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Sodium tanshinone IIA sulfonate depresses angiotensin II-induced cardiomyocyte hypertrophy through MEK/ERK pathway

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Abbreviations: Ang II, angiotensin II; ANP, atrial natriuretic peptide; ERK, extracellular signal-regulated kinase; SBP, systolic blood pressure; STS, Sodium tanshinone IIA sulfonate

Abstract

Cardiomyocyte hypertrophy is a major cause of morbidity and mortality worldwide. The aim of this study is to determine the effects of sodium tanshinone IIA sulfonate (STS) on cardiomyocyte hypertrophy induced by angiotensin II (Ang II) *in vivo* and *in vitro*. In long-term treatment, adult Wistar rats were infused with Ang II for three weeks via osmotic mini-pumps and some of them were given intragastrically of STS. Left ventricle was isolated; the ratio of left ventricular weight to body weight and systolic blood pressure (SBP) were determined and heart morphometry was assessed after hematoxylin and eosin staining. Results indicated STS inhibited Ang II-induced increases in myocyte diameter and decreased the LVW/BW ratio independent of decreasing systolic blood pressure. *In vitro*, treatment of cultured cardiomyocytes with STS inhibited Ang II-induced increase in cell size, protein synthesis, *ANP* expression, activation of extracellular signal-regulated kinase (ERK) and ERK kinase (MEK). Then we reexamined the mechanism of STS-induced anti-hypertrophic effects. Results revealed MEK inhibitor U0126 (20 μM) markedly enhanced STS-induced depressions in [^{3}H]leucine incorporation and *ANP* expression. In conclusion, MEK/ERK pathway plays a significant role in the anti-hypertrophic effects of STS.

Keywords: angiotensin II; extracellular signal-regulated MAP kinases; hypertrophy; myocytes, cardiac; tanshinone

Introduction

Cardiomyocyte hypertrophy, 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 (Levy *et al.*, 1990). One of the most important factors contributing to the development of cardiomyocyte hypertrophy is angiotensin II (Ang II) which increases protein synthesis and cell diameter in cultured neonatal rat myocytes through the type 1 Ang II receptor (Baker *et al.*, 1990). These processes directly lead to cardiomyocyte hypertrophy .

Ang II is a multifunctional agonist for cardiomyocyte hypertrophy, stimulating protein phosphorylation, contractility, gene expression and cell growth (Kagiyama *et al.*, 2002). Many of the signaling events stimulated by Ang II are mediated by members of the mitogen-activated protein kinase (MAPK) family, including the extracellular signal-regulated kinase (ERK), the p38 and the c-Jun NH2-terminal kinase (JNK) (Thorburn *et al.*, 1995). Among the MAPKs, ERK has been focused on the essential regulators of a hypertrophic response although JNK and p38 were recently studied in regulating cardiac hypertrophy (Sugden, 2001; Maller, 2003; Ptashne

and Gann, 2003).

Sodium tanshinone IIA sulfonate (STS) 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). In this paper, we used long-term treatment experiments and cultured neonatal rat cardiomyocytes to investigate the influence of STS on Ang II-induced cardiomyocyte hypertrophy *in vivo* and *in vitro*; examined effects of STS on MEK/ERK signaling pathway; then raised the experimental basis for using STS to cardiomyocyte hypertrophy in clinic.

Materials and Methods

Materials

Adult Wistar rats (200-230 g) and 1-day-old Wistar rats were obtained from Experimental Animal Center of Tongji Medical College, Grate II, and Certificate No 19-050. This study complies 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 of 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 was obtained from Sigma-Aldrich (Saint Louis). U0126 was obtained from Alexis Biochemicals (Lausen, Switzerland). Antibodies for phosphorylated ERK, MEK and α-actin were purchased from Santa Cruz Biotechnology Inc (CA). Texas Red-X phalloidin was obtained from Molecular Probes Inc. All other chemicals used were of the highest grade available commercially. The molecule structure of STS is shown in Figure 1.

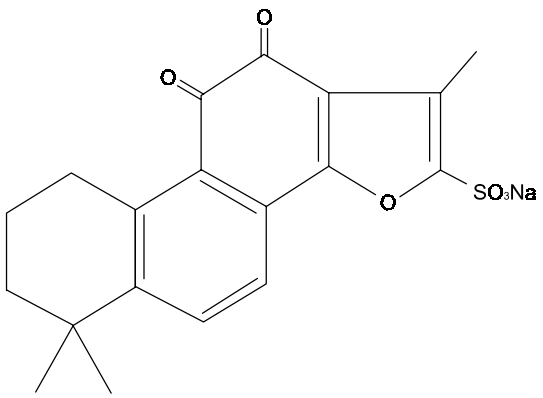

Figure 1. The molecular structure of STS.

Experimental protocol in long-term treatment

For Ang II stimulation, adult Wistar rats weighing 200 to 230 *g* were anesthetized with isoflurane. Osmotic mini-pumps (Alza Corp.) containing either saline or Ang II (25 ng/kg/min, dissolved in saline), were implanted subcutaneously for three weeks. Some Ang II-exposed rats were given intragastrically of PBS, others were given intragastrically of STS (50 mg/kg/d) in PBS for 3 weeks. At the end of experiments, morphology of left ventricle was examined.

Determination of systolic blood pressure

In all rats, systolic blood pressure (SBP) was measured by the tail-cuff method using the plethysmographic tail-cuff method. The blood pressure was measured twice a week in each rat by the same person and at the same time of day (Sen, 1983).

Morphological examination

After 3 weeks of the treatment, animals were weighed and killed by decapitation. The thoracic cavity was immediately opened. With the blood squeezed out, the heart was excised and rinsed in cold physiological saline. The atria were removed and all the epicardial fat was removed. The left ventricle was isolated, blotted, and weighed in an analytical balance. The hypertrophy index was determined using the ratio of left ventricular weight over body weight (LVW/BW) (Hayakawa and Raij, 1997). Histopathological observation was also carried out as described previously (Miao *et al.*, 2001). Briefly, immediately after gross detection, all samples of left ventricles in 2- to 3-mm-thick slices were immersed in formalin solution for more than 1 week, dehydrated in ethanol, cleared in dimethylbenzene, and embedded in paraffin. Then the 5-μm-thick sections were prepared and stained with hematoxylin and eosin for light microscopic evaluation. Mean myocyte diameter was calculated by measuring 100 cells from sections stained with hematoxylin and eosin.

Neonatal cardiomyocyte cultures

Primary cultures of 1-day old neonatal Wistar rat myocytes were prepared as previously described (Hannan *et al.*, 1996). Minced ventricular myocardium was placed into D-Hanks' salt solution, pH 7.4. The cells were dissociated by a trypsin (0.125%) digestion in D-Hanks' salt solution. After each of five successive 8 min incubations, the dissociated cells were mixed with DMEM containing 10% FBS and were centrifuged and pooled. The dissociated cells were enriched for cardiomyocytes by the technique

of differential adhesion for 90 min and plated at a concentration of 10^6 cells/well. Cultures were incubated in a humidified 5% CO_2-95% O_2 at 37°C. Bromodeoxyuridine (0.1 mM) was added into the medium to inhibit proliferation of nonmyocytes. This procedure yielded cultures with 90-95% myocytes, as assessed by microscopic observations of cellular contractions. After a 2 overnight incubation in DMEM containing 10% FBS the attached cells were rinsed and maintained in DMEM containing 0.1% FBS. After 48 h of serum starvation, cardiomyocytes were treated with various agents.

Immunofluorescence

After treated with 0.1 μM Ang II alone or with 10 μM STS for 24 h, cardiomyocytes were fixed in 3.7% paraformaldehyde, and permeabilized with 0.3% Triton X-100. Cells were incubated with Texas Red-X phalloidin (1:500). Immunostained cardiomyocytes were viewed by fluorescence microscopy. Quantitation of cell surface area was performed on actin-stained cardiomyocytes. The cell size of cardiomyocytes was measured by directly tracing the stained areas on a photograph.

Protein synthesis measurement ([^{3}H]Leucine incorporation)

Protein synthesis was determined by assessing the incorporation of labelled leucine from the extracellular medium into total TCA-precipitable cell proteins (Yamazaki *et al.*, 1995). Cardiomyocytes cultured for 48 h in DMEM with 10% FBS were starved in 0.1% FBS-DMEM for 24 h. Then, cells were treated with 0.1 μM Ang II alone or with STS (2, 10, 50 μM) for 24 h. [^{3}H] Leucine (1 μCi/ml) was added 2 h before harvest. At the end of the labeling incubation, plates were placed on ice, quickly washed twice with ice-cold phosphate-buffered saline, incubated for 30 min with 5% TCA, and washed again. Precipitates were solubilized for 30 min in 0.5 M NaOH and neutralised. Radioactivity was measured by a liquid scintillation counter.

Northern blot analysis

After a 24 h incubation with various agents, the cultured myocytes were submitted to RNA extraction. Total RNA was extracted from cultured cells with TRIzol Reagent (Sigma Chemical Co.). Northern blot analysis was performed according to the procedure previously described (Wollert *et al.*,1994). The cDNA probes used were as follows: rat atrial natriuretic peptide (*ANP*) cDNA, a 0.825-kb fragment and rat GAPDH, a 1.3-kb fragment. The cDNA probes were labeled with [^{32}P]dCTP (3 mCi/mmol; New England Nuclear) by random primer extension. Autoradiography was performed on a Kodak XAR-5 film with an intensifying screen at -80°C. Autoradiograms were quantified by an image analyzer (LEICA, DC200, Germany). Results were normalized to GAPDH gene expression.

Western blot analysis

To explore the molecular mechanisms of the anti-hypertrophic effect of STS, we determined whether STS inhibits signaling through MEK/ERK pathway. After various treatments, myocytes were harvested and lysed for 20 min in 200 μl lysis buffer (10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 μg/ml pepstatin). Equal protein was loaded in each lane, resolved by SDS-PAGE, blotted on nitrocellulose membrane. Membranes were blocked in 5% nonfat milk powder in Tris-buffered saline (TBS)/0.1% Tween-20 for 1 h at room temperature, and then incubated with specific antibodies in 5% BSA in TBS for another 1 h. Membranes were incubated with peroxidase conjugated second antibody in blocking buffer for 1 h. The labeled proteins were detected with enhanced chemiluminescence.

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 $P < 0.05$.

Results

Effects of STS on Ang II-induced left ventricular hypertrophy

After 3 weeks of experiment, Ang II rats exhibited left ventricular hypertrophy, as evidenced by the increase of the LVW/BW ratio, when compared with controls (Figure 2). Meanwhile, there were significant differences in the LVW/BW ratio between STS-treated and untreated Ang II rats. In STS-treated Ang II rats, the LVW/BW ratio was decreased compared with untreated Ang II rats, and similar to the levels in control. However, there was no obvious difference in SBP between STS-treated and untreated Ang II rats, which means anti-hypertrophic effect of STS is independent of decreasing SBP. Under microscope, left ventricular tissues from Ang II-treated rats demonstrated obvious cardiomyocyte hypertrophy (Figure 3). These characteristic changes induced by Ang II were markedly inhibited by long-term treatment of STS.

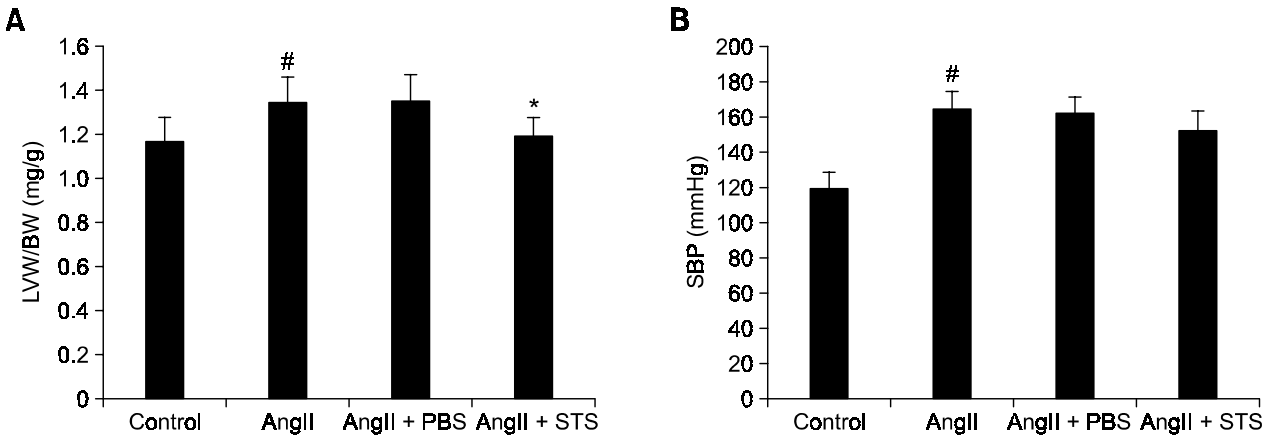

Figure 2. The LVW/BW ratio and SBP after the long term treatment. After 3 weeks of treatment, the LVW/BW ratio and the SBP were measured. Data represent means ± S.D. #*P* < 0.01 versus control group; **P* < 0.01 versus Ang II group. The experiments were repeated five times with reproducible results.

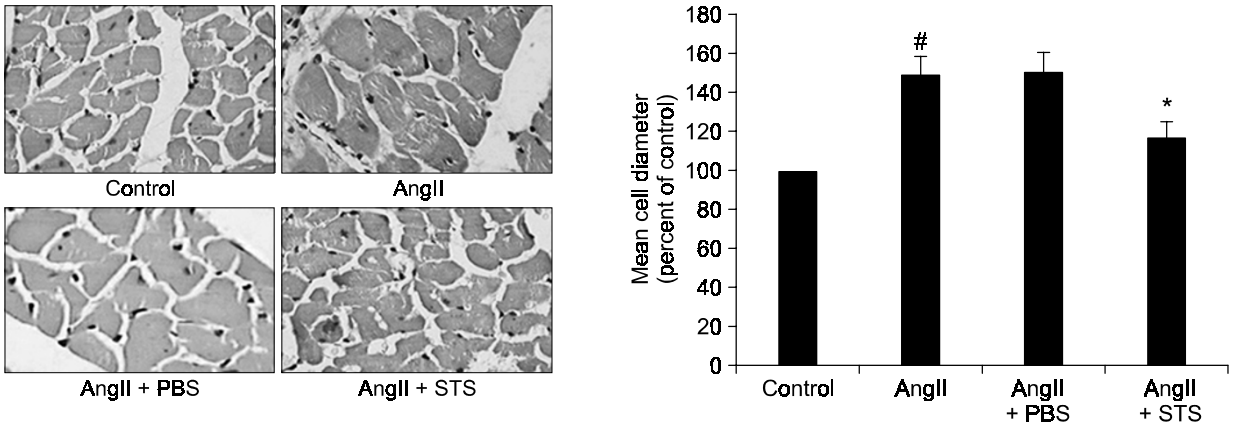

Figure 3. Long-term treatment of STS (50 mg/kg/d) inhibited Ang II-induced cardiomyocyte hypertrophy *in vivo*. Representative hematoxylin and eosin-stained high magnification (original magnification ×200) of left ventricles from different groups. Mean myocyte diameter was calculated by measuring 100 cells from sections. Data represent means ± S.D. #*P* < 0.01 versus control group; **P* < 0.01 versus Ang II group. The experiments were repeated six times with reproducible results.

Effects of STS on the Ang II-induced cardiomyocyte hypertrophy

In addition to increased LVW/BW ratio, cardiomyocyte hypertrophy is characterized by the increase in cell size, protein synthesis and induction of fetal-type genes (e.g., *ANP*). Therefore, we examined the effects of STS on these parameters. First, cardiomyocytes were treated with 0.1 μM Ang II alone or with 10 μM STS for 24 h. Ang II induced a marked increase in cell size which was markedly inhibited by STS (Figure 4). Next, inhibition of Ang II-induced protein synthesis by treatment with STS was analyzed by measurement of [^{3}H] Leucine incorporation into the cardiomyocytes. As shown in Figure 5, exposure of cells to STS (2, 10, 50 μM) for 24 h significantly depressed Ang II-induced increases in [^{3}H] Leucine incorporation. Furthermore, *ANP* mRNA expression was examined by Northern Blot. Results indicated Ang II induced expression of *ANP* gene in myocytes and the induction was markedly decreased by treatment with STS (Figure 5).

Effect of STS on ERK activation

We further investigated whether STS inhibited ERK pathway in Ang II-treated cardiomyocytes. To investigate effects of STS on ERK, myocytes were treated with 0.1 μM Ang II alone or with STS (2, 10, 50 μM) for 20 min. The activation of ERK was examined by Western blot with the phosphospecific

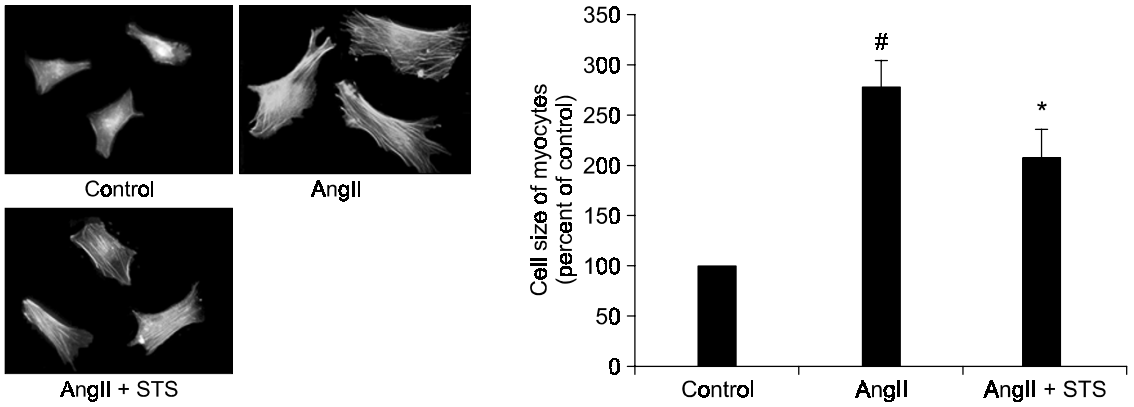

Figure 4. Inhibition of STS on Ang II-induced increase in the cell size. Cells were treated with 0.1 μM Ang II alone or with 10 μM STS for 24 h. Immunofluorescence was performed with Texas Red-X pholloidin. The cell size of cardiomyocytes was measured by directly tracing actin-stained cardiomyocytes. #*P* < 0.01 versus control group; **P* < 0.01 versus Ang II group. The experiments were repeated five times with reproducible results.

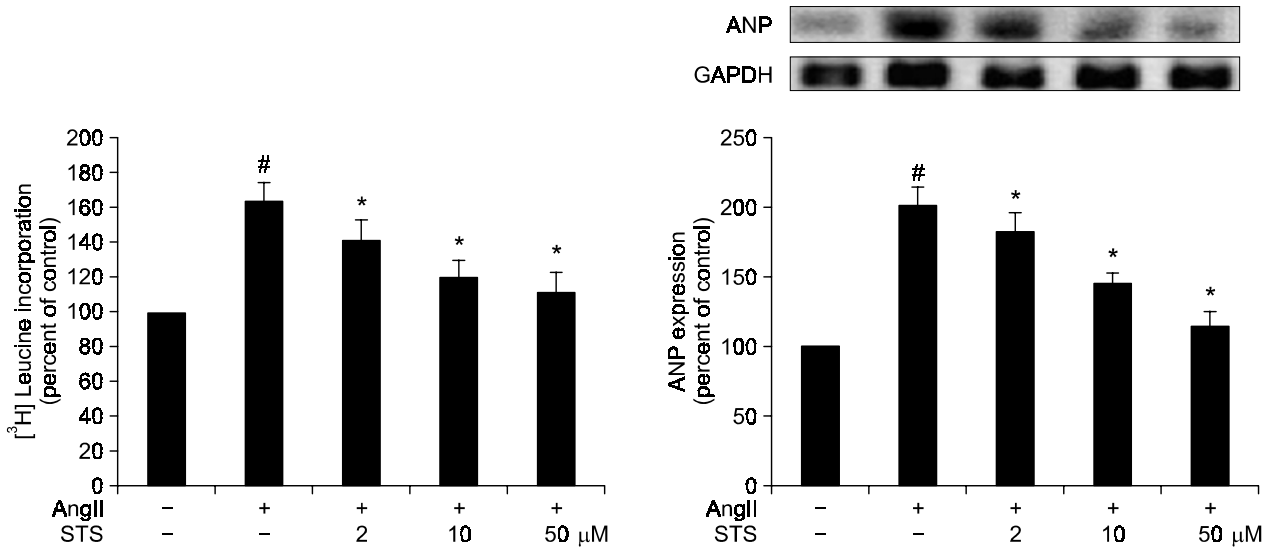

Figure 5. Inhibition of STS on Ang II-induced increase in [^{3}H]Leucine incorporation and *ANP* expression. Myocytes were treated with 0.1 μM Ang II alone or with STS (2, 10, 50 μM) for 24 h. Protein synthesis rate was measured as [^{3}H]Leucine incorporation. *ANP* mRNA expression was measured by Northern blot. Control group were treated with saline. #*P* < 0.01 versus control group; **P* < 0.01 versus Ang II group. The experiments were repeated five times with reproducible results.

antibody. The data suggest that STS markedly inhibited ERK1/2 activations with increasing dose of STS (Figure 6). Thus, STS inhibited the activations of ERK1/2 signaling pathway in a dose-dependent manner.

Effect of STS on MEK activation

MAPK/ERK kinase-1/2 (MEK1/2) phosphorylates and activates ERK1/2 in response to mitogenic stimuli (Solit *et al.*, 2005). To elucidate the mechanism by which STS inhibited ERK activation, we examined effect of STS on MEK activation in cardiomyocytes. The protein content of p-MEK1/2 was also examined by Western blot analysis. As shown in Figure 6, Ang II stimulation increased the activity of MEK1/2, which was inhibited by STS.

Moreover, to confirm the significance of MEK/ERK pathway in the anti-hypertrophic effect of STS, we examined the effects of MEK1/2 inhibitor U0126 (Favata *et al.*, 1998) on hypertrophic response such as [^{3}H] Leucine incorporation and *ANP* mRNA ex-

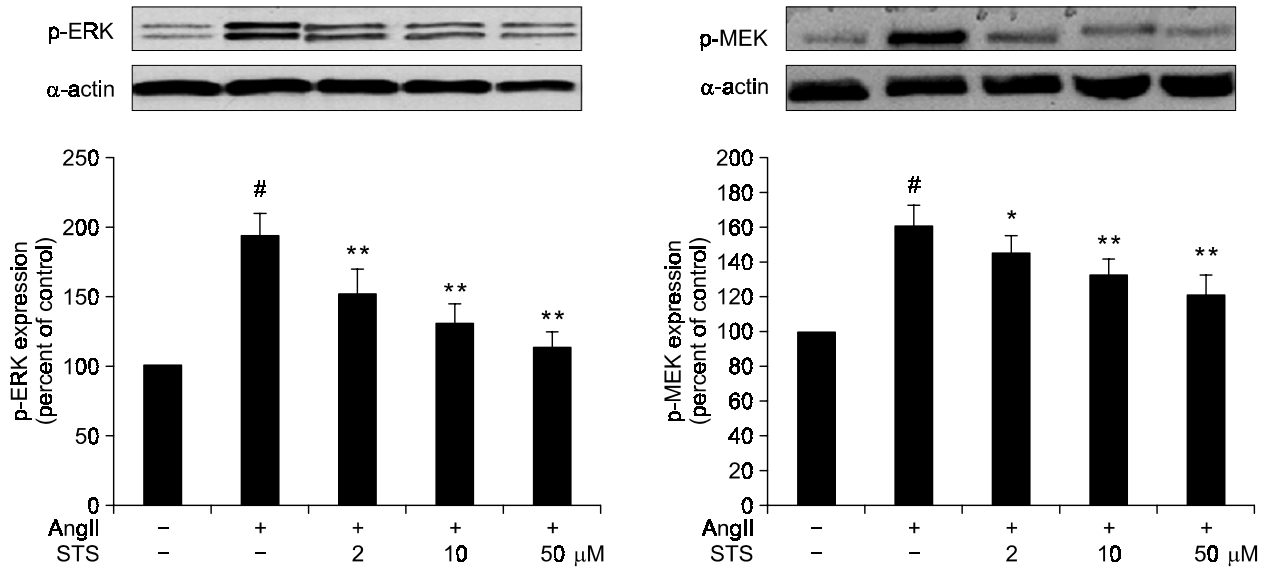

Figure 6. Inhibition of STS on Ang II-induced ERK and MEK activation. Myocytes were treated with 0.1 μM Ang II alone or with STS (2, 10, 50 μM) for 20 min. The activations of ERK and MEK were examined by Western blot with the phosphospecific antibody. Data represent means ± S.D. #*P* < 0.01 versus control group; **P* < 0.05 and ***P* < 0.01 versus Ang II group. The experiments were repeated five times with reproducible results.

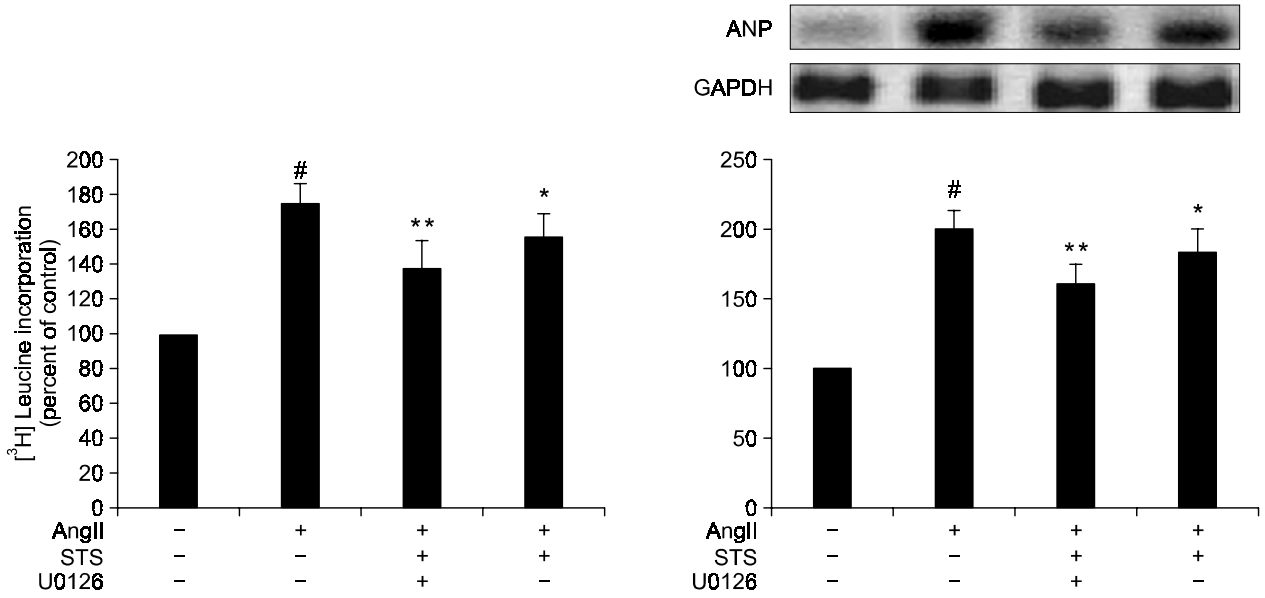

Figure 7. Inhibition of U0126 on STS-induced depressions of [^{3}H]Leucine incorporation and *ANP* expression. U0126 was dissolved in DMSO. Control cultures were supplemented with DMSO vehicle alone (0.1% final concentration). In each group, concertration of Ang II was 0.1 μM and that of STS was 10 μM. After myocytes were treated differently for 24 h, [^{3}H] Leucine incorporation and *ANP* expression were measured. Data represent means ± S.D. #*P* < 0.01 versus control group; **P* < 0.01 versus Ang II group and ***P* < 0.05 versus (Ang II + STS) group. The experiments were repeated five times with reproducible results.

pression. U0126 was dissolved in DMSO. Control cultures were supplemented with DMSO vehicle alone (0.1% final concentration). In each group, concentration of Ang II was 0.1 μM and that of STS was 10 μM. After myocytes were treated differently for 24 h, [^{3}H] Leucine incorporation and *ANP* mRNA expression were measured.

STS-induced depression in [^{3}H] Leucine incor-

poration was significantly enhanced by U0126 (20 μM). Moreover, U0126 makedly enhanced STS-induced depression in *ANP* mRNA expression (Figure 7). These findings suggested the importance of MEK/ERK pathway in the anti-hypertrophic effects of STS.

Discussion

Cardiomyocyte hypertrophy refers to the increase of cell's volume and myocomma; and changes of types of contracting proteins at the same time. In its early stage, it is a compensatory response, but if prolonged, the heart may undergo a transition to heart failure. Therefore, it is important to prevent the process of cardiomyocyte hypertrophy induced by extracellular signals for any proposed therapy to regulate the myocardial hypertrophic responses (Cohn *et al.*, 2000; Frey *et al.*, 2004). As an important neuroendocrine factor, Ang II can not only regulate the physiological functions of cadiovascular system, but also make a vital role in physiopathologic processes such as myocardial hypertrophy or heart failure (Wassmann *et al*., 2004).

The root of *Salvia miltiorrhiza Bunge*, known as Danshen in Chinese, is a herbal plant widely used to cure myocarditis and myocardial infarction (Chen *et al.*, 1979). Tanshinone IIA is the most abundant component and structurally representative of the tanshinones of *Salvia miltiorrhiza* (Tang and Eisenbrand, 1992). In China, Sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA, is usually administrated to patients suffering myocardial infarction and angina cordis (Chen *et al.*, 1979; Tao *et al.*, 1996). Although STS has been used as a medicinal agent in the treatment of many diseases, its role in cardiomyocyte hypertrophy remains unknown. Recently, tanshinone IIA was shown to be a promising new anti-hypertrophic drug that reduced cardiomyocyte hypertrophy (Takahashi *et al.*, 2002). Nevertheless, up to date, little is known about the cellular and molecular mechanisms of STS-mediated anti-hypertrophic effects in cardiomyocytes after Ang II stimulation and no work has been done to investigate the effect of STS on the MEK/ERK pathway. In this study, we attempted to explore the possible anti-hypertrophic effect of STS on Ang II-induced cardiomyocyte hypertrophy and its molecular events.

In this research, *in vivo*, we used Ang II infused by mini-pump to establish cardiomyocyte hypertrophy. After our long-term treatment studies, STS markedly inhibited Ang II-induced increases in the LVW/BW ratio, independent of decreasing SBP (Figure 2). As previously reported, *in vivo* Ang II via AT_1 Receptor, causes not only ventricular hypertrophy, independently of blood pressure, but also a shift to the fetal phenotype of the myocardium (Lijnen and Petrov, 1999). Therefore, STS may have some pharmacological properties similar to angiotensin-converting enzyme inhibitors or Ang II receptor antagonists, which needs further research. Under microscope, the characteristic hypertrophic changes induced by Ang II were markedly inhibited by long-term treatment of STS (Figure 3). Furthermore, in cultured cardiomyocytes, STS significantly depressed Ang II-induced marked increases in the cell size, protein synthesis and induction of *ANP* gene (Figure 4 and 5). Then we tried to explore the mechanism through which STS inhibited cardiomyocyte hypertrophy.

The mechanism underlying the attenuation of Ang II-induced actions by STS is an area of considerable interest. Several points in the Ang II signal transduction pathways are possible sites of STS action (Force and Bonventre, 1998; Miyazaki *et al.*, 1998; Dostal and Baker, 1999). Although the mechanism by which AngII induces cardiomyocyte hypertrophy is not fully understood, protein kinases especially the MAPK family have been reported to play a pivotal role in the development of cardiac hypertrophy (Kim *et al.*, 1999; Seko *et al.*, 1999; Fischer *et al.*, 2001; Takeishi *et al.*, 2001). Among the MAPK superfamily, ERK1/2 has been focused on the essential regulators of a hypertrophic response although the role of JNK and p38 were recently studied in regulating cardiac hypertrophy (Sugden, 2001). ERK1/2 phosphorylation targets substrates in the nucleus (e.g. c-*myc*, c-*jun*, and ATF-2), leading to transcriptional reprogramming that is likely responsible, at least partly, for altered gene expression associated with hypertrophy. In agreement with previous findings (Shih *et al.*, 2001), we found that Ang II enhanced ERK activation which was dramatically decreased after treatment with STS in a dose- dependent manner (Figure 6). This result demonstrates that inhibition of ERK1/2 pathway might be important for protective effects of STS. However, any site regulating ERK1/2 could also be implicated.

MEK1/2, an immediate upstream regulator of ERK, phosphorylates and activates ERK1/2, but not JNK or p38 in response to Ang II stimulation (Solit *et al.*, 2005). Transgenic overexpression of MEK1/2 results in considerable cardiac hypertrophy (Bueno *et al.*, 2000). Our results indicated the inhibition pattern of MEK activation by STS was consistent with that of ERK1/2 activation, indicating that MEK/ERK pathway plays a significant role in the anti-hypertrophic effect of STS (Figure 6). The signal cascade of angiotension-II coupling to ERK pathway was reported (Pinzar *et al*., 2005). In the paper, it was found that 10 μM U0126 can completely abo-

lishes ERK1/2 phosphorylation. As an inhibitor of MEK, U0126 was discovered in a screen for compounds that inhibited AP-1 dependent transcription, and was subsequently characterized as an inhibitor of both MEK1 and MEK2 (Favata *et al.*, 1998). As the latest report, U0126 has marked anti-hypertrophic effects on cardiomyocyte hypertrophy and downregulated some hypertrophic gene expressions (Kennedy *et al.*, 2006). U0126 also abolishes Prostaglandin E_2-induced cardiac hypertrophy in a dose-dependent manner (Frias *et al.*, 2007). Furthermore, U0126 inhibits endothelin-1 and phenylephrine-induced protein synthesis and increased cell size, sarcomeric reorganization, and expression of beta-myosin heavy chain in myocytes (Yue *et al.*, 2000). Therefore, to further elucidate the significance of MEK/ERK pathway in the anti-hypertrophic effect of STS, we examined the effect of U0126 on [^{3}H] Leucine incorporation and *ANP* mRNA expression. STS-induced depressions in [^{3}H] Leucine incorporation and *ANP* mRNA expression were largely enhanced by U0126 (20 μM) (Figure 7). It was clearly shown that U0126 and STS affects synergeticlly. This finding confirms that MEK/ERK pathway plays a significant role in the anti-hypertrophic effect of STS. Nevertheless, we can not rule out the possibilities that some other mechanisms are also involved in the growth-inhibitory effects of STS in cardiomyocytes. Then the molecular mechanism underlying anti-hypertrophic effect of STS remains to be further explored.

Taken together, the present study delivers important new insights to the molecular mechanisms of action of STS on cardiomyocytes. Our results indicated that STS markedly depresses Ang II-induced cardiomyocyte hypertrophy *in vivo* and *in vitro*. The inhibition of MEK/ERK pathway may be involved in the anti-hypertrophic effect. It provides a possible mechanism mediating the inhibitory effect of STS on cardiomyocyte hypertrophy. Although the precise mechanism by which STS inhibits the development of cardiac hypertrophy remains to be further clarified, understanding the pharmacological actions of STS on cardiac cells may allow the development of novel therapeutic strategies for modulating the hypertrophy of cardiomyocytes and the overall remodeling of the myocardium. The next steps we need to take are to investigate the effects of STS on other mediators and effectors of cardiomyocyte hypertrophy.

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