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Effects of Chinese herb medicine Radix Scrophulariae on ventricular remodeling

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The effects and mechanism of the extract of *Radix Scrophulariae* (ERS), a traditional Chinese herb, on experimental ventricular remodeling in rats was studied Rats were separated randomly into 5 groups: sham, model, captopril (40 mg·kg^{-1}) and ERS (8, 16 g·kg^{-1}). The experimental ventricular remodeling was induced with ligating the left anterior descending branch of the coronary artery of the rats. The sham group was conducted the same procedure without ligation. After 4 weeks treatment with intragastric administration of the corresponding drugs, the left ventricular weight index (LVWI) and heart weight index (HWI) were determined. The concentrations of angiotensin II (Ang II) and hydroxyproline (Hyp) in myocardium were detected. Myocardium tissue was stained with HE and picric acid/Sirius red for cardiocyte cross-section area and collagen content measurements. Real-time RT-PCR was used to detect the gene expressions of AT_1R, TNF-α and TGF-β_1 mRNA. ERS could significantly reduce the LVWI, HWI, decrease the content of Ang II, Hyp, diminish cardiocyte cross-section area and ameliorate collagen deposition. In addition, ERS could down regulate the gene expressions of AT_1R, TNF-α and TGF-β_1 mRNA in myocardium. ERS has beneficial effect against ventricular remodeling. The mechanism may be related to decreasing the level of Ang II and cardiac fibrosis, modulating some gene expressions associated with cardiac hypertrophy.

1. Introduction

Radix Scrophulariae, the dried root of *Scrophularia ningpoensis* Hemsl., is a traditional Chinese herb recorded in a classic source (Shen Nong Ben Cao Jing). This means that the herb has been used for more than two thousand years. In TCM theory, *Radix Scrophulariae* has the effects of clearing away heat from the blood, nourish yin, purging fire, and relieving toxins, which increase patients' ability to keep the balance of yin and yang and protect them from invasion by internal or external pathogenic influences. Indications include: consumption of yin caused by febrile disease, deep-red tong and polydipsia, maculae caused by virulent pathogen, constipation due to impairment of body fluid, hectic fever and phthisical cough, conjunctivitis, sore throat, carbuncles, sores and other pyogenic skin infections (Jialin 2007).

Myocardial infarction (MI) is one of the leading causes of death throughout the world. Left ventricular (LV) remodeling after MI involves cardiocyte hypertrophy, chamber dilation, and interstitial fibrosis. Alterations in cardiocytes and collagen matrix lead to ventricular enlargement, contractile dysfunction and heart failure (HF). Hence, LV remodeling is regarded as a major risk factor of heart disease.

In the present study, we investigated whether long-term treatment with ERS could prevent the progression of LV remodeling after MI in rats and revealed the underline mechanism of its action to a certain extent.

2. Investigations and results

2.1. Effects on left verticals weight index and heart weight Index

The degree of cardiac hypertrophy was assessed by the increase of left ventricular weight index (LVWI) and heart weight index (HWI). As shown in Table 1, LVWI and HWI were increased significantly in the model group compared with those in the sham-operated group ($p < 0.01$). ERS treatment significantly decreased LVWI and HWI. The reference drug captopril also significantly reduced LVWI and HWI.

2.2. Effects on angiotensin II and hydroxyproline

LV remodeling is associated with the renin-angiotensin-aldosterone system. As shown in Table 1, angiotensin II (Ang II) and hydroxyproline (Hyp) concentrations were significantly higher in the model than in the sham-operated rats ($p < 0.01$), but they were attenuated in ERS and captopril groups ($p < 0.01$).

2.3. Effects on cardiocyte cross-section area

The ventricles were fixed with 10% formaldehyde, embedded in paraffin and sectioned. The sections were stained with HE and photographed under 400 × magnification, as shown in Fig. 1. HE staining showed about a 2-fold increase in the cardiocyte

Table 1: Effect of ERS on cardiac weight indexes, myocardium Ang II and Hyp concentrations in rats with ventricular remodeling induced by coronary artery ligation ($\bar{x} \pm s$, n = 11)

Group	Dose	LVWI (mg/g)	HWI (mg/g)	Ang II pg/mg·prot	Hyp (μg/mg·prot)
Sham	−−	2.34 ± 0.14**	2.98 ± 0.17*	278.21 ± 19.94**	0.957 ± 0.168**
Model	−−	2.53 ± 0.16	3.18 ± 0.23	348.34 ± 18.95	1.162 ± 0.090
Captopril	40 mg/kg	2.28 ± 0.17**	2.87 ± 0.19**	315.83 ± 23.91**	0.696 ± 0.349**
ERS-L	8 g/kg	2.44 ± 0.14	3.08 ± 0.15	319.97 ± 27.20*	0.745 ± 0.209**
ERS-H	16 g/kg	2.38 ± 0.12*	2.97 ± 0.11*	307.84 ± 25.49**	0.569 ± 0.301**

*$P < 0.05$, **$P < 0.01$ *vs* model group

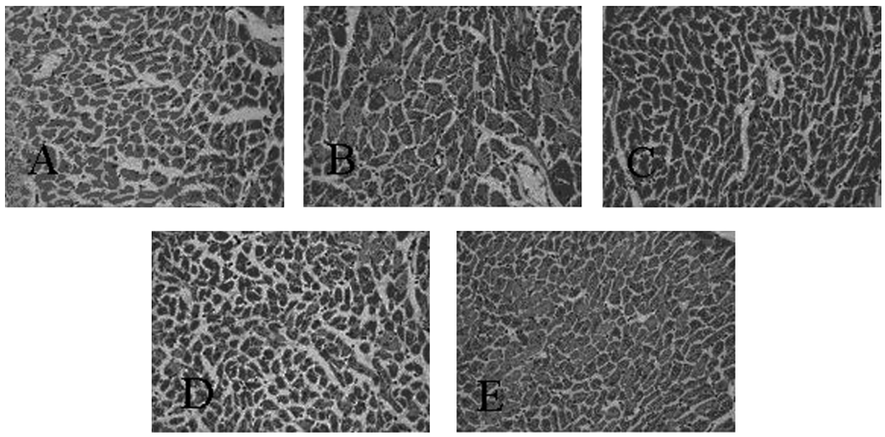

Fig. 1: Effect of ERS on cardiocyte cross-section area in rats with ventricular remodeling induced by coronary artery ligation. A: sham-operated group; B: model group; C: captopril group; D: ERS 8 g·kg^{-1} group; E: ERS 16 g·kg^{-1} group

cross-section area in model rats compared with that in the sham-operated rats. Cardiocyte cross-section area was smaller in ERS and captopril treated groups than in the model group ($p < 0.01$), as shown in Table 2.

2.4. Effects on collagen deposition

2.4.1. Perivascular collagen area and collagen volume fraction

The ventricular sections were stained with Sirius red in aqueous saturated picric acid for examination of perivascular and interstitial fibrosis in myocardium. As shown in Figs. 2, 3 and Table 2, the perivascular collagen area (PVCA) and collagen volume fraction (CVF) were markedly increased in the model group, compared to that in the control group. The treatment with ERS or captopril significantly decreased PVCA and CVF. ($p < 0.01$)

2.4.2. Effects on collagen distributions of type I and III

The contents of subtype I and III collagen in myocardium were determined using a microscope with polarimetric filter analysis at the same time. As shown in Figs. 4, 5 and Table 2, collagen distributions of type I and III were significantly increased in model rats compared with that in the sham-operated rats. However, those were significantly decreased in ERS or captopril treated groups ($p < 0.01$).

2.5. AT_1R, TNF-α and TGF-β1 mRNA expression

To further evaluate the effect of ERS on ventricular remodeling, we investigated some gene expressions associated with cardiac hypertrophy such as AT_1R, TNF-α and TGF-β1. As shown in Table 3, the AT_1R, TNF-α and TGF-β1 mRNA expression were increased significantly in the LV of model rats compared to that of sham-operated rats. ERS remarkably reduced these gene expression levels ($p < 0.01$). Captopril also significantly reduced these gene expressions ($p < 0.01$).

$$\text{relative expression of mRNA} = 2^{-\Delta ct} \times 100\%$$

$$\Delta ct = \text{CT(objective gene)} - \text{CT(GAPDH)}$$

Table 2: Effects of ERS on cardiocyte cross-section area and collagen deposition in rats with ventricular remodeling induced by coronary artery ligation ($\bar{x} \pm s$, n = 8)

Group	Dose	Cardiocyte cross-section area (pixel)	PVCA	CVF (%)	Collagen I (%)	Collagen III (%)
Sham	−−	6340.0 ± 531.1**	4.49 ± 1.50**	2.95 ± 1.37**	0.25 ± 0.20**	0.15 ± 0.09**
Model	−−	12957.6 ± 523.9	9.31 ± 4.57	5.97 ± 2.44	0.56 ± 0.38	0.27 ± 0.14
Captopril	40 mg/kg	7497.4 ± 430.4**	3.55 ± 1.65**	2.99 ± 0.81**	0.26 ± 0.16**	0.16 ± 0.10*
ERS-L	8 g/kg	8124.4 ± 243.5**	4.17 ± 2.00**	3.25 ± 0.81**	0.28 ± 0.15**	0.15 ± 0.09*
ERS-H	16 g/kg	8785.6 ± 296.3**	3.35 ± 1.02**	3.03 ± 0.96**	0.27 ± 0.12**	0.15 ± 0.07**

*$P < 0.05$, **$P < 0.01$ *vs* model group

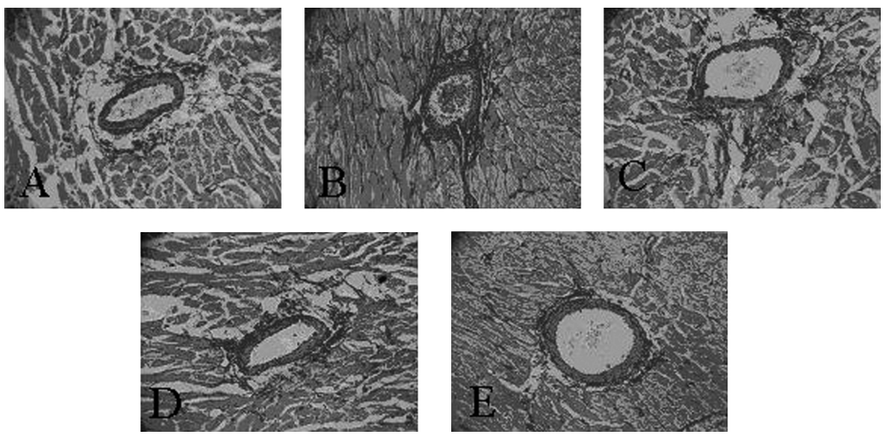

Fig. 2: Effect of ERS on myocardial perivascular collagen area in rats with ventricular remodeling induced by coronary artery ligation. A: sham-operated group; B: model group; C: captopril group; D: ERS 8 g·kg^{-1} group; E: ERS 16 g·kg^{-1} group

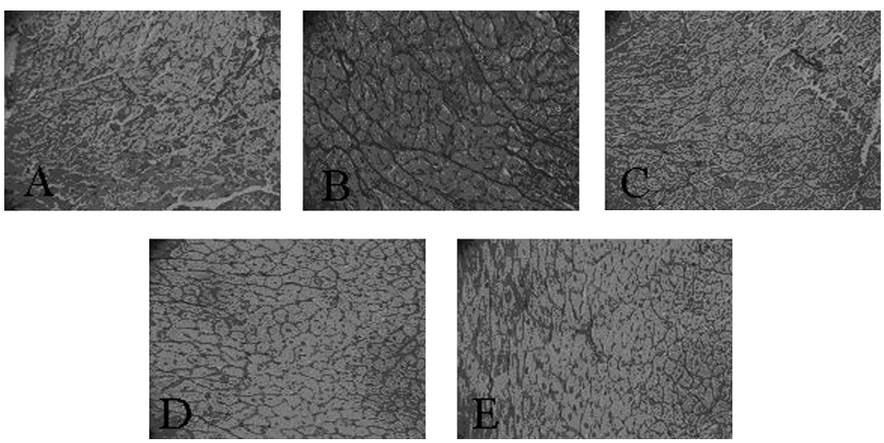

Fig. 3: Effect of ERS on myocardial collagen volume fraction in rats with ventricular remodeling induced by coronary artery ligation. A: sham-operated group; B: model group; C: captopril group; D: ERS 8 g·kg^{-1} group; E: ERS 16 g·kg^{-1} group

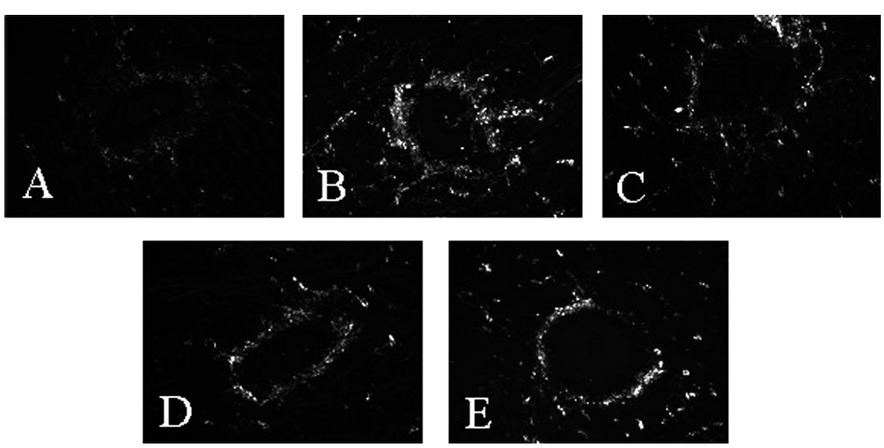

Fig. 4: Effect of ERS on myocardial perivascular collagen distributions of type I and III in rats with ventricular remodeling induced by coronary artery ligation. A: sham operated group; B: model group; C: captopril group; D: ERS 8 g·kg^{-1} group; E: ERS 16 g·kg^{-1} group

3. Discussion

In the last 20 years, the role of MI and the subsequent alteration in ventricular architecture of the infarcted and non-infarcted myocardium have become increasingly associated with a phenomenon known as ventricular remodeling. This process consists of left ventricular wall thinning in the infarction area, ventricular chamber dilatation, and compensatory hypertrophy of the noninfarcted portion of the myocardium (Paul, 1995). Because collagen deposition occurs early after infarction and tends to make the shape deformation permanent (Jugdutt and Amy 1986), it would seem logical to begin therapy before collagen deposition as soon as possible. In this study, the therapy was begun next day after ligation.

Left ventricular remodeling marked by inflammation, interstitial fibrosis, cardiocyte hypertrophy, systolic and diastolic dysfunction (Tenhunen et al. 2006).

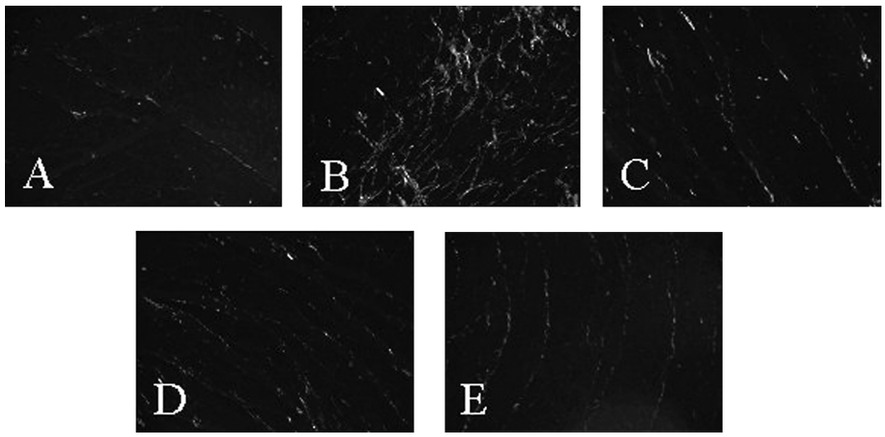

Fig. 5: Effect of ERS on myocardial collagen distributions of type I and III in rats with ventricular remodeling induced by coronary artery ligation. A: sham-operated group; B: model group; C: captopril group; D: ERS 8 g·kg^{-1}group; E: ERS 16 g·kg^{-1}group

Table 3: Effects of ERS on AT_1R, TNF-α and TGF-β1mRNA expressions in rats with ventricular remodeling induced by coronary artery ligation. ($\bar{x} \pm s$, n = 5)

Group	Dose	AT_1R mRNA	TNF-α mRNA	TGF-β1 mRNA
Sham	– –	1.49 ± 0.89**	3.14 ± 2.00**	1.51 ± 0.86**
Model	– –	36.54 ± 9.82	33.48 ± 9.74	40.05 ± 18.99
Captopril	40 mg/kg	4.38 ± 4.91**	6.86 ± 6.04**	2.94 ± 1.33**
ERS-L	8 g/kg	13.90 ± 8.83**	9.84 ± 3.17**	6.56 ± 2.99**
ERS-H	16 g/kg	3.70 ± 0.48**	5.60 ± 2.74**	3.01 ± 2.07**

*P < 0.05, **P < 0.01 *vs* model group

The present study demonstrated that administration of ERS could significantly reduce the LVWI, HWI; decrease the content of Ang II, Hyp; diminish cardiocyte cross-section area and ameliorate collagen deposition. In addition, ERS could down regulate the gene expressions of AT_1R, TNF-α and TGF-β_1 mRNA in myocardium. The mechanism may be versatile and complex.

There is considerable evidence that the renin-angiotensin-aldosterone system is activated after acute myocardial infarction and that activation of this system has important physiological and long-term morphological consequences.

In the study, the degree of cardiac hypertrophy was assessed by the increase of LVWI, HWI and the transverse area of cardiocytes. Cardiocyte hypertrophy was demonstrable microscopically, with an up to 100% increase in cell transverse area. The result is similar to the report (Anversa et al. 1985). Treatment for four weeks with low and high dose of ERS prevented cardiac hypertrophy.

There is now evidence that LV hypertrophy is associated with the induction of gene expression for angiotensin converting enzyme (ACE) and increased local synthesis of Ang II within the ventricular myocardium (Schunkert et al. 1990). Inhibition of ACE is beneficial for suppressing ventricular remodeling (Schieffer et al. 1994). ERS significantly attenuated the increase of Ang II concentration, it may be one of the mechanisms of its action against cardiac hypertrophy.

Many reports underscored the importance of the interaction between cytokines and the RAS in ventricular remodeling in the progression of CHF (Frolkis et al. 2001; Gurlek et al. 2001; Tsutamoto et al. 2000). The hearts that have suffered a MI have an increase in the expression of all the pro-inflammatory cytokines. TNF-α is among the most important inflammatory cytokines (Testa et al. 1996; Irwin et al. 1999). Ang II and TNF-α can potentiate the effects of each other, resulting in a vicious cycle towards CHF. Ang II secreted from cytoplasmic granules and the corresponding hypertrophic response is mediated by AT1 receptors (AT_1R). In the present study, treatment with ERS significantly decreased the elevated gene expression of AT_1R alongside a decrease in TNF-α gene expression in the heart.

Ang II and the cytokine TGF-β_1 are connected in the pathogenesis of cardiac remodeling. Ang II can up-regulate TGF-β_1 expression via activation of AT_1R in cardiocyte and fibroblast (Diniz et al. 2007). TGF-β_1 also can mediate Ang II induced structural ventricular remodeling in an autocrine/paracrine manner (Gray et al. 1998). TGF-β1 increases early in the infarct zone, stimulating macrophage and fibroblast chemotaxis and fibroblast proliferation (Desmouliere et al. 1993). Proliferation of fibroblasts during cardiac hypertrophy is a major mechanism contributing to the development of fibrosis, and fibroblasts accumulate in fibrotic and scarred areas (Deblois et al. 2005). In the present study, we observed that ERS inhibited myocardial fibrosis in the non-infarcted myocardium. Like captopril (van Krimpen et al. 1991), ERS given early during healing postinfarction but not later, inhibited collagen deposition in the non-infarcted myocardium as well.

Additionally, the major components of cardiac extracellular matrix (ECM) proteins are collagens, of which collagen type I composes approximately 85% (Heeneman et al. 2003). In particular, the accumulation of collagen type I which is characterized by tensile strength (Weber 1989), may contribute to myocardial stiffness by limiting the motion of cardiocytes, and may promote arrhythmias by electrical isolation of adjacent cardiocytes. Type III collagen, which is deposited during healing, has little tensile strength. The type I/III collagen ratio might increase between 21 and 90 days after MI in rats (Jugdutt et al. 1996). Therefore, the suppressive effect of ERS on collagen type I and III observed in the present study may provide a molecular substrate for the beneficial effects on LV structure and function, and the antifibrotic effects appeared to be attributable to the suppressive effect on the TNF-α secretion.

ACEI blocks local angiotensin II and TGF-β_1, the latter being a potent modulator of the extracellular matrix and a stimulator of collagen synthesis (Sadoshima and Izumo 1993). So ACEI can reduce the fibrosis and inhibit collagen deposition that accompanies LV remodeling in a variety of experimental models (Brilla et al. 1990). ACEI's inhibition of Ang II and TGF-β_1 might be especially important mediators of antagonizing myocardial hypertrophy and fibrosis.

ERS has beneficial effects against ventricular remodeling. The mechanism may be related to decreasing the level of Ang II and cardiac fibrosis, modulating AT_1R, TNF-α and TGF-β1 mRNA expressions associated with cardiac hypertrophy.

The effects of ERS are similar to those of ACEI, which are well known to prevent ventricular remodeling after MI. These results suggest that administration of a sufficient dose of ERS may have the same cardioprotective effects as ACEI in this animal model. However, the mechanism of ERS in detail and the effective components of ERS remain to be further explored.

4. Experimental

4.1. Herb extract and drug

Radix Scrophulariae was purchased from Yanghetang Medical Material Company (Shanghai, China), authenticated by Dr. Zhao, Department of Pharmacognosy, Shanghai University of TCM. It was decocted with distilled water, and the water soluble extract yield was obtained for the experiment (60% yield of prepared extracts in terms of starting crude material). Captopril tablets (batch number: 070704) were purchased from Shanghai Hengshan Pharmaceutical Co. Ltd., China. It was dissolved in distilled water.

4.2. Animals

Male Sprague Dawley rats weighing 220–250 g were obtained from the Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The rats were maintained on 12-h light/12-h dark cycle in the room at a temperature of $23 \pm 1\,^{\circ}C$, humidity of $40 \pm 5\%$. These rats received humane care and had free access to a standard diet and drinking water. The animal experiment was carried out according to the Guide for Care and Use of Laboratory Animals, published by US guidelines (NIH publication #85-23, revised in 1985).

4.3. Animal model and protocol

Rats were anesthetized by intraperitoneal administration of pentobarbital ($40\,mg\,kg^{-1}$), intubated and artificially ventilated. After left thoracotomy and pericardiotomy, the left coronary artery was ligated approximately 2 mm from its origin with a 4-0 silk suture. Then the thorax was closed immediately. The survival rate of the surgery was about 60%. After coronary ligation, all rats were randomly assigned to following 4 groups: model, captopril ($40\,mg\,kg^{-1}$), ERS ($8, 16\,g\,kg^{-1}$). The sham-operated rats underwent the same operation without ligation. After the surgery, the rats of all groups were given antibiotic (penicillin, $1 \times 10^4\ U{\cdot}kg^{-1}$, i.m.) and allowed to recover.

The rats were orally administrated with the corresponding extract or drug at the above described doses once a day. And distilled water was administered in the same manner for the sham-operated and model control groups. Treatment started from the next day after operation and continued for four weeks.

4.4. LVWI and HWI

Four weeks after the surgery the rats were weighed and then sacrificed by decapitation. The hearts were taken out and the left ventricle was separated from the atria, aorta and adipose tissue. The heart weight and left ventricle weight were measured, then, LVWI and HWI were estimated by calculating HW-to-BW and LVW-to-BW ratio.

The LV was divided by a cross section in the middle. The upper half part was fixed with 10% formaldehyde for pathological sectioning. The lower half part was further dissected into 3 parts and then quickly frozen in liquid nitrogen, and preserved at $-70\,^{\circ}C$ for biochemical and gene expression analysis.

4.5. Angiotensin II and hydroxyproline determination

Tissue homogenates (2%) were made for determination of Ang II and Hyp concentrations. The homogenized tissue was centrifuged ($4\,^{\circ}C$, 1780 g, 15 min.) and the supernatant liquid was collected for measurement. Ang II concentrations were quantified by radioimmunoassay with an iodine [^{125}I] Ang II radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing, China). Hyp concentrations were detected by ultraviolet spectrophotometry with the Hyp kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). Protein concentrations of myocardial homogenates were assayed with the Coomassie Brilliant Blue Kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). Ang II and Hyp concentrations were corrected for protein concentrations.

4.6. Histological analysis

The ventricles were fixed with 10% formaldehyde, embedded in paraffin, sectioned at 5 μm in thickness, stained with hematoxylin and eosin (HE) for measurement of cardiocyte cross section size, and stained with Sirius red in aqueous saturated picric acid for examination of perivascular and interstitial fibrosis in myocardium. Each sample slice was photographed with the digital camera (Olympus BX51, Japan) connected to microscope ($400 \times$ magnification) and computer. They were analyzed with image-Pro Plus 6.0 analyzing software (Media Cybernetics, U.S.A.).

Three fields per sample slice were randomly selected and 20 myocardial cells per-field were chosen to calculate cell size. Another 3 fields were randomly selected for calculating the ratio of picric Sirius red stained fibrosis area to total myocardial area. The perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area (Shinzato et al. 2007). The contents of subtype I and III collagen in myocardium were determined by using microscope with polarimetric filter analysis at the same time.

4.7. Real-time RT-PCR determination

Total RNA was extracted from the tissues by using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA yields and purity were assessed by spectrophotometric analysis. Total RNA (1 μg) from each well was subjected to reverse transcription with random hexamer primers, deoxynucleoside triphosphates (dNTPs), and Maloney murine leukemia virus (M-MLV) reverse transcriptase in a total reaction volume of 20 μl.

The real-time RT-PCR reactions (50 μl) consisted of 32.5 μl SYBR Green Mix, 1 μl mixed primers, 2 μl cDNA and 14.5 μl double-distilled water. A typical protocol included incubation at $50\,^{\circ}C$ for 2 min. and taq activation at $95\,^{\circ}C$ for 10 min. followed by 40 cycles with $95\,^{\circ}C$ denaturation for 20 s, $55\,^{\circ}C$ annealing for 30 s, and $72\,^{\circ}C$ extension for 30 s. The sequences of primer were as follows: AT_1R mRNA sense: 5'-GCACACTGGCAATGTAATGC-3', AT_1R mRNA anti-sense: 5'-GTTGAACAGAACAAGTGACC-3'; TNF-α mRNA sense: 5'-TGACTTTCTCCTGGTATGAAATGG-3', TNF-α mRNA anti-sense: 5'-TGACTTTCTCCTGGTATGAAATGG-3'; TGF-β_1 mRNA sense: 5'-GCTGCTGACCCCCACTGAT-3', TGF-β_1 mRNA anti-sense: 5'-TGCCGGACAACTCCAGTGA-3'; GAPDH sense: 5'-CCGAGGGCCCACTAAAGG-3', GAPDH anti-sense: 5'-GCTGTTGAAGTCACAGGAGACAA-3'.

Real-time RT-PCR was carried out with 7300 Sequence Detection System and data analyzed by ABI Prism 7300 SDS Software.

All values obtained with the AT_1R, TNF-α or TGF-β1 primers were normalized to the values obtained with the GAPDH primers. The results were expressed as the relative integrated intensity.

4.8. Statistics

All data were expressed as the mean $\pm$ SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparisons, followed by Dunnett's test to evaluate the difference between two groups through the software of SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, USA).

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