

Effect of the Shensong Yangxin Capsule on Energy Metabolism in Angiotensin II-Induced Cardiac Hypertrophy

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Abstract

Background: Shensong Yangxin Capsule (SSYX), traditional Chinese medicine, has been used to treat arrhythmias, angina, cardiac remodeling, cardiac fibrosis, and so on, but its effect on cardiac energy metabolism is still not clear. The objective of this study was to investigate the effects of SSYX on myocardium energy metabolism in angiotensin (Ang) II-induced cardiac hypertrophy.

Methods: We used 2 μl (10^{-6} mol/L) AngII to treat neonatal rat cardiomyocytes (NRCMs) for 48 h. Myocardial α -actinin staining showed that the myocardial cell volume increased. Expression of the cardiac hypertrophic marker-brain natriuretic peptide (BNP) messenger RNA (mRNA) also increased by real-time polymerase chain reaction (PCR). Therefore, it can be assumed that the model of hypertrophic cardiomyocytes was successfully constructed. Then, NRCMs were treated with 1 μl of different concentrations of SSYX (0.25, 0.5, and 1.0 $\mu\text{g}/\text{ml}$) for another 24 h. To explore the time-depend effect of SSYX on energy metabolism, 0.5 $\mu\text{g}/\text{ml}$ SSYX was added into cells for 0, 6, 12, 24, and 48 h. Mitochondria was assessed by MitoTracker staining and confocal microscopy. mRNA and protein expression of mitochondrial biogenesis-related genes – Peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), energy balance key factor – adenosine monophosphate-activated protein kinase (AMPK), fatty acids oxidation factor – carnitine palmitoyltransferase-1 (CPT-1), and glucose oxidation factor – glucose transporter- 4 (GLUT-4) were measured by PCR and Western blotting analysis.

Results: With the increase in the concentration of SSYX (from 0.25 to 1.0 $\mu\text{g}/\text{ml}$), an increased mitochondrial density in AngII-induced cardiomyocytes was found compared to that of those treated with AngII only (0.25 $\mu\text{g}/\text{ml}$, 18.3300 ± 0.8895 vs. 24.4900 ± 0.9041 , $t = 10.240$, $P < 0.0001$; 0.5 $\mu\text{g}/\text{ml}$, 18.3300 ± 0.8895 vs. 25.9800 ± 0.8187 , $t = 12.710$, $P < 0.0001$; and 1.0 $\mu\text{g}/\text{ml}$, 18.3300 ± 0.8895 vs. 24.2900 ± 1.3120 , $t = 9.902$, $P < 0.0001$; $n = 5$ per dosage group). SSYX also increased the mRNA and protein expression of PGC-1 α (0.25 $\mu\text{g}/\text{ml}$, 0.8892 ± 0.0848 vs. 1.0970 ± 0.0994 , $t = 4.319$, $P = 0.0013$; 0.5 $\mu\text{g}/\text{ml}$, 0.8892 ± 0.0848 vs. 1.2330 ± 0.0564 , $t = 7.150$, $P < 0.0001$; and 1.0 $\mu\text{g}/\text{ml}$, 0.8892 ± 0.0848 vs. 1.1640 ± 0.0755 , $t = 5.720$, $P < 0.0001$; $n = 5$ per dosage group), AMPK (0.25 $\mu\text{g}/\text{ml}$, 0.8872 ± 0.0779 vs. 1.1500 ± 0.0507 , $t = 7.239$, $P < 0.0001$; 0.5 $\mu\text{g}/\text{ml}$, 0.8872 ± 0.0779 vs. 1.2280 ± 0.0623 , $t = 9.379$, $P < 0.0001$; and 1.0 $\mu\text{g}/\text{ml}$, 0.8872 ± 0.0779 vs. 1.3020 ± 0.0450 , $t = 11.400$, $P < 0.0001$; $n = 5$ per dosage group), CPT-1 (1.0 $\mu\text{g}/\text{ml}$, 0.7348 ± 0.0594 vs. 0.9880 ± 0.0851 , $t = 4.994$, $P = 0.0007$, $n = 5$), and GLUT-4 (0.5 $\mu\text{g}/\text{ml}$, 1.5640 ± 0.0599 vs. 1.7720 ± 0.0660 , $t = 3.783$, $P = 0.0117$; 1.0 $\mu\text{g}/\text{ml}$, 1.5640 ± 0.0599 vs. 2.0490 ± 0.1280 , $t = 8.808$, $P < 0.0001$; $n = 5$ per dosage group). The effect became more obvious with the increasing concentration of SSYX. When 0.5 $\mu\text{g}/\text{ml}$ SSYX was added into cells for 0, 6, 12, 24, and 48 h, the expression of AMPK (6 h, 14.6100 ± 0.6205 vs. 16.5200 ± 0.7450 , $t = 3.456$, $P = 0.0250$; 12 h, 14.6100 ± 0.6205 vs. 18.3200 ± 0.9965 , $t = 6.720$, $P < 0.0001$; 24 h, 14.6100 ± 0.6205 vs. 21.8800 ± 0.8208 , $t = 13.160$, $P < 0.0001$; and 48 h, 14.6100 ± 0.6205 vs. 23.7400 ± 1.0970 , $t = 16.530$, $P < 0.0001$; $n = 5$ per dosage group), PGC-1 α (12 h, 11.4700 ± 0.7252 vs. 16.9000 ± 1.0150 , $t = 7.910$, $P < 0.0001$; 24 h, 11.4700 ± 0.7252 vs. 20.8800 ± 1.2340 , $t = 13.710$, $P < 0.0001$; and 48 h, 11.4700 ± 0.7252 vs. 22.0300 ± 1.4180 , $t = 15.390$; $n = 5$ per dosage group), CPT-1 (24 h, 15.1600 ± 1.0960 vs. 18.5800 ± 0.9049 , $t = 6.048$, $P < 0.0001$, $n = 5$), and GLUT-4 (6 h, 10.2100 ± 0.9485 vs. 12.9700 ± 0.8221 , $t = 4.763$, $P = 0.0012$; 12 h, 10.2100 ± 0.9485 vs. 16.9100 ± 0.8481 , $t = 11.590$, $P < 0.0001$; 24 h, 10.2100 ± 0.9485 vs. 19.0900 ± 0.9797 , $t = 15.360$, $P < 0.0001$; and 48 h, 10.2100 ± 0.9485 vs. 14.1900 ± 0.9611 , $t = 6.877$, $P < 0.0001$; $n = 5$ per dosage group) mRNA and protein increased gradually with the prolongation of drug action time.

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Conclusions: SSYX could increase myocardial energy metabolism in AngII-induced cardiac hypertrophy. Therefore, SSYX might be considered to be an alternative therapeutic remedy for myocardial hypertrophy.

Key words: AMP-Activated Protein Kinase; Cardiac Hypertrophy; Energy Metabolism; Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 Alpha; Shensong Yangxin Capsule

INTRODUCTION

It is well known that myocardial hypertrophy is often accompanied by cardiac energy metabolism disorders and that myocardial metabolic disorders aggravate the pathological progression of cardiac hypertrophy to heart failure. Energy metabolism includes mitochondrial metabolism, carbohydrate metabolism, and lipid metabolism. Mitochondria are essential and critical regulators of oxidative phosphorylation.^[1-3] Peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), a transcription coactivator of nuclear receptors and master regulator of metabolism, plays an important role in cardiac metabolism regulation. Previous study^[4] had demonstrated that PGC-1 α played an important role in the development of pathological cardiac hypertrophy, metabolic disorders, and reactivation of the fetal genetic program on animals models of chronic heart failure. PGC1- α was also involved in mitochondrial biogenesis, which was vital for cell survival. Experimental evidence^[5] supported the roles of mitochondrial dysfunction and oxidative stress as determinants of neuronal death as well as endogenous protective mechanisms after stroke. Accumulating evidence suggested that activation of 5' AMP-activated protein kinase (AMPK) during myocardial ischemia increased both glucose uptake and glycolysis as well as fatty acid oxidation (FAO) during reperfusion. Gain-of-function mutations of AMPK in cardiac muscle might also be causally related to the development of hypertrophic cardiomyopathies.^[6] Mitochondrial FAO is an important energy provider for cardiac work, and changes in cardiac substrate preference are associated with different heart diseases. Carnitine palmitoyltransferase-1 (CPT-1) is thought to be the rate-limiting enzyme in FAO and is inhibited by malonyl-CoA.^[7] Insulin-regulated glucose transporter-4 (GLUT-4) is immunolocalized in rat cardiac muscle under conditions of basal and stimulates glucose uptake, which are achieved by fasting and a combined exercise/insulin stimulus, respectively.^[8] Mitochondrial dysfunction has been demonstrated to contribute to heart disease and its clinical manifestations, and damaged mitochondrial homeostasis might lead to heart failure over time.^[9] Consequently, exploration of a new therapeutic strategy to improve mitochondrial function and energy metabolism may have great clinical significance.

The Shensong Yangxin Capsule (SSYX) was designed and carefully formulated in accordance with the rules of traditional Chinese medicine (TCM) and includes *Panax ginseng*, *Salvia miltiorrhiza*, *Nardostachys jatamansi*, and so on.^[10] According to TCMs, SSYX has long been used in China as a traditional Chinese remedy to treat

a variety of cardiac diseases, especially to treat cardiac arrhythmias and various arrhythmias in coronary heart disease and chronic heart failure,^[6,11-13] angina in coronary heart disease,^[14,15] cardiac remodeling, cardiac fibrosis, regulation of lipid levels, and so on. The above studies have revealed the positive role of SSYX in cardiac energy metabolism, providing the experimental basis for our research. SSYX might block multiple ion channels,^[16] which might change the action potential duration and contribute to its antiarrhythmic effects.^[17,18] SSYX suppressed transforming growth factor- β 1/Smad signaling to inhibit fibrosis in diabetic cardiomyopathy and improved cardiac function.^[19] SSYX could block the Smad signaling pathway to attenuate cardiac remodeling after myocardial infarction.^[11] However, the influence of SSYX on myocardium energy metabolism remains unclear. In this study, we studied the regulatory effects of different SSYX drug concentrations on myocardial energy metabolism during AngII-induced cardiac hypertrophy using neonatal rat cardiomyocytes (NRCMs). We particularly focused on the mitochondrial density, glucose metabolism, fatty acid metabolism, and deciphering the underlying molecular mechanisms. Our results might provide a significant therapeutic strategy for ameliorating mitochondrial function through the use of SSYX.

METHODS

Cell culture and treatment

All experiments involving animals were conducted in accordance with the 8th Edition of the Guide for the Care and Use of Laboratory Animals (Guide NRC, 2011) published by the US National Institutes of Health and were approved by the Animal Care and Use Committee of Renmin Hospital at Wuhan University (Production license: SCXK 2015-0018). All animals used in this study were postnatal days 1-3 Sprague-Dawley (SD) rats, which were supplied by the Experiment Animal Centre of Wuhan University. We also followed the methods reported in the literature;^[20] NRCMs were isolated from the hearts of SD rat pups on postnatal days 1-3 and were cultured to <98% purity.^[21] Briefly, SD rats were sacrificed by swift decapitation, and the hearts were excised and minced immediately. Heart tissues were digested in a solution containing 0.08% collagenase Type II and 0.125% trypsin, and the supernatant was collected. The process was repeated 7-10 times. Then, cardiomyocytes were enriched via differential attachment, plated, and cultured in a culture dish with DMEM/F₁₂ medium (Gibco, Grand Island, NY, USA) containing 15% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin, and 0.1 mmol/L BrdU (Sigma-Aldrich, St. Louis, MO, USA).^[22,23]

After culturing for 48 h, the culture medium was replaced with complete medium for 12 h. Then, cardiomyocytes were randomly assigned into five groups and subsequently subjected to different treatments. These groups were as follows: control, 10^{-6} mol/L angiotensin (Ang) II (Sigma-Aldrich, St. Louis, MO, USA), 10^{-6} mol/L AngII + SSYX (0.25 $\mu\text{g/ml}$), 10^{-6} mol/L AngII + SSYX (0.5 $\mu\text{g/ml}$), and 10^{-6} mol/L AngII + SSYX (1.0 $\mu\text{g/ml}$). SSYX, provided by Shijiazhuang Yiling Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China), was diluted in culture media to various concentrations (0.25, 0.5, and 1.0 $\mu\text{g/ml}$).

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed on a StepOne™ Real-Time PCR Instrument (Life technologies, Shanghai, China). Each sample was used for 3 replicates using a SYBR® Premix Ex Taq™ Kit (TaKaRa). The primers were prepared by Wuhan Jin Kairui Bioengineering Co., Ltd. For synthesis, the following primers were used: *β -actin* forward: 5'-CACGATGGAGGGCCGGACTCATC-3'; reverse: 5'-TAAAGACCTCTATGCCAACACAGT-3'; the gene information of the other factors is shown in Table 1.

Western blotting analysis

TBS buffer solution was used to moisten the adherent cells 2–3 times, and the last time, the residual liquid was removed. A suitable volume of total protein extraction reagent (with proteinase inhibitor added for a few minutes) was added for 3–5 min to the culture plate/bottle. The plate/bottle was shaken repeatedly during this period to ensure that the reagent fully contacted the cells. The cells and reagents were scraped with cell scrapers and then collected in 1.5 ml centrifuge tubes. The sample was placed on an ice bath 30 min, and a pipette was used repeatedly to ensure that the cells were completely lysed. Centrifuged for 5 minutes at 4°C and 13000×g with the following primary antibodies: AMPK α 1 (1:200 dilution, sc-130394, Santa Cruz Biotechnology), p-AMPK (1:1000 dilution, YT639-FIW, Baiao Bolai, BeiJing), PGC-1 α (1:500 dilution, sc-518025, Santa Cruz Biotechnology), CPT-1 (1:200 dilution, sc-514555, Santa Cruz Biotechnology), and GLUT-4 (1:1000 dilution, sc-53566, Santa Cruz Biotechnology). The supernatant was collected and was considered the total protein solution. A BCA protein (Beyotime, Nanjing, China) concentration assay kit was used to determine the concentration of the sample protein. The protein concentration was also

determined by SDS-PAGE electrophoresis. The film was scanned and archived, and the AlphaEaseFC software (Alpha Innotech, California, USA) processing system was used to analyze the optical density of the target band.

Alpha-actinin staining detects myocardial cell size

The NRCM cell suspension was added to a cover glass and cultured in an incubator with a CO₂ concentration of 5% at 37°C for 2 h. Then, 2 ml of the cell culture solution was added and incubated for approximately 6 h. Cells were then fixed with 4% polyoxymethylene for 30 min. To prevent the flow of antibodies, the creeping piece was dried slightly, and then, a uniform position in the middle cell of the cover glass was drawn with a histochemical pen, adding 50–100 μl of working fluid to break the membrane, followed by incubation for 10 min at room temperature. A 3% hydrogen peroxide solution was added to the ring and was incubated for 20 min at room temperature. The glass was washed with PBS (pH 7.4) 3 times and placed on a shaking table. After drying, the cells were covered with 5% bovine serum albumin (BSA). The cells were laid in a wet box at 4°C overnight. The glass was washed in PBS (pH 7.4) 3 times on a shaking table and washed for 5 min each time. After dripping, the glass was incubated for 50 min at room temperature with two anti-covering cells from a kit. Each slice was placed in 50–100 μl of DAPI dye and incubated at room temperature for 5 min. A proper amount of anti-fluorescence quenching agent was added to the cells, and the cover glass slide was sealed and observed under a fluorescence microscope.

MitoTracker staining and confocal microscopy

NRCMs were plated in chamber slides (BD Bioscience, Sparks, MD, USA) at a density of 1×10^6 cells/well and treated with or without SSYX for 48 h. Cells were then stained with MitoTracker® Red CMXRos (Yisheng Biological Technology, Shanghai, China) at a concentration of 300 nmol/L diluted in prewarmed culture media for 30 min. MitoTracker® Red CMXRos contains a weak thiol-reactive chloromethyl functional group labeled with mitochondria that can label mitochondria in cardiomyocytes and is used to observe the number of mitochondria in cells. Then, cells were fixed in 4% formaldehyde in prewarmed culture media and stained with DAPI staining solution (Beyotime, China) to mark cell nuclei. A total of 20 fields/well (×400) were taken by a confocal microscope (Carl Zeiss, Thuringia, Germany). MitoTracker® Red CMXRos make mitochondria to emit a

Table 1: Gene information for some factors

| Gene | Species | Forward | Reverse |
|---------------------------------|---------|-----------------------------------|-------------------------------------|
| <i>BNP</i> | Rat | 5'-AGCCAGTCTCCAGAACAATCC-3' | 5'-CGGTCTATCTTCTGCCAAAA-3' |
| <i>Capase-3</i> | Rat | 5'-TGGAATGTCAGCTCGCAATG-3' | 5'-CAGGTCCGTTCTGTTCCAAAA-3' |
| <i>PGC-1α</i> | Rat | 5'-GGAGCAATAAAGCAAAGCGCA-3' | 5'-GTGTGAGGAGGGTTCATCGTT-3' |
| <i>AMPK</i> | Rat | 5'-ACA GAA GCC AAA TCA GGG ACT-3' | 5'-CAC GGA TGA GGT AAG AGA GAC T-3' |
| <i>CPT-1</i> | Rat | 5'-CCA GGC AAA GAG ACA GAC TTG-3' | 5'-GCCAAACCTTGAAGAAGCGA-3' |
| <i>GLUT-4</i> | Rat | 5'-GTATGTTGCCGATGCTATGG-3' | 5'-CCTCTGGTTTCAGGCACTCT-3' |

BNP: Brain natriuretic peptide; *Capase-3*: Cysteiny aspartate specific proteinase-3; *PGC-1 α* : Peroxisome proliferator-activated receptor coactivator 1 α ; *AMPK*: AMP-activated protein kinase; *CPT-1*: Carnitine acyltransferase enzyme-1; *GLUT-4*: Glucose transporter protein-4.

red fluorescence under a confocal microscope. Therefore, we were able to judge the number and density of mitochondria based on the amount of red fluorescence. The cell size and fluorescence intensity were measured by ImageJ (National Institutes of Health, NIH, USA) and Zeiss software (Carl Zeiss Jena, Germany), respectively.

Statistical analysis

Statistical analyses and all calculations were performed using GraphPad Prism version 5.0 statistical software (GraphPad Software, San Diego, CA, USA). All experimental data were expressed as the mean \pm standard deviation. One-way analysis of variance (ANOVA; three or more groups) was used for intergroup comparison. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Shensong Yangxin Capsule attenuates the myocardial hypertrophy and myocardial apoptosis induced by Angiotensin II *in vivo*

To evaluate the effect of the SSYX on the myocardial hypertrophy and apoptosis induced by AngII *in vivo*, NRCMs were cultured for 48 h, 10^{-6} mol/L AngII was added to the experimental group for 24 h, and α -actinin staining was used to observe myocardial hypertrophy in each group [Figure 1a and 1b]. PCR was used to detect the mRNA expression of the cardiomyocyte hypertrophy marker – brain natriuretic peptide (*BNP*) [Figure 1c] and cardiomyocyte apoptosis factor marker – *Capase-3* [Figure 1d]. Comparing with the control group, cell volume in AngII group ($28,369 \pm 1783 \mu\text{m}^2$ vs. $222,400 \pm 12,602 \mu\text{m}^2$, $t = 24.380$, $P < 0.0001$, $n = 5$) increased. Comparing with the control group, the expression of *BNP* and *Capase-3* mRNA in AngII group (0.8654 ± 0.0542 vs. 1.2060 ± 0.0206 , $t = 10.730$, $P < 0.0001$; 0.5400 ± 0.0758 vs. 1.1170 ± 0.0160 , $t = 11.740$, $P < 0.0001$, $n = 5$ per dosage group) increased. The cardiac hypertrophy model was successfully constructed. Then, the cells were treated with 0.25, 0.5, and 1.0 $\mu\text{g/ml}$ SSYX for 12 h. Comparing with the AngII group, cell volume in 0.25 $\mu\text{g/ml}$ SSYX ($222,400 \pm 12,602 \mu\text{m}^2$ vs. $190,000 \pm 15,811 \mu\text{m}^2$, $t = 4.070$, $P = 0.0024$, $n = 5$), 0.5 $\mu\text{g/ml}$ SSYX ($222,400 \pm 12,602 \mu\text{m}^2$ vs. $100,000 \pm 15,811 \mu\text{m}^2$, $t = 15.380$, $P < 0.0001$, $n = 5$), and 1.0 $\mu\text{g/ml}$ SSYX ($22,2400 \pm 12,602 \mu\text{m}^2$ vs. $64,000 \pm 11,402 \mu\text{m}^2$, $t = 19.900$, $P < 0.0001$, $n = 5$) groups decreased. Comparing with the AngII group, the expression of *BNP* and *Capase-3* mRNA in 0.25 $\mu\text{g/ml}$ SSYX (1.2060 ± 0.0206 vs. 1.0770 ± 0.0581 , $t = 4.072$, $P = 0.0024$; 1.1170 ± 0.0160 vs. 1.0880 ± 0.0739 , $t = 0.5942$, $P > 0.9999$; $n = 5$, respectively), 0.5 $\mu\text{g/ml}$ SSYX (1.2060 ± 0.0206 vs. 0.7814 ± 0.0684 , $t = 13.370$, $P < 0.0001$; 1.1170 ± 0.0160 vs. 0.8724 ± 0.1181 , $t = 4.977$, $P = 0.0007$; $n = 5$, respectively), and 1.0 $\mu\text{g/ml}$ SSYX (1.2060 ± 0.0206 vs. 0.5992 ± 0.0348 , $t = 19.110$, $P < 0.0001$; 1.1170 ± 0.0160 vs. 0.8364 ± 0.0692 , $t = 5.710$, $P = 0.0001$; $n = 5$, respectively) decreased. Studies have shown that only when the concentration of SSYX drug reached 0.5 $\mu\text{g/ml}$ could reduce the expression of *Capase-3*

mRNA and resist the apoptosis of cardiomyocytes induced by AngII. However, low concentrations of SSYX (0.25 $\mu\text{g/ml}$) have been able to reduce the expression of *BNP* mRNA and alleviate AngII-induced cardiomyocyte hypertrophy, and its effect was positively related to the drug concentration.

Shensong Yangxin Capsule protects the mitochondrial density in the process of Angiotensin II-induced cardiac hypertrophy

To evaluate the protective effect of SSYX on mitochondria, AngII-induced cells were treated with SSYX at different concentrations of 0.25, 0.5, and 1.0 $\mu\text{g/ml}$ for 12 h. Then, 300 $\mu\text{mol/L}$ preheated mitochondrial MitoTracker Red (40741ES50, Yisheng Biological Technology Co., Ltd. Shanghai) was added to the cell plates for 30 min, and the cells were observed with a confocal microscope [Figure 2a]. We found that the number of mitochondria decreased after adding 10^{-6} mol/L AngII compared with the control group (21.3700 ± 0.7222 vs. 18.3300 ± 0.8895 , $t = 5.045$, $P = 0.0002$, $n = 5$; Normalized MitoTracker Red content, Figure 2b). However, when different concentrations of SSYX were added to AngII-induced cells, the number of mitochondria in cardiomyocytes began to increase. When the different concentrations (0.25 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, and 1.0 $\mu\text{g/ml}$) of SSYX were compared, it was interesting to note that the number of mitochondria increased with increasing concentrations of SSYX (0.25 $\mu\text{g/ml}$, 18.3300 ± 0.8895 vs. 24.4900 ± 0.9041 , $t = 10.240$, $P < 0.0001$; 0.5 $\mu\text{g/ml}$, 18.3300 ± 0.8895 vs. 25.9800 ± 0.8187 , $t = 12.710$, $P < 0.0001$; and 1.0 $\mu\text{g/ml}$, 18.3300 ± 0.8895 vs. 24.2900 ± 1.3120 , $t = 9.902$, $P < 0.0001$; $n = 5$ per dosage group). Therefore, we concluded that SSYX had a protective effect on the mitochondrial density in AngII-induced cardiac hypertrophy. This effect became more obvious as the concentration of SSYX increased.

Shensong Yangxin Capsule regulates mitochondrial biogenesis and energy balance with peroxisome proliferator-activated receptor gamma coactivator-1 alpha and AMP-activated protein kinase activation

To explore the effects of SSYX on mitochondrial biogenesis and energy balance, we examined the mRNA and protein expression of the mitochondrial biological origin and energy balance key factors – *PGC-1 α* and *AMPK* – after treating cells with the above concentrations of SSYX (0.25 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, and 1.0 $\mu\text{g/ml}$) [Figure 3a–3d]. *PGC-1 α* and *AMPK* mRNA were detected by PCR. *PGC-1 α* , p-*AMPK*, and *AMPK* protein expressions were detected by Western blotting analysis. As shown in Figure 3c, the expression of *AMPK* mRNA decreased in AngII group compared with the control group (1.1940 ± 0.0447 vs. 0.8872 ± 0.0779 , $t = 8.450$, $P < 0.0001$, $n = 5$), while it increased in 0.25 $\mu\text{g/ml}$ SSYX (0.8872 ± 0.0779 vs. 1.1500 ± 0.0507 , $t = 7.239$, $P < 0.0001$, $n = 5$), 0.5 $\mu\text{g/ml}$ SSYX (0.8872 ± 0.0779 vs. 1.2280 ± 0.0623 , $t = 9.379$, $P < 0.0001$, $n = 5$), and 1.0 $\mu\text{g/ml}$ SSYX (0.8872 ± 0.0779 vs. 1.3020 ± 0.0450 , $t = 11.400$, $P < 0.0001$, $n = 5$) groups compared with AngII group. The expression of *PGC-1 α* mRNA decreased in AngII group

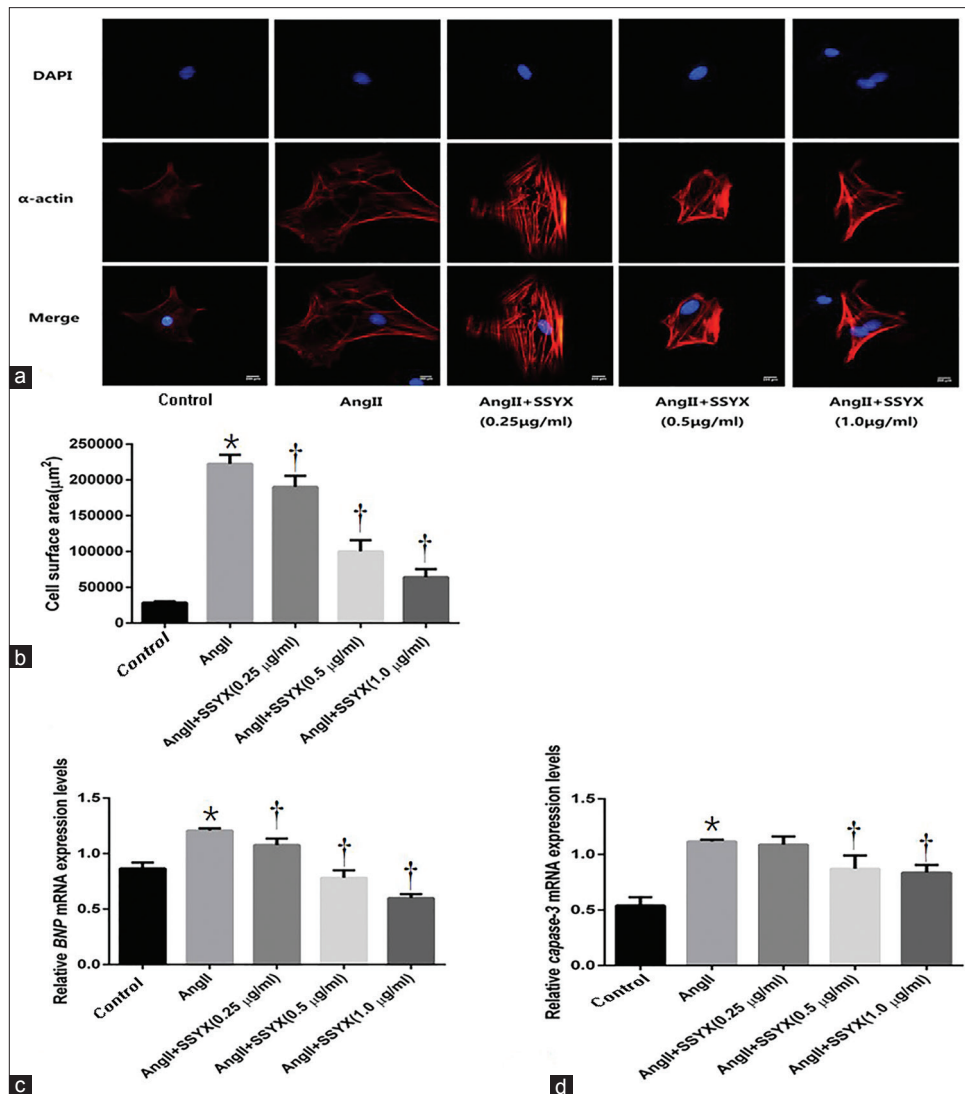


Figure 1: Myocardial hypertrophy and apoptosis in the control, AngII, SSYX (0.25, 0.5, and 1.0 µg/ml drug concentration) groups. (a) Representative immunofluorescent (α -actinin) images of NRCMs in each group. Bar = 200 µm. Red staining denotes α -actinin-stained cardiomyocytes. Blue staining denotes DAPI-stained myocardial cell nuclei. (b) Summarized data of surface area of myocardial cells in each group. (c) Relative expression levels of *BNP* mRNA in each group. (d) Relative expression levels of *Capase-3* mRNA in each group. * $P < 0.0001$ versus the control group; † $P < 0.05$ versus the AngII group. The data are expressed as the mean \pm SD ($n = 5$, samples were collected from five different passages). The symbols *, † indicate that the value of cell volume and *BNP* and *Capase-3* mRNA expression significantly differed from different groups. *BNP*: Brain natriuretic peptide; *Capase-3*: Cysteiny aspartate specific proteinase-3; mRNA: Messenger RNA; Ang: Angiotensin; SSYX: Shensong Yangxin Capsule; NRCMs: Neonatal rat cardiomyocytes; SD: Standard deviation.

compared with the control group (1.3640 ± 0.0545 vs. 0.8892 ± 0.0848 , $t = 9.865$, $P < 0.0001$, $n = 5$), while it increased in 0.25 µg/ml SSYX (0.8892 ± 0.0848 vs. 1.0970 ± 0.0994 , $t = 4.319$, $P = 0.0013$, $n = 5$), 0.5 µg/ml SSYX (0.8892 ± 0.0848 vs. 1.2330 ± 0.0564 , $t = 7.150$, $P < 0.0001$, $n = 5$), and 1.0 µg/ml SSYX (0.8892 ± 0.0848 vs. 1.1640 ± 0.0755 , $t = 5.720$, $P < 0.0001$, $n = 5$) groups compared with AngII group. To explore time-depend effect of SSYX on mitochondrial biogenesis and energy balance, 0.5 µg/ml SSYX was added into cells for 0, 6, 12, 24, and 48 h [Figure 3e–3h]. The expression of *AMPK* and *PGC-1 α* mRNA presented an upward trend in different hours (*AMPK*: 6 h, 14.6100 ± 0.6205 vs. 16.5200 ± 0.7450 , $t = 3.456$, $P = 0.0250$; 12 h, 14.6100 ± 0.6205 vs. 18.3200 ± 0.9965 ,

$t = 6.720$, $P < 0.0001$; 24 h, 14.6100 ± 0.6205 vs. 21.8800 ± 0.8208 , $t = 13.160$, $P < 0.0001$; and 48 h, 14.6100 ± 0.6205 vs. 23.7400 ± 1.097 , $t = 16.530$, $P < 0.0001$; $n = 5$ per dosage group; *PGC-1 α* : 12 h, 11.4700 ± 0.7252 vs. 16.9000 ± 1.0150 , $t = 7.910$, $P < 0.0001$; 24 h, 11.4700 ± 0.7252 vs. 20.8800 ± 1.2340 , $t = 13.710$, $P < 0.0001$; and 48 h, 11.4700 ± 0.7252 vs. 22.0300 ± 1.4180 , $t = 15.390$, $P < 0.0001$; $n = 5$ per dosage group). In summary, SSYX increased the mRNA and protein expression of *AMPK* and *PGC-1 α* in a dose-dependent manner. Compared with different hours (from 0 to 48 h), the effect was becoming more and more remarkable. Therefore, SSYX could improve mitochondrial biogenesis and energy balance in cardiac hypertrophy.

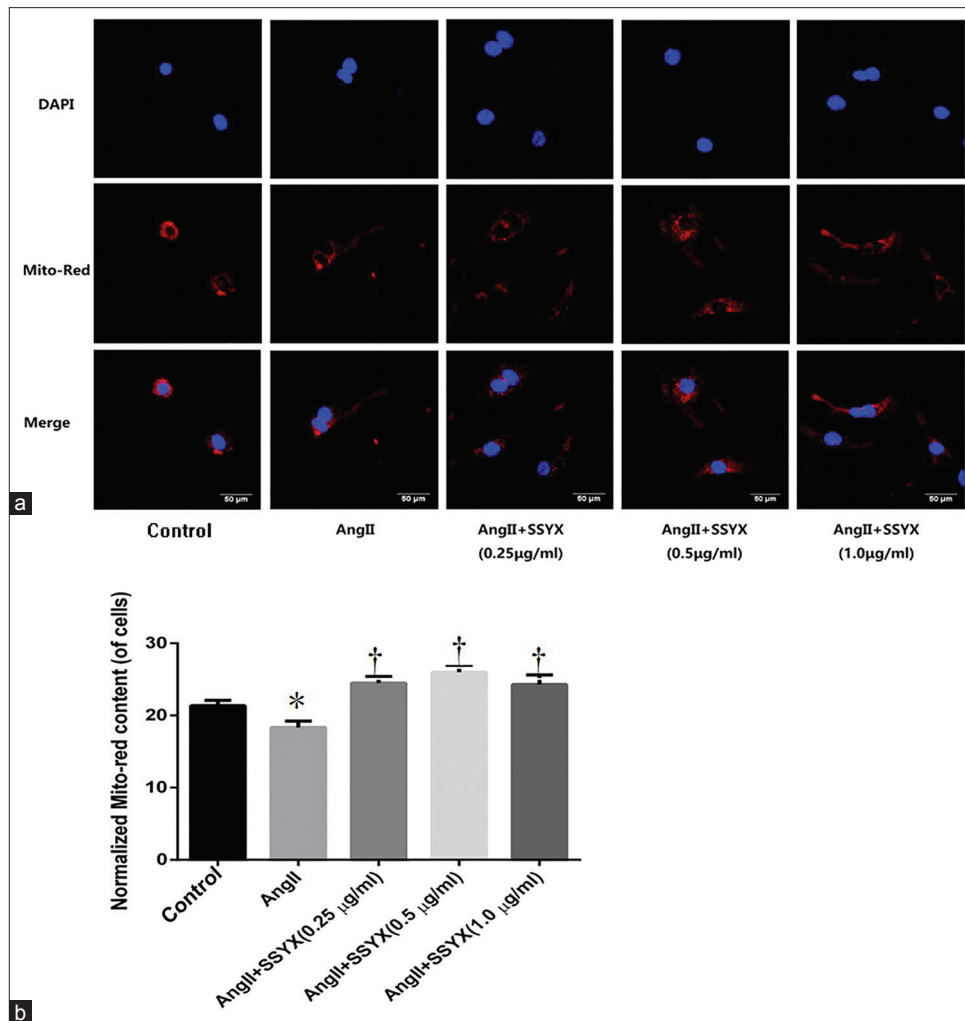


Figure 2: Mitochondrial density in AngII-induced cardiomyocyte hypertrophy 12 h after SSYX treatment with different drug concentrations. (a) Representative fluorescence microscope image of cardiomyocytes costained with mitochondrial Mito-Red Tracker and myocardial cell nuclei DAPI staining at 12 h after treatment with 0.25, 0.5, and 1.0 µg/ml SSYX. Scale bars = 50 µm. Red staining denotes Mito-Red Tracker-stained mitochondria. Blue staining denotes DAPI-stained myocardial cell nuclei. (b) Normalized MitoTracker Red content. * $P = 0.0002$ versus control group; † $P < 0.0001$ versus AngII group. The data were expressed as the mean \pm SD ($n = 5$, samples were collected from five different passages), the symbols “*,” “†” indicate that the value of Mito-Red Tracker content significantly differed from different groups. Ang: Angiotensin; SSYX: Shensong Yangxin Capsule; SD: Standard deviation.

Shensong Yangxin Capsule regulates fatty acids and glucose oxidation during the process of cardiac hypertrophy

To explore the effects of SSYX on fatty acids and glucose oxidation, we examined the mRNA and protein expression of the fatty acid and glucose oxidation key factors – *CPT-1* and *GLUT-4* – after treating cells with 0.25 µg/ml, 0.5 µg/ml, and 1.0 µg/ml SSYX [Figure 4a–4d]. *CPT-1* and *GLUT-4* mRNA were detected by PCR. *CPT-1* and *GLUT-4* protein expression were detected by Western blotting analysis. As shown in Figure 4c, adding AngII into cardiomyocytes, the decrease of *CPT-1* mRNA expression was not statistically significant compared with the control group. During the process of AngII-induced cardiac hypertrophy, the increase of *CPT-1* mRNA expression in 0.25 and 0.5 µg/ml SSYX group was not statistically significant compared with AngII group. Studies have shown that lipid metabolism was only

affected when the concentration of SSYX reached 1.0 µg/ml (0.7348 ± 0.0594 vs. 0.9880 ± 0.0851 , $t = 4.994$, $P = 0.0007$, $n = 5$), 0.25, and 0.5 µg/ml SSYX had less effect on lipid metabolism. Different from fatty acid metabolism, in the process of AngII-induced cardiomyocyte hypertrophy, the expression of *GLUT-4* mRNA was decreased and glucose metabolism was weakened. When 0.25 µg/ml SSYX was used to treat cardiac hypertrophy, the effect on glucose metabolism was small, and the increase of *GLUT-4* mRNA expression was not statistically significant compared with AngII group. Only when the concentration of SSYX reached 0.5 µg/ml, its effect on glucose metabolism would appear. At this time, the expression of *GLUT-4* mRNA in the 0.5 µg/ml SSYX group (1.5640 ± 0.0599 vs. 1.7720 ± 0.0660 , $t = 3.783$, $P = 0.0117$, $n = 5$) and 1.0 µg/ml SSYX group (1.5640 ± 0.0599 vs. 2.0490 ± 0.1280 , $t = 8.808$, $P < 0.0001$, $n = 5$) increased. SSYX increased mRNA

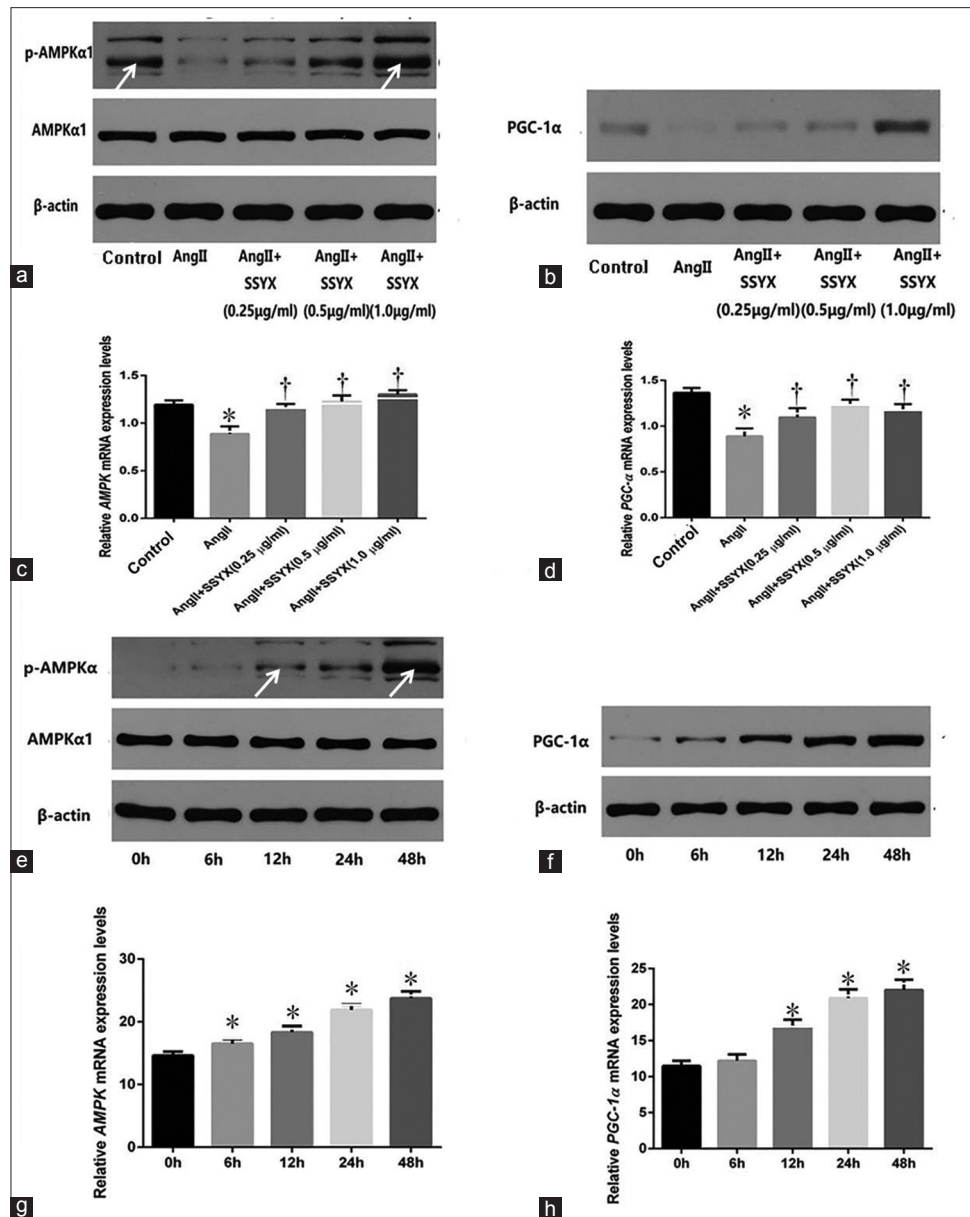


Figure 3: Expression of PGC-1 α and AMPK mRNAs and protein in AngII-induced cardiac hypertrophy. (a) Expression of p-AMPK and AMPK protein in the control, AngII, and SSYX (0.25, 0.5, and 1.0 $\mu\text{g/ml}$) groups. (b) Expression of PGC-1 α protein in each group. (c) Relative expression levels of AMPK mRNA in groups. (d) Relative expression levels of PGC-1 α mRNA in groups. (e) Expression of p-AMPK and AMPK protein treated with 0.5 $\mu\text{g/ml}$ SSYX for 0, 6, 12, 24, and 48 h. (f) Expression of PGC-1 α protein in groups. (g) Relative expression levels of AMPK mRNA in groups; (h) relative expression levels of PGC-1 α mRNA in groups. * $P < 0.0001$ versus control group; † $P < 0.05$ versus AngII group; * $P < 0.0001$ versus 0 h; the data are expressed as the mean \pm SD ($n = 5$, samples were collected from five different passages). The symbols “*,” “†” indicate that the value of PGC-1 α and AMPK mRNA expression significantly differed from those of different groups and different durations. p-AMPK: Activity AMP-activated protein kinase; AMPK: AMP-activated protein kinase; PGC-1 α : Peroxisome proliferator-activated receptor coactivator 1 alpha; mRNA: Messenger RNA; Ang: Angiotensin; SD: Standard deviation; SSYX: Shensong Yangxin Capsule.

and protein expression of CPT-1 and GLUT-4 with the increasing drug concentration. To explore time-dependent effect of SSYX on fatty acids and glucose oxidation, 0.5 $\mu\text{g/ml}$ SSYX was added into cells for 0, 6, 12, 24, and 48 h [Figure 4e–4h]. The results showed that the effect of 0.5 $\mu\text{g/ml}$ SSYX on the fatty acid metabolism of cardiomyocytes had no obvious regularity with time, only when the action time reached 24 h, and the expression of CPT-1 mRNA was statistically significant (15.1600 \pm 1.0960 vs. 18.5800 \pm 0.9049, $t = 6.048$, $P < 0.0001$, $n = 5$). The

effect of 0.5 $\mu\text{g/ml}$ SSYX on the glucose metabolism of cardiomyocytes became more and more significant with time, and the expression of GLUT-4 mRNA was measured at 0, 6 (10.2100 \pm 0.9485 vs. 12.9700 \pm 0.8221, $t = 4.763$, $P = 0.0012$, $n = 5$), 12 (10.2100 \pm 0.9485 vs. 16.9100 \pm 0.8481, $t = 11.590$, $P < 0.0001$, $n = 5$), 24 (10.2100 \pm 0.9485 vs. 19.0900 \pm 0.9797, $t = 15.360$, $P < 0.0001$, $n = 5$), and 48 (10.2100 \pm 0.9485 vs. 14.1900 \pm 0.9611, $t = 6.877$, $P < 0.0001$, $n = 5$) h. Compared with different hours (from 0 to 48 h), the effect was

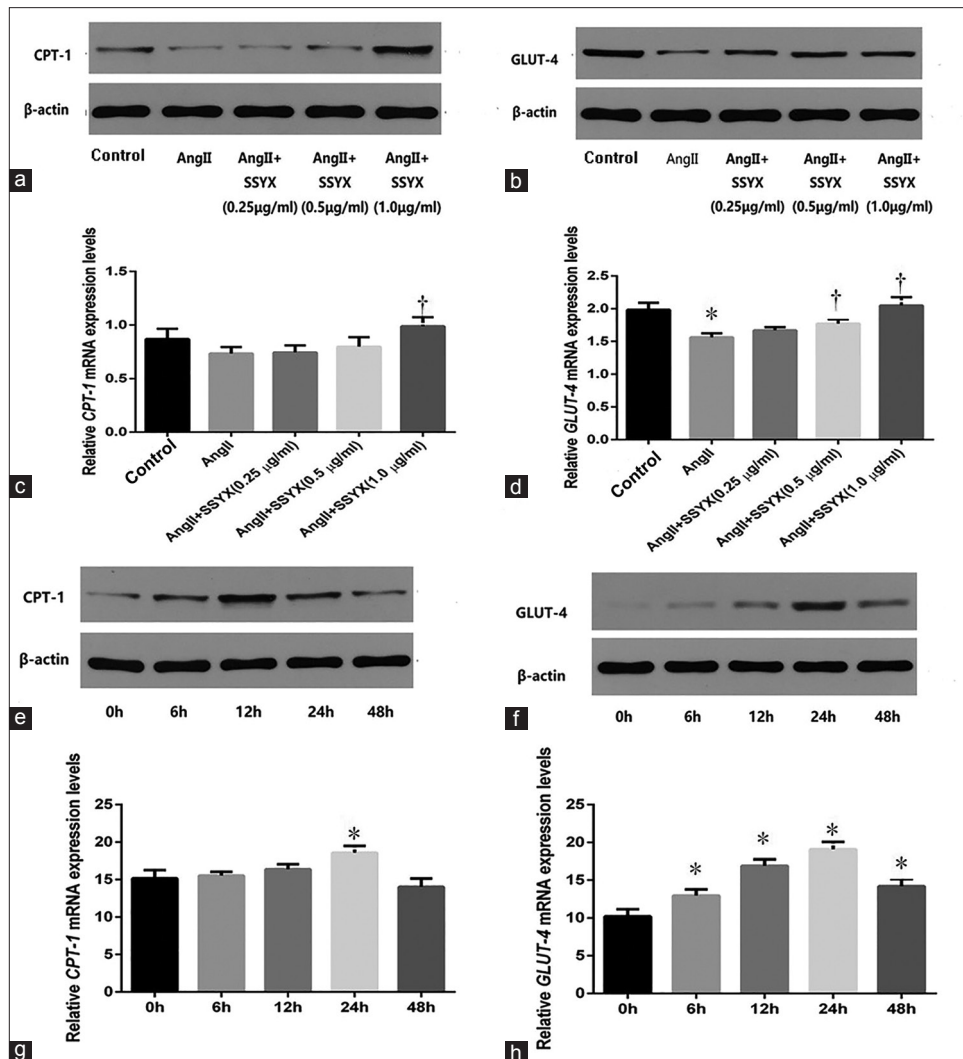


Figure 4: Expression of CPT-1 and GLUT-4 mRNAs and protein in AngII-induced cardiac hypertrophy. (a) Expression of CPT-1 protein in the control, AngII, and SSYX (0.25, 0.5, and 1.0 $\mu\text{g/ml}$) groups. (b) Expression of GLUT-4 protein in each group. (c) Relative expression levels of *CPT-1* mRNA in each group. (d) Relative expression levels of *GLUT-4* mRNA in groups. (e) Expression of CPT-1 protein treated with 0.5 $\mu\text{g/ml}$ SSYX for 0, 6, 12, 24, and 48 h. (f) Expression of GLUT-4 protein in each group. (g) Relative expression levels of *CPT-1* mRNA in each group. (h) Relative expression levels of *GLUT-4* mRNA in each group. * $P < 0.0001$ versus control group; † $P < 0.05$ versus AngII group; * $P < 0.05$ versus 0 h; the data are expressed as the mean \pm SD ($n = 5$, samples were collected from five different passages). The symbols “*,” “†” indicate that the value of *CPT-1* and *GLUT-4* mRNA expression significantly differed from different groups and durations. CPT-1: Carnitine acyltransferase enzyme-1; GLUT-4: Glucose transporter protein-4; mRNA: Messenger RNA; Ang: Angiotensin; SSYX: Shensong Yangxin Capsule; SD: Standard deviation.

becoming more and more remarkable, but when the action time reached 48 h, both *CPT-1* and *GLUT-4* mRNA and protein expression decreased, which suggested that glucose metabolism and fatty acid metabolism were both weakened at this time. In conclusion, SSYX can improve fatty acid metabolism and glucose metabolism in cardiac hypertrophy and it needs to reach the appropriate dose and time.

DISCUSSION

This study revealed that SSYX not only can reduce the cardiomyocyte hypertrophy and apoptosis induced by AngII but also can protect the mitochondrial density in hypertrophic cardiomyocytes in a dose-dependent manner (from 0.25 to 1.0 $\mu\text{g/ml}$). In addition, SSYX improved energy metabolism by activating the expression of related factors in

AngII-induced cardiac hypertrophy, such as mitochondrial biogenesis factor – *PGC-1 α* , energy balance factor – *AMPK*, fatty acid metabolism factor – *CPT-1*, and glucose metabolism factor – *GLUT-4*. Moreover, these effects need appropriate drug action time and drug concentration. We found that only when the concentration of SSYX reached 1.0 $\mu\text{g/ml}$, lipid metabolism was affected, and 0.25 and 0.5 $\mu\text{g/ml}$ SSYX had less effect on it. Unlike fatty acid metabolism, when the concentration of SSYX reached 0.5 $\mu\text{g/ml}$, the expression of *GLUT-4* mRNA was increased and its effect on glucose metabolism had appeared. SSYX not only increased mRNA and protein expression of *CPT-1* and *GLUT-4*, but the effect increased with the increasing drug concentration. In order to observe the best action time, we chose the same concentration of SSYX (0.5 $\mu\text{g/ml}$) for different time

(from 0 to 48 h); the effect on mitochondrial biogenesis and energy balance was becoming more and more remarkable. However, when the action time reached 48 h, both *CPT-1* and *GLUT-4* mRNA and protein expression decreased, which suggests that glucose and fatty acid metabolism were both weakened at this time. It was gratifying that our findings were not only consistent with our expectations but also consistent with previous experiments.

Mitochondrial biogenesis is regulated by a complex regulatory system. Peroxisome proliferator-activated receptor coactivator 1 alpha (*PGC-1α*) is a critical regulator that participates in mitochondrial biogenesis and oxidative metabolism in the heart.^[24,25] Some reports have shown that the mitochondrial function is increased by the activation of *PGC-1α*.^[26] AMP-activated protein kinase (*AMPK*) is a key modulator of lipid and glucose metabolism and energy balance. When the ATP levels are reduced, *AMPK* is rapidly activated and becomes involved in cell energy regulation.^[27] The current study confirmed that SSYX activated *AMPK* and upregulated the expression of *PGC-1α* compared with the control group. Myocardial remodeling can reduce the oxygen level in myocardial tissue and affects substrate oxidation, thus resulting in a reduction of the oxidative phosphorylation efficiency.^[2] In this experiment, SSYX increased the expression levels of carnitine acyltransferase enzyme-1 (*CPT-1*) and glucose transporter protein-4 (*GLUT-4*) in cardiac hypertrophy, which were key factors of fatty acid and glucose oxidation, respectively. These studies illustrated that SSYX promoted the oxidation of fatty acids and glucose and protected cardiac myocytes from energy deficiency.

However, the experiments still have many limitations. This study did not deeply investigate the specific mechanism SSYX regulated the mitochondria, glucose metabolism, and fatty acid metabolism. We did not conduct animal experiments to verify our results. Although SSYX at different drug concentrations was used in this study, due to the limited number of experimental groups, the optimal concentration of SSYX for regulating myocardial energy metabolism was not explored. In addition, SSYX itself is a compound preparation of TCM, so we did not study which specific component of SSYX regulates energy metabolism. These experiments will be the next stage of our research. We are fully aware that many issues still need to be resolved, but this study is still significant.

We proposed and demonstrated for the first time that SSYX regulated myocardial energy metabolism. SSYX not only reduced AngII-induced cardiac hypertrophy and cardiomyocyte apoptosis but also regulated the myocardial mitochondrial density and energy metabolism in cardiac hypertrophy. The mitochondrion is the main organelle of oxidative phosphorylation. Mitochondrial density is relatively reduced during cardiac hypertrophy, so oxidative phosphorylation of the myocardium produces insufficient energy to meet the needs of hypertrophic cardiomyocytes, which leads to myocardial dysfunction and aggravates

myocardial injury. We demonstrated that SSYX increased the myocardial mitochondrial density while cardiac hypertrophy, which implied that SSYX increased the capacity of the oxidative phosphorylation of cells during myocardial hypertrophy, improved myocardial function and slow or even reversed the progression of cardiac hypertrophy to heart failure. These results provided evidence of a new treatment for cardiac hypertrophy and heart failure.

In summary, SSYX can increase myocardial energy metabolism in AngII-induced cardiac hypertrophy. SSYX can be considered to be an alternative therapeutic remedy for myocardial hypertrophy.

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Conflicts of interest

There are no conflicts of interest.

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参松养心胶囊在血管紧张度 II 诱导的心肌肥大中对心肌细胞能量代谢的影响

摘要

背景: 作为中国的传统中药, 参松养心胶囊 (Shensong Yangxin Capsule, SSSYX) 可以用来治疗心律失常、心绞痛、心肌重构、心肌纤维化等等, 但是它对心脏能量代谢的影响还尚不明确, 本研究的目的主要是探究参松养心胶囊在血管紧张度 (Angiotensin) II 诱导的心肌肥大过程中对心肌细胞能量代谢的影响。

方法: 我们用 $2 \mu\text{l}$ (10^{-6}mol/L) Ang(Angiotensin) II 诱导 Sprague-Dawley (SD) 大鼠的乳鼠心肌细胞 (Neonatal rat cardiomyocytes, NRCMs) 48 小时, 用心肌细胞的 α -actinin 染色观察细胞体积大小, 用实时定量聚合酶链反应 (Real-time Polymerase chain Reaction, PCR) 检测心肌肥厚标记物 BNP (Brain natriuretic peptide) mRNA 表达以判断心肌细胞肥大模型构建是否成功。然后, 用 $1 \mu\text{l}$ 浓度梯度为 0.25, 0.5, and $1.0 \mu\text{g/ml}$ 的参松养心胶囊溶液处理细胞 24 小时。同时, 为了探究参松养心胶囊对心肌细胞能量代谢影响的时间效应, 我们用 $0.5 \mu\text{g/ml}$ 参松养心胶囊溶液分别作用细胞 0, 6, 12, 24 和 48 小时。用线粒体 Mito-Red Tracker 探针标记心肌细胞中的线粒体并用共聚焦显微镜观察线粒体密度, 用 PCR 检测线粒体生物起源相关因子- *PGC-1 α* (peroxisome proliferator-activated receptor coactivator-1 alpha), 能量平衡关键因子- *AMPK* (AMP-activated protein kinase), 脂肪酸代谢因子- *CPT-1* (carnitine acyltransferase enzyme-1), 葡萄糖氧化关键因子- *GLUT-4* (glucose transporter protein 4) 的 mRNA 表达。

结果: 随着参松养心胶囊溶液药物浓度的增加, SSSYX 处理组的线粒体密度逐渐增多 ($0.25 \mu\text{g/ml}$, 18.3300 ± 0.8895 vs. 24.4900 ± 0.9041 , $t = 10.240$, $P < 0.0001$; $0.5 \mu\text{g/ml}$, 18.3300 ± 0.8895 vs. 25.9800 ± 0.8187 , $t = 12.710$, $P < 0.0001$; $1.0 \mu\text{g/ml}$, 18.3300 ± 0.8895 vs. 24.2900 ± 1.3120 , $t = 9.902$, $P < 0.0001$; 每组 n 均等于 5); 而且, SSSYX 也可以促进 AngII 诱导心肌细胞肥大过程中能量代谢相关因子- *PGC-1 α* ($0.25 \mu\text{g/ml}$, 0.8892 ± 0.0848 vs. 1.0970 ± 0.0994 , $t = 4.319$, $P = 0.0013$; $0.5 \mu\text{g/ml}$, 0.8892 ± 0.0848 vs. 1.2330 ± 0.0564 , $t = 7.150$, $P < 0.0001$; $1.0 \mu\text{g/ml}$, 0.8892 ± 0.0848 vs. 1.1640 ± 0.0755 , $t = 5.720$, $P < 0.0001$; 每组 n 均等于 5), *AMPK* ($0.25 \mu\text{g/ml}$, 0.8872 ± 0.0779 vs. 1.1500 ± 0.0507 , $t = 7.239$, $P < 0.0001$; $0.5 \mu\text{g/ml}$, 0.8872 ± 0.0779 vs. 1.228 ± 0.0623 , $t = 9.379$, $P < 0.0001$; $1.0 \mu\text{g/ml}$, 0.8872 ± 0.0779 vs. 1.3020 ± 0.0450 , $t = 11.400$, $P < 0.0001$; n 均等于 5), *CPT-1* ($1.0 \mu\text{g/ml}$, 0.7348 ± 0.0594 vs. 0.9880 ± 0.0851 , $t = 4.994$, $P = 0.0007$, $n = 5$) 和 *GLUT-4* ($0.5 \mu\text{g/ml}$, 1.5640 ± 0.0599 vs. 1.7720 ± 0.0660 , $t = 3.783$, $P = 0.0117$; $1.0 \mu\text{g/ml}$, 1.5640 ± 0.0599 vs. 2.0490 ± 0.1280 , $t = 8.808$, $P < 0.0001$; 每组 n 均等于 5) 的 mRNA 和蛋白质表达, 并且随着 SSSYX 药物浓度的增加, 这种作用效果越发显著; 当用 $0.5 \mu\text{g/ml}$ 相同剂量的参松养心胶囊溶液分别处理细胞 0, 6, 12, 24 和 48 小时后, *AMPK* (6 h, 14.6100 ± 0.6205 vs. 16.5200 ± 0.7450 , $t = 3.456$, $P = 0.0250$; 12 h, 14.6100 ± 0.6205 vs. 18.3200 ± 0.9965 , $t = 6.720$, $P < 0.0001$; 24 h, 14.6100 ± 0.6205 vs. 21.8800 ± 0.8208 , $t = 13.160$, $P < 0.0001$; 48 h, 14.6100 ± 0.6205 vs. 23.7400 ± 1.0970 , $t = 16.530$, $P < 0.0001$; 每组 n 均等于 5), *PGC-1 α* (12 h, 11.4700 ± 0.7252 vs. 16.9000 ± 1.0150 , $t = 7.910$; 24 h, 11.4700 ± 0.7252 vs. 20.8800 ± 1.234 , $t = 13.710$, $P < 0.0001$; 48 h, 11.4700 ± 0.7252 vs. 22.0300 ± 1.4180 , $t = 15.390$, $P < 0.0001$; n 均等于 5), *CPT-1* (24 h, 15.1600 ± 1.0960 vs. 18.5800 ± 0.9049 , $t = 6.048$, $P < 0.0001$, $n = 5$), *GLUT-4* (6 h, 10.2100 ± 0.9485 vs. 12.9700 ± 0.8221 , $t = 4.763$, $P = 0.0012$; 12 h, 10.2100 ± 0.9485 vs. 16.9100 ± 0.8481 , $t = 11.590$, $P < 0.0001$; 24 h, 10.2100 ± 0.9485 vs. 19.0900 ± 0.9797 , $t = 15.360$, $P < 0.0001$; 48 h, 10.2100 ± 0.9485 vs. 14.1900 ± 0.9611 , $t = 6.877$, $P < 0.0001$; 每组 n 均等于 5) 的 mRNA 和蛋白质表达均随着时间的延长而逐渐增多。

结论: 参松养心胶囊可以促进血管紧张度 II 诱导的心肌肥大过程中心肌细胞的能量代谢, 这可能成为治疗心肌肥大的新的治疗方法。