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Sodium tanshinone IIA sulfonate derived from Danshen (*Salvia miltiorrhiza*) attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells

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Abstract

Sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA, is isolated from the root of *Salvia miltiorrhiza* known as "Danshen." Although injection of *S. miltiorrhiza* extract and STS is used widely and successfully in clinics in China for treating cardiovascular diseases, the exact mechanism for its therapeutic basis is poorly understood. The present study was undertaken to characterize the effect of STS on angiotensin II-induced hypertrophy on cultured myocytes and cardiac fibroblasts (nonmyocytes) prepared from neonatal rat hearts. Angiotensin II (1 nM) increased protein synthesis and surface area in myocytes, and DNA synthesis and cell number in nonmyocytes, respectively. Exposure of the myocytes to STS (5–80 μM) for 24 hr produced no cytotoxicity as evaluated by the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT) assay. Although STS (10 μM) alone showed no effect on the growth of cultured cardiac cells, it markedly suppressed angiotensin II-induced enlargement of cells and [^{3}H]phenylalanine incorporation, proceeding from the induction of immediate early gene (c-*jun*) expression in myocytes. Furthermore, STS prevented the rise in $[Ca^{2+}]_i$ mediated by angiotensin II in myocytes. In contrast, STS (10 μM) was without effect on hyperplasia and c-*jun* expression induced by angiotensin II in nonmyocytes. The present *in vitro* findings support the interpretation that STS is a substance that may be beneficial in protecting the myocardium against hypertrophy.

Keywords: *Salvia miltiorrhiza*; Sodium tanshinone IIA sulfonate; Angiotensin II; Cardiac hypertrophy; Hyperplasia; Immediate early gene

1. Introduction

STS, a derivative of tanshinone IIA, is isolated from the root of *Salvia miltiorrhiza* known as "Danshen" in traditional Chinese medicine. Although the injection of *S. miltiorrhiza* extract and STS is an effective drug treatment used widely in China for patients hospitalized with cardiovascular diseases, the exact mechanism of its therapeutic action is poorly understood [1,2]. One of the most important factors contributing to the development of heart failure is the neurohumoral peptide angiotensin II [3,4]. Evidence that angiotensin II is a stimulator of cardiomyocyte hypertrophy [5,6] has led to the widespread use of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II AT_1 receptor antagonists in patients with myocardial infarction and congestive heart failure. In the present study, we investigated the effect of STS on cardiac hypertrophy, and assessed its ability to protect the myocardium against hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells.

2. Materials and methods

2.1. Materials

The root of *S. miltiorrhiza* was purchased from the Tochimoto Tenkaido Co. A voucher specimen was deposited

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Abbreviations: STS, sodium tanshinone IIA sulfonate; MTT, 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan; Ca-T, calcium transient.

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in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University (TMPW No. 16950). STS was prepared from tanshinone IIA, which was isolated from the root of *S. miltiorrhiza*, according to the method of Chien *et al.* [7]. Their structures were identified by comparing the spectral findings with observations reported previously [7,8]. [Sar^1]-Ang II was purchased from the Sigma Chemical Co. All other chemical reagents used were of the highest grade commercially available.

2.2. Preparation of cardiac myocyte and nonmyocyte cultures

Primary cardiac myocyte and nonmyocyte cultures from 1-day-old Wistar rats were prepared according to the procedure described by Sadoshima *et al.* [9]. For selective enrichment with cardiac myocytes, dissociated cells were preplated for 1 hr, during which period the nonmyocytes attached readily to the bottom of the culture dish [10]. Nonadherent cells, mostly myocytes, were plated at a density of $2-5 \times 10^6$ cells/mL/dish. Bromodeoxyuridine (0.1 mM) was added during the first 2 days to prevent the proliferation of nonmyocytes. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observations of cellular contractions. Both myocytes and nonmyocytes were maintained in Dulbecco's modified Eagle's medium/F-12 (Dainippon Pharmaceutical Co; 1:1, v/v) supplemented with newborn calf serum (5 or 10%; Dainippon), 3 mM pyruvic acid, 100 μM ascorbic acid, 5 μg/mL of insulin, 5 μg/mL of transferrin, and 5 ng/mL of selenium (Boehringer Mannheim) for 48 hr followed by serum-free medium. All experiments were performed 24 hr after transferring the cells to serum-free medium.

2.3. Evaluation of cytotoxicity

Cardiac myocytes were cultured in a 96-well microplate. Increasing concentrations of STS in PBS were added to cultures after medium renewal. After a 24-hr incubation, cytotoxicity was assessed by the MTT assay [11]. MTT solution (5 mg/mL) was added to each well (10 μL/100 μL medium) in the assay, and plates were incubated at 37° for 4 hr. Acid–isopropanol (100 μL; 0.04 N HCl in isopropanol) was added to each well and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Dynatech MR580 micro-ELISA reader (Shimazu), using a test wavelength of 570 nm, and a reference wavelength of 630 nm.

2.4. Measurement of [^{3}H]thymidine uptake and cell density

During the course of the experiment, bromodeoxyuridine was omitted from the culture medium. Cells were grown in serum-free medium for 24 hr and then stimulated with angiotensin II (1 nM). After 24 hr in [*methyl*-^{3}H]thymidine-containing buffer, the cells were washed three times with PBS and harvested with 10% trichloroacetic acid at 4° for 60 min. The trichloroacetic acid-precipitate was washed three times with 95% ethanol, and then was dissolved in 0.15 N NaOH. Radioactivity was determined using a scintillation counter. The protein concentration was determined by the method of Lowry *et al.* [12] using bovine serum albumin as a standard. Cells were washed three times with PBS, detached with 0.5 mL trypsin (Difco Laboratories)–EDTA solution, and dissociated by trituration. Cell numbers were counted with a hemocytometer.

2.5. Evaluation of cell size and incorporation of [^{3}H]phenylalanine

Myocyte morphology was monitored with an inverted phase contrast microscope (IX70; Olympus) and a videomonitor equipped with a color video copy processor (SCT-CP710; Mitsubishi). The cell images were introduced into an intensified charged couple device camera (INT-11A; INTER DEC) and videotaped using a VHS recorder (INTER DEC). The shape and location of each myocyte were recorded by photography before initiating the experiment and again after STS treatment. Cell size was quantified by measuring the cell surface area using image processing software (NIH Image 1.59/Power Macintosh 7200; National Institutes of Health). The alterations of surface area were estimated for each cell and are expressed as a percentage of the observed cells by planimetry-enlarged photographs. As an index of protein synthesis, cellular accumulation of L-[2,6-^{3}H]phenylalanine (Amersham) was measured as described by Sadoshima *et al.* [9]. After incubation in serum-free medium for 24 hr, the cells were stimulated with angiotensin II (1 nM) for 24 hr in the presence of [^{3}H]phenylalanine (1 μCi/mL). The cells were then washed three times with PBS and harvested as above. Aliquots were prepared, and radioactivity was measured as described previously [13].

2.6. Analysis of RNA

Total RNA was isolated from cardiac cells using the acid guanidinium thiocyanate–phenol–chloroform extraction method [14]. The RNA concentration was determined spectrophotometrically by absorbance at 260 nm. Northern blot analysis was performed according to the procedure described by Kim *et al.* [15]. The cDNA probes used were as follows: c-*jun*, 2 kb *Eco*RI fragment of rat c-*jun* cDNA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1.3 kb *Pst*I–*Pst*I fragment of rat GAPDH cDNA. The cDNA probes were labeled with [^{32}P]dCTP (specific activity, 3 mCi/mmol; New England Nuclear) by random primer extension using a Bca BEST Labeling Kit (Takara). Auto-

radiography was performed on Kodak XAR-5 film with an intensifying screen at −80°. Autoradiograms were quantified using an image analyzer (BAS 1500, Fuji Film). Results were normalized to the expression of GAPDH mRNA.

2.7. Measurement of $[Ca^{2+}]_i$

The method of measuring $[Ca^{2+}]_i$ has been described previously by Takahashi *et al.* [13]. An INTER DEC M-1000 fluorescent spectromicroscope system was used to evaluate $[Ca^{2+}]_i$. During the course of a cycle of contraction and relaxation, the 340/380 nm fluorescence ratio increased to a maximum value during systole (Max), and fell to a minimum value during diastole (Min). A calcium transient (Ca-T) represents the difference between the Max and the Min values. Data are expressed as Ca-T and represent the mean of 10 beats.

2.8. Statistics

Depending upon the design of the experiments, statistical significance was determined by Student's *t*-test, ANOVA combined with Bonferroni's method, and the χ^2-test to compare individual data points for significance. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated *P*-value was less than 0.05.

3. Results

3.1. Influence of STS on myocyte viability and nonmyocyte proliferation

Exposure of cultured cardiomyocytes to STS (5–80 μM) for 24 hr produced marginal cytotoxicity, as assessed by the MTT assay (Fig. 1). Additionally, myocyte monolayers continued to contract synchronously in the presence of STS (data not shown). Similar findings were obtained when nonmyocyte proliferation was determined as a parameter for cellular growth (Table 1-a and -b). As shown in Table 1-a, there was no significant change (96 ± 6%) in the density of nonmyocytes treated with 10 μM STS for 24 hr.

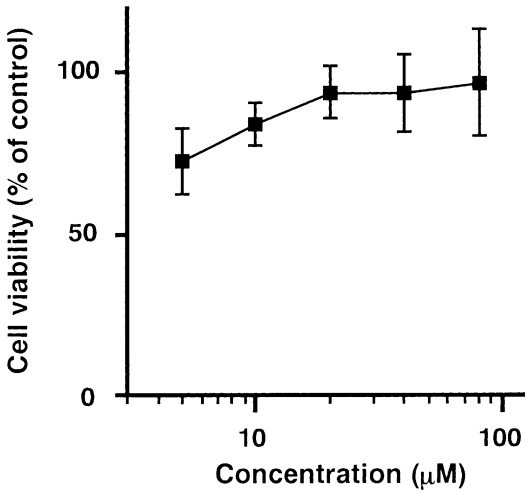

Fig. 1. Influence of STS on the viability of cardiac myocytes. Cardiomyocytes were cultured in microwells and incubated with increasing concentrations of STS. After 24 hr, cell viability was determined using the MTT assay. Values are expressed as a percent of control, in which the control cells were not exposed to STS. Values are the means ± SEM of 4–8 samples obtained from three different cultures.

3.2. Effect of STS on angiotensin II-induced hypertrophic responses in myocytes and nonmyocytes

In cardiac nonmyocyte cultures, angiotensin II (1 nM) treatment caused a significant increase in [^{3}H]thymidine uptake over a 24-hr period (Table 1-b). The mitogenic effect of angiotensin II on nonmyocytes was also confirmed by counting the number of cells (Table 1-a). Additional angiotensin II (1 nM) caused a 2.9-fold increase in the expression of an immediate early gene (c-*jun*) after a 0.5-hr incubation (Table 1-c). However, STS (10 μM) treatment failed to attenuate angiotensin II-induced hyperplastic growth in cultured nonmyocytes (Table 1).

Table 2 summarizes the effects of STS on angiotensin II-induced hypertrophic responses of myocytes. Angiotensin II (1 nM) mediated a significant increase (61%) in protein synthesis in myocytes, as measured by [^{3}H]phenylalanine

Table 1
Influence of STS on angiotensin II-induced hyperplastic responses of cardiac nonmyocytes

STS (10 μM)	Ang II (1 nM)	Nonmyocytes (% of control)		
		(a) Cell number (N = 12)	(b) [^{3}H]Thymidine incorporation (N = 9–15)	(c) c-*jun* Expression (N = 4–8)
−	−	100	100	100
−	+	130 ± 7*	155 ± 8*	285 ± 16*
+	−	96 ± 6		105 ± 12*
+	+	126 ± 4*	161 ± 11*	275 ± 35*

Nonmyocytes were stimulated with angiotensin II (1 nM). For cell number (a) and [^{3}H]thymidine incorporation (b), cells were stimulated for 24 hr. For c-*jun* expression (c), cells were stimulated for 0.5 hr. The average control values were: (a) 8.9×10^5 cells/mL, (b) 613 dpm/μg protein and (c) 0.05, respectively. Values are means ± SEM. Figures in parentheses represent the number of samples obtained from three different cultures.

* $P < 0.05$ versus control.

Table 2
Effect of STS on angiotensin II-induced hypertrophic response of cardiac myocytes

STS (10 μM)	Ang II (1 nM)	Myocytes (% of control)			
		(a) [^{3}H]Phenylalanine incorporation (N = 5–8)	(b) Cell size (96–121 cell)	(c) c-*jun* Expression (N = 5–6)	(d) Ca^{2+}-T (N = 4–11)
−	−	100	100	100	100
−	+	161 ± 9*	141 ± 4*	144 ± 17*	118 ± 5*
+	−	112 ± 4	114 ± 54	86 ± 17	104 ± 9
+	+	127 ± 8**	113 ± 4**	79 ± 7**	99 ± 3**

Myocytes were stimulated with angiotensin II (1 nM). For [^{3}H]phenylalanine incorporation (a) and cell size (b), cells were stimulated for 24 hr. For c-*jun* expression (c) and Ca^{2+}-T (d), cells were stimulated for 30 and 5 min, respectively. Values of (a) and (c) are expressed as percent of controls, in which the control cells were exposed to neither STS nor angiotensin II. Values of (b) and (d) are expressed as percent of initial values (the same cells at 0 hr). The average control values were: (a) 327 dpm/μg protein, (b) 118, (c) 0.5 and (d) 1.6, respectively. Values are means ± SEM. Figures in parentheses represent number of samples obtained from three different cultures.

* $P < 0.05$ versus controls.

** $P < 0.05$ versus angiotensin II.

incorporation over 24 hr (Table 2-a). Fig. 2 is a pictorial representation of the hypertrophic response of myocytes to angiotensin II stimulation. The surface area of the cardiomyocytes treated with 1 nM angiotensin II had increased by 41% at 24 hr (Table 2-b). STS reversed these effects, decreasing [^{3}H]phenylalanine incorporation into myocytes from 161 to 127% (Table 2-a) and myocyte cell size from 141 to 113% (Table 2-b). Moreover, STS suppressed c-*jun* expression induced by angiotensin II in myocytes (Table 2-c), whereas [^{3}H]phenylalanine incorporation, cell size, and c-*jun* expression in myocytes were not altered significantly by treatment with STS alone. It has been established that most hypertrophic stimuli, including angiotensin II, enhance similar signal transduction pathways, in which

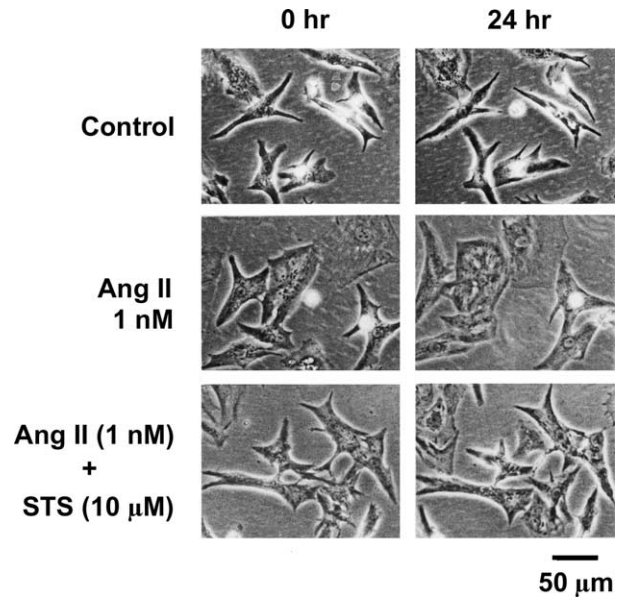

Fig. 2. Typical changes in cardiomyocyte size induced by angiotensin II (Ang II) in the presence and absence of STS. Rat neonatal cardiomyocytes were exposed to medium containing STS (10 μM) and Ang II (1 nM) for 24 hr. The cell sizes were then examined for changes in cell surface area using phase contrast micrographs observed at a magnification of 200×.

$[Ca^{2+}]_i$ rises and protein kinase C is activated [4,16,17]. Therefore, we examined the effect of STS on angiotensin II-induced changes in $[Ca^{2+}]_i$. The control, unstimulated myocytes formed monolayer sheets of synchronously contracting cells and exhibited typical Ca-T, defined as the difference between the maximum (Max) and minimum (Min) values. In the absence of STS, angiotensin II (1 nM) increased the magnitude of Ca-T (Table 2-d). Within 5 min of exposure to angiotensin II, the Ca-T increased to 118%. By contrast, myocytes treated with 10 μM STS exhibited little change in Ca-T upon addition of angiotensin II (1 nM).

4. Discussion

The root of *S. miltiorrhiza* BUNGE, known as Danshen in Chinese, is an herbal plant that has been used widely in traditional Chinese medicine for the treatment of myocarditis and myocardial infarction [1,2,18–20]. Tanshinone IIA is most abundant and structurally representative of the tanshinones of *S. miltiorrhiza* [21,22]. In China, STS, a derivative of tanshinone IIA, is usually administered intravenously (40–160 mg doses daily) to patients with myocardial infarction or angina pectoris. These patients have exhibited significant improvements with respect to subjective clinical symptoms, objective clinical signs, and electrocardiogram (ECG) parameters [18–20]. In the present study, cultured cardiomyocytes when exposed to STS (5–80 μM) for 24 hr showed no signs of toxicity (Fig. 1).

We confirmed the effects of angiotensin II on (a) hypertrophy of myocytes, (b) hyperplasia of nonmyocytes, and (c) c-*jun* expression of both cell types. Namely, in myocytes, angiotensin II was found to increase protein synthesis and cell size (Table 2) without changing the rate of DNA synthesis [13]. However, in nonmyocytes, angiotensin II increased both DNA synthesis and cell number. It also induced an increase in c-*jun* expression within 0.5 hr in both cultured myocytes and nonmyocytes [13]. The most

important finding of this study was the observed interaction between STS and angiotensin II in the cultured cardiomyocytes, but not in the nonmyocytes. Although exposure of the isolated myocytes to STS in the absence of angiotensin II had no effect on protein synthesis or cell size, treatment with 10 μM STS reduced angiotensin II responsiveness relative to growth and c-*jun* expression. Accordingly, STS exerted a beneficial effect relative to angiotensin II-induced changes in both the morphology and the molecular status of the cardiomyocytes, whereas angiotensin II-induced hyperplasia of nonmyocytes was not blocked by STS.

The mechanism underlying the attenuation of angiotensin II-induced actions by STS remains an area of considerable interest. Several steps in the angiotensin II signal transduction pathway are logical sites of STS action [3,4,16,17]. The angiotensin II (AT_1) receptor is coupled through a G protein to a membrane-bound phospholipase C enzyme [16,17], which is subsequently activated. The resulting products of phospholipase C action, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, are important second messengers in hypertrophic growth [3,4,16,17]. IP_3 promotes the release of Ca^{2+} from the sarcoplasmic reticulum, thereby increasing $[Ca^{2+}]_i$. Moreover, angiotensin II promotes calcium influx through the calcium channel and calcium handling by the Na^+/Ca^{2+} exchanger [23]. The subsequent increase in $[Ca^{2+}]_i$ plays a potentiating role in the growth process by activating several signaling pathways [16,17]. Diacylglycerol serves as an activator of protein kinase C, which in turn promotes protein synthesis [17,24]. Danshen or STS has been investigated for different pharmacological actions in the cardiovascular system [19–22,25,26]. They include a dose-dependent hypotensive action [25], a positive inotropic effect, a negative chronotropic effect, vasodilatation of coronary arteries, and inhibition of platelet aggregation *in vitro* [19–22,26–28]. Patmore and Whiting [29] demonstrated that STS had a negative inotropic effect on isolated papillary muscle of the guinea-pig heart. This negative inotropic effect was reversible by increasing the extracellular calcium concentration [29]. Zhou and Ruigrok [27] also reported similar findings using isolated rat hearts and indicated that they should be considered as a naturally occurring calcium antagonist, although they did not examine the $[Ca^{2+}]_i$ concentration directly. Interestingly, we found that STS suppressed the activation of c-*jun* and the increase in $[Ca^{2+}]_i$ mediated by angiotensin II in cardiomyocytes (Table 2). Because $[Ca^{2+}]_i$ is thought to be an important hypertrophic messenger and because the rise of $[Ca^{2+}]_i$ activates pathways that contribute to the hypertrophic response of angiotensin II, the modulation of the angiotensin II-mediated changes in $[Ca^{2+}]_i$ provides a rationale for the reversal of some angiotensin II effects by STS.

Another question arising from the present study is: why does STS treatment fail to suppress angiotensin II-induced hyperplasia in nonmyocytes? One possibility is that the differences in the response to angiotensin II of the myocytes and nonmyocytes relate to function and morphology. Cardiomyocytes are terminally differentiated and lose their ability to duplicate soon after birth, whereas nonmyocytes can multiply [30]. While myocytes in culture actively contract, nonmyocytes lack this characteristic [31]. This may be important since $[Ca^{2+}]_i$ undergoes dramatic changes during the contraction–relaxation cycle [31]. In fact, STS attenuated the angiotensin II-induced elevation in $[Ca^{2+}]_i$ (Table 2). One possible explanation for this action is that contracting cells may be more sensitive to the modulating actions of $[Ca^{2+}]_i$ than quiescent cells. Furthermore, although both rat cardiomyocytes and cardiac nonmyocytes have a functional angiotensin II receptor, the receptor subtype (angiotensin II types 1a, 1b, and 2 receptor) expression in the rat heart is cell-type specific, which may affect the signaling pathway taken [32]. The expression of AT1b-receptor mRNA in cardiomyocytes, unlike cardiac nonmyocytes, predominates over the AT1a-receptor mRNA level [32]. Zou *et al.* [33] recently suggested that angiotensin II-evoked signal transduction pathways differ among cell types. In cardiac fibroblasts, angiotensin II activates extracellular signal-regulated kinases (ERKs) through a pathway including the $G_{\beta\gamma}$ subunit of G_i protein, tyrosine kinases including Src family tyrosine kinases, Shc, Grb2, Ras, and Raf-1 kinase, whereas G_q and protein kinase C are important in cardiac myocytes [33]. Therefore, there is the possibility that these distinctive signaling pathways between the myocytes and nonmyocytes may result in different downstream responses to STS with regard to angiotensin II action(s).

The present findings indicate that STS is an effective inhibitor of angiotensin II action. It is plausible that the beneficial effect of STS in the treatment of heart failure could relate to its suppression of angiotensin II-mediated cellular responses.

References

[1] Chiang Su New Medical College, editors. Dictionary of Chinese crude drugs. Shanghai: Shanghai Scientific Technologic Publisher, 1977. p. 478–82.
[2] Namba T. The encyclopedia of Wakan-Yaku (traditional Sino-Japanese medicines) with color pictures, vol. I. Osaka, Japan: Hoikusha Publishing Co. Ltd., 1993. p. 24–5.
[3] Dostal DE, Baker KM. The cardiac renin–angiotensin system: conceptual, or a regulator of cardiac function? Circ Res 1999;85:643–50.
[4] Dostal DE, Hunt RA, Kule CE, Bhat GJ, Karoor V, McWhinney CD, Baker KM. Molecular mechanisms of angiotensin II in modulating cardiac function: intracardiac effects and signal transduction pathways. J Mol Cell Cardiol 1997;29:2893–902.
[5] Friedrich SP, Lorell BH, Rousseau MF, Hayashida W, Hess OM, Douglas PS, Gordon S, Keighley CS, Benedict C, Krayenbuehl HP, Grossman W, Pouleur H. Intracardiac angiotensin-converting enzyme inhibition improves diastolic function in patients with left ventricular hypertrophy due to aortic stenosis. Circulation 1994;90:2761–71.
[6] Gottlieb SS, Dickstein K, Fleck E, Kostis J, Levine TB, LeJemtel T, DeKock M. Hemodynamic and neurohormonal effects of the

angiotensin II antagonist losartan in patients with congestive heart failure. Circulation 1993;88:1602–9.
[7] Chien MK, Young PT, Ku WH, Chen X, Chen HT, Yeh HC. Studies on the active principles of Danshen. I. The structure of sodium tanshinone IIA sulfonate and methylene tanshinquinone. Acta Chim Sin 1978;36: 199–206.
[8] Honda G, Koezuka Y, Tabata M. Isolation of an antidermatophytic substance from the root of *Salvia miltiorrhiza*. Chem Pharm Bull (Tokyo) 1988;36:408–11.
[9] Sadoshima J, Jahn L, Takahashi T, Kulik TJ, Izumo S. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. J Biol Chem 1992;267:10551–60.
[10] Polinger IS. Separation of cell types in embryonic heart cell cultures. Exp Cell Res 1970;63:78–82.
[11] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[12] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol regent. J Biol Chem 1951;193:265–75.
[13] Takahashi K, Azuma M, Taira K, Baba A, Yamamoto I, Schaffer SW, Azuma J. Effect of taurine on angiotensin II-induced hypertrophy of neonatal rat cardiac cells. J Cardiovasc Pharmacol 1997;30:725–30.
[14] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
[15] Kim S, Ohta K, Hamaguchi A, Yukimura T, Miura K, Iwao H. Angiotensin II induces cardiac phenotypic modulation and remodeling *in vivo* in rats. Hypertension 1995;25:1252–9.
[16] Jalili T, Takeishi Y, Walsh RA. Signal transduction during cardiac hypertrophy: the role of $G\alpha_q$, PLC βI, and PKC. Cardiovasc Res 1999;44:5–9.
[17] Sadoshima J, Izumo S. Signal transduction pathways of angiotensin II-induced c-*fos* gene expression in cardiac myocytes *in vitro*. Roles of phospholipid-derived second messengers. Circ Res 1993;73:424–38.
[18] Shanghai Cooperative Group for the study of Tanshinone IIA. Therapeutic effect of sodium tanshinone IIA sulfonate in patients with coronary heart disease: a double blind study. J Tradit Chin Med 1984;4:20–4.
[19] Wang YP, Chen YH, Xu DZ, Jiang WD. The effect of sodium tanshinone IIA sulfonate on hemodynamics and the size of myocardial infarction in dogs. J Shanghai Med Coll 1980;7:347.
[20] Shanghai Cooperative Group for the study of Danshen 201. Clinical report: therapeutic effect of sodium tanshinone IIA sulfonate in 108 patients with coronary artery disease. Chin Med Rep 1978;1:37–9.
[21] Xu R, editor. Danshen: biological application. Beijing, China: Science Publisher, 1990.
[22] Tang W, Eisenbrand G. *Salvia miltiorrhiza* Bge. Chinese drugs of plant origin. Berlin/Heidelberg, Germany: Springer, 1992. p. 891–902.
[23] Ballard C, Schaffer S. Stimulation of the Na^+/Ca^{2+} exchanger by phenylephrine, angiotensin II and endothelin 1. J Mol Cell Cardiol 1996;28:11–7.
[24] Allo SN, Carl LL, Morgan HE. Acceleration of growth of cultured cardiomyocytes and translocation of protein kinase C. Am J Physiol 1992;63:C319–25.
[25] Li CP, Yung KH, Chiu KW. Hypotensive action of *Salvia miltiorrhiza* cell culture extracts. Am J Chin Med 1990;18:157–66.
[26] Chen YC. Pharmacological actions of Danshen. Acta Pharm Sin 1984;19:876–80.
[27] Zhou W, Ruigrok TJC. Protective effect of Danshen during myocardial ischemia and reperfusion: an isolated rat heart study. Am J Chin Med 1990;18:19–24.
[28] Zhao BL, Jiang W, Zhao Y, Hou JW, Xin WJ. Scavenging effects of *Salvia miltiorrhiza* on free radicals and its protection for myocardial mitochondrial membranes from ischemia-reperfusion injury. Biochem Mol Biol Int 1996;38:1171–82.
[29] Patmore L, Whiting RL. Calcium entry blocking properties of tanshinone II-A sulphonate, an active principal of the antianginal extract, Danshen. Br J Pharmacol 1982;75(Suppl):149P.
[30] Anversa P, Palackal T, Sonnenblick EH, Olivetti G, Megga LG, Capasso JM. Myocyte cell loss and myocyte cellular hyperplasia in the hypertrophied aging rat heart. Circ Res 1990;67:871–85.
[31] Harary I, Farley B. *In vitro* studies of isolated beating heart cells. Science 1960;131:1674–5.
[32] Matsubara H, Kanasaki M, Murasawa S, Tsukaguchi Y, Nio Y, Inada M. Differential gene expression and regulation of angiotensin II receptor subtypes in rat cardiac fibroblasts and cardiomyocytes in culture. J Clin Invest 1994;93:1592–601.
[33] Zou Y, Komuro I, Yamazaki T, Kudoh S, Aikawa R, Zhu W, Shiojima I, Hiroi Y, Tobe K, Kadowaki T, Yazaki Y. Cell type-specific angiotensin II-evoked signal transduction pathways: critical roles of $G_{\beta\gamma}$ subunit, Src family and Ras in cardiac fibroblasts. Circ Res 1998; 82:337–45.