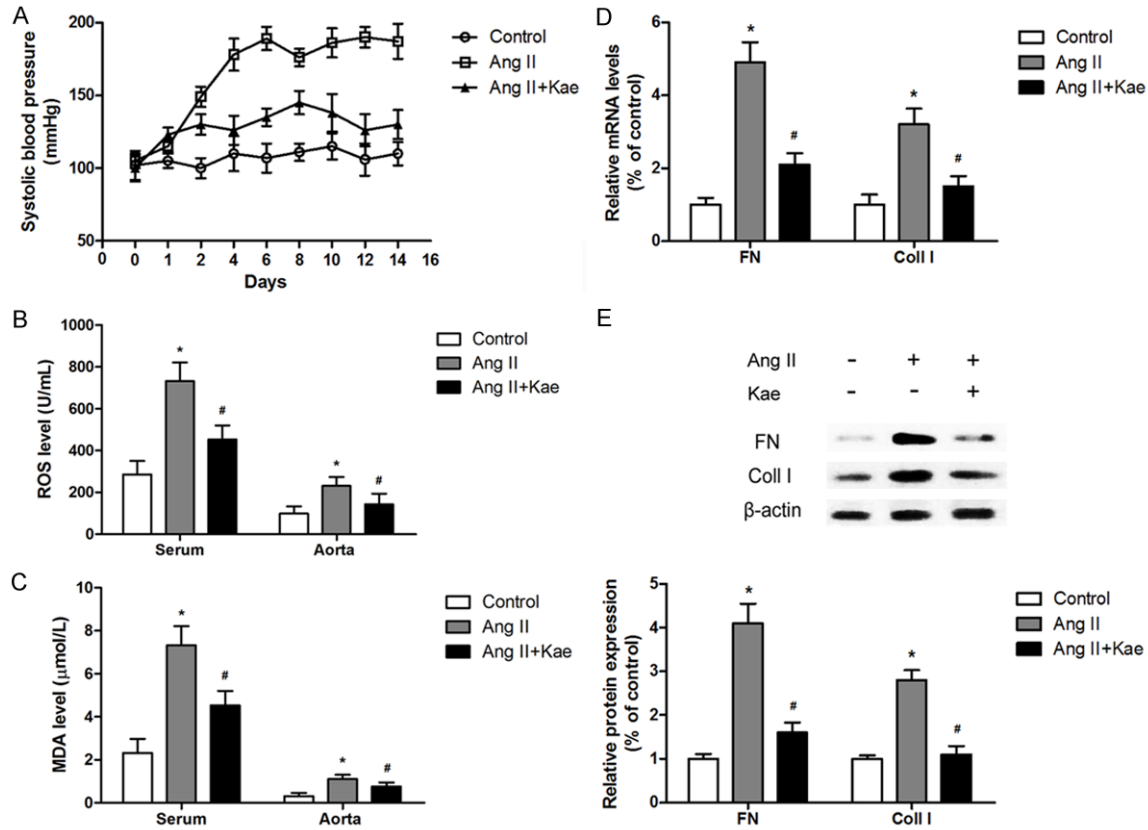


Figure 4. Kaempferol attenuated Ang II-induced vascular fibrosis in rats. The rats were infused with Ang-II (100 ng/kg/min) and administrated with kaempferol (120 mg/kg/d) for 14 days. SBP was recorded on 0, 1, 2, 4, 6, 8, 10, 12 and 14 days. After 14 days, the rats were anesthetized with pentobarbital, blood and descending aorta were collected for further use. A. Blood pressure was detected by tail-cuff method. B. ROS levels in serum and aorta. C. MDA levels in serum and aorta. D. mRNA levels of FN and Coll I were detected by RT-PCR. E. Protein levels of FN and Coll I were detected by western blotting. * $P < 0.05$ vs. control group, # $P < 0.05$ vs. Ang II group. Data are represented as mean \pm SD of five animals.

ways involved in the protective effect of kaempferol on Ang II treated VSMCs was explored. We found that Ang II significantly increased the ratio of p-JNK/JNK, p-p38/p38, and p-ERK/ERK in VSMCs, and the activation of JNK and ERK1/2 were blocked by kaempferol (Figure 3), indicating that JNK and ERK1/2 pathways were involved in the effect of kaempferol on VSMCs.

Kaempferol attenuated Ang II-induced hypertension in rats

The effect of kaempferol on Ang II induced hypertension was evaluated in rats. The results in Figure 4 showed that blood pressure of the rats was elevated by treatment with Ang II, while kaempferol alleviated the blood pressure. The mRNA and protein levels of Coll I and FN in aorta were increased in the Ang II-infused rats, compared to the rats in control group.

Kaempferol attenuated the induction of Coll I and FN. ROS and MDA levels in serum and aorta were significantly increased after treatment with Ang II, suggesting that Ang II induced oxidative stress in rats. Treatment with kaempferol alleviated the oxidative stress in Ang II-infused rats.

Discussion

Vascular fibrosis involves accumulation of collagen, FN, and other ECM in the vessel wall during hypertension process. All of these processes are influenced by Ang II, which is an octapeptide hormone [10]. Ang II-induced vascular fibrosis contributes to vascular remodeling. Excessive proliferation of VSMCs is involved in the vascular remodeling and results in a thickened vessel wall, and lead to the exacerbation of hypertension. It has been reported that Ang

II is reported to induce cell proliferation of VSMC [16-18]. Coll I and FN are typical member of ECM, and Ang II is proved to stimulate the expression of Coll I and FN in VSMCs [15, 19-21]. In addition, Ang II was proved to mediate many of its physiological and pathological effects by inducing ROS production through activation of vascular NADPH oxidase [10]. Consistent with these studies, our results showed that Ang II stimulated VSMCs proliferation and expression of Coll I and FN. ROS production and gp^{91phox} expression were also induced by Ang II.

Previous study proved that kaempferol may reduce the risk of cardiovascular diseases [11, 22]. Kaempferol possesses the activity to inhibit cell proliferation, migration, apoptosis of VSMCs [11, 23, 24]. We found that kaempferol inhibited the cell proliferation induced by Ang II in a dose- and time-dependent manner. Besides, our results indicated that kaempferol reduced the accumulation of Coll I and FN in VSMCs, suggesting that kaempferol attenuated Ang II-induced vascular fibrosis. It has been reported that the protective effect of kaempferol on cardiovascular diseases is attributed to its antioxidant and anti-inflammatory effect [25, 26]. Treatment with kaempferol protects endothelial cells from lysophosphatidylcholine induced endothelial damage via its antioxidant effect [27]. In the present study, the increased ROS generation and gp^{91phox} expression in Ang II-induced VSMCs was inhibited by kaempferol.

Ang II mediates its effects in the vascular System via complex signalling pathways, including MAP kinases, tyrosine kinases, and RhoA/Rho kinases [10]. Among those pathways, MAPK family plays an important role in hypertension-associated vascular fibrosis [28-30]. MAPK is also closely related to oxidative stress [31]. Ang II has the ability to activate all three major MAPKs, including JNK, p38 and ERK1/2 in VSMCs [15, 32], which is consistent with our results. It has been shown that kaempferol possesses an inhibitory effect on the phosphorylation of JNK, p38 and ERK1/2 [33, 34]. In the present study, we tested the hypothesis that MAPK pathway is involved in the effect of kaempferol on vascular fibrosis. The results showed that kaempferol suppressed the activation of JNK and ERK1/2 induced by Ang II, while its effect on phosphorylation of p38 is not

obvious. The results indicated that JNK and ERK1/2 pathways were involved in the effect of kaempferol on Ang II-induced vascular fibrosis in VSMCs.

In summary, the present study evaluated the effect of kaempferol on Ang II induced vascular fibrosis both *in vitro* and *in vivo*. The results showed that Ang II induced cell proliferation and expression of Coll I and FN in VSMCs, while kaempferol attenuated the effect of Ang II on VSMCs. The increased ROS generation and gp^{91phox} expression in Ang II-induced VSMCs was inhibited by kaempferol. We also found that kaempferol inhibited the phosphorylation of JNK and ERK1/2 in Ang II-induced VSMCs. The animal model of hypertension was built in rats by infusion of Ang II. Treatment with kaempferol prevented vascular fibrosis of Ang II induced hypertension in rats. These findings indicated that JNK and ERK1/2 pathways partially contributed to the protective effect of kaempferol on Ang II-induced hypertension and associated vascular fibrosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiangdong Xu, Department of Cardiology, Shanghai Jiading District Central Hospital, Chengbei Rd. 1, Jiading District, Shanghai 201800, China. Tel: +86 21 69987008; Fax: +86 21 69987008; E-mail: xiangdongxu721@163.com

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