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Asiatic acid inhibits cardiac fibrosis throughNrf2/HO-1 and TGF-β1/Smads signaling pathways in spontaneous hypertension rats☆

Zhe Meng[a], Hai-yu Li[a,*], Chun-ying Si[b], Yu-zhou Liu[a], Shuai Teng[a]

[a] Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China
[b] Department of Cardiology, The First Affiliated Hospital of Henan University of TCM, Zhengzhou, Henan, China

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ABSTRACT

Objective: Asiatic acid (AA) has been suggested to inhibit pulmonary and hepatic fibrosis, while its influence on cardiac fibrosis remains unclear. We aimed to investigate whether AA could inhibit overpressure-induced cardiac fibrosis in spontaneous hypertension rats (SHRs).

Method: SHRs were treated with AA (20 mg kg^{-1} day^{-1}) for 12 weeks and cultured cardiac fibroblasts (CFs) were treated with Ang II (10^{-7} mol/L) in vitro. Markers of oxidative stress were measured and extent of cardiac fibrosis was evaluated with Sirius Red staining. Levels of Superoxide Dismutase (SOD), Malondialdehyde (MDA), reactive oxygen spices (ROS) and Glutathione (GSH) were measured by using commercial assay kits. Collagen deposition was detected. The expression of relative protein and mRNA was measured by Western blot and real-time PCR, respectively.

Results: AA reduced systolic blood pressure, attenuated myocardial hypertrophy, reduced college deposition and the expression of collagen I and III, connective tissue growth factor, and plasminogen activator inhibitor-1, in mRNA and protein levels, with inhibition of TGF-β1 expression, phosphorylation of Smad2/3, and increase of Smad7 expression. AA reduced malondialdehyde and reactive oxygen spices, while increased the activities of superoxide dismutase and glutathione, accompanied with elevation of nuclear translocation of nuclear-factor erythroid 2-related factor 2 (Nrf2) and expression of heme oxygenase (HO-1) and NAD(P)H dehydrogenase [quinone] 1 (NQO-1) in vivo and in vitro. Moreover, pretreating CFs with siRNA for Smad7 or Nrf2 both partially reversed the inhibition of AA on Ang II-induced cardiac fibrosis.

Conclusion: AA attenuates pressure overload-induced cardiac fibrosis via enhancing of Nrf2/HO-1 and suppressing TGF-β1/Smads phosphorylation.

1. Introduction

Cardiac fibrosis, characterized by the proliferation of cardiac fibroblasts (CFs) and the deposition of extracellular matrix (ECM), has been demonstrated to play an important role in the pathogenesis of heart failure caused by various etiologies, including coronary heart disease, hypertension, and valvular heart diseases et al. [1,2]. Accordingly, treatments targeting the attenuation of the progression of cardiac fibrosis have been established as potential strategies for the prevention of heart failure. Pathophysiologically, the proliferation and activation of CFs have been suggested to contribute to the progression of cardiac fibrosis, through secreting collagen proteins and other pro-fibrotic factors, including connective tissue growth factor (CTGF) and plasminogen activator inhibitor (PAI)-1, finally leading to the deposition of ECM in the heart [1,3,4]. Overactivated neurohormonal factors, including the angiotensin II (Ang II), has been recognized as a critical stimulator of cardiac fibrosis via inducing proliferation and migration of CFs, cardiomyocyte hypertrophy, and over-secretion of the main components of ECM, including collagen I (Col I), collagen III (Col III), and fibronectin (FN) [3,5,6].

Nuclear-factor erythroid 2-related factor 2 (Nrf2), which belongs to the CNC (cap “n” collar) family of transcription factors, has been indicated to play an important role in the regulation of the oxidative response via modulating the expressions of various antioxidant factors, such as heme oxygenase-1 (HO-1) and antioxidant enzymes NAD (P) H dehydrogenase [quinone] 1 (NQO1), after it is activated and bound to the antioxidant-response elements (ARE) [7,8]. Activation of Nrf2 has been regarded to be beneficial for the attenuation of the oxidative

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* Corresponding author.
E-mail address: lihaiyu156239@126.com (H.-y. Li).

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stress-mediated diseases, including cardiac fibrosis [9–11]. In addition, inhibiting the activation of Nrf2 led to cardiac hypertrophy related left ventricular diastolic dysfunctionin mice [12]. Moreover, growing evidence has proved that TGF-β/Smads signaling also plays a critical role in the procession of cardiac fibrosis. TGF-β1 induces the activation of Smad2/3 via binding to its receptor and therefore exerting the downstream effect of TGF-β1-mediatedc cardiac fibrosis [13]. On the other hand, the activation of Smad7 has been suggested to down-regulate TGF-β1-induced cardiac fibrosis [4]. The increased phosphorylation of Smad2/3 has been proved to up-regulate the deposition of ECM. However, inhibiting the activation of Smad2/3 may be an effective way to attenuate cardiac fibrosis caused by different stimuli [3,4]. In contrast, Smad7 is a negative regulator of TGF-β/Smads signaling pathway, and the protein expression Smad7 is significantly decreased during the pathogenesis of cardiac fibrosis [14]. Some previous studies have shown that enhanced activation of Smad7 can effectively inhibit TGF-β1-mediated fibrosis in heart and other organs [17–19]. Therefore, disturbance of the activation of TGF-β/Smads, especially upregulation of Smad2/3 activation and downregulation of Smad7 expression, plays a critical role in the progression of cardiac fibrosis.

Asiatic acid (AA), is a triterpenoid extracted from *Centella asiatica*, which is a daily drink or vegetable in Southeast Asia and has been used as a treatment for many diseases since ancient times. Currently, accumulating evidence suggests that Asiatic acid confers multiple beneficial effects, including anti-inflammation [20], anti-fibrosis [14–16], and anti-oxidation [21]. Privious studies have reported that asiatic acid ameliorates pulmonary fibrosis via supressing pro-fibrotic and inflammatory signaling pathways, and furthermore, asiatic acid attenuates liver fibrosis by regulating the P13K/AKT/mTOR and Bcl-2/Bax signaling pathways. In the cardiovascular system, AA has been suggested to inhibit left ventricular remodeling and improves cardiac function in the rat model of myocardial infarction and overpressure-induced heart failure [22]. Moreover, in rats treated with doxorubicin, AA has been observed to prevent cardiac function and inhibit ventricle remodeling by elevating Nrf2 activity, accompanied with the suppression of fibrosis in lung and liver via regulating the TGF-β/Smads signaling pathway [23–25]. However, the potential effect of AA on the cardiac fibrosis induced by pressure overload has not been determined, and whether its potential benefits involving the regulation of Nrf2 activation and TGF-β/Smads signal pathways remain unclear. Therefore, we aimed to investigate whether AA could inhibit overpressure-induced cardiac fibrosis in spontaneous hypertension rats (SHRs) and explore the potential molecular pathways involved in this study.

2. Materials and methods

2.1. Chemicals and reagents

Asiatic acid ($C_{30}H_{48}O_5$, MW: 488.70) was purchased from Guangxi Changzhou Natural Products Development Co. Ltd. (Guangxi, China), and the purity is over 95%. 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) penicillin, streptomycin, Tris, and Ang II, were obtained from Sigma Chemical Co (St Louis, MO, USA). Antibodies against Nrf2, HO-1, NQO-1, Histone, Col I, Col III, CTGF, PAI-1, Smad7, TGF-β1, Smad2/3, and phospho-Smad2/3 (p-Smad3) were purchased from Abcam CO (Cambridge, UK). Fetal bovine serum (FBS), EasyS-cript Reverse Transcriptase, Transzol, TransStrat Green QpcrSuperMix, and theβ-actin antibody were purchased from TransGen Biotechnology (Beijing, China).

2.2. Animal experiments

Eight-week-old male Wistar Kyoto (WKY) rats and age-matched male spontaneously hypertensive rats (SHRs) were purchased from Shanghai Laboratory Animal Technique Corp. Normal WKY rats were divided randomly into the control group (n = 10) and the AA group (n = 10), and the latters were treated with AA (20 mg·kg^{-1}·day^{-1}) only. The SHRs were randomly divided into 2 groups (n = 10 each) for treatments with oral AA (20 mg·kg^{-1}·day^{-1}) or saline for 12 weeks, respectively. All rats were housed under similar conditions with a 12 h light/dark cycle at 23 ± 1 °C and humidity 55% ± 5%, with free access to an ordinary diet and water. Systolic blood pressure (SBP) was measured by tail-cuff method in conscious rats. Body weight was measured weekly during the study period. At the end of the 12th week, rats were sacrificed by intra-aortic administration of 10% potassium chloride and an excess amount of pentobarbital. Hearts were subsequently excised and weighed. One portion of the left ventricles were fixed in 4% for maldehyde solution and embedded in paraffin for collagen evaluation, and the remaining left ventricles were snap-frozen in liquid nitrogen and stored at −70 °C for subsequent biochemical assays.

Table 1
Sequences of primers used for the real-time PCR analysis.

Gene	Oligonucleotide primer sequences (5′–3′)
CTGF	Forward: AAGAAGACTCAGCCAGACC
	Reverse: TGGAAAGAAGTCTGAGGAAGG
PAI-1	Forward: CCTCCTCATCCTGCCTAAGTTC
	Reverse: GCCGCTCTCGTTCACCTC
Col I	Forward: ACTCAGCCCTCTGTGCCT
	Reverse: CCTTCGCTTCCATACTCG
Col III	Forward: AGATGCTGGTGCTGAGAAG
	Reverse: TGGAAAGAAGTCTGAGGAAGG
TGF-β1	Forward: GCGACTCCTGCTGCTTTCTC
	Reverse: GTTGTTGCGGTCCACCATTA
GAPDH	Forward: GCCTTCTCCATGGTGGTGAA
	Reverse: GGTCGGTGTGAACGGATTTG

2.3. Histological analysis

The left ventricle were fixed in 10% formalin, paraffin-embadded and sectioned. Left ventricle sections were stained with haematoxylin/eosin (H&E) for histological examination. The collagen deposition in the left ventricle was detected by Sirius Red staining and Masson staining. Ten fields in each region of the heart were selected randomly from four nonconsecutive serial sections, and collagen content was quantified as Sirius Red positive areas by using fluorescence confocal microscopy (Leica TCS SP2-AOB).

2.4. Cell culture

Sprague-Dawley rats (1–2 days old) were purchased from the Key Laboratory of Animal Institute in the School of Medicine ofZhengzhou University. The collagenase and trypsin were used to digest the left ventricle tissue to obtain CFs. Cells were cultured in DMEM with 15% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at37 °C. Both morphologicand immunocytochemicalmethods were used to identify CFs, and the cells within fourpassages were used in the subsequent experiments. When the CFs reached 80–90% confluence, the medium was replaced with serum-free medium, and the CFs were cultured for another24 h before other analyses.

2.5. Cell viability assay

CFs were cultured at a density of 5000 cells/well in 96-well plates, containing 100 μL of DMEM medium with 15% FBS, and then incubated for 12 h. Asiatic acid was dissolved in DMSO. Cell viability was determined by the MTT reduction assay. Following various indicated treatments for 24 or 72 h, the medium was removed and the cells were incubated with MTT solution (5 mg/mL) for 4 h at 37 °C. The dark blue formazan crystals that formed from the intact cells were solubilized with 150 μL of DMSO, and then the absorbance was measured at

Table 2
Effects of AA on SBP and cardiac function in rats from different groups.

Parameter	Control	SHRs	SHRs + AA	Wky + AA
SBP (mmHg)	131 ± 4	198 ± 7[a]	170 ± 5[b]	133 ± 5
BW (g)	367 ± 6	374 ± 7	363 ± 4	371 ± 5
LVW/BW (mg/g)	2.17 ± 0.04	2.31 ± 0.06[a]	2.24 ± 0.05[b]	2.16 ± 0.05
HW/BW (mg/g)	3.20 ± 0.07	3.44 ± 0.08[a]	3.31 ± 0.05[b]	3.22 ± 0.04
Ang II (pg/mL)	121.4 ± 13.5	157.3 ± 14.8	142.6 ± 13.2	124.1 ± 12.6

Data are present as means ± SEM of 10 rats in each group.
WKY: Wistar Kyoto rats; SHR: spontaneously hypertension rats; AA: Asiatic acid; SBP: systolic blood pressure; BW: body weight; LVW/BW: left ventricle weight/body weight; HW/BW: heart weight/body weight; Ang II: angiotensin II.
[a] $P < 0.01$ vs Control.
[b] $P < 0.01$ vs SHRs.

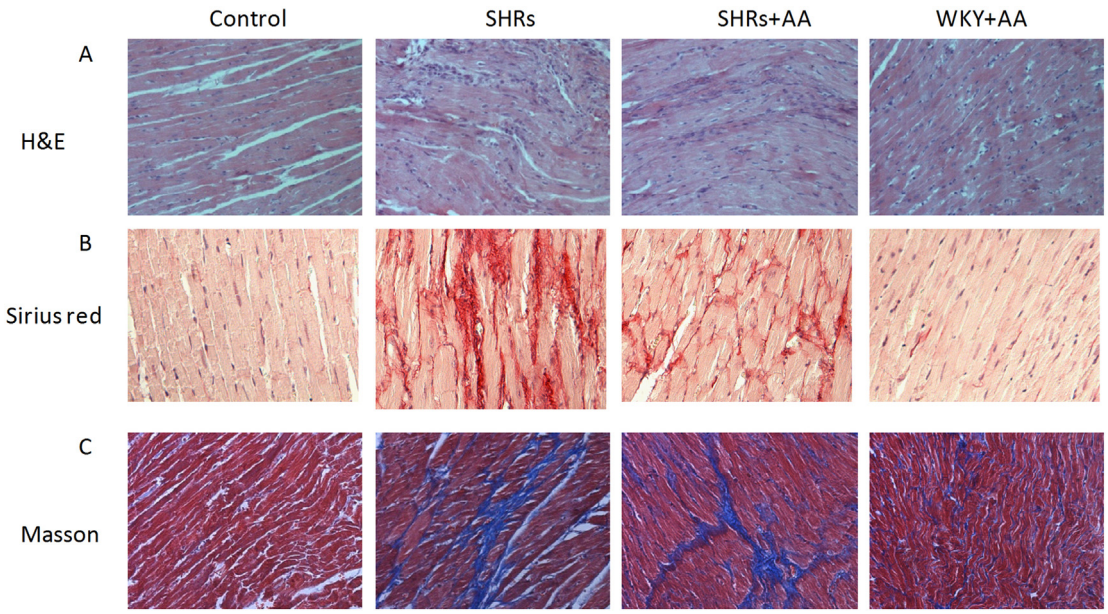

Fig. 1. Effect of AA on collagen deposition in the left ventricle of rats from different groups. WKY rats were used as control group, and SHRs were treated with saline or AA (20 mg kg^{-1} day^{-1}) for 12 weeks by oral gavage. H&*E*-staining (A)、 Sirius red staining (B) and Masson-staining (C) were used to detect the levels of collagen deposition in the left ventricle of different groups. ($n = 10$ rats, each group). Scale bar: 100 μm. WKY: Wistar Kyoto rats; SHRs: spontaneously hypertensive rats; AA: Asiatic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

490 nm witha microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Western blot

Both CFs and the left ventricle tissue were lysed by using 200 μL ice-cold lysis buffer (pH 7.4, 50 mmol/L HEPES, 5 mmol/L EDTA, 100 mmol/L NaCl, 1% Triton X-100, protease inhibitor cocktail; Roche, Mannheim, Germany) in the presence of phosphatase inhibitors (50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 1 nmol/L microcystin). The protein concentration was determinedusing a BCA protein assay kit. Samples underwent 10 or 12% SDS-PAGE and were transferred onto a polyvinylidene difluoride membrane in a semi-dry system, which was subsequentlyblocked with 5% fat-free milk in TBST buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl and 0.1% Tween20), and incubated with primary antibodies for Col I (1:500), Col III (1:500), CTGF (1:400), PAI-1 (1:500), Nrf2 (1:500), HO-1 (1:500), NQO-1 (1:500), TGF-β (1:500), Smad2/3 (1:400), p-Smad2/3 (1:400), Smad7 (1:400), p-Smad7 (1:500), Histone (1:2000) and β-actin (1:4000) respectively. After incubation in TBST buffer overnight, the membranes were washed and incubated with secondary antibodies for 90 min. The optical density of the bands was scanned and quantified using a Gel-Pro Analyzer v4.0 (Media Cybernetics, L.P.). β-actin was used as an endogenous control. Data was normalized to β-actin levels.

2.7. Real-time PCR

After CFs were treated with different stimuli, total RNA was extracted using a Transzol reagent, and the DNA was removed using a DNA-free kit (Ambion, Austin, TX, USA). The quality of mRNA was verified by performing denaturing agarose gel electrophoresis containing 1.5% formaldehyde. The total RNA concentration and purity were determined by UV–Vis spectroscopy using the Bio-Rad SmartSpec 5000 system (Bio-Rad, Hercules, CA, USA). To synthesize cDNA, 1 μg of total RNA was used in a 20 μL reaction system containing oligo(dT)18 Primer and TransScript Reverse Transcriptase. Primers for rat Col I, Col III, CTGF, PAI-1, and GADPH were designed using the Beacon designer v4.0 (Premier Biosoft, CA, USA) (Table 1). GADPH was used as an endogenous control. The mRNA levels of Col I, Col III, CTGF, PAI-1, and GADPH were examined by real-time PCR using the ABI PRISM 7000 sequence detection PCR system (Applied Biosystems, Foster City, CA, USA). A single melting curve peak confirmed the presence of a single product. Results were expressed as fold differences relative to β-actin using the $2^{-\Delta\Delta CT}$ method.

2.8. Small-interfering RNA

CFs (5×10^6) were cultured in 6-well plates and grown to about 70% to 80% confluence, and then transiently transfected with

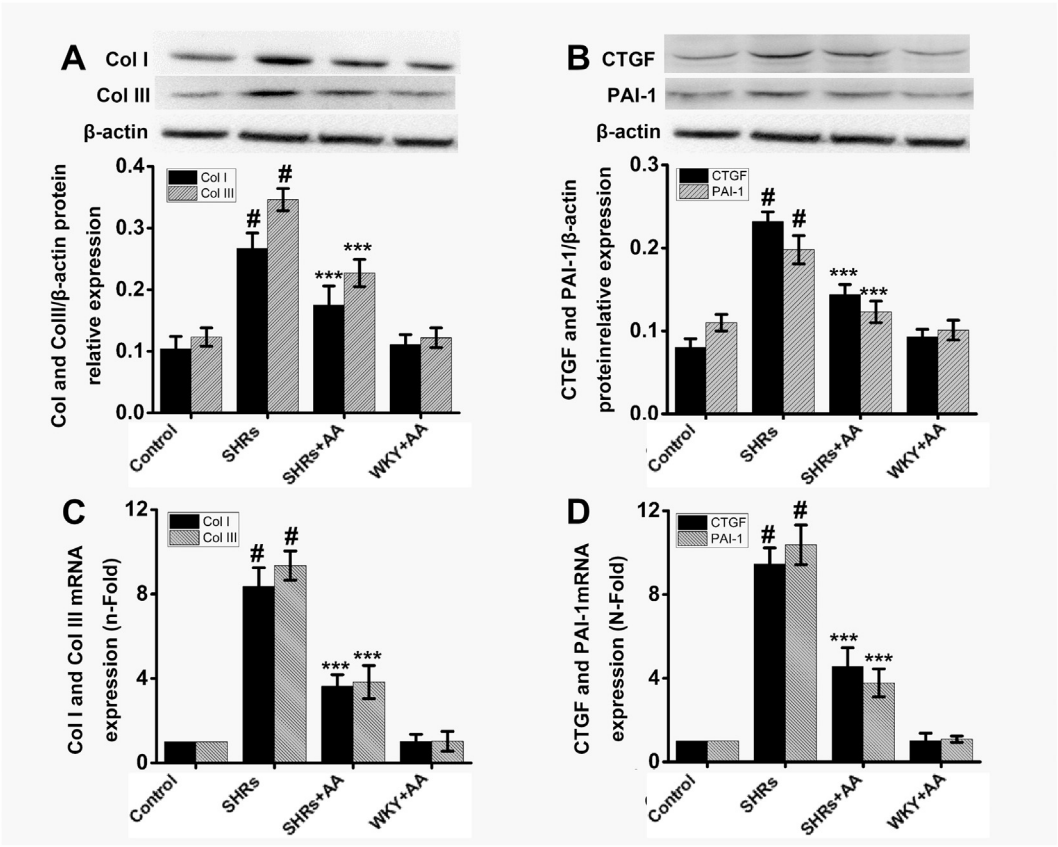

Fig. 2. Effects of AA on the mRNA and protein expressions of Col I, Col III, CTGF, and PAI-1, in rats from different groups. WKY rats were used as control group, and SHRs were treated with saline or AA (20 mg kg^{-1} day^{-1}) for 12 weeks by oral gavage. Western-blotting was used to measure the expressions of Col I (A), Col III (A), CTGF (B), and PAI-1 (B). The mRNA expressions of Col I (C), Col III (C), CTGF (D), and PAI-1 (D) were detected by real-time PCR (n = 10 rats, each group). Data arepresented as the means ± SEMs of six independent experiments. $^{\#}P < 0.01$ vs WKY; $^{***}P < 0.001$ vs SHRs.

100 pmol/L of Smad7 small interfering (siRNA) duplex oligonucleotides (on-TARGET plus SMART pool, Thermo Fisher Scientific, Rockford, IL) or non-target duplex oligo nucleotides (on-TARGET plus siCONTROL, Thermo Fisher Scientific), as a negative control siRNA using the TransLipidTM transfection agent (Transgen Biotech, Beijing, China) according to the manufacturer's instruction. After 24 h, the protein expression of Smad7 was analyzed. Transfection rates of 60% to 70% were used for the other experiments.

2.9. Measurements of oxidative stress

Cardiac tissues and cells were homogenized at 4 °C by using cold Tris buffer (0.01 M Tris-HCl, 0.1 mM EDTA-Na2, 0.01 M sucrose, 0.9% saline; pH 7.4), and then the resultant homogenates were centrifuged (14,000 × *g*, 15 min) at 4 °C. After centrifuged, the supernatant was collected for the measurements of the activities of GSH, SOD and levels of MDA and ROS generation by using commercial assay kits (Nanjing Jiancheng Bio-Corporation, Nanjing, China).

2.10. Statistical analysis

Continuous data were presented as mean ± standard deviation (SD). The comparisons among continuous variables among the groups wereanalyzed using ANOVA, and the difference between each of the 2 groups was detected using the Post hoc test withStatistic version 8.0software (Statsoft Inc., USA). A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of AA on SBP and cardiac function in rats from different groups

At the end of our experiment, the body weight (BW) was not significantly different among the rats from each group. Compared with the control group, the SBP, Ang II concentration, left ventricle weight/body weight (LVW/BW) and heart weight / body weight (HW/BW) ratios in SHRs group were elevated, while treatment with AA (20 mg kg^{-1} day^{-1}) for 12 weeks significantly attenuated the elevations of SBP and Ang II concentration, as well as pressure overload-induced elevation of LVW/BW and HV/BW ratios (Table 2). Treating the WKY rats with AA (20 mg kg^{-1} day^{-1}) for 12 weeks did not significantly affect these parameters (Table 2).

3.2. Effect of AA on collagen deposition in the left ventricle of SHRs

According to the results of Sirius Red staining and Masson staining, we observed that the collagen content in the left ventricle of SHRs was significantly higher than that of WKY rats (Fig. 1B). However, treating SHRs with AA (20 mg kg^{-1} day^{-1}) for 12 weeks could partially reduce the collagen content in the left ventricle (Fig. 1C). Treating the WKY rats with AA (20 mg kg^{-1} day^{-1}) for 12 weeks did not significantly affect collagen deposition in the left ventricle (Fig. 1D).

3.3. Effect of AA on expression of Col I, Col III, CTGF, and PAI-1 in the left ventricle of SHRs

As shown in Fig. 2, compared with the control groups, the protein

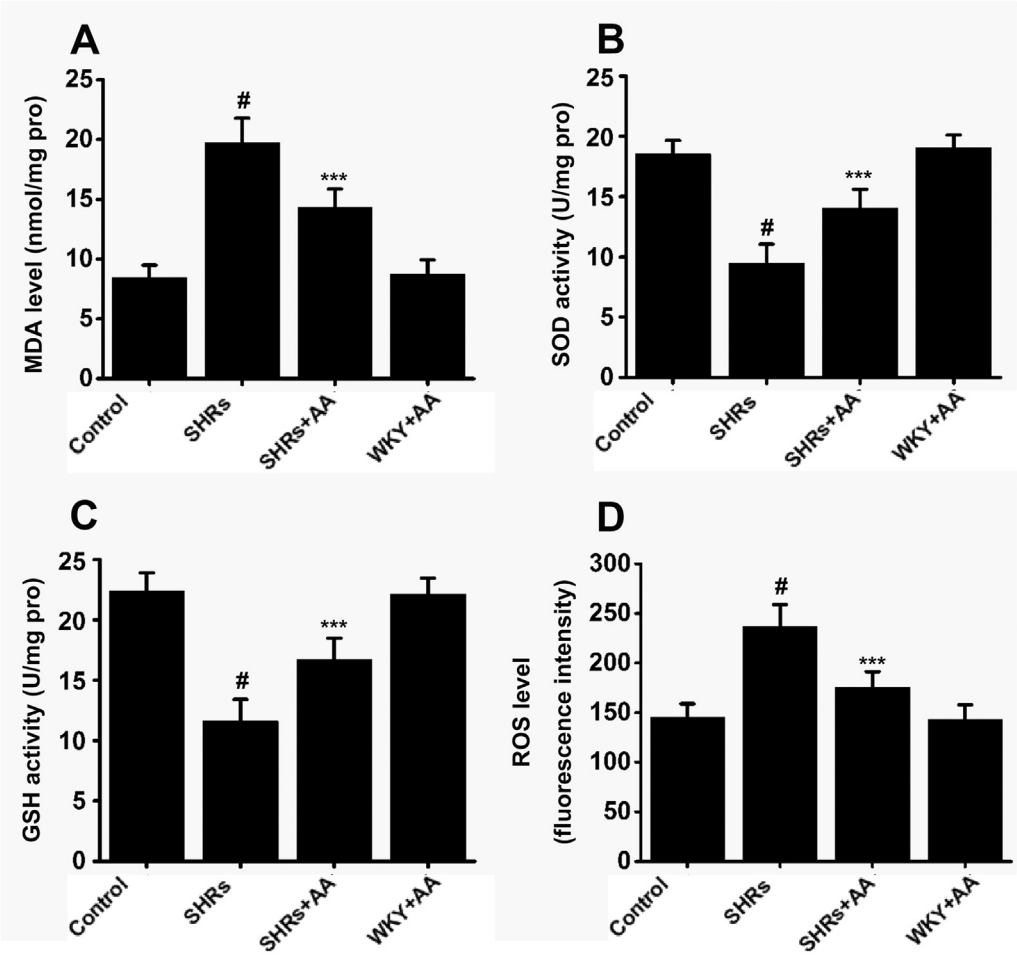

Fig. 3. AA inhibited oxidative stress in the left ventricle of SHRs.WKY rats were used as control group, and SHRs were treated with saline or AA (20 mg kg^{-1} day^{-1}) for 12 weeks by oral gavage. Measurements of malondialdehyde (MDA) levels (A), superoxide dismutase (SOD) and glutathione (GSH) activities (B, C), and reactive oxygen species (ROS) generation were performed in myocardial tissues (D). $^{\#}P < 0.01$ vs Wky; $^{***}P < 0.001$ vs SHRs.

expressions of Col I, Col III, CTGF, and PAI-1 wereincreased in SHRs group. However, treating the SHRs with AA (20 mg kg^{-1} day^{-1}) for 12 weeks significantly suppressed the protein expressions of Col I, Col III, CTGF, and PAI-1 in the left ventricle. However, treating WKY rats with AA (20 mg kg^{-1} day^{-1}) did not affect the protein expressions of Col I, Col III, CTGF, and PAI-1 in the left ventricle (Fig. 2A and B). The mRNA expressions of Col I, Col III, CTGF, and PAI-1 exhibited the similar change among the different groups (Fig. 2C and D).

3.4. Effect of AA on oxidative stress in the left ventricle of SHRs

As shown in Fig. 3, the levels of MDA and ROS production wereincreased, while the activities of SOD and GSH were significantly decreased in the SHRs group. Pretreatment with AA (20 mg·kg^{-1}·day^{-1}) significantly inhibited the pressure overload-induced elevations of MDA and ROS production and reductions of SOD, and GSH, in SHRs (Fig. 3). Treating WKY rats with AA (20 mg·kg^{-1}·day^{-1}) for 12 weeks did not affect these biomarkers (Fig. 3). These results suggested that AA could partially inhibit the extent of pressure overload-induced oxidative stress in SHRs.

3.5. Effect of AA on the expressions of Nrf2, HO-1, NQO-1 in the left ventricle of SHRs

As shown in Fig. 4, compared with the control group, the nuclear translocation of Nrf2 and the expressions of HO-1, NQO-1 were significantly reduced in the left ventricle of SHRs. Pretreatment with AA (20 mg·kg^{-1}·day^{-1}) for 12 weeks could partially increase the nuclear translocation of Nrf2 and the expressions of HO-1 and NQO-1 in the left ventricle of SHRs. Treating WKY rats with AA (20 mg·kg^{-1}·day^{-1}) for 12 weeks did not significantly affectthe protein expressions of Nrf2, HO-1, NQO-1 (Fig. 4).

3.6. Effect of AA on the expressions of TGF-β1, phosphorylation of Smad2/3, and Smad7 production in the left ventricle of SHRs

As shown in Fig. 5, the expression of TGF-β1 and phosphorylation of Smad2/3 were increased in SHRs, which could be attenuated by treatment with AA (20 mg·kg^{-1}·day^{-1}) for 12 weeks (Fig. 5A and B). The expression of Smad7 was decreased in SHRs group, while treating the SHRs with AA (20 mg·kg^{-1}·day^{-1}) could attenuate this effect (Fig. 5C). Treating the WKY rats with AA (20 mg·kg^{-1}·day^{-1}) did not significantly affect the activation of TGF-β1/Smads signal pathway (Fig. 5).

3.7. Effect of AA on Ang II-induced proliferation of CFs in vitro

Firstly, we detected the cytotoxicity of AA on CFs. AA exhibited minimal cytotoxicity on CFs of the concentrations between0 and 160 μmol/L, during which the cellular viability was over 90% (Fig. 6A). Then, we observed whether AA could inhibit Ang II-induced proliferation of CFs. As shown in Fig. 6 B, treating CFs with Ang II

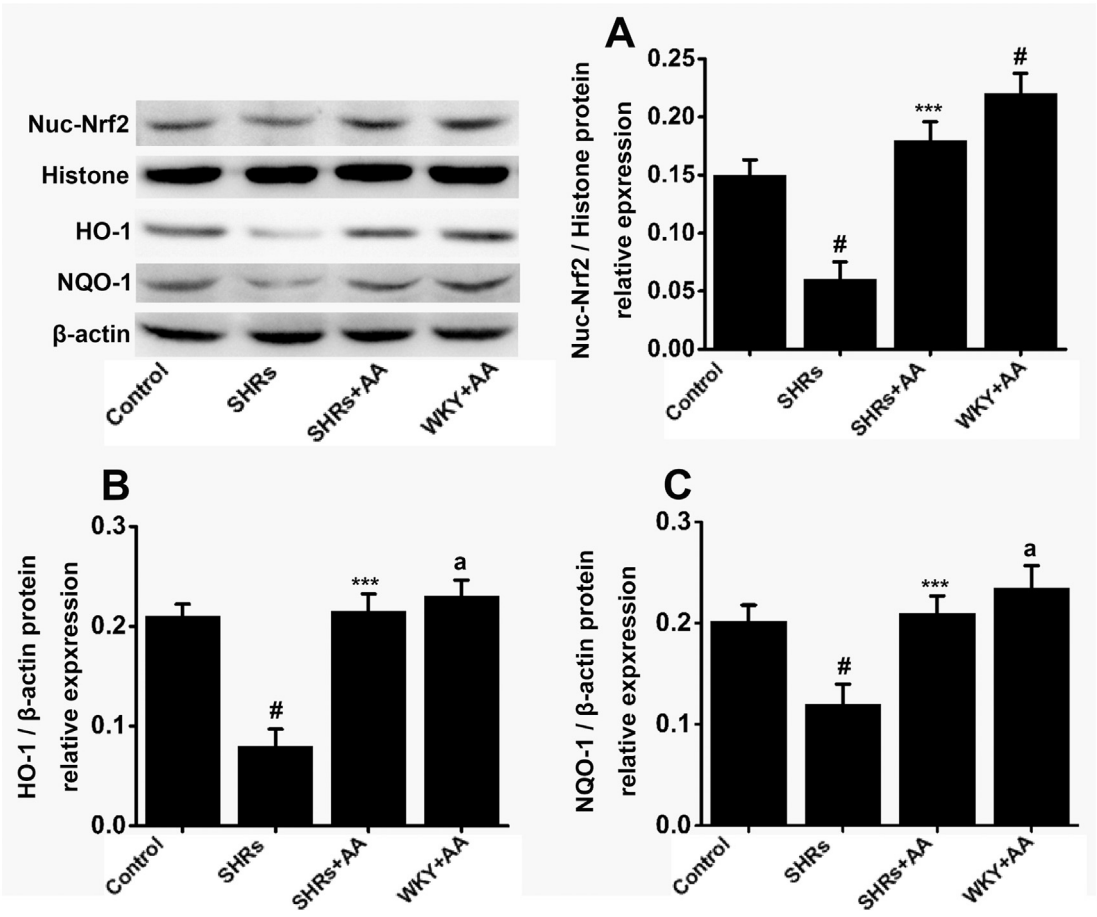

Fig. 4. AA enhanced the activation of Nrf2-mediated anti-oxidative signaling pathways in SHRs. WKY rats were used as control group, and SHRs were treated with saline or AA (20 mg kg^{-1} day^{-1}) for 12 weeks by oral gavage. Western blotting was used to measure the nuclear translocation of Nrf2 (A) and expressions of HO-1 (B) and NQO-1 (C). $^{\#}P < 0.01$ vs Wky; $^{***}P < 0.001$ vs SHRs.

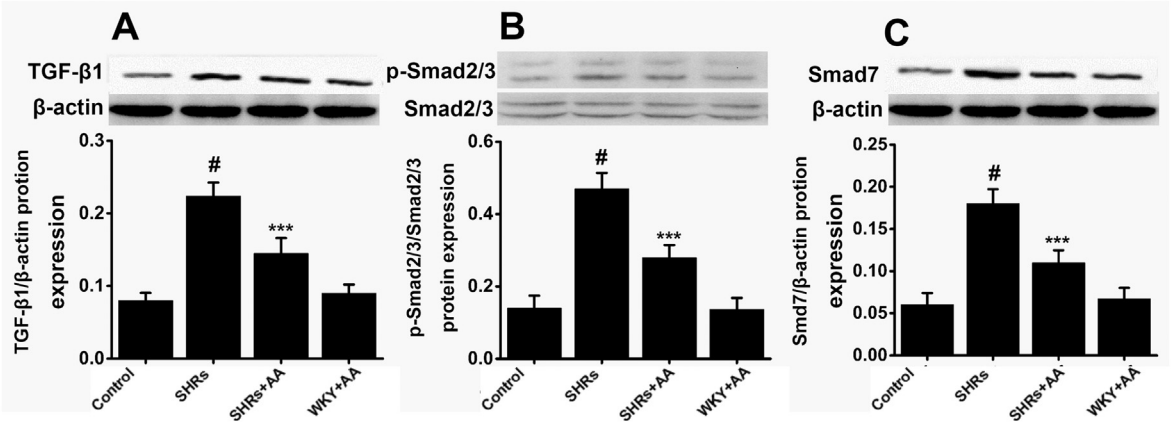

Fig. 5. Effects of AA on the expressions of TGF-β1, Smad7, and phosphorylation of Smad2/3 in rats from different groups. WKY rats were used as control group, and SHRs were treated with saline or AA (20 mg kg^{-1} day^{-1}) for 12 weeks by oral gavage. Western blotting was used to measure the expressions of TGF-β1 (A), Smad7 (C), and phosphorylation of Smad2/3 (B), in different groups (n = 10 rats, each group). Data arepresented as the means ± SEMs of six independent experiments. $^{\#}P < 0.01$ vs Wky; $^{***}P < 0.001$ vs SHRs.

(10^{-7} mol/L) for 72 h was associated with increased number of CFs, while pretreatment with AA (40, 80, and 160 μmol/L) could inhibit Ang II-induced proliferation of CFs in a concentration-dependent manner. Furthermore, pretreating CFs with si-RNA for Smad7 or Nrf2 both partially reversed inhibitory effect of AA on Ang II-induced CFs proliferation (Fig. 6B).

3.8. Effect of AA on the expression of Col I, Col III, CTGF, and PAI-1 in Ang II-stimulated CFs

As shown in Fig. 7, after treating the CFs with Ang II (10^{-7} mol/L) for 24 h, the protein expressions of Col I, Col III, CTGF, and PAI-1 were significantly increased, while pretreatment with AA (40, 80, 160 μmol/L) could concentration-dependently attenuate this effect (Fig. 7A and

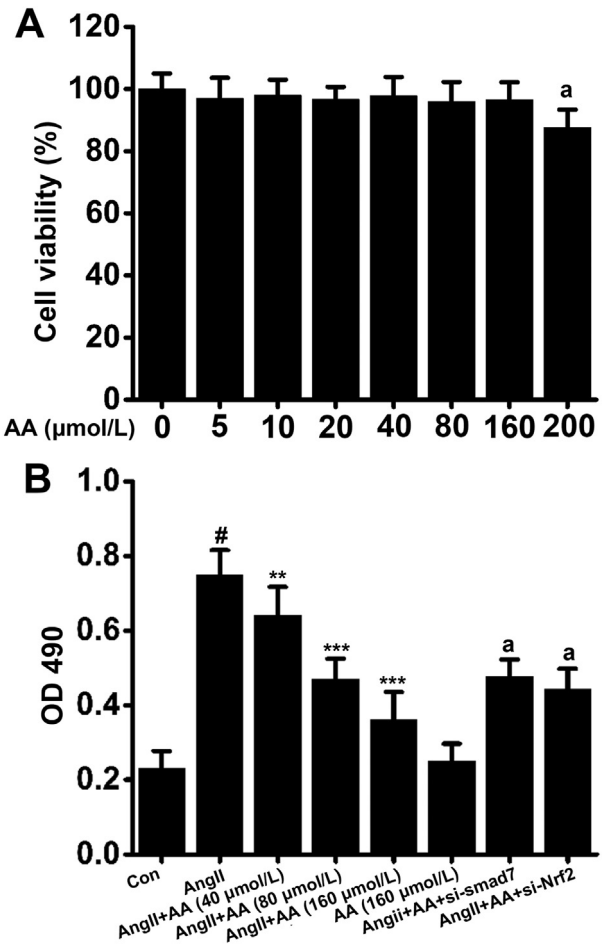

Fig. 6. Effects of AA on cell viability and CFs proliferation. CFs were treated with different concentrations of AA (0, 5, 10, 20, 40, 80, 160, and 200 μmol/L) for 24 h, and then the cell viability was measured by MTT assay (A). CFs were pretreated with AA (40, 80, and 160 μmol/L) for 1 h, and then treated with Ang II (10^{-7} mol/L) for another 72 h. After successful transfection with si-RNA for Smad7 or Nrf2, the cells were pretreated with AA (160 μmol/L), and then stimulated with Ang II (10^{-7} mol/L) for another 72 h. The proliferation of CFs was measured by MTT assay (B). Data are presented as the means ± SEMs of six independent experiments. $^{\#}P < 0.01$ vs Control; $^{***}P < 0.001$ vs Ang II; $^{**}P < 0.01$ vs Ang II; $^{a}P < 0.001$ vs Ang II + AA (160 μmol/L).

B). AA also inhibited Ang II-induced mRNA expressions of Col I, Col III, CTGF, and PAI-1 in CFs in a concentration-dependent manner (Fig. 7C and D). Treating CFs with AA(160 μmol/L) only did not significantly affect the expressions of Col I, Col III, CTGF, and PAI-1, in the levels of mRNA and protein (Fig. 7).

3.9. Inhibition of Nrf2 partially abolished the anti-fibrotic effect of AA in CFs

To observe whether the activity of Nrf2 played an important role in anti-fibrotic effect of AA in CFs, the si-RNA for Nrf2 was used. As shown in Fig. 8, compared with the group treated with AngII plus AA (160 μmol/L), the group pretreated with a combination of AA and Nrf2-si-RNA exhibited higher expressions of Col I, Col III, CTGF, and PAI-1, which suggested Nrf2-siRNA could partially abolish AA's inhibitory effects on the expressionsof Col I, Col III, CTGF, and PAI-1, in Ang II-stimulated CFs (Fig. 8). These results suggested that enhanced activity of Nrf2may be involved in the AA's inhibitory effect on pressureoverload-induced cardiac fibrosis.

3.10. Enhancing Smad7 and suppressing TGF-β/Smads are critical mechanisms underlying theinhibition of AA againstAng II-induced cardiac fibrosis in CFs

As shown in Fig. 9, treating the CFs with Ang II (10^{-7} mol/L) for 24 h increased the phosphorylation of Smad2/3, which could be inhibited by pretreatment with AA (40, 80, 160 μmol/L), in a concentration-dependent manner. Treating the CFs with AA (160 μmol/L) did not affect the phosphorylation of Smad2/3 (Fig. 9 A). Opposite to the increased in phosphorylation of Smad2/3, after treating CFs with Ang II (10^{-7} mol/L) for 24 h, the protein expression of Smad7 was decreased, which could also be partially inhibited by pretreatment with AA (40, 80, 160 μmol/L) in a concentration-dependent manner (Fig. 9 B). To evaluate whether the activation of Smad7 plays an important role for regulation of TGF-β1/Smads signaling during the inhibitory effect of AA on pressure overload-induced cardiac fibrosis, si-RNA for Smad7 was used. As shown in Fig. 9, pretreating CFs with Smad7-si-RNA and then stimulated with Ang II (10^{-7} mol/L) for 24 h partially abolished AA's inhibitory effect on the protein expressions of Col I, Col III, CTGF, and PAI-1. These results indicated that enhanced activation of Smad7 might be involved in the inhibitory effect of AA on pressure overload-induced cardiac fibrosis.

4. Discussion

In the present study, we demonstrated that AA inhibited the pressureoverload-induced cardiac fibrosis in vivo and in vitro. We found that AA was capable of reducing collagen deposition, suppressing the expressions of Col I, Col III, CTGF and PAI-1, and preventing the deterioration of cardiac function. In addition, AA preserved the activity of SOD and GSH, and reduced the level of MDA and ROS generation, accompanied with enhanced activation of Nrf2-mediated antioxidative signaling. Moreover, AA also inhibited the expression of TNF-β1 and phosphorylation of Smad2/3, while increased expression of Smad7.Pretreating CFs with si-RNA for Smad7 or Nrf2 both partially abolished the inhibitory effect of AA on Ang II-induced expressions of pro-fibrotic factors in CFs. These results provided a novel linkage between the anti-fibrotic effect of AA and Nrf2/HO-1 and TNF-β1-Smads signaling pathways in pressure overload-induced cardiac fibrosis in SHRs. These results may be important for the understanding of the potential molecular mechanisms underlying the beneficial effect of its anti-fibrotic pharmacologic activity.

Despite of significant progression in the treatments advancement of many cardiovascular diseases, cardiac fibrosis caused by multiple diseases including coronary heart disease, hypertension, and heart valvular disease, remains the main cause of heart failure, which is associated with considerable morbidity and mortality for people from both the developed and the developing countries [26]. The abnormal deposition of ECM in the left ventricle and pathological proliferation of CFs are key characteristics of the progression of cardiac fibrosis. Although there are four types of collagen within heart, the Col I and Col III are the main components, which have been considered to beaccounted for about 85% and 11% of total collagen in the heart, respectively. Normally, Col I helps ventricle to maintain intensity, and Col III is important for the extension and elasticity of the myocardium [27]. The balance between the metabolisms of the collagens forms a very complex network, including a number of pro-fibrotic and anti-fibrotic factors. During the procession of cardiac fibrosis, the levels of pro-fibrotic factors such as CTGF, PAI-1, and TNF-β1, arebecoming higher, while the levels of anti-fibrotic factors are becoming lower, leading to an disturbance of the homeostasis [3,28]. In our present study, we found the collagen deposition and expression of Col I and Col III in the left ventricle of SHRs were significantly higher than those in WKY rats. AA has been proved to inhibit the expression of Col I during renal fibrosis [29] and left ventricular remolding [30]. We also observed that treating SHRs with AA could attenuate the pressure overload-induced

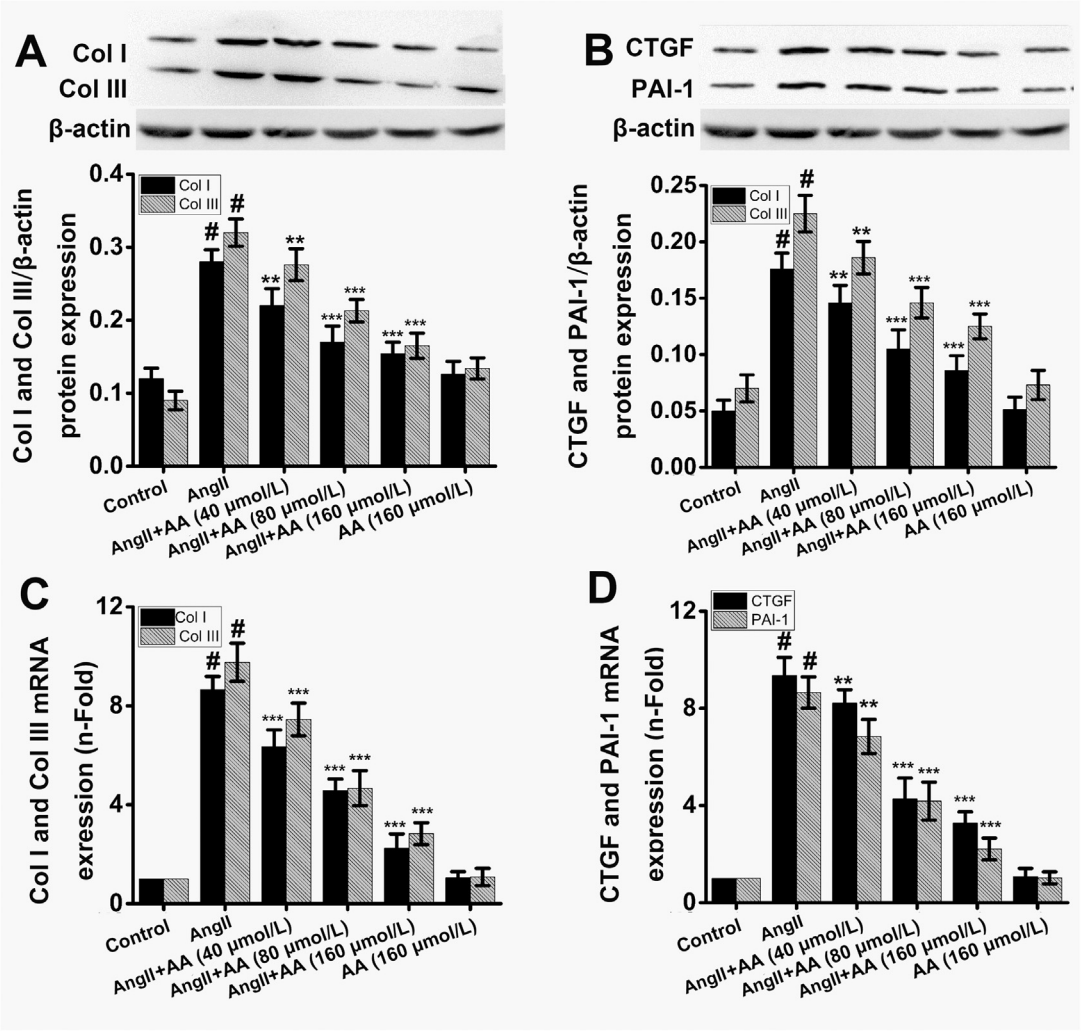

Fig. 7. Effects of AA on the expressions of Col I, Col III, CTGF, and PAI-1 in TNF-β1-stimulated CFs. CFs were pretreated with AA (40, 80, and 160 μmol/L) for 1 h, and then treated with Ang II (10^{-7} mol/L) for another 24 h. Western blotting was used to measure the expressions of Col I (A), Col III (A), CTGF (B), and PAI-1 (B). Some cells were with AA (40, 80, and 160 μmol/L) for 1 h, and then treated with Ang II (10^{-7} mol/L) for another 6 h. The mRNA expressions of Col I (C), Col III (C), CTGF (D), and PAI-1 (D) were detected by real-time PCR. Data arepresented as the means ± SEMs of six independent experiments. $^{\#}P < 0.01$ vs Control; $^{***}P < 0.001$ vs Ang II; $^{**}P < 0.01$ vs AngII.

collagen deposition and expressions of Col I and Col III in left ventricle, and in CFs. Moreover, AA inhibited AngII-induced expressions of Col I and Col III in a concentration-dependent manner. These results suggested that AA might attenuate the progression of cardiac fibrosis via preserving the balance of the collagen metabolism. Of note, proliferation of CFs leads to the disturbance of the balance of collagen metabolism, which causes increased collagen deposition and harm the cardiac function [31,32]. In the current study, we found AA could inhibit Ang II-induced proliferation of CFs, which indicated that the anti-fibrotic effect of AA might be pleiotropic.

CTGF plays a critical role in regulating EMC in heart via stimulating collagen deposition and inducing CFs proliferation, which are recognized as markers for interstitial fibrosis [33]. As a downstream autocrine or paracrine factor regulated by TGF-β1, the level of CTGF is significantly increased, during pathological conditions including heart failure [34]. Growing evidence has indicated that reducing the level of CTGF might slow the procession of fibrosis, not only in the heart but also in other organs like kidney and liver [4,35,36]. Therefore, we hypothesized that inhibiting the expression of CTGF might be beneficial for the suppression of cardiac fibrosis. AA was also observed to inhibit expression of CTGF during carbon tetrachloride-induced hepatic fibrosis, via the modulation of the TNF-β1/Smads signaling pathway [24]. PAI-1 is another potential pro-fibrotic factor which can stimulate collagen production, and induce the CFs proliferation and myocardium apoptosis, during cardiac fibrosis [37,38]. Our previous study has shwon that the level of PAI-1 is significantly increased in SHRs, while targeting the reducing of PAI-1 production may inhibit cardiac fibrosis [3]. In our present study, we found AA could reduce the productions CTGF and PAI-1 in the left ventricle of SHRs and also inhibit Ang II-induced expressions of CTGF and PAI-1, in CFs. These results exhibited that reducing the expressions of these pro-fibrotic factors might be a novel molecular mechanism underlying the pharmacologic benefits of AA on myocardial fibrosis.

Oxidative stress has been regarded as a critical contributor to the overpressure-induced ventricular remodeling and cardiac dysfunction [39]. Nrf2, as a powerful redox regulator, plays an important role in inhibiting pathological oxidative stress and reducing the production of reactive oxygen species (ROS), through controlling the basal and inducible expressions of various intrinsic antioxidant genes and phase II detoxifying enzymes [7]. Compared to mice with overexpression of Nrf2 which exhibited significantly inhibitedcardiac fibrosis and dysfunction, Nrf2 knockout mice was observed to have prominent cardiac fibrosis and dysfunction, after transverse aortic constriction surgery [40]. These results suggested that enhancing the Nrf2 activation might

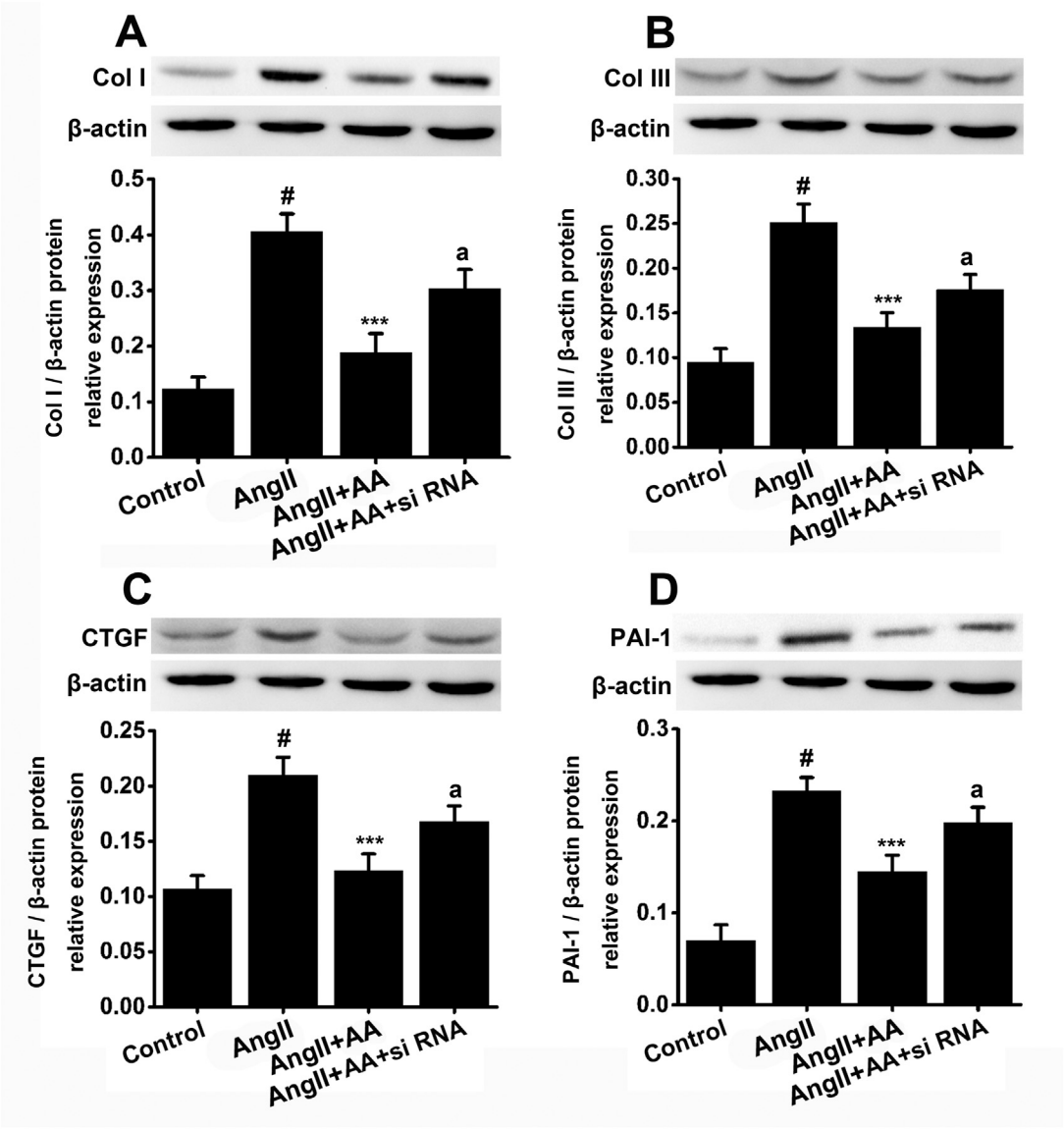

Fig. 8. Inhibition of Nrf2 partially reversed the anti-fibrotic effect of AA on SHRs. CFs were pretreated with AA (160 μmol/L) or a combination with si-RNA-Nrf2 and AA (160 μmol/L) for 1 h, and then treated with Ang II (10^{-7} mol/L) for another 24 h. Western blotting was used to measure the expressions of Col I (A), Col III (B), CTGF (C), and PAI-1 (D). $^{\#}P < 0.01$ vs Control; $^{***}P < 0.001$ vs Ang II; $^{a}P < 0.01$ vs AA.

reverse cardiac fibrosis, directly. Notably, growing evidence showing that overactivated ROS accelerated cardiac fibrosis and induced cardiac dysfunction, and inhibiting ROS production could partially prevent cardiac function [41]. After Nrf2 is translocated into the nucleus and bind with the AREs, it upregulatedthe antioxidative-relative genetic expressions of HO-1, SOD, and GSH, which are capable of reducing ROS production [7]. In our present study, it was shown that AA down-regulated the level of MDA, but increased the activitiesof SOD, GSH, and CAT, accompanied with significantly enhanced Nrf2 activation, which partially reversed pressure overload-induced cardiac fibrosis and prevented cardiac function. Moreover, by using a si-RNA for Nrf2, we confirmed that the anti-fibrotic effect of AA was at least partially dependent on the enhanced Nrf2 activation. Our results provided a notable linkage between the cardiac protective effect of AA and the activation of Nrf2-mediated antioxidative signaling in overpressure-induced cardiac dysfunction.

TGF-β1, belonging to the family of pro-fibrotic cytokine, plays a key role in the pathogenesis of tissue fibrosis, and growing evidence has indicated that during hypertension-induced cardiac fibrosis, the transcription, secretion, and activation of TGF-β1 is significantly increased [5,42]. Except for CFs and myocardium, almost all type of cells in the heart, such as endothelium and macrophage, can produce TGF-β1, via a paracrine or autocrine-mediated mechanism. Our previous studies have also shown that the mRNA and protein expressions of TGF-β1 are elevated in SHRs and in Ang II-stimulated CFs [3]. When the production and activity of TGF-β1 are elevated, it can induce cardiac fibrosis by promoting CFs proliferation, increasing collagen production, and suppressing the activity of MMPs [42–44]. AA has been proved to attenuate carbon tetrachloride-induced liver fibrosis and myocardial infarction-induced left ventricle remodeling, by inhibiting TGF-β1 production, which suggested that the anti-fibrotic activity of AA might be related to the inhibition of TGF-β1 [24,30]. In our present study, we found that treatment with AA could significantly reduce TGF-β1 production in the heart of SHRs, which further confirmed that the anti-fibrotic activity of AA is related to the inhibited TGF-β1 production in the left ventricle.

Smads family is a group of critical downstream molecular involved in TGF-β1-mediated tissue fibrosis-relative signaling pathway, their activation leads to the upregulated expressions of a diversity of pro-fibrotic factors, including collagen, CTGF, and PAI-1 [3,4,45]. Smads family is normally recognized to have 3 types, including: TGF-β1

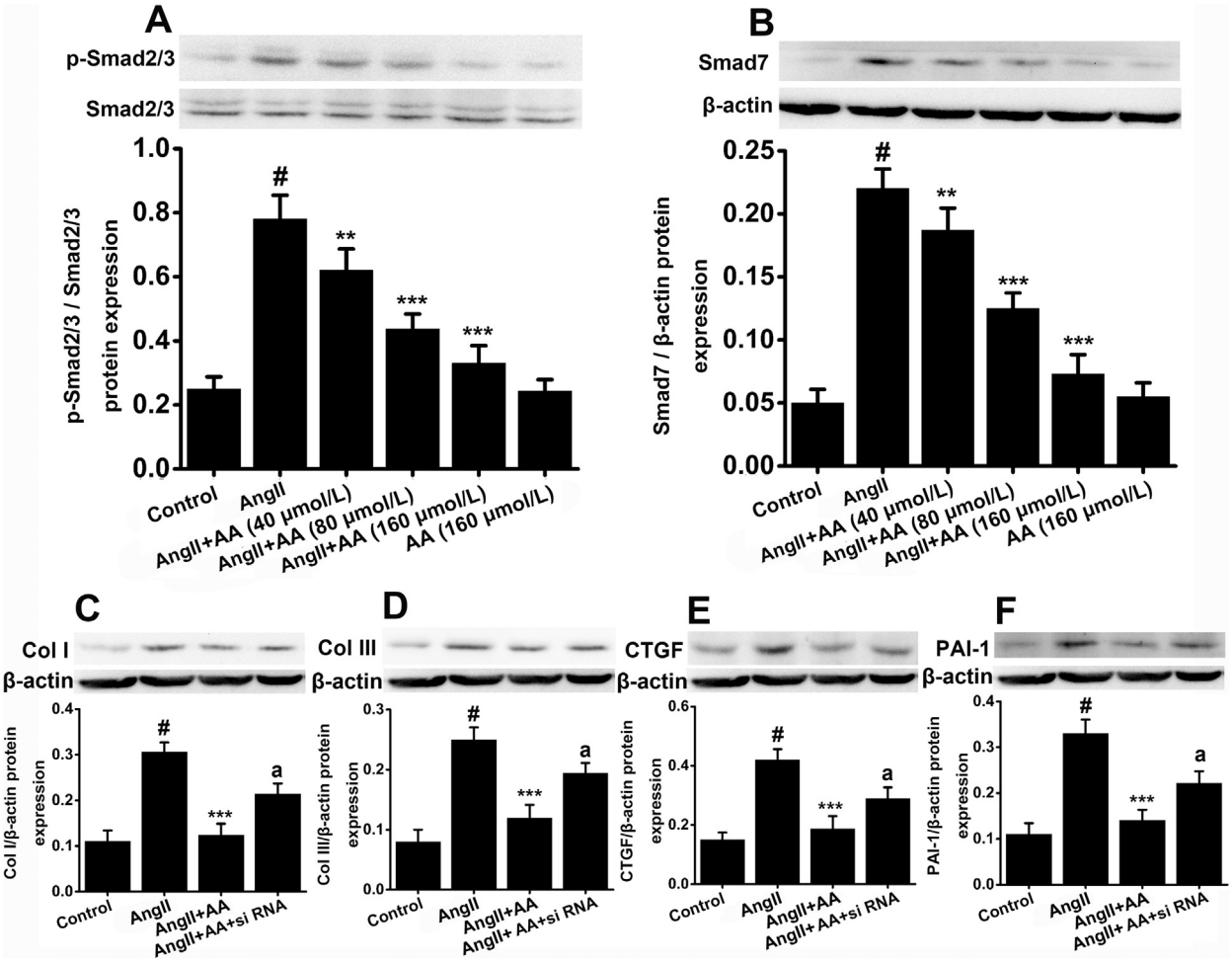

Fig. 9. Enhancing of Smad7 activation and suppressing of TGF-β/Smads signaling, are critical mechanisms by which AA inhibited Ang II-induced cardiac fibrosis in CFs. CFs were pretreated with AA (40, 80, and 160 μmol/L) for 1 h, and then treated with Ang II (10^{-7} mol/L) for another 24 h. Western blotting was used to measure the expression of Smad7 (B) and phosphorylation of Smad2/3 (A). Some cells were pretreated with AA (160 μmol/L) or si-RNA of Smad7 for 1 h, and then treated with Ang II (10^{-7} mol/L) for another 24 h. Western blotting was used to measure the expressions of Col I (C), Col III (D), CTGF (E), and PAI-1 (F). $^{\#}P < 0.01$ vs Control; $^{***}P < 0.001$ vs Ang II; $^{a}P < 0.01$ vs AA.

receptor activated Smads (Smad1, Smad2, Smad3, Smad5 and Smad8), common mediator Smads (Smad4 and Smad10), and inhibitory Smads (Smad6 and Smad7). Normally, after TGF-β1 contacts with its cellular surface receptor, Smad2 firstly forms a complex with Smad3 and Smad4, then the complex travels from the cytoplasm into the nucleus, and finally TGF-β1-mediated fibrosis-relative signaling pathway is activated to exert the regulatory effects of subsequent targeting genes in heart [46]. When the inhibitory Smad6 and Smad7 are aviated, they can negatively regulate the activity of TGF-β1-mediated signaling pathway, by suppressing Smad2/3 phosphorylation and then blocking the formation of Smad complex [47]. Our previous study has indicated that inhibiting Smad2/3 phosphorylation could reduce collagen deposition in the heart of SHRs [4]. AA has been proved to reduce collagen production via enhancing the expression of Smad7, in keloid fibroblasts [14]. Therefore, we hypothesized that inhibition of Smad2/3 phosphorylation and elevation of Smad7 activity might be beneficial ways to attenuate TGF-β1-indcued cardiac fibrosis. However, whether AA could reduce collagen production in SHRs and in CFs has not been explored. In our present study, we firstly found that AA could effectively inhibit Smad2/3 phosphorylation and upregulate the expression of Smad7, in heart of SHRs, and then the similar results were retrieved in CFs, accompanied with the reduction of collagen expression. For further understanding of the relationship between the anti-fibrotic effect of AA and the TGF-β1/Smads signaling pathway, we used si-RNA for Smad7 to reduce the expression of Smad7. As expected, accompanied with the reduction of Smad7 expression, the inhibitory effect of AA on Ang II-induced collagen production was attenuated. Accordingly, these results suggested that the inhibitory effect of AA on mediating collagen metabolism is at least partially depending on inhibiting Smad2/3 phosphorylation and enhanced expression of Smad7 in CFs.

In conclusion, our present study indicated that treatment with AA attenuates overpressure-induced cardiac fibrosis and improves cardiac function in SHRs. The potential cardioprotective effect of AA may at least partially depend on the enhancement of Nrf2/HO-1 activation and suppressing TGF-β1/Smads signaling, which lead to inhibited cardiac fibroblasts proliferation and preserved balance of collagen metabolism. These results may be important for the understanding of the potential molecular mechanisms underlying the beneficial effect of its anti-fibrotic pharmacologic activity.

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