Introduction

Cardiovascular disease (CVD) continues to be one of the main causes of death in the world (1). Hypertension is a highly prevalent disorder and a major risk factor for cardiovascular diseases (2). Vascular remodeling (VR) occurs during normal angiogenesis and under various pathological situations, including hypertension (3). VR is an active process of structural alteration that involves changes in at least four cellular processes — cell growth, cell death, cell migration, and the production or degradation of the extracellular matrix — and is the common pathological basis of a variety of cardiovascular diseases (4). The adventitia plays an essential role in the regulatory systems that control VR (5). As the main cell types in the adventitia, adventitial fibroblasts (AFs) can differentiate into myofibroblasts (MFs), migrate, proliferate, and secrete cytokines and play a critical role in the adventitial response to injury (6). Activated AFs exhibit different functional characteristics that contribute significantly to VR. Therefore, the study of AFs bioactivity and the possible mechanisms underlying adventitial activation may provide a potential therapeutic strategy for vascular diseases.

Safflower yellow (SY), a natural chalcone compound extracted from Carthamus tinctorius L., is used extensively in traditional Chinese medicine for the treatment of ischemic cardiocerebrovascular disease (7). Many investigations have found that SY has numerous pharmacological effects; for example, it can inhibit the conglomeration of hematoblasts efficaciously and exhibit anti-inflammatory, anti-allergic, and anti-cancer activities, as well as a protective effect against cardiovascular diseases (8). Injectable SY has been widely used in Chinese medicine and was approved as a new drug by the State Food and Drug Administration (SFDA) for treating patients with ischemic cardiocerebrovascular disease in 2005 (9).
Previous studies have shown that safflower yellow B could significantly protect endothelial cells from angiotensin II (Ang II)-induced cell damage (10). SY has been shown to reverse VR in spontaneous hypertensive rats and influence plasma renin activity and Ang II levels (11). However, its detailed mechanisms require further study to provide more scientific evidence for the clinical treatment of VR.

Ang II is well known to play an important role in cardiovascular diseases through the regulation of cell growth, inflammation, and fibrosis (12). Ang II, acting through the local renin-angiotensin system, may be involved in the regulation of vascular growth associated with hypertension, atherosclerosis, and vascular injury. In cultured AFs, Ang II stimulates the proliferation (13) and migration of AFs (14) and myofibroblast formation (15). Ang II also regulates extracellular matrix (ECM) remodeling in cardiac fibroblasts by increasing the synthesis of collagen and fibronectin, promoting the accumulation of proteoglycans and increasing the expression of TGF-β (16).

The aim of this study was to investigate the effects of SY on cell proliferation, migration, apoptosis, and the expression of ERK1/2, p-ERK1/2, and collagens I and III in Ang II–stimulated AFs. This study provides the first direct evidence in VR that SY inhibits Ang II–induced AF proliferation and migration, thereby regulating ECM synthesis.

**Materials and Methods**

**Antibodies and reagents**

Safflower yellow was purchased from Yongning Pharma (Zhejiang, China) with 98.0% purity. The AT1-receptor blocker losartan and the AT2-receptor blocker PD123319 were purchased from Sigma (St. Louis, MO, USA). Mouse anti-ERK monoclonal antibody, mouse anti-Collagen-I monoclonal antibody, mouse anti-Collagen-III monoclonal antibody, mouse anti-pERK monoclonal antibody, mouse anti-AP-1 monoclonal antibody, and mouse anti-β-actin monoclonal antibody were obtained from Abcam (Cambridge, MA). HRP-conjugated goat anti-mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Fluorescein isothiocyanate (FITC)-Annexin V / Propidium iodide (PI) apoptosis assay kit was from Bio-Rad (Hercules, CA, USA). The enhanced chemiluminescence Western blot detection reagents were from Pierce (Rockford, IL, USA). Dimethyl sulfoxide (DMSO), propidium iodide (PI), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Abcam (Cambridge, UK).

**Adventitial fibroblasts culture**

Animal experiments conformed to the guidelines issued by the Institute of Henan Provincial People’s Hospital for Laboratory Animals. The present study was performed with approval from the Animal Ethics Committee of the Institute of Henan Provincial People’s Hospital. All surgery was performed under sodium pentobarbital anesthesia (Sigma), and all efforts were made to minimize suffering. AFs were harvested from thoracic aortas of male Sprague-Dawley rats (Shanghai Laboratory Animal Center, the Chinese Academy of Sciences) as described previously (17), with minor modifications. They were grown in DMEM containing 110 mg/L of sodium pyruvate (Gibco, Rockville, MD, USA) and 1000 mg/L of l-glutamine and d-glucose supplemented with 10% FBS (heat-inactivated; Millipore, Boston, MA, USA) and 1% penicillin/streptomycin (Abcam). The medium was changed every 2 – 3 days, and the cells were passaged with trypsin-EDTA (Sangon, Shanghai, China) when they became confluent. All experiments were performed with fibroblasts at passages 2 – 3. AFs were grown to 80% confluence and serum starved for 24 h in serum-free medium before treatment with SY.

**Experimental groups**

The AFs were divided into seven groups as follows: –Control, AFs treated with serum-free DMEM; Ang II group, AFs induced by Ang II at 1.9 × 10^{-11} M; positive control group (PC), AFs treated with SY (25 μg/mL) added to serum-free DMEM; losartan + Ang II group, AFs treated with losartan (10 μM) + Ang II (1.9 × 10^{-11} M) added to serum-free DMEM; PD123319 + Ang II group, AFs treated with PD123319 (10 μM) + Ang II (1.9 × 10^{-11} M) added to serum-free DMEM; SY (12.5 μg/mL) + Ang II group, AFs treated with SY (12.5 μg/mL) + Ang II (1.9 × 10^{-11} M) added to serum-free DMEM; SY (25 μg/mL) + Ang II group, AFs treated with SY (25 μg/mL) + Ang II (1.9 × 10^{-11} M) added to serum-free DMEM.

**Determination of AF proliferation**

AF proliferation was measured using the previously described MTT assay. The AFs were seeded in 96-well flat-bottom microtiter plates (Pierce) at a density of 4 × 10^4 cells per well for 48 h, and AFs were treated with various concentrations of SY, losartan (10 μM), or PD123319 (10 μM) for 24 h, respectively; and then then stimulated with 1.9 × 10^{-11} M Ang II for an additional 24 h. An MTT solution (5 mg/mL) was added at the end of incubation, and the AFs were incubated for 4 h at 37°C. The medium was removed, and dimethyl sulfoxide (DMSO) was added to each well. The absorbance value in the 96-well plate was read spectrophoto-
metrically at 570 nm on a microtiter plate reader (Merek, Whitehouse Station, NJ, USA).

**AFs migration assay**

The migration assay was performed using the transwell system (8.0 μm; Millipore, BD Biosciences, San Jose, CA, USA). AFs were grouped and treated as described above. The cells were trypsinized and counted, and then 5 × 10⁴ cells were seeded in each cell culture insert. Serum-free cell culture medium (400 μL) was added to each insert. One milliliter of medium with 5% FBS was added to the 6-well plate, and then the insert was transferred to the well and cultured at 37°C with 5% CO₂. After 6 h of incubation, the number of cells that had migrated through the pores was quantified by counting 5 independent visual fields under the microscope (Olympus, Tokyo) using a 10 × objective. Migrated cells were stained with Giemsa dye for observation (18).

**Analysis of AFs apoptosis**

The cell apoptotic ratio was measured by Annexin V-FITC and PI staining followed by analysis with flow cytometry (Biovision, Milpitas, CA, USA) according to the manufacturer’s instructions. After starvation and synchronization for 4 h, cells were treated with 12.5 mg/mL SY, 25 mg/mL SY, losartan (10 μM) or PD123319 (10 μM) for 24 h and then stimulated with 1.9 × 10⁻¹¹ M Ang II for an additional 24 h. Cells were trypsinized and harvested by centrifugation and then incubated with Annexin V and PI for 15 min at room temperature. After incubation in the dark for 1 h, cells were subjected to flow cytometry and the rate of cell apoptosis was determined.

**RT-PCR**

AFs were treated with (12.5 μg/mL, 25 μg/mL) SY, losartan (10 μM), or PD123319 (10 μM) for 24 h and then stimulated with 1.9 × 10⁻¹¹ M Ang II for an additional 24 h. The PC group was treated only with 25 μg/mL SY. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The primer sequences used for PCR amplification were Collagen I: forward, 5′-TCC CCAGCCACAAAGAGTCTACA-3′ and Reverse, 5′-GTGATTTGGTGGGATGTCTTCGTC-3′; Collagen III: forward, 5′-CTGCCATCCTGAACTCAAGAGTGG-3′ and reverse, 5′-CCATCCCTCAGAACATGTGTAGG-3′ (19). Reverse transcription and PCR were performed using a Takara RNA PCR (AMV) kit with primers designed for murine genes. The real-time measurements were performed with a Rotor-Gene 2000 Real-Time Cycler instrument (Invitrogen). Samples were analyzed in triplicate. The amplification protocol used was as follows: an initial 3 min denaturation at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 74°C for 45 s. Concentrations of samples were calculated by using Rotor-Gene 2000 software (Invitrogen).

**Western blotting**

Western blot analysis was performed as previously described. After treatment, cells were lysed in cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Extracts or proteins were separated by SDS-PAGE followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes and probed with appropriate primary and secondary antibodies. An enhanced ECL kit and a film were used to visualize protein bands that had reacted with antibodies. Protein levels were semi-quantified using the Image-Pro Plus software (Pierce) and adjusted by a loading control beta-actin. Each experiment was repeated at least 3 times.

**Statistical analyses**

The results are presented as the mean and standard error of the mean (S.E.M.); data were analyzed with SPSS16.0 (SPSS, Inc., Chicago, IL, USA). Statistical analyses involved the use of Student’s t-test for the comparison of 2 groups or 1-way ANOVA for multiple comparisons. P < 0.05 was considered to be significant. All experiments were repeated at least 3 times.

**Results**

**SY treatment suppresses AFs proliferation**

To evaluate the effects of the SY on cell proliferation, AFs were treated with Ang II for 24 h. Cell proliferation was measured with the MTT assay. The number of surviving cells is directly proportional to the level of formazan product. As shown in Fig. 1, the OD₅₇₀ was significantly higher in the Ang II group than in the control group (P < 0.01). However, a significant down-regulation of OD₅₇₀ was observed in the various concentrations of SY + Ang II groups compared with the Ang II group (P < 0.01 or 0.05) in a dose-dependent manner. Ang II–induced increases of OD₅₇₀ was blocked by losartan (10 μM), an AT₁-receptor antagonist, but not PD123319 (10 μM), an AT₂-receptor antagonist. The value of OD₅₇₀ was statistically similar between the control and PC groups (P > 0.05). These observations indicate that SY could effectively, at least in part, suppress the proliferation of AFs.

**SY suppresses AFs migration**

To evaluate the effects of SY on cell migration, AFs were treated with Ang II for 24 h. As shown in Fig. 2, the number of migratory AFs was significantly higher
in the Ang II group (125 ± 15) than in the control (60 ± 5) group (P < 0.01). However, a significant down-regulation was observed in the SY (12.5 µg/mL) (82 ± 6), SY (25 µg/mL) (75 ± 10), and losartan groups (61 ± 12) compared with the Ang II group (P < 0.05). Losartan (10 µM) but not PD123319 (10 µM) attenuated Ang II-induced migration of AFs. The number of migratory AFs were statistically similar in both control and PC groups.

**SY induces AFs apoptosis**

We next examined whether the growth inhibitory effect of SY was due to the induction of cell apoptosis. The percentages of apoptotic cells were further determined by flow cytometric analysis, following FITC-annexin V/PI staining. Figure 3 shows that the percentage of apoptotic cells was significantly decreased in the Ang II group compared to the control group (P < 0.01). There was a noticeable up-regulation of apoptosis in the SY (25 µg/mL, 12.5 µg/mL) + Ang II groups and losartan group but not the PD123319 group compared to the Ang II group (P < 0.05). The percentage of apoptotic cells was not significantly different between the control and PC groups (P > 0.05).

**Effects of SY on ECM**

Several mechanisms have been implicated in VR increased cell proliferation and altered ECM turnover (20). To evaluate the effects of SY on the ECM synthesis, we measured the protein and mRNA expression of collagen I and collagen III, which constitute the bulk of the ECM. As shown in Fig. 4, the levels of the collagen I and collagen III proteins and mRNA were increased in the Ang II group compared to the control group (P < 0.01). In the SY (25 µg/mL, 12.5 µg/mL) + Ang II groups and losartan group, this increase was significantly inhibited compared to the Ang II group (P < 0.05). The levels of the collagen I and collagen III proteins and mRNA were not significantly different between the control and PC groups, and no significant difference was also observed between the Ang II group and PD123319 group (P > 0.05) (data was not shown).

**Effects of SY on Ang II/ERK/AP-1 signaling pathway**

A previous study showed that activated ERK leads to collagen I and collagen III and AP-1 gene expression (21). To determine the mechanism of SY on the Ang II–induced cell proliferation, migration, and expression of collagen I and collagen III, ERK1/2, p-ERK1/2, and AP-1 protein expression levels were examined by western blot analysis. Figure 5 shows that the protein levels of p-ERK1/2 and AP-1 were increased in the Ang II group compared to the control group (P < 0.01). There was a noticeable down-regulation of p-ERK1/2 and AP-1 protein expression in the SY (25 µg/mL, 12.5 µg/mL) + Ang II groups and losartan group compared to the control group (P < 0.05). The level of ERK1/2 protein was statistically similar in all groups.
Fig. 3. The effects of SY on AF apoptosis. AFs were treated with Ang II for 24 h. Cell apoptosis was measured by flow cytometry. A) Graph of apoptosis. B) Columns, mean of data obtained from 3 independent experiments. Bars indicate the mean ± S.E.M. PC group was treated only with 25 μg/mL SY. Losartan (10 μM), an AT₁-receptor antagonist; PD123319 (10 μM), an AT₂-receptor antagonist. *P < 0.01, compared to the Control; *P < 0.05, compared to Ang II; #P > 0.05, compared to the Control; †P > 0.05, compared to Ang II.

Fig. 4. Collagen I and collagen III expression. A) The protein level of collagen I and collagen III were determined by western blot analysis; β-actin was used as a loading control. B) Collagen I and collagen III mRNA expression is detected by RT-PCR. All experiments were repeated at least 3 times. The values shown represent the mean ± S.E.M., *P < 0.01, compared to the control; *P < 0.05, compared to Ang II.
Discussion

This study demonstrated the regulatory effects of Ang II on downstream mediators expressed in cultured AFs. After stimulation with Ang II, ERK1/2 phosphorylation was induced, which is consistent with the observed changes in collagen I and collagen III gene and protein expressions and AP-1 protein expression. In addition, we showed that Ang II up-regulated cell proliferation and migration and decreased apoptosis in AFs. However, all of these changes were reversed by treatment with SY.

AFs play prominent roles in the pathogenesis of neointima formation (22). Ang II is an important vascular peptide involved in VR in the adventitia (23). Ang II promotes proliferation, migration, adhesion, and ECM synthesis in adventitial cells and myofibroblast formation in fibrosis (24). Ang II may play an important role in regulating AF function during VR following arterial injury (25).

SY has a wide range of pharmacological activities, including coronary dilatation, anti-oxidation, myocardial, and cerebral protection and immunosuppressive activity (26). SY for injection was approved as a new drug by the State Food and Drug Administration in 2005 and contains hydroxysafflor yellow A at about 785 mg/g, which is one of the major active chemical components in SY. However, the pharmacological role of SY in the VR has not been studied at a cellular level. This study was designed to assess the role of SY on Ang II–induced AF proliferation and migration and the subsequent regulation of ECM synthesis.

In the present study, we evaluated the effects of SY on the inhibition of Ang II–induced proliferation and migration and the promotion of Ang II–inhibited apoptosis in AFs, along with its mechanism of action. Our studies showed that SY could both inhibit Ang II–induced AF proliferation and migration and induce cell apoptosis. In addition, the mechanisms underlying collagen synthesis and AFs migration during VR might involve the Ang II/ERK/AP-1 signaling pathway.

The roles of ECM (collagen I and collagen III) in VR have been recently studied. However, the exact mechanisms behind the regulators and the underlying mechanisms controlling the expression and activation of these proteins in AFs are poorly understood. It has been shown that Ang II induces an increase in the synthesis of collagen I and collagen III in fibroblasts (27) and ERK-induced collagen I and collagen III expression in cultured renal interstitial fibroblasts (28). In this study, we found that Ang II significantly induced the expression of collagen I and collagen III at both the protein and mRNA level and increased the expression of AP-1 protein in AFs. Therefore, we speculate that Ang II induces the synthesis and secretion of collagens I and III via the AP-1 activation pathway. Further evidence suggests that ERK1/2 signaling is primarily responsible for Ang II–induced AP-1, collagen I, and collagen III expression (29). In this study, we found that expression of the
p-ERK1/2 protein was up-regulated in the Ang II group compared to the control group. The results obtained revealed that the ERK/AP-1 signaling pathway is activated after Ang II stimulation in vitro.

Our results revealed that the proliferation and migration of AFs are upregulated and apoptosis is decreased in the Ang II group compared to the control group. However, these trends were reversed by treatment with SY or losartan. Furthermore, we observed that SY can reduce Ang II–induced effects and the expression of collagen I and collagen III is simultaneously downregulated. Moreover, increased collagen I and collagen III production induced by ERK/AP-1 was abrogated by SY or losartan.

In conclusion, our study indicates that SY exhibits anti-proliferative, anti-migrative, and pro-apoptotic activity in rat aortic AFs, perhaps through the Ang II/ERK/AP-1 signaling pathway. The present findings may provide new clues regarding the potential function of SY to treat or prevent VR.

References