Sodium tanshinone IIA sulfonate attenuates angiotensin II-induced collagen type I expression in cardiac fibroblasts *in vitro*

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Abstract

Cardiac fibrosis occurs after pathological stimuli to the cardiovascular system. One of the most important factors that contribute to cardiac fibrosis is angiotensin II (Ang II). Accumulating studies have suggested that reactive oxygen species (ROS) plays an important role in cardiac fibrosis and sodium tanshinone IIA sulfonate (STS) possesses antioxidant action. We therefore examined whether STS depresses Ang II-induced collagen type I expression in cardiac fibroblasts. In this study, Ang II significantly enhanced collagen type I expression and collagen synthesis. Meanwhile, Ang II depressed matrix metalloproteinase-1 (MMP-1) expression and activity. These responses were attenuated by STS. Furthermore, STS depressed the intracellular generation of ROS, NADPH oxidase activity and subunit p47[phox] expression. In addition, N-acetylcysteine the ROS scavenger, depressed effects of Ang II in a manner similar to STS. In conclusion, the current studies demonstrate that anti-fibrotic effects of STS are mediated by interfering with the modulation of ROS.

Keywords: angiotensin II; collagen; fibrosis; heart; reactive oxygen species; tanshinone

Introduction

Cardiac fibrosis is characterized by a disproportionate accumulation of extracellular matrix (ECM) proteins that occurs after myocyte death, inflammation, hypertrophy, and stimulation by a number of hormones, cytokines, and growth factors (Wood, 2002; Kass *et al.*, 2004; Jaana *et al.*, 2005). Among the ECM proteins, collagens constitute up to 85% (Brown, 2005). Collagens are proteins that consist of a triple helix of polypeptide chains and globular domains, and comprise a family of proteins of at least 19 genetically distinct types, among which type I and type III constitute two-thirds. As in the form of thick fiber with a high tensile strength, collagen type I content is considered a major determinant of myocardial stiffness. Cardiac fibroblasts (CFs) are the major source of collagen in the myocardium. Collagen type I accumulation in the heart depends not only on its production, but also on its degradation, which is performed by proteinases, such as matrix metalloproteinase-1 (MMP-1) (Heeneman *et al.*, 2003).

Angiotensin II (Ang II) plays a critical role in cardiac fibrosis (Weber *et al.*, 1991; Gibbons, 1998). In CFs, Ang II can upregulate collagen type I expression through stimulation oxidative stress (Cohn *et al.*, 2000). Oxidative stress induces remodeling of the myocardium and regulates collagen metabolism in a variety of non-cardiac cells, such as lung and skin fibroblasts and human venous endothelial cells. Moreover, Ang II stimulation of the type 1 Ang II receptor has been shown to regulate collagen type I degradation by attenuating the activity and expression of collagen degrading enzyme MMP-1 in rat CFs (Chen *et al.*, 2004a). Despite the physiopathologic significance of fibrosis,
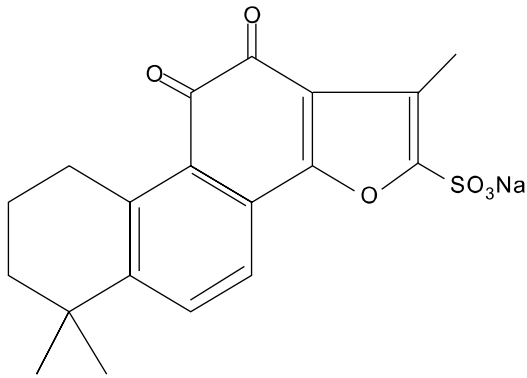
Figure 1. The molecular structure of STS.

no effective treatment strategies exist.

Sodium tanshinone IIA sulfonate (STS, Figure 1) is a derivative of tanshinone IIA which is a lipid-soluble pharmacologically active component isolated from the rhizome of the Chinese herb *Salvia miltiorrhiza*, a well-known traditional Chinese medicine used for the treatment of cardiovascular diseases such as coronary heart disease (Zhao *et al.*, 1996). Recently, STS was shown to possess antioxidant action (Wang *et al.*, 2008; Yang *et al.*, 2008). In this paper, we explored the effects and mechanisms of STS on Ang II-induced collagen type I expression in CFs *in vitro*; then raised the experimental basis for using STS to cardiac fibrosis in clinic.

Results

Effect of STS on Ang II-Induced ROS generation

A previous study showed that there was minimal ROS generation in CFs in the control state (Lijnen *et al.*, 2006). However, on treatment with Ang II, there was a marked increase in intracellular production of ROS, which played a key role in cardiac fibrosis. As STS was shown to possess some antioxidant action, we examined the effect of STS (3, 10, 30 μM) on Ang II-increased intracellular ROS generation. STS suppressed ROS formation in CFs as measured after Ang II treatment for 12 h, while STS alone had no effect on ROS generation (Figure 2). These data, therefore, clearly demonstrated that STS could decrease ROS production.

Effects of STS on the Ang II-induced collagen type I expression and collagen synthesis

As it is shown in Figure 3, Ang II significantly enhanced collagen type I expression, which was attenuated by the pretreatment with STS (3, 10, 30 μM).

Consistent with the increase of collagen expression, Ang II (0.1 μM) caused a significant increase in the rate of [^{3}H]proline incorporation, which was also significantly depressed by the pretreatment with STS. To determine whether the inhibition of Ang II-induced collagen type I expression and collagen synthesis by STS is via a decrease in ROS production, we employed N-acetyl-L-cysteine (NAC), a known antioxidant. The results showed that NAC (8 mM) significantly reduced collagen type I expression and collagen synthesis induced by Ang II, qualitatively similar to STS (Figure 3). These findings implied that STS inhibited Ang II-induced fibrotic responses via attenuation of ROS in CFs.

Effects of STS on the Ang II-induced MMP-1 expression and activity

Collagen type I accumulation in the heart depends not only on its production, but also on its degradation, which is performed by proteinases, such as matrix metalloproteinase-1 (MMP-1). Therefore, we explored the effects of STS on the Ang II-induced MMP-1 expression and MMP-1 activity. As it is shown in Figure 4, Ang II significantly depressed MMP-1 expression and activity. These effects were significantly attenuated by pretreatment with STS (3, 10, 30 μM). To determine whether these effects of STS were via a decrease in ROS production, we employed NAC. As shown in Figure 4, the effects of Ang II on MMP-1 expression and activity were significantly suppressed by NAC in CFs. These findings implied that STS promoted collagen degradation via attenuation of ROS in CFs.
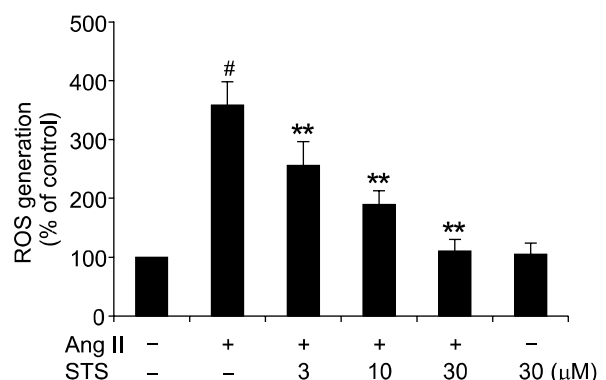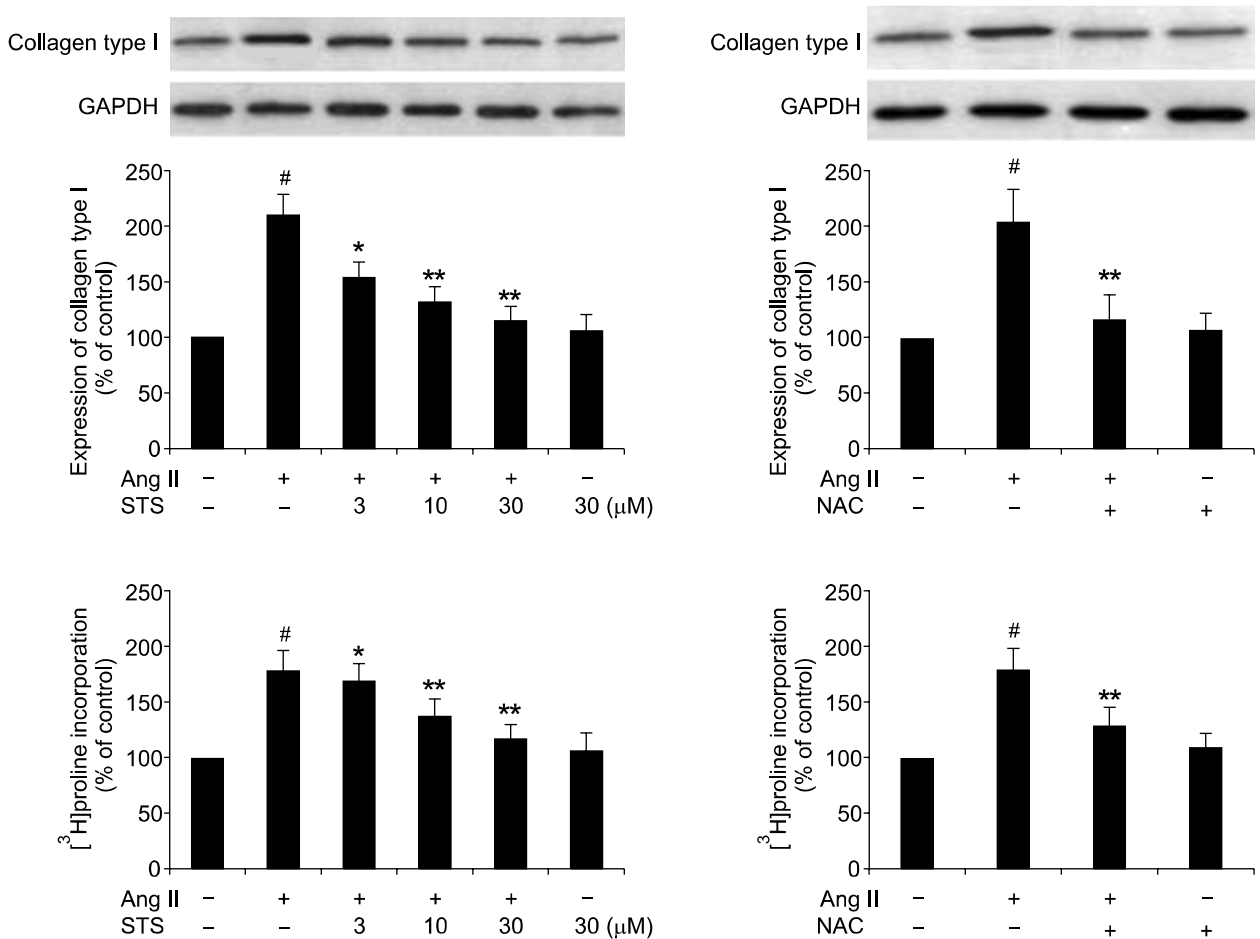

Figure 2. Effects of STS on Ang II-induced production of ROS. Cardiac fibroblasts were pretreated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II. After 12 h, DCF fluorescence was measured by fluorospectrophotometer analysis. The experiments were repeated five times with reproducible results. $^{\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. Ang II group.

Figure 3. Effects of STS on the Ang II-induced collagen type I expression and collagen synthesis. Cardiac fibroblasts were pretreated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II. After 24 h, collagen type I expression was determined with the Western Blot and collagen synthesis was determined with the [^{3}H]proline incorporation. The experiments were repeated five times with reproducible results. $^\#P < 0.01$ vs. control; $^*P < 0.05$ and $^{**}P < 0.01$ vs. Ang II group.

Effects of STS on Ang II-induced fibroblast proliferation

The effect of STS on the fibroblast proliferation was assessed by MTT assay and DNA synthesis assay ([3H]thymidine incorporation). Recent studies reported that Ang II was associated with fibroblast proliferation (Chen *et al.*, 2004b; Chen and Mehta, 2006; Zhou *et al.*, 2007). In the present study, we examined fibroblast proliferation in response to Ang II and its modulation by the pretreatment of STS. The data showed that, Ang II did induce CFs proliferation, whereas STS (3, 10, 30 μM) did not induce obvious inhibitory effect on fibroblast proliferation (Figure 5).

Effect of STS on Ang II-induced NADPH oxidase activity and subunit p47^{phox} expression

To determine the mechanisms of STS-induced de-

crease in ROS generation, we examined NADPH oxidase activity and subunit p47^{phox} expression in CFs with the treatment of Ang II and STS using cytochrome c reduction assay. Ang II (0.1 μM) increased NADPH oxidase activity and subunit p47^{phox} expression. These responses were markedly depressed by treatment of STS.

Discussion

Cardiac fibrosis, a common complication of hypertension, increases the risk for cardiac ischemia, left ventricular dysfunction and sudden cardiac death, represents a very strong predictor of cardiovascular mortality and death of all causes and is recognized as a risk factor for the development of congestive heart failure (Weber *et al.*, 1994, 1995; Fedak *et al.*, 2005). The regulation of the ECM of the heart is essential to understanding the chronic
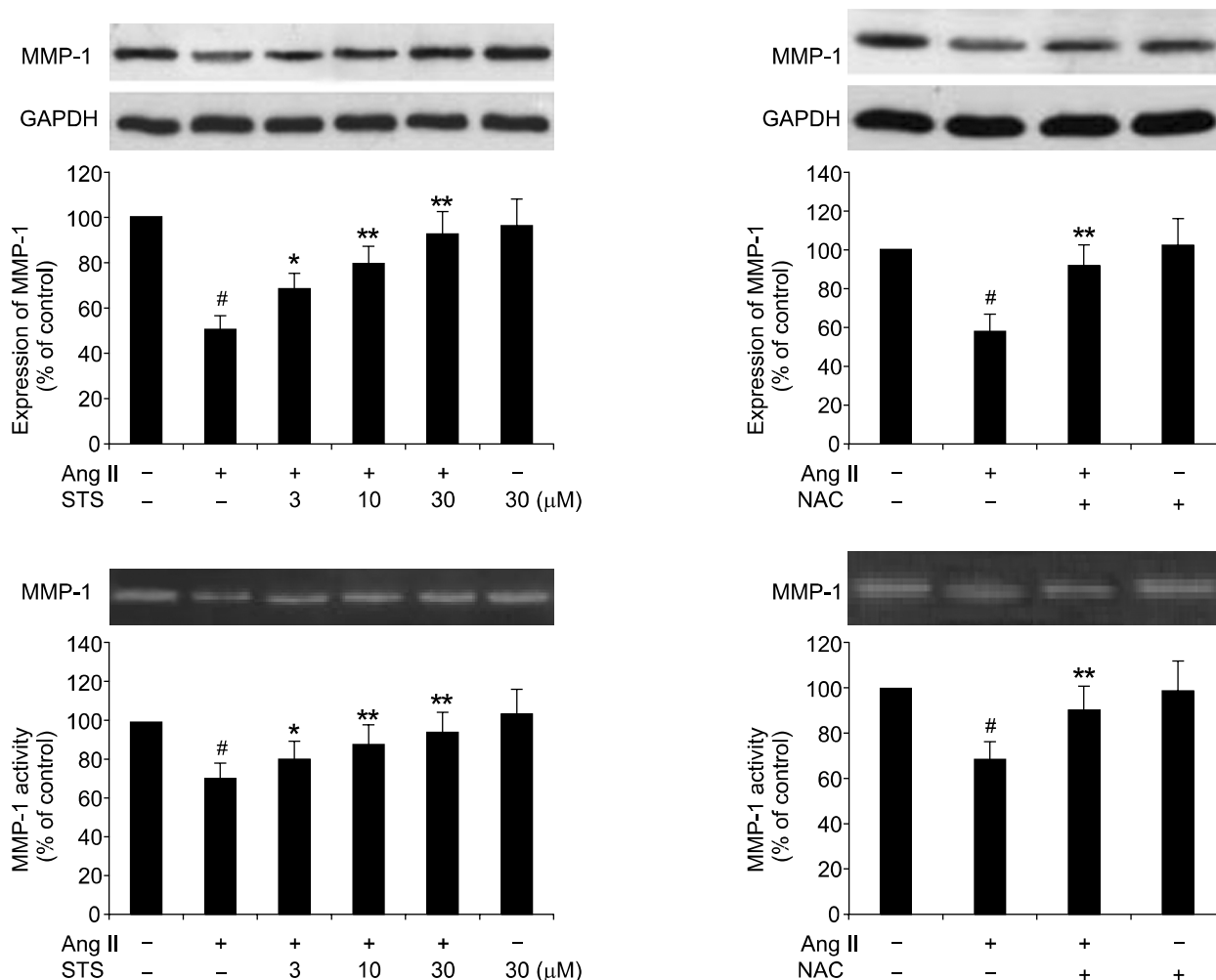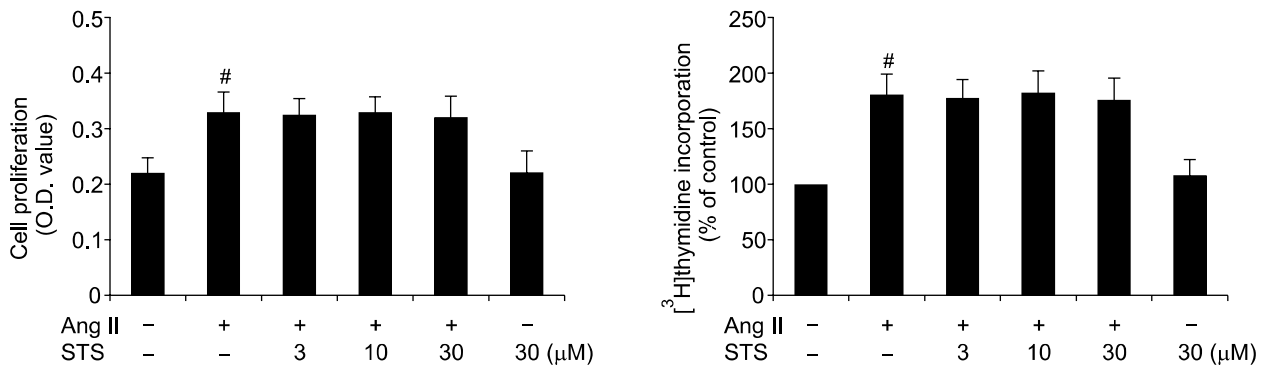
Figure 4. Effects of STS on the Ang II-induced MMP-1 expression and activity. Cardiac fibroblasts were pretreated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II. After 24 h, MMP-1 expression was determined with the Western Blot and MMP-1 activity was determined with the zymography. The experiments were repeated five times with reproducible results. [#]$P < 0.01$ vs. control; [*]$P < 0.05$ and [**]$P < 0.01$ vs. Ang II group.

changes in heart function in the above disease states (Brown *et al.*, 2007). Excessive collagen is clearly detrimental to cardiac function (Kawano *et al.*, 2000). One of the most important factors contributing to the development of cardiac fibrosis is Ang II (Sun and Weber, 2003; Kumar *et al.*, 2008). Most of the known effects of Ang II were mediated by AT1 receptors, the expression of which in CFs far exceeded that in cardiomyocytes (Lijnen *et al.*, 2001). Stimulation with Ang II resulted in myocardial fibrosis *in vivo* (Kim *et al.*, 1995) as well as CFs proliferation and collagen deposition *in vitro* (Ashizawa *et al.*, 1996).

The root of *Salvia miltiorrhiza* BUNGE, known as Danshen in Chinese, is an herbal plant widely used to cure myocarditis and myocardial infarction (Chen *et al.*, 1979). The traditional Chinese medicine Danshen, derived from the dried root or rhizome of

Salviae miltiorrhizae Bge, has been widely used for treatment of cardiovascular and cerebrovascular diseases. More than 30 diterpene compounds have been separated and identified from Danshen. Actually, Danshen-derived compounds have many important pharmacology effects in basic experiments or clinic, such as anti-tumor, immunoloregulation and cardioprotective effects, and so on (Kang *et al.*, 2000; Su and Lin, 2008; Zhou *et al.*, 2008). Tanshinone IIA is most abundant and structurally representative of the tanshinones of *Salvia miltiorrhiza* (Tang and Eisenbrand, 1992). Recently, STS was shown to be a promising drug that reduced cardiac remodeling through depressing cardiomyocyte hypertrophy (Yang *et al.*, 2007). Moreover, STS was shown to possess antioxidant action (Zhou *et al.*, 1999, 2003). Evidence shows that STS is an effective antioxidant that inhibits the
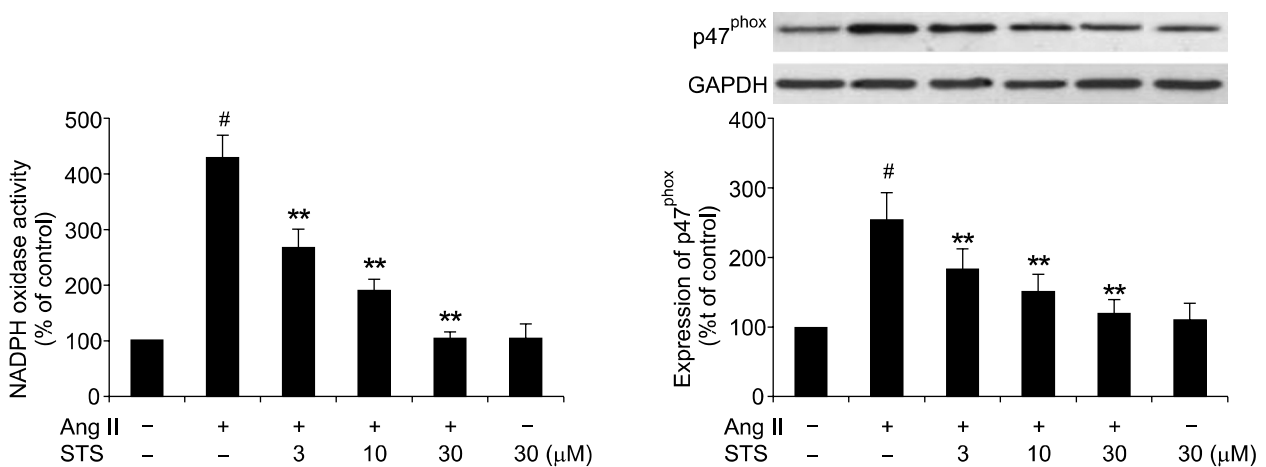
Figure 5. Effects of STS on Ang II-induced fibroblast proliferation. Cardiac fibroblasts were cultured in microplate and were incubated with STS (3, 10, 30 μM). After 24 h, cell proliferation was determined with the MTT assay and DNA synthesis assay ([^{3}H]thymidine incorporation). The experiments were reated five times with reproducible results. $^{\#}P < 0.01$ vs. control.

formation of reactive oxygen radicals in rat heart mitochondria (Yang *et al.*, 2008), breaks the chain reactions of peroxidation by scavenging lipid-free radicals and increases the activity of superoxide dismutase (Wang *et al.*, 2008). Nevertheless, up to date, little is known about the cellular and molecular mechanisms of STS-mediated anti-fibrotic effects in cardiac fibroblasts after Ang II stimulation. In this study, we attempted to explore the effects and mechanisms of STS on Ang II-induced collagen type I expression in cultured CFs.

In this research, *in vitro*, we proved that Ang II (0.1 μM) increased the expression and synthesis of collagen type I in CFs. In concordance with the increase of collagen expression, Ang II caused a significant increase in the rate of [^{3}H]proline incorporation. These results were consistent with the previous reports (Lijnen *et al.*, 2001; Chen *et al.*,

2004c). The above effects were significantly depressed by the pretreatment with different concentrations of STS. Therefore, STS can depress the Ang II-induced collagen type I expression through attenuating collagen synthesis. Moreover, it is known that collagen type I accumulation in the heart depends not only on its production, but also on its degradation, which is performed by proteinases, such as matrix metalloproteinase-1 (MMP-1) (Brilla *et al.*, 1994; D'Armiento *et al.*, 2002; Heeneman *et al.*, 2003). As it is shown in Figure 4, Ang II significantly depressed MMP-1 expression and activity, which accorded with the previous reports (Chen *et al.*, 2004a, b; Lijnen *et al.*, 2006; Pan *et al.*, 2008). The pretreatment of STS can depress these effects of Ang II. However, the present study showed that STS did not induce obvious inhibitory effect on fibroblast proliferation, which is consistent

Figure 6. Effect of STS on Ang II-induced NADPH oxidase activity and subunit p47^{phox} expression. Cardiac fibroblasts were pretreated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II. After 24 h, NADPH oxidase activity was measured by SOD-inhibitable cytochrome c reduction and p47^{phox} expression was measured by Western blot. The experiments were repeated five times with reproducible results. $^{\#}P < 0.01$ vs. control; **P $<$ 0.01 vs. Ang II group.

with the previous report (Takahashi *et al.*, 2002). Interestingly, the effects of Ang II on CFs are different from the effects of Ang II in endothelial cells (Seeger *et al.*, 2001) and cardiomyocytes (Rouet-Benzineb *et al.*, 2000), wherein Ang II enhances MMP expression/activity. Therefore, Ang II affects MMP expression/activity differently in different cell types. The effects of STS on these areas are the fields we would explore in the future.

It is shown that Ang II enhances the ROS generation in CFs (Chen *et al.*, 2006), which is confirmed in this study. In our research, STS depressed Ang II-stimulated ROS generation and depressed the effects of Ang II on collagen as well as MMP-1 expression in cardiac fibroblasts. Furthermore, the pretreatment of NAC, the ROS scavenger, exerted qualitatively similar effects as STS on collagen type I and MMP-1 expression in response to Ang II. From the above evidence, attenuation of ROS generation is, to a great extent, the basis of the observed effects of STS. NADPH oxidase is a major source of ROS and subunit p47^{phox} plays a key role in Ang II-mediated NADPH oxidase activation (Lambeth *et al.*, 2004; Murdoch *et al.*, 2006). To determine the mechanisms of the anti-fibrotic effects of STS, we examined NADPH oxidase activity and subunit p47^{phox} expression in CFs with the treatment of Ang II and STS. Ang II increased NADPH oxidase activity and subunit p47^{phox} expression. These responses were markedly attenuated by treatment of STS.

Taken together, the present study delivers important new insights to the molecular mechanisms of actions of STS on cardiac fibroblasts. Our results indicated that STS markedly depresses Ang II-induced collagen type I expression *in vitro*. Moreover, STS could modulate the redox-sensitive steps involved in the Ang II signaling pathway. Although the precise mechanism by which STS depresses the development of cardiac fibrosis remains to be further clarified, understanding the pharmacologic actions of STS on cardiac fibroblasts may contribute to the choice of STS for the people who suffer from cardiovascular diseases.

Methods

Materials

One-day-old Wistar rats were obtained from Experimental Animal Center of Tongji Medical College, Grate II, and Certificate No 19-050. This research complied with the European Community Guidelines for the Care and Use of Experimental Animals and was approved by the Animal Research Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). STS (99.5%) were obtained from the Research Center of Traditional Chinese Medicine (Wuhan, China) and dissolved in PBS. Ang II and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (Saint Louis). Antibodies for collagen type I, MMP-1 and NADPH oxidase subunit p47^{phox} were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). All other chemicals used were of the highest grade available commercially. The molecule structure of STS is shown in Figure 1.

Cell cultures

Rat cardiac fibroblasts were prepared and cultured as described earlier. Briefly, cardiac ventricles from one-day-old Wistar rats were separated and minced with scissors into small pieces in ice-cold balanced salt solution. Ventricular CFs were dispersed in 10 ml Spinner solution of the following compositions (in mM): NaCl 116, HEPES 20, NaH$_2$PO$_4$ 12.5, glucose 5.6, KCl 5.4, and MgSO$_4$ 0.8 (pH 7.35) containing 0.1% collagenase with agitation for 10 min at 37°C. The digestion steps were repeated five to seven times until the tissues were completely digested. The cells were then combined, centrifuged, and re-suspended in a chilled fetal calf serum. After final collection, the cells were pelleted by centrifugation (800 × *g* for 10 min at room temperature). The supernatant was discarded, and the cells were re-suspended in DMEM. The resulting cell mixture was prep-plated for 1 h in a 5% CO$_2$-containing incubator at 37°C to plate out CFs. After removal of the myocyte-enriched medium, DMEM was then added to the pre-plated CFs which were cultured for 2 days before being passaged. Experiments were performed with cells from passage 3.

Western blot analysis

The expressions of collagen type I, MMP-1 and subunit p47^{phox} were determined by Western Blot. Fibroblasts from each group were pelleted and extracted in iced cell lysis buffer (Cell Signaling Technologies). Cell lysates were centrifuged at 15,000 *g* for 15 min at 4°C and the supernatants from each group were separated by 10% SDS-PAGE (for MMP-1) and 8% nondenatured-PAGE (for collagen type I) and then transferred to nitrocellulose membranes. After incubation in blocking solution (5% nonfat milk, Sigma), membranes were incubated with primary antibodies (Sigma-Aldrich) overnight at 4°C. Membranes were washed with 1 × TBST solution and then incubated with secondary antibody (1:5,000 dilution, Amersham Life Sciences) for 2 h. The membranes were detected with the ECL system (Amersham Life Sciences) and relative intensities of protein bands analyzed by Scan-gel-it software.

Collagenase activity assay

Active MMP-1 secreted into culture medium can be identified and quantified through gelatin zymography. Essentially, the conditioned culture medium was collected from the dishes and 10 µl of the medium was subjected to electrophoresis in SDS polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. The gels were soaked in 2.5% Triton-X100 for 60 min and then washed

with water for 60 min to remove SDS. The gels were then incubated in a developing buffer containing 50 mM Tris, pH 7.4, 5 mM CaCl$_2$, and 0.02% sodium azide for 18 h at 37°C. After incubation, the gels were stained with Coomassie blue and photographed.

MTT assay

When achieving 50% confluence, CFs were starved for 24 h with DMEM and treated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II. After 24 h, cell proliferation was assessed by the MTT assay. The assay is based on the transformation of the tetrazolium salt MTT by active mitochondria to an insoluble formazan salt. MTT was added to each well under sterile conditions (with a final concentration of 5 mg/ml), and the plates were incubated for 4 h at 37°C. Untransformed MTT was removed by aspiration, and formazan crystals were dissolved in DMSO (150 μl/well). Formazan was quantified at 540 nm using a Bio-Rad automated EIA Analyzer.

DNA and collagen synthesis assay

DNA synthesis was evaluated by measuring [³H]thymidine incorporation and collagen synthesis was evaluated by measuring [³H]proline incorporation as described earlier (Zhang *et al*., 2007). In brief, cardiac fibroblasts were made quiescent by culture in serum-free DMEM for 24 h. Then these cells were pretreated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II for 24 h. DNA synthesis was assessed by the addition of 1 μCi/ml of [³H]thymidine (Amershan Biosciences) for a period of 4 h prior to the end of the treatment protocol. Collagen synthesis was assessed by the addition of 1 μCi/ml [³H]proline (Amershan Biosciences) for a period of 6 h. Cells were washed with PBS and ice-cold 10% TCA. The cells were solubilized and the cell extracts analyzed in a liquid scintillation counter.

Measurement of ROS production

The determination of intracellular ROS production was based on the oxidation of 2',7'-dichlorodihydrofluorescein (DCFH) to a fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, CFs were loaded with 20 μM DCFH (Molecular Probes) for 30 min at 37°C in the dark. Cells loaded with DCFH were treated with STS (3, 10, 30 μM) for 30 min before stimulation with 0.1 μM Ang II. After 12 h, the cells were washed twice with PBS and detached by trypsin, then measured for DCF fluorescence intensity by fluorospectrophotometer analysis at an excitation wavelength of 485 nm and at an emission wavelength of 530 nm. The cell number in each sample was counted and utilized to normalize the fluorescence intensity of DCF.

NADPH oxidase activity

NADPH oxidase-dependent superoxide production was measured by SOD-inhibitable cytochrome c reduction as described previously (Lijnen *et al*., 2006). Cell homoge-

nates were distributed in 96-well flat-bottom culture plates (200 μl/well). Cytochrome c (500 μM) and NADPH (100 μM) were added in the presence or absence of SOD (200 U/ml) and incubated at room temperature for 30 min. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader. Superoxide production in nanomoles per milligram of protein was calculated from the difference between absorbance with and without SOD and extinction coefficient for change of ferricytochrome c to ferrocytochrome c.

Statistical analysis

Results were expressed as mean ± S.D. Statistical significance was determined using one-way ANOVA. The differences were considered statistically significant at a value of $P < 0.05$.

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