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Original Article

Hydroxysafflor yellow A mitigates myocardial fibrosis induced by isoproterenol and angiotensin II

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Abstract: Aims: To investigate the potential inhibitory effect of Hydroxysafflor yellow A (HSYA) on myocardial fibrosis induced by isoproterenol (ISO) and angiotensin II (Ang II) and the possible underlying mechanism. Methods: Mice were injected subcutaneously with ISO and given HSYA by gavage in vivo. Masson's trichrome staining, immunohistochemical staining and immunofluorescence assays were conducted to evaluate the expression and localization of collagen and inflammatory cytokines, respectively. In vitro, cardiac fibroblasts (CFs) were treated with various doses of HSYA and induced with Ang II. Cell proliferation and migration were assessed using wound healing assay. Cell counting kit-8 was used to measure the cell viability. Collagen I, collagen III, phosphorylation of Smad2/3, Smad2/3, TGFβ1, interleukin (IL)-1β, IL-18, NLRP3 inflammasome-associated proteins were detected by Western blotting. Levels of reactive oxygen species (ROS) were evaluated using 2',7'-dichlorofluorescein diacetate assay. Results: HSYA significantly inhibited ISO-induced myocardial fibrosis, NLRP3 inflammasome activation as well as IL-18 and IL-1β expressions in mice. HSYA significantly reduced the proliferation and migration of CFs, and suppressed the accumulation of collagen I and collagen III. TGFβ1 and P-Smad2/3 induced by Ang II was repressed by HSYA. HSYA downregulated IL-1β and IL-18, blocked NLRP3 activation, and reduced ROS in CFs. Conclusion: HSYA may inhibit myocardial fibrosis by blocking NLRP3 pathway in CFs.

Keywords: Hydroxysafflor yellow A, myocardial fibrosis, NLRP3, reactive oxygen species, TGFβ1

Introduction

Myocardial fibrosis is a protective mechanism in patients with acute or chronic heart injury. However, excessive myocardial fibrosis can contribute to heart stiffness, cardiac diastolic and systolic dysfunction, electrical conduction disorder, heart failure and death [1, 2]. Fibrosis is characterized by the differentiation of fibroblasts into myofibroblasts and increased synthesis and secretion of collagens (mainly collagen I and III in the heart), leading to an imbalance in extracellular matrix (ECM) metabolism [3].

Angiotensin II (Ang II) represents the major effector hormone of the renin-angiotensin-aldosterone system and promotes vasoconstriction, cell proliferation, inflammation, oxidative stress, and fibrosis by inducing the angiotensin type 1 receptor (AT1R) [4]. Recently, Ang II has been reported to stimulate the NLRP3 inflammasome pathway [5, 6]. NLRP3 refers to a pattern recognition receptor that is involved in the pathogenesis of inflammation and chronic disorders [7]. NLRP3 can assemble with apoptosis-associated speck-like protein containing CARD (ASC) and pro-caspase-1 to form inflammasomes up to microns in diameter; subsequently, pro-caspase-1 produces mature caspase-1 with self-shearing activity. Mature caspase-1 cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 [8]. NLRP3 deletion alleviates cardiomyopathy and myocardial fibrosis by inhibiting mitochondrial dysfunction [5].

Significant myocardial fibrosis can be triggered by subcutaneous injection of the nonspecific β-adrenergic receptor agonist isoproterenol (ISO) *in vivo* [9]. ISO can induce cardiotoxic effects in rats and mice, initiate alternative fibrosis by causing cardiomyocyte necrosis, and stimulate cardiomyocytes or immune cells to release pro-fibrotic mediators [10, 11], which is

the most widely used way of establishing model of myocardial injury and myocardial fibrosis [12]. It has been found that ISO can activate TLR4/NF-κB/NLRP3 pathway [13].

Hydroxysafflor yellow A (HSYA) represents the major component of the traditional Chinese medicine safflower extract. Safflower yellow for injection (its main active ingredient is HSYA) is clinically applied in the treatment of cardiovascular disease to relieve angina pectoris [14-16]. Studies have found that HYSA reduces fibrosis in the lung, liver and kidney, but its role in cardiac fibrosis has not been well described [17-20]. HSYA has been reported to reduce the myocardial injury induced by hypoxia/reoxygenation via the NLRP3 inflammasome pathway in H9c2 cell line [21]. In this study, ISO and Ang II were used to mimic myocardial fibrosis both *in vivo* and *in vitro*, so as to investigate whether HSYA could alleviate myocardial fibrosis, and to explore whether its underlying mechanism is related to NLRP3 inflammasome pathway.

Materials and methods

Establishment of myocardial fibrosis and administration of HSYA in mice

All the animal protocols were approved by the Ethics Committee of Heilongjiang Provincial Hospital. The use and care of the animals conformed to the Guide for Care and Use of Laboratory Animals (NIH). Male 8-week-old C57BL/6 mice were purchased from the Animal Center of the Second Affiliated Hospital of Harbin Medical University. The mice were allocated into 3 groups (n = 12). ISO (5 mg/kg/day) was injected subcutaneously daily into mice in the ISO group and ISO + HSYA group [22, 23], while saline (0.2 ml) was injected subcutaneously into those in the control group for 2 weeks. Further, mice in the ISO + HSYA group were gavaged with HSYA (100 mg/kg/day) daily for 2 weeks, while mice in the control and ISO groups were gavaged with the same volume of sterile water daily [24]. All the mice were sacrificed on the 15th day.

Masson's trichrome staining

The hearts of mice were fixed in 4% paraformaldehyde for 24 h, paraffin-embedded and sectioned. The sections were subjected to Masson's trichrome staining as per the manufacturer's procedure (Solarbio, Beijing, China) and analyzed with Image Pro Plus 6.0 software. The integrated optical density of the blue stained part was calculated.

Immunohistochemistry

After the paraffin-embedded sections were dewaxed, the antigen retrieval was performed using citric acid repair solution. The sections were sealed with 10% ready-to-use goat serum for 0.5 h and stained with antibodies specific for IL-1β (Abcam, Cambridge, UK) and IL-18 (Boster, Wuhan, Hubei, China) to determine the level of inflammation. Images were acquired with an Olympus microscope and analyzed with Image Pro Plus 6.0 software.

Immunofluorescence staining

Frozen sections were immersed in 4% paraformaldehyde fixation solution and fixed for 10 min at room temperature. After being treated with 0.1% Triton X-100 (prepared with PBS) for 5 min, the sections were sealed for 20 min in 10% ready-to-use goat serum. Primary antibodies to ASC (Bioss, Beijing, China) and α-SMA (Bioss) were diluted at 1:200 in 1% BSA for an overnight incubation at 4°C. Following washing 3 times with PBS, FITC goat anti-mouse fluorescent secondary antibody and TRITC goat anti-rabbit fluorescent secondary antibody were diluted at 1:400 in PBS for a 1-h incubation at room temperature. The nucleus was stained with DAPI for 10 min, and the sections were sealed with AntiFade Mounting Medium and photographed with fluorescence microscope (Olympus, Tokyo, Japan).

Isolation and culture of primary cardiac fibroblasts (CFs)

Newborn C57BL/6 mice (within three days) were euthanized by intraperitoneal injection of sodium pentobarbital, and the hearts were isolated under aseptic conditions. The heart was place in serum-free DMEM and cut open, and blood was rinsed from the heart cavity with PBS. The hearts were soaked with 0.25% trypsin until completely digested. Trypsin solution was supplemented to DMEM containing 10% FBS (complete medium). The mixture was centrifuged at 1500 rpm for 10 min and resuspended in the complete medium. After 1-1.5 h, the culture medium was refreshed with the complete medium, and the adherent cells were CFs. The isolated CFs were identified by immu-

nofluorescence assay with Vimentin antibody (Abcam).

HSYA and Ang II treatment in vitro

HSYA (HY-N0567, MedChemExpress, Monmouth Junction, NJ, USA) was diluted in sterile 0.9% NaCl. CFs were treated with HSYA (50, 100, and 200 μM) for 1 h before Ang II (100 nM, HY-13948, MedChemExpress) treatment.

Cell counting kit-8 (CCK8)

CFs were seeded into 96-well plates at 1×10^4 cells/well, then incubated with HSYA (0, 50, 100, 200, and 400 μM) for 24 h and with CCK8 working solution (MA0218, Meilunbio, Dalian, Liaoning, China) for 1-4 h. The optical density value at 450 nm was obtained using a microplate reader (Tecan, Switzerland) to evaluate the cell viability.

Wound healing assay

CFs were cultured in labelled plates until an 80-90% confluency. After serum starvation for 12 h, the cell layer was scratched perpendicular to the lines using a p200 pipette tip to form wounds. Subsequently, the CFs were treated with HSYA for 1 h before Ang II treatment. Images of the wounds were acquired at 0^{th} and 24^{th} h after treatment using an inverted microscope (Olympus). ImageJ software was applied to calculate the area and length of the rectangular wounds. The Migration $rate_{24\,h}$ (%) = $[(width_{0\,h} - width_{24\,h})/width_{0\,h}] \times 100\%$.

Western blotting

Proteins from whole-cell lysates were extracted using RIPA lysis buffer (Beyotime, Shanghai, China), followed by denaturation at 100°C for 10 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and sealed with 5% BSA or milk. PVDF membranes were probed with antibodies to collagen I (1:1000, bs-10423R, Bioss), collagen III (1:1000, bs-0549R, Bioss), TGF-β1 (1:500, BM3901, Boster), Smad2/3 (1:500, E-AB-32920, Elabscience, Wuhan, Hubei, China), P-Smad2/3 (1:500, E-AB-21040, Elabscience), IL-1β (1:1000, ab9722, Abcam), IL-18 (1:800, BA14935, Boster), NLRP3 (1:800, ab214185, Abcam), ASC (1:500, bs-6741R, Bioss), cleaved caspase-1 (1:100, sc-56036, Santa Cruz, CA, USA), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000, TA-08, ZSGB-BIO, Beijing, China) at 4°C overnight. GAPDH served as the internal reference protein. The membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 h. The enhanced chemiluminescence (Meilunbio) was used for visualization, and Bio-Rad ChemiDoc™ imaging system (Hercules, CA, USA) for analysis.

Detection of reactive oxygen species (ROS)

ROS were detected using DCFH-DA (S0033S, Beyotime). CFs (2×10^5 cells/ml) were plated on cell slides cultured in 35 mm dishes and treated with HSYA and Ang II the next day. After 24 h of Ang II treatment, the CFs were treated with DCFH-DA (1:1000) and Hoechst 33342 (1:1000) for 20 min. The cells were photographed using a fluorescence microscope (Olympus). Meanwhile, CFs (2×10^4 cells/well) were seeded in 96-well plates and treated with Ang II and HSYA as described above. CFs were loaded with DCFH-DA for 20 min. The intensity of DCF was immediately detected using an infinite M200 multifunctional microplate reader (Tecan, Switzerland) at 488/525 nm.

Statistical analyses

All experiments were repeated at least three times. The measurement data were expressed as mean ± standard error of mean (SEM). The Shapiro-Wilk test was applied to test the normality of the data, and the data that met the normal distribution were compared among multiple groups using one-way ANOVA. Tukey's test was used to compare the data between the groups. For data that did not meet the normal distribution, the Kruskal-Wallis test was used for inter-group comparisons, and Dunn's test was used for inter-group comparisons. In this study, data were analyzed statistically using GraphPad Prism software (version 7.0, GraphPad, San Diego, CA, USA), and p values less than 0.05 were considered statistically significant.

Results

HSYA inhibits myocardial fibrosis and IL-18 expression in mouse hearts

Masson's trichrome staining was conducted to evaluate the production of collagen in the hearts of mice to validate the impact of HSYA on myocardial fibrosis *in vivo*. Collagen fibers

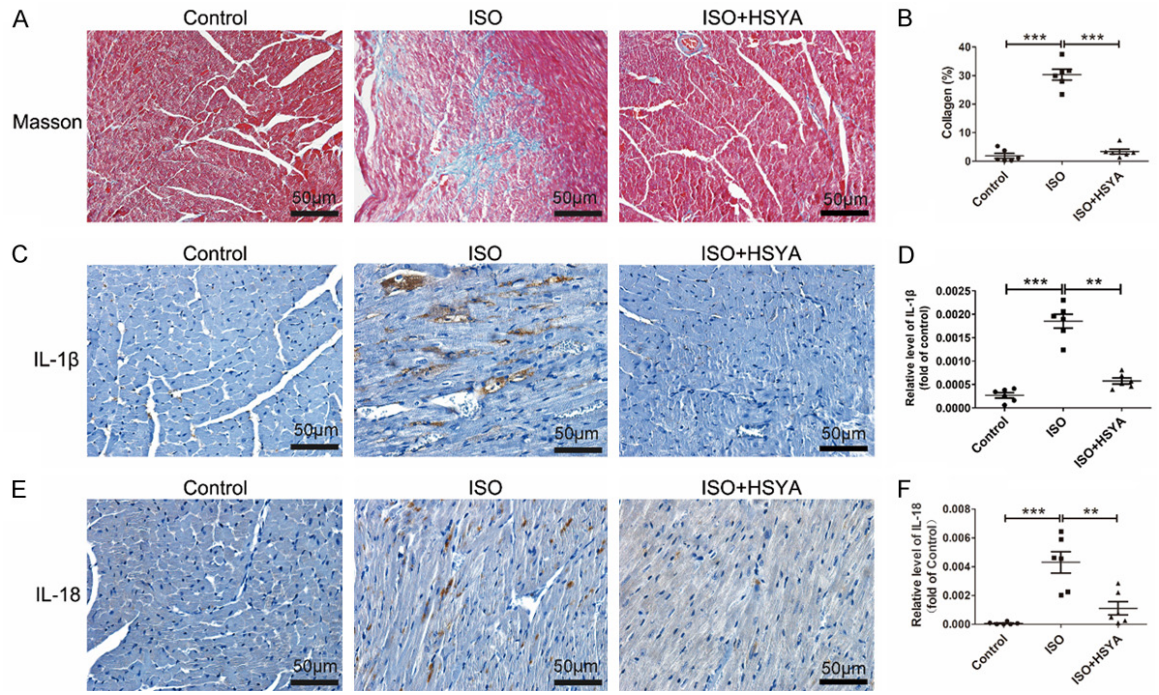

Figure 1. Masson's trichrome staining and immunohistochemistry of inflammatory cytokines in mouse heart. A, B. The production of cardiac collagen analyzed using Masson's trichrome staining. C, D. Immunohistochemical staining of IL-1β. E, F. Immunohistochemical staining of IL-18. $^{**}P < 0.01$, $^{***}P < 0.001$. Data are shown as the mean ± SEM, n = 6 per group. Scale bar: 50 μm. ISO, Isoproterenol; HSYA, Hydroxysafflor Yellow A; IL, Interleukin; SEM, Standard Error of Mean.

were stained in blue and muscle fibers in red after staining. HSYA significantly inhibited the ISO-induced cardiac collagen in mice (**Figure 1A**, **1B**). We also examined the intensity of IL-1β and IL-18 staining in the mouse heart by immunohistochemistry, and found that HSYA significantly repressed IL-1β and IL-18 levels induced by ISO in mouse hearts (**Figure 1C-F**).

HSYA inhibits NLRP3 inflammasome activation in vivo

To detect NLRP3 inflammasome activation and its cellular localization in mouse hearts, we performed ASC and α-SMA immunofluorescence staining on frozen sections of mouse hearts. α-SMA is a marker for fibroblasts to differentiate into myofibroblasts under the context of fibrosis. We found that the intensities of ASC and α-SMA staining in mouse heart were increased by ISO, and the co-localization of these two markers. However, HSYA significantly inhibited ASC and α-SMA expressions. These findings indicated that the cardiac myofibroblast activation and NLRP3 inflammasome activation were significantly reduced after administration of HSYA (**Figure 2**).

HSYA did not affect the survival of isolated CFs

CFs isolated from mouse heart tissues were identified by immunofluorescence of Vimentin with a purity of more than 95% (**Figure 3A**). To determine whether HSYA affected the viability of CFs, CFs were induced with HSYA (0, 50, 100, 200 and 400 μM) for 24 h, then cell viability was assessed. There was no substantial difference between the groups (**Figure 3B**), indicating that all HSYA concentrations less than or equal to 400 μM were safe. Therefore, we selected 50, 100 and 200 μM of HSYA for subsequent experiments.

HSYA inhibits Ang II-induced migration and collagen synthesis of CFs

To dissect the impact of HSYA on Ang II-induced fibrosis, we first assessed CF migration and the secretion and deposition of ECM. A wound-healing assay was conducted to assess the effects of HSYA on Ang II-induced cell migration. The initial width of the wound was the same in all the groups. After 24 h of Ang II treatment, cell migration was significantly inhibited

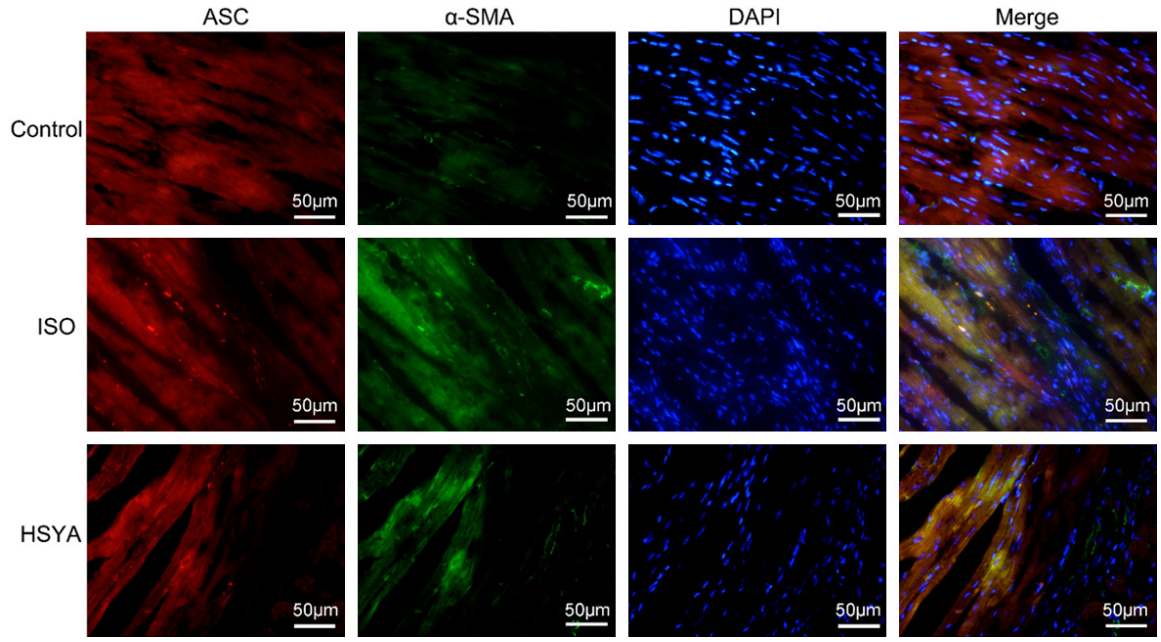

Figure 2. Immunofluorescence staining of ASC and α-SMA in the mouse heart. The red fluorescence was ASC, the green fluorescence was α-SMA, and the blue fluorescence was DAPI staining of the nucleus. Scale bar: 50 μm. ISO, Isoproterenol; HSYA, Hydroxysafflor Yellow A; ASC, Apoptosis-Associated Speck-Like Protein Containing CARD; α-SMA, α-Smooth Muscle Actin; DAPI, 4',6-Diamidino-2-Phenylindole Dihydrochloride.

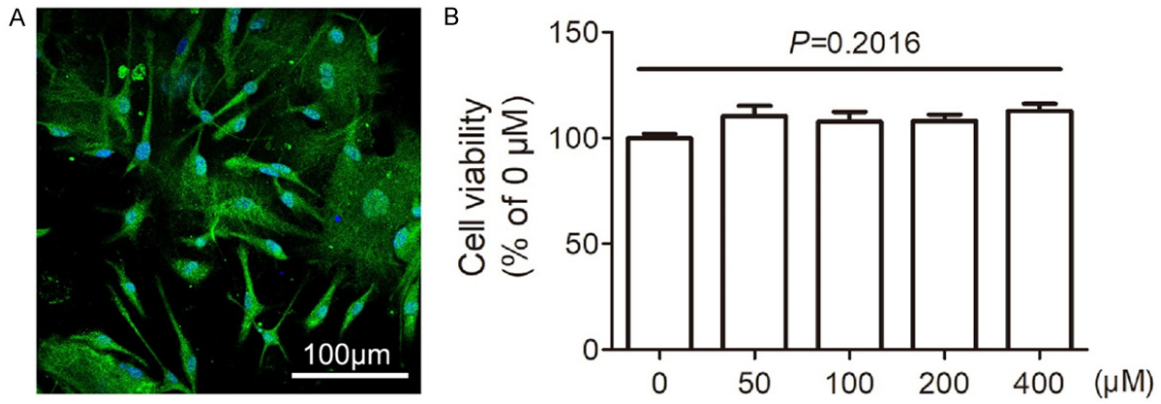

Figure 3. Effects of HSYA on cell viability of CFs. A. Vimentin immunofluorescence identification of CFs (Scale bar: 100 μm). B. The viability of CFs was determined using the CCK-8 assay. Data are shown as the mean ± SEM, n = 6 per group. HSYA, Hydroxysafflor Yellow A; CFs, Cardiac Fibroblasts; SEM, Standard Error of Mean.

by different concentrations of HSYA (50, 100, and 200 μM) (**Figure 4A**, **4B**). Collagen I and collagen III protein levels, as evaluated by Western blotting, were increased when CFs were treated with Ang II for 48 h. The expression of collagen I was inhibited by different concentrations of HSYA (50 μM, 100 μM and 200 μM). Collagen III was significantly inhibited by 200 μM HSYA, but there was no statistical difference at 50 and 100 μM (**Figure 4C-E**).

HSYA inhibits the TGFβ1-Smad2/3 pathway activated by Ang II

TGFβ1 is significant during the progression of myocardial fibrosis [25, 26]. In vivo, Ang II cannot induce cardiac hypertrophy and fibrosis without TGFβ1 [27]. The effects of HSYA on TGF-β1 activation and P-Smad2/3 expression were therefore determined. Western blotting was implemented to evaluate the protein ex-

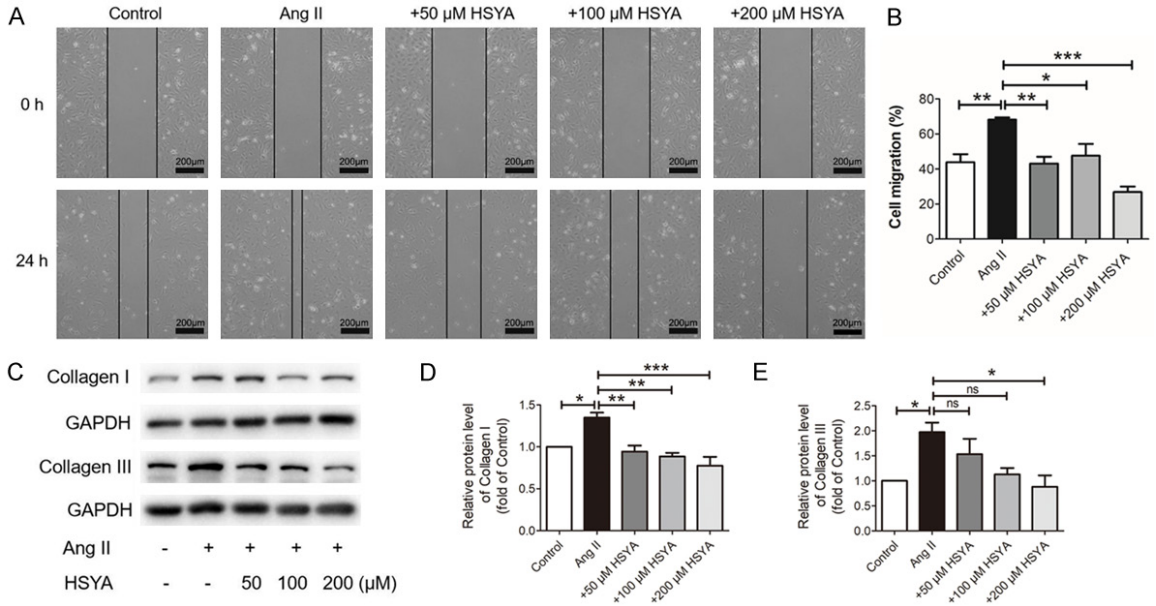

Figure 4. Effects of HSYA on the migration and collagen synthesis of CFs in the presence of Ang II. A, B. The migration of CFs at the 0th and 24th h in the wound-healing assay and the migration rate. Scale bar: 200 μm. C-E. Expressions of Collagen I and Collagen III determined by Western blotting (normalized to GAPDH). Data presented as mean ± SEM, n = 3 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. HSYA, Hydroxysafflor Yellow A; CFs, Cardiac Fibroblasts; Ang II, Angiotensin II; GAPDH, Lyceraldehyde-3-Phosphate Dehydrogenase; SEM, Standard Error of Mean; ns, not significant.

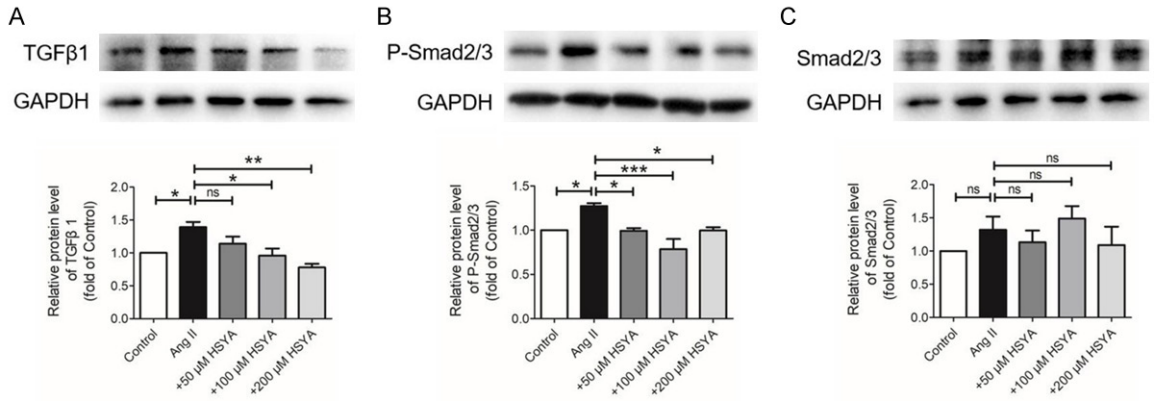

Figure 5. Effects of HSYA on Ang II-induced activation of the TGFβ1-Smad2/3 pathway. A. Expression of TGFβ1 was measured by Western blotting, and the ratio of TGFβ1 normalized to GAPDH was calculated. B. Expression of P-Smad2/3 was measured by Western blotting, and the ratio of P-Smad2/3 normalized to GAPDH was calculated. C. Expression of Smad2/3 was measured by Western blotting, and the ratio of Smad2/3 normalized to GAPDH was calculated. Data presented as mean ± SEM, n = 3 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. HSYA, Hydroxysafflor Yellow A; Ang II, Angiotensin II; TGFβ1, Transforming Growth Factor β1; GAPDH, Lyceraldehyde-3-Phosphate Dehydrogenase; SEM, Standard Error of Mean; ns, not significant.

pressions of TGFβ1, P-Smad2/3 and Smad2/3. TGFβ1 and P-Smad2/3 protein expressions were increased when CFs were induced with Ang II for 48 h. The expression of TGFβ1 was inhibited by HSYA at 100 and 200 μM, but there was no statistical difference at 50 μM (**Figure 5A**). The expression of P-Smad2/3 was significantly inhibited by different concentrations of HSYA (50, 100 and 200 μM) (**Figure 5B**). Ang II and HSYA had insignificant effect on the protein expression of Smad2/3 (**Figure 5C**). These data indicated that HSYA alleviated myocardial fibro-

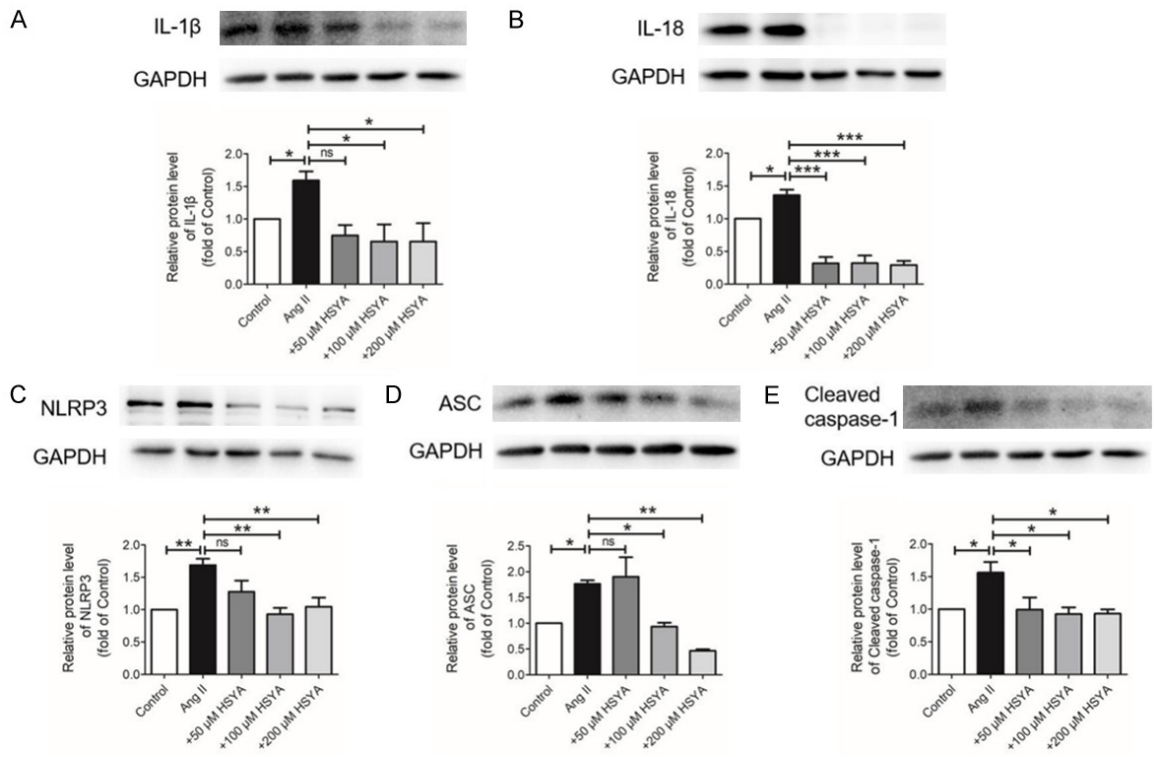

Figure 6. Effects of HSYA on NLRP3 inflammasome activation and inflammatory cytokine expression in CFs. A-E. Expressions of IL-1β, IL-18, NLRP3, ASC and Cleaved caspase-1 were analyzed by Western blotting, and the ratio of IL-1β, IL-18, NLRP3, ASC and Cleaved caspase-1 normalized to GAPDH respectively were calculated. Data presented as mean ± SEM, n = 3 per group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. HSYA, Hydroxysafflor Yellow A; CFs, Cardiac Fibroblasts; IL, Interleukin; NLRP3, Nucleotide-Binding Domain and Leucine-Rich Repeat Containing PYD-3; ASC, Apoptosis-Associated Speck-Like Protein Containing CARD; GAPDH, Lyceraldehyde-3-Phosphate Dehydrogenase; SEM, Standard Error of Mean; ns, not significant.

sis by terminating the TGFβ1-Smad2/3 pathway activation.

HSYA inhibits Ang II-induced NLRP3 inflammasome activation and the levels of inflammatory cytokines in CFs

NLRP3 deficiency weakens Ang II-induced renal fibrosis and cardiomyopathy [5, 28]. Therefore, Western blotting was performed to evaluate the protein expressions of IL-1β, IL-18, NLRP3, ASC and cleaved caspase-1. The expressions of these proteins were increased when CFs were induced with Ang II for 5 h. The expressions of IL-1β, NLRP3 and ASC were inhibited by 100 and 200 μM HSYA, but no statistical difference was observed at 50 μM (**Figure 6A**, **6C**, **6D**). The expressions of IL-18 and cleaved caspase-1 were significantly suppressed by different concentrations of HSYA (50, 100 and 200 μM) (**Figure 6B**, **6E**). It was indicated that HSYA inhibited the Ang II-induced NLRP3 activation and downstream inflammatory cytokines.

HSYA prevents Ang II-induced oxidative stress in CFs

ROS are important mediators of Ang II-induced fibrosis [4, 29], and DCFH-DA was used to detect the impact of HSYA on Ang II-induced ROS generation. CFs were treated with HSYA (50, 100 or 200 μM) for 1 h and induced with Ang II. The CFs were stained with DCFH-DA and Hoechst 33342 after 24 h of Ang II stimulation. The intracellular DCF fluorescence was promoted in Ang II-stimulated CFs, but reduced by HSYA (50, 100 and 200 μM) treatment (**Figure 7A**). The results obtained using the multifunctional microplate reader were similar to those of the fluorescence images. Ang II-induced DCF fluorescence elevation was significantly reversed by HSYA (100 and 200 μM). HSYA at 50 μM showed an inhibitory trend, but the difference showed no statistical significance (**Figure 7B**). These data indicated that HSYA mitigated the ROS production by Ang II, suggesting that HSYA

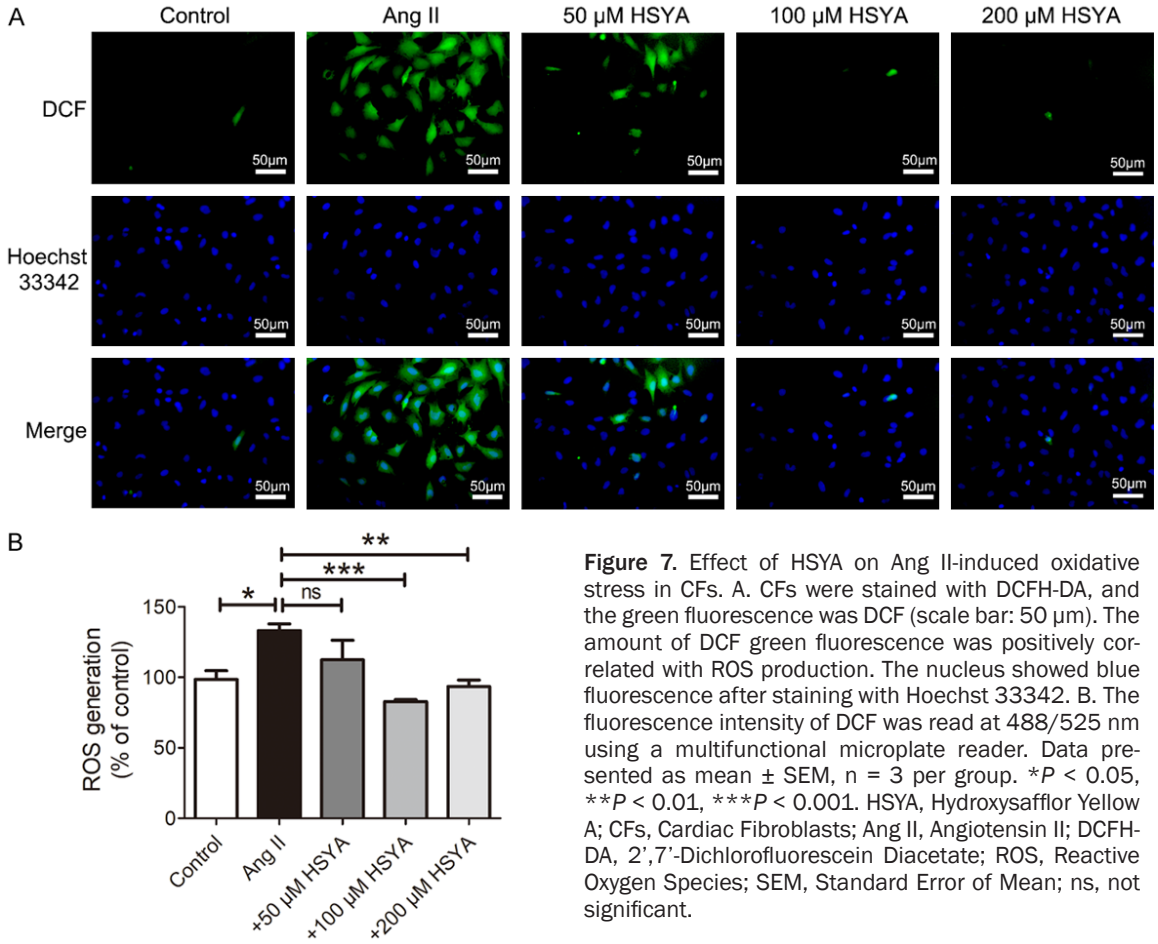

Figure 7. Effect of HSYA on Ang II-induced oxidative stress in CFs. A. CFs were stained with DCFH-DA, and the green fluorescence was DCF (scale bar: 50 μm). The amount of DCF green fluorescence was positively correlated with ROS production. The nucleus showed blue fluorescence after staining with Hoechst 33342. B. The fluorescence intensity of DCF was read at 488/525 nm using a multifunctional microplate reader. Data presented as mean ± SEM, n = 3 per group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. HSYA, Hydroxysafflor Yellow A; CFs, Cardiac Fibroblasts; Ang II, Angiotensin II; DCFH-DA, 2',7'-Dichlorofluorescein Diacetate; ROS, Reactive Oxygen Species; SEM, Standard Error of Mean; ns, not significant.

prevented Ang II-induced oxidative stress in CFs.

Discussion

This study found that HSYA alleviated myocardial fibrosis, NLRP3 inflammasome activation, and IL-18 and IL-1β expressions in mice induced by ISO and suppressed Ang II-induced collagen synthesis by inhibiting the TGFβ1-Smad2/3, ROS, NLRP3 inflammasome pathways in isolated CFs. These findings indicated that HSYA might be an effective drug for myocardial fibrosis.

TGFβ1 is a necessary factor for Ang II to induce myocardial fibrosis, and there are two types of TGFβ receptors (TβRs). The activation of the heterodimers (TβRI and TβRII) could progress to the downstream signaling pathways, which includes classical and non-classical pathways. The classic signaling pathway of TGFβ1 promotes fibrosis, involving the phosphorylation of Smad2/3, which is then combined with Smad4 to form a complex and transported to the nucleus. The complex serves as a transcription factor to initiate the transcription of a large number of pro-fibrotic genes. The non-classical pathway is involved in a variety of mitogen-activated protein kinase signal transduction pathways, including Jun N-terminal kinase and P38 [30]. In this study, the classical pathway involved TGFβ1 and P-Smad2/3 was verified using Western blotting. The results indicated that HSYA could inhibit Ang II-induced collagen synthesis by inhibiting the TGFβ1-Smad2/3 pathway. Intriguingly, HSYA has been demonstrated to suppress the nuclear translocation of Smad2 and Smad3 and the binding activity of Smad3 to collagen I promoter in human fetal lung fibroblasts [31]. Moreover, HSYA targeted TβRII to reduce the phosphorylation of Smad2 and Smad3 in TGFβ1-treated human fetal lung fibroblasts cells [32]. The above evidence partially explained how HSYA modulates the TGFβ1-

Smad2/3 pathway, which deserves validation in CFs.

Ang II-induced myofibroblast differentiation is impaired in NLRP3$^{-/-}$ CFs [4, 5]. Therefore, NLRP3 plays an important role in myocardial fibrosis. According to previous reports, the mechanism by which the NLRP3 inflammasome pathway promotes myocardial fibrosis may involve the generation of inflammatory cytokines and NLRP3 protein itself. Both IL-18 and IL-1β promote fibrosis by enhancing the inflammatory response and inducing TGFβ1 expression [11, 33]. IL-1β and TNF-α synergize to induce AT1R synthesis in the heart post-myocardial infarction, thus contributing to extracellular matrix remodeling and fibrosis [34]. TGFβ induced an increase in NLRP3 protein expression in CFs, and the central nucleotide-binding domain of NLRP3 enhanced the Smad2/3 signaling. The mitochondrial localization of NLRP3 can also increase ROS production to enhance Smad2/3 signaling, thereby promoting the differentiation of CFs into myofibroblasts [35]. In this study, we observed that HSYA inhibited NLRP3 inflammasome activation and the protein expressions of IL-1β and IL-18. Studies have reported that a single subcutaneous injection of ISO in mice can activate cardiac IL-18 rather than IL-1β through β1-AR-ROS signaling pathway, and then increase the expression of chemokines, recruit macrophage infiltration, and promote the activation of cardiac inflammation and fibrosis [11]. It has also been reported that ISO can simultaneously induce the increase of IL-1β and IL-18 expressions in vivo and in vitro [13, 36].

ROS enhancement impair the dynamic balance, resulting in oxidative stress-related damage, which is implicated in the pathogenesis of myocardial infarction [37, 38]. Treatment of atrial fibroblasts with the ROS scavenger N-acetyl-L-cysteine repressed Ang II-induced α-SMA expression and ECM deposition [39]. Consistently, oxidative stress was alleviated by HSYA at 80 μM via inhibiting ROS generation and modulating the activities of SOD and MDA in H9c2 cardiomyocytes [40]. Our study demonstrated that HSYA inhibited Ang II-induced ROS production in a dose-dependent manner. In H9c2 cardiomyocytes and RAW264.7, HSYA plays a protective role by inhibiting NLRP3 [21, 41]. This is the first study to confirm that HSYA also plays such a role in CFs.

Conclusions

In summary, we defined the protective effect of HSYA on ISO- and Ang II-induced myocardial fibrosis and the possible molecular mechanisms. Our data demonstrated that HSYA prevents myocardial fibrosis by blocking the ROS, NLRP3, IL-18/IL-1β-TGFβ1 pathways. But the present study has certain limitations. First, only a single dose of HSYA was used for *in vivo* experiments. Second, no intervention of the signaling pathways has been performed, and the upstream and downstream relationships of these pathway need to be further investigated. Nevertheless, our findings suggest the possible application of HSYA for the treatment of myocardial fibrosis in clinic.

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Disclosure of conflict of interest

None.

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References

[1] Lopez B, Ravassa S, Moreno MU, Jose GS, Beaumont J, Gonzalez A and Diez J. Diffuse myocardial fibrosis: mechanisms, diagnosis and therapeutic approaches. Nat Rev Cardiol 2021; 18: 479-498.

[2] Plitt GD, Spring JT, Moulton MJ and Agrawal DK. Mechanisms, diagnosis, and treatment of heart failure with preserved ejection fraction and diastolic dysfunction. Expert Rev Cardiovasc Ther 2018; 16: 579-589.

[3] Frangogiannis NG. Cardiac fibrosis. Cardiovasc Res 2021; 117: 1450-1488.

[4] Forrester SJ, Booz GW, Sigmund CD, Coffman TM, Kawai T, Rizzo V, Scalia R and Eguchi S. Angiotensin II signal transduction: an update on mechanisms of physiology and pathophysiology. Physiol Rev 2018; 98: 1627-1738.

[5] Chen Y, Zeng M, Zhang Y, Guo H, Ding W and Sun T. Nlrp3 deficiency alleviates angiotensin II-induced cardiomyopathy by inhibiting mito-

chondrial dysfunction. Oxid Med Cell Longev 2021; 2021: 6679100.
[6] Gan W, Ren J, Li T, Lv S, Li C, Liu Z and Yang M. The SGK1 inhibitor EMD638683, prevents angiotensin II-induced cardiac inflammation and fibrosis by blocking NLRP3 inflammasome activation. Biochim Biophys Acta Mol Basis Dis 2018; 1864: 1-10.
[7] Pinar AA, Scott TE, Huuskes BM, Tapia Caceres FE, Kemp-Harper BK and Samuel CS. Targeting the NLRP3 inflammasome to treat cardiovascular fibrosis. Pharmacol Ther 2020; 209: 107511.
[8] Chen J and Chen ZJ. PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. Nature 2018; 564: 71-76.
[9] Nguyen MN, Kiriazis H, Ruggiero D, Gao XM, Su Y, Jian A, Han LP, McMullen JR and Du XJ. Spontaneous ventricular tachyarrhythmias in beta2-adrenoceptor transgenic mice in relation to cardiac interstitial fibrosis. Am J Physiol Heart Circ Physiol 2015; 309: H946-957.
[10] Nuamnaichati N, Sato VH, Moongkarndi P, Parichatikanond W and Mangmool S. Sustained beta-AR stimulation induces synthesis and secretion of growth factors in cardiac myocytes that affect on cardiac fibroblast activation. Life Sci 2018; 193: 257-269.
[11] Xiao H, Li H, Wang JJ, Zhang JS, Shen J, An XB, Zhang CC, Wu JM, Song Y, Wang XY, Yu HY, Deng XN, Li ZJ, Xu M, Lu ZZ, Du J, Gao W, Zhang AH, Feng Y and Zhang YY. IL-18 cleavage triggers cardiac inflammation and fibrosis upon β-adrenergic insult. Eur Heart J 2018; 39: 60-69.
[12] Allawadhi P, Khurana A, Sayed N, Kumari P and Godugu C. Isoproterenol-induced cardiac ischemia and fibrosis: plant-based approaches for intervention. Phytother Res 2018; 32: 1908-1932.
[13] Li M, Tan H, Gao T, Han L, Teng X, Wang F and Zhang X. Gypensapogenin I ameliorates isoproterenol (ISO)-induced myocardial damage through regulating the TLR4/NF-kappaB/NLRP3 pathway. Molecules 2022; 27: 5298.
[14] Xu H, Liu T, Wang W, Su N, Yang L, Yang Z, Dou F, Cui J, Fei F, Ma J, Wen A and Ding Y. Proteomic analysis of hydroxysafflor yellow A against cerebral ischemia/reperfusion injury in rats. Rejuvenation Res 2019; 22: 503-512.
[15] Li Z, Sun X, Guo S, Wang L, Wang T, Peng C, Wang W, Tian Z, Zhao R, Cao W and Tian Y. Rapid stabilisation of atherosclerotic plaque with 5-aminolevulinic acid-mediated sonodynamic therapy. Thromb Haemost 2015; 114: 793-803.
[16] Zhang Q, Peng JH and Zhang XN. A clinical study of Safflower Yellow injection in treating coronary heart disease angina pectoris with Xin-blood stagnation syndrome. Chin J Integr Med 2005; 11: 222-225.
[17] Jin M, Wang L, Wu Y, Zang BX and Tan L. Protective effect of hydroxysafflor yellow A on bleomycin-induced pulmonary inflammation and fibrosis in rats. Chin J Integr Med 2018; 24: 32-39.
[18] Jin M, Wu Y, Wang L, Zang B and Tan L. Hydroxysafflor yellow A attenuates bleomycin-induced pulmonary fibrosis in mice. Phytother Res 2016; 30: 577-587.
[19] Hu N, Duan J, Li H, Wang Y, Wang F, Chu J, Sun J, Liu M, Wang C, Lu C and Wen A. Hydroxysafflor yellow A ameliorates renal fibrosis by suppressing TGF-beta1-induced epithelial-to-mesenchymal transition. PLoS One 2016; 11: e0153409.
[20] Zhang Y, Guo J, Dong H, Zhao X, Zhou L, Li X, Liu J and Niu Y. Hydroxysafflor yellow A protects against chronic carbon tetrachloride-induced liver fibrosis. Eur J Pharmacol 2011; 660: 438-444.
[21] Ye JX, Wang M, Wang RY, Liu HT, Qi YD, Fu JH, Zhang Q, Zhang BG and Sun XB. Hydroxysafflor yellow A inhibits hypoxia/reoxygenation-induced cardiomyocyte injury via regulating the AMPK/NLRP3 inflammasome pathway. Int Immunopharmacol 2020; 82: 106316.
[22] Pan XC, Liu Y, Cen YY, Xiong YL, Li JM, Ding YY, Tong YF, Liu T, Chen XH and Zhang HG. Dual role of triptolide in interrupting the NLRP3 inflammasome pathway to attenuate cardiac fibrosis. Int J Mol Sci 2019; 20: 360.
[23] Ding YY, Li JM, Guo FJ, Liu Y, Tong YF, Pan XC, Lu XL, Ye W, Chen XH and Zhang HG. Triptolide upregulates myocardial forkhead helix transcription factor p3 expression and attenuates cardiac hypertrophy. Front Pharmacol 2016; 7: 471.
[24] Shi XM, Zhang H, Zhou ZJ, Ruan YY, Pang J, Zhang L, Zhai W and Hu YL. Effects of safflower yellow on beta-amyloid deposition and activation of astrocytes in the brain of APP/PS1 transgenic mice. Biomed Pharmacother 2018; 98: 553-565.
[25] Hanna A and Frangogiannis NG. The role of the TGF-beta superfamily in myocardial infarction. Front Cardiovasc Med 2019; 6: 140.
[26] Kim KK, Sheppard D and Chapman HA. TGF-beta1 signaling and tissue fibrosis. Cold Spring Harb Perspect Biol 2018; 10: a022293.
[27] Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR and Doetschman T. TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. J Clin Invest 2002; 109: 787-796.
[28] Zhang Y, Liu Y, Bi X, Hu C and Ding W. NLRP3 deletion attenuated angiotensin II-induced renal fibrosis by improving mitochondrial dys-

function and endoplasmic reticulum stress. Nephron 2021; 145: 518-527.
[29] Chen RR, Fan XH, Chen G, Zeng GW, Xue YG, Liu XT and Wang CY. Irisin attenuates angiotensin II-induced cardiac fibrosis via Nrf2 mediated inhibition of ROS/TGFbeta1/Smad2/3 signaling axis. Chem Biol Interact 2019; 302: 11-21.
[30] Ong CH, Tham CL, Harith HH, Firdaus N and Israf DA. TGF-beta-induced fibrosis: a review on the underlying mechanism and potential therapeutic strategies. Eur J Pharmacol 2021; 911: 174510.
[31] Pan R, Zhang Y, Zang B, Tan L and Jin M. Hydroxysafflor yellow A inhibits TGF-beta1-induced activation of human fetal lung fibroblasts in vitro. J Pharm Pharmacol 2016; 68: 1320-1330.
[32] Pan R, Zhang Y, Zheng M, Zang B and Jin M. Hydroxysafflor yellow A suppresses MRC-5 cell activation induced by TGF-beta1 by blocking TGF-beta1 binding to TbetaRII. Front Pharmacol 2017; 8: 264.
[33] Zhang WJ, Chen SJ, Zhou SC, Wu SZ and Wang H. Inflammasomes and fibrosis. Front Immunol 2021; 12: 643149.
[34] Gurantz D, Cowling RT, Varki N, Frikovsky E, Moore CD and Greenberg BH. IL-1beta and TNF-alpha upregulate angiotensin II type 1 (AT1) receptors on cardiac fibroblasts and are associated with increased AT1 density in the post-MI heart. J Mol Cell Cardiol 2005; 38: 505-515.
[35] Bracey NA, Gershkovich B, Chun J, Vilaysane A, Meijndert HC, Wright JR Jr, Fedak PW, Beck PL, Muruve DA and Duff HJ. Mitochondrial NLRP3 protein induces reactive oxygen species to promote Smad protein signaling and fibrosis independent from the inflammasome. J Biol Chem 2014; 289: 19571-19584.
[36] Caceres FT, Gaspari TA, Samuel CS and Pinar AA. Serelaxin inhibits the profibrotic TGF-beta1/IL-1beta axis by targeting TLR-4 and the NLRP3 inflammasome in cardiac myofibroblasts. FASEB J 2019; 33: 14717-14733.
[37] Cai H, Liu Y, Men H and Zheng Y. Protective mechanism of humanin against oxidative stress in aging-related cardiovascular diseases. Front Endocrinol (Lausanne) 2021; 12: 683151.
[38] Liu L, Gong W, Zhang S, Shen J, Wang Y, Chen Y and Meng G. Hydrogen sulfide attenuates angiotensin II-induced cardiac fibroblast proliferation and transverse aortic constriction-induced myocardial fibrosis through oxidative stress inhibition via sirtuin 3. Oxid Med Cell Longev 2021; 2021: 9925771.
[39] Chen T, Li M, Fan X, Cheng J and Wang L. Sodium tanshinone IIA sulfonate prevents angiotensin II-induced differentiation of human atrial fibroblasts into myofibroblasts. Oxid Med Cell Longev 2018; 2018: 6712585.
[40] Ni B, Zhou D, Jing Y and Liu S. Hydroxysafflor yellow A protects against angiotensin II-induced hypertrophy. Mol Med Rep 2018; 18: 3649-3656.
[41] Xu X, Guo Y, Zhao J, Wang N, Ding J and Liu Q. Hydroxysafflor yellow A inhibits LPS-induced NLRP3 inflammasome activation via binding to xanthine oxidase in mouse RAW264.7 macrophages. Mediators Inflamm 2016; 2016: 8172706.