

Suxiao Jiuxin Pill protects cardiomyocytes against mitochondrial injury and alters gene expression during ischemic injury

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Abstract. Suxiao Jiuxin Pill (SX), a traditional Chinese medicine compound consisting primarily of tetramethylpyrazine and borneol, has been reported to protect against ischemic heart disease. However, the effects of SX on mitochondrial injury and gene expression in various signaling pathways are unclear. The aim of the present study was to investigate the effects of SX on mitochondrial injury and to screen the expression of genes potentially altered by SX using a cell culture model of ischemic injury. Simulated ischemia was established by culturing HL-1 cardiomyocytes in Dulbecco's modified Eagle's medium without glucose or serum in a hypoxic chamber containing 95% N₂ and 5% CO₂ for 24 h. HL-1 cardiomyocytes were divided into 3 groups: Control, ischemic injury and ischemic injury + SX (100 µg/ml; n=3 wells/group). Mitochondrial membrane potential was detected by staining with JC-1 dye. The mRNA expression levels of adenylyl cyclase (Adcy) 1-9, adrenoceptor β₁, Akt1, ATPase Na⁺/K⁺ transporting subunit β₂, calcium voltage-gated channel auxiliary subunit α_{2δ} (Cacna2d2), Cacna2d3, calcium channel voltage-dependent γ subunit 8, cytochrome C oxidase subunit 6A2 (Cox6a2), fibroblast growth factor receptor (Fgfr) 4, Fgf8, Fgf12, Gnas complex locus, glycogen synthase kinase 3β (Gsk3b), mitogen-activated protein kinase (Mapk)11-14, Mapk kinase kinase 1 (Map4k1), Mas1, nitric oxide synthase 3 (Nos3), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (Pik3ca), phospholipase A2 group 4A, rap guanine nucleotide exchange factor 4 and ryanodine receptor 2 were detected using reverse transcription-quantitative polymerase chain reaction. The protein expression levels of phosphoinositide 3-kinase (PI3K), MAS-1 and phosphorylated-endothelial NOS were also examined by immunofluorescence staining. The

decrease in mitochondrial membrane potential in the cell culture model of ischemic injury (P<0.001) was significantly attenuated by SX treatment (P<0.001). Furthermore, increases in the mRNA expression levels of Adcy2 (P<0.05), 3 (P<0.01) and 8 (P<0.05) in the ischemic injury model were significantly attenuated by SX treatment (P<0.01), and SX treatment significantly decreased the mRNA expression levels of Adcy1 (P<0.01) and 6 (P<0.05) in ischemic cells. Decreases in the mRNA expression levels of Cox6a2 (P<0.001), Gsk3b (P<0.01) and Pik3ca (P<0.001) in the ischemic injury model were also significantly attenuated by SX treatment (P<0.05, P<0.01 and P<0.001, respectively). In addition, the decrease in the protein expression of PI3K (P<0.001) was significantly attenuated by SX treatment (P<0.001). The present findings indicate that SX may protect cardiomyocytes against mitochondrial injury and attenuate alterations in the gene expression of Adcy2, 3 and 8, Cox6a2, Gsk3b and Pik3ca during ischemic injury.

Introduction

Ischemic heart disease (IHD), also known as coronary artery disease, includes conditions such as stable angina, unstable angina, myocardial infarction and sudden cardiac arrest (1). IHD is the most common type of cardiovascular disease, and its mortality rate has recently been increasing (2). In 2010, IHD was the cause of 7 million mortalities worldwide; an increase of 35% since 1990 (3,4). Current management of IHD includes lifestyle changes, coronary interventions, coronary artery bypass grafting and medication, including cholesterol lowering agents, beta-blockers, nitroglycerin and calcium channel blockers (5).

Suxiao Jiuxin Pill (SX), a traditional Chinese medicine compound that primarily consists of tetramethylpyrazine and borneol, is commonly prescribed alongside standard management strategies for IHD in China (6). SX has been reported to protect against IHD in clinical and animal studies (7,8). Among patients with acute coronary syndrome undergoing early percutaneous coronary intervention, the incidence of perioperative myocardial infarction was significantly lower in those who received SX treatment when compared with a placebo group (6). In addition, SX improved hemodynamic and myocardial oxygen metabolism, and reduced the degree and scope of myocardial ischemia in a dog model of coronary artery occlusion (8). However, the molecular mechanisms underlying

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the protective effects of SX on IHD and cardiomyocytes are largely unknown.

Mitochondria are a major target in hypoxic injury (9). The organelles are the final regulators of cell survival and death due to their role in ATP generation and ability to trigger apoptosis and necrosis (10). However, the effects of SX on mitochondrial injury in hypoxic injury remain to be fully elucidated. Membrane-related structures, including receptors, G protein and enzymes, which are present in the plasma, mitochondrial and sarcoplasmic reticulum membranes, serve an important role in signal transduction in cardiomyocytes (11). However, it is currently unclear whether SX exerts effects on the proliferation of cardiomyocytes or nitric oxide (NO)-associated muscle relaxation during ischemic injury.

The present study used a cell culture model of ischemic injury to examine the effects of SX on mitochondrial membrane potential and the expression of genes associated with membrane structures and signals, namely adenylyl cyclase (*Adcy*), adrenoceptor β 1 (*Adrb1*), ATPase Na⁺/K⁺ transporting subunit β 2 (*Atp1b2*), calcium voltage-gated channel auxiliary subunit α 2 δ (*Cacna2d2*), *Cacna2d3*, calcium channel voltage-dependent γ subunit 8 (*Cacng8*), cytochrome C oxidase subunit 6A2 (*Cox6a2*), *Gnas*, phospholipase A2 group 4A (*Pla2g4a*) and ryanodine receptor 2 (*Ryr2*) (12-14). In addition, the expression of genes associated with cell proliferation, namely *Akt1*, fibroblast growth factor receptor (*Fgfr*)4, 8 and 12, glycogen synthase kinase 3 β (*Gsk3b*), mitogen-activated protein kinase (*Mapk*)11-14, *Mapk* kinase kinase 1 (*Map4k1*), *Mas1* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*Pik3ca*) was evaluated (15-17). Furthermore, genes associated with NO-associated muscle relaxation, including nitric oxide synthase 3 (*Nos3*) and rap guanine nucleotide exchange factor 4 (*Rapgef4*), were investigated (18,19). These methods aimed to determine the effects of SX on mitochondrial injury and gene expression in a cell model of ischemic injury.

Materials and methods

Cells, reagents and equipment. A mitochondria membrane potential kit (Beyotime Institute of Biotechnology, Shanghai, China); Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); fetal bovine serum (FBS; Bio-west, Inc., North Logan, UT, USA); anti-MAS1 (ab197992), anti-phosphoinositide 3-kinase (PI3K; ab40776) and anti-phosphorylated (p)-endothelial(e)NOS primary antibodies (ab95254; all from Abcam, Cambridge, MA, USA); primers (Invitrogen; Thermo Fisher Scientific, Inc.); RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA); TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.); chloroform; isopropanol; 75% anhydrous ethanol (all from Sinopharm Chemical Reagent, Inc., Shanghai, China); diethyl pyrocarbonate H₂O (used for RNA extraction); SuperScript III Reverse Transcriptase; SYBR Green I (all from Invitrogen; Thermo Fisher Scientific, Inc.); RNase inhibitor (Fermentas; Thermo Fisher Scientific, Inc.); oligo dT/primer; Platinum Taq DNA Polymerase; and 100 mM dNTPs (all Invitrogen; Thermo Fisher Scientific, Inc.) were used in the present study.

Equipment used in the present study included a cell incubator (Thermo Fisher Scientific, Inc.); light and

fluorescence microscopes (Olympus Corporation; Tokyo, Japan); a table-type refrigerated centrifuge (Zhengzhou Nanbei Instrument Equipment Inc., Zhengzhou, China); and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

HL-1 murine cardiomyocytes (Novobio Scientific, Inc., Shanghai, China) were cultured in DMEM supplemented with 10% FBS, 0.1 mM norepinephrine, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 mg/ml amphotericin B at 37°C in a cell incubator containing 5% CO₂. SX (40 mg/tablet; Tianjin Zhongxin Pharmaceutical Inc., Tianjin, China) was principally composed of *Chuan Xiong* (*Rhizoma Chuanxiong*) and *Borneolum Syntheticum*. SX was ground into a powder and dissolved in phosphate buffered saline (PBS) to a final concentration of 100 μ g/ml.

Experimental design and cell culture model of ischemic injury. Simulated ischemia was established by culturing HL-1 cardiomyocytes (1x10⁴ cells/ml) in DMEM without glucose or serum in a hypoxic chamber containing 95% N₂ and 5% CO₂ for 24 h at 37°C. HL-1 cardiomyocytes were divided into 3 groups: Control, ischemic injury, and ischemic injury + SX (n=3 wells/group). To determine the optimal SX dosage, SX (10, 50, 100, 200 and 400 μ g/ml, respectively) was added to the cell culture medium for 24 h at 37°C immediately before hypoxia. Cell morphology was observed using light microscopy. Among all the dosages, 100 μ g/ml SX induced optimal growth (fewest dead cells per microscopic field) of the HL-1 cardiomyocytes, indicating that the protective effects of SX against ischemic injury were greatest at this dosage (Fig. 1). Therefore, 100 μ g/ml of SX was used in the following experiments. Cells in the control group were cultured in DMEM supplemented with 10 mM glucose and 10% FBS in a normoxic chamber containing 5% CO₂ for 24 h at 37°C.

Detection of mitochondrial membrane potential. HL-1 cardiomyocytes in the 3 groups were cultured in 24-well plates, and treated as described above. A mitochondrial membrane potential kit was utilized to detect mitochondrial membrane potential. Cells were washed with DMEM medium once, and 0.25 ml fresh DMEM medium supplemented with 10 mM glucose and 10% FBS was added into each well. JC-1 dye (0.25 ml; from mitochondria membrane potential kit) was added into each well and mixed thoroughly. Cells were subsequently incubated in an incubator at 37°C for 20 min. At the end of incubation, culture supernatant was discarded and cells were washed with PBS (3 times, 30 sec/wash). Cell culture medium (0.5 ml) was added, and cells were observed using a fluorescence microscope. Integrated optical density (IOD) was calculated by multiplying the area (size) and average density of fluorescence (20). Samples were evaluated using Image-Pro Plus 7 software (Media Cybernetics Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs from HL-1 cardiomyocytes in the control, ischemic injury, and ischemic injury + SX groups were extracted and purified using TRIzol, following the manufacturer's instructions. RNase inhibitor was utilized in order to reduce the degradation of RNA. A universal cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized for

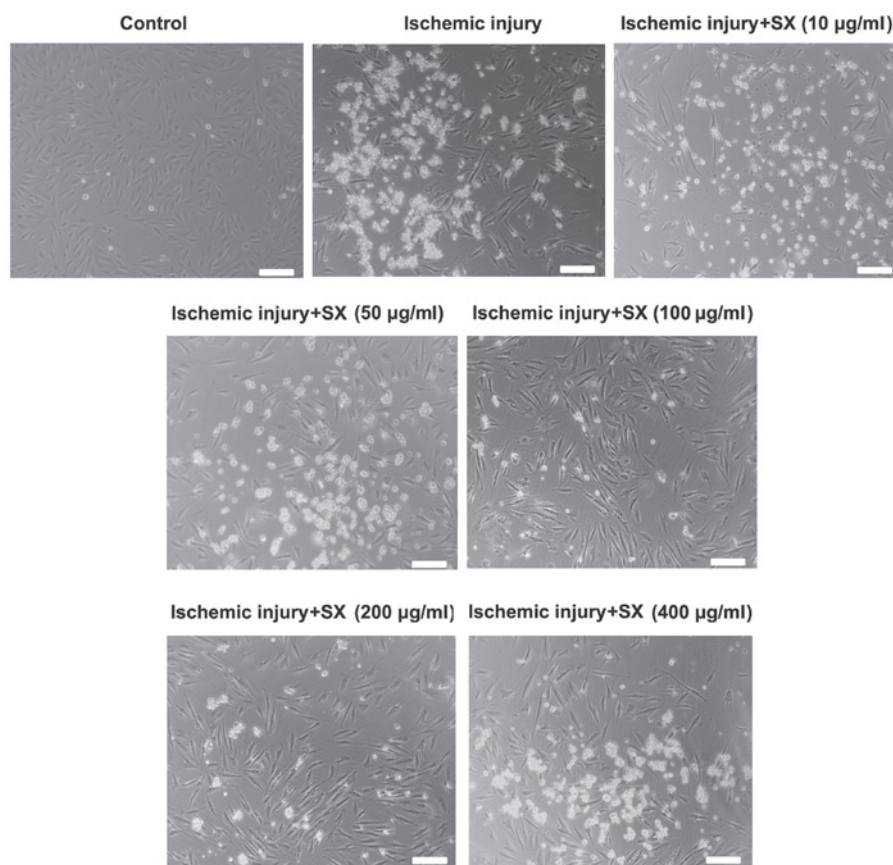


Figure 1. Determination of the optimal dosage of SX. HL-1 cardiomyocytes were divided into 3 groups: Control, ischemic injury, and ischemic injury + SX. Induced ischemia was established by culturing HL-1 cardiomyocytes in glucose- and serum-free Dulbecco's modified Eagle's medium in a hypoxic chamber with 95% N₂ and 5% CO₂ for 24 h. Cells in the control group were cultured in Dulbecco's modified Eagle's medium containing glucose and serum in a normoxic chamber with 5% CO₂ for 24 h. To determine the optimal SX dosage, SX (10, 50, 100, 200 and 400 µg/ml, respectively) was added to cell culture medium at the start of hypoxia. Cell morphology was observed using light microscopy. The growth of HL-1 cardiomyocytes was optimal following treatment with 100 µg/ml SX, which indicated that 100 µg/ml SX exerted the greatest protective effects against ischemic injury. Therefore, 100 µg/ml SX was used for subsequent experiments. Magnification, x100; scale bar=100 µm. SX, Suxiao Jiuxin Pill.

reverse transcription according to the manufacturer's instructions. Each reaction contained 0.5 µl of oligo dT/primers (0.2 µg/µl) and 1 µl of SuperScript III reverse transcriptase (200 U/µl). The specific primers used for qPCR for Adcy1, Adcy2, Adcy3, Adcy4, Adcy5, Adcy6, Adcy7, Adcy8, Adcy9, Adrb1, Akt1, Atplb2, Cacna2d2, Cacna2d3, Cacng8, Cox6a2, Fgfr4, Fgf8, Fgf12, Gnas, Gsk3b, Mapk11, Mapk12, Mapk13, Mapk14, Map4k1, Mas1, Nos3, Pik3ca, Pla2g4a, Rapgef4, Ryr2 and β-actin genes are listed in Table I. qPCR was performed by utilizing mRNA qPCR Detection kit. SYBR Green I, Platinum Taq DNA Polymerase and 100 mM dNTPs were utilized in the experiment. The following PCR conditions were used: Pre-denaturing at 95°C for 2 min; denaturing at 95°C for 10 sec; and annealing and polymerization at 60°C for 30 sec and 70°C for 45 sec. A total of 40 PCR cycles were performed. PCR was conducted using a CFX96 Touch™ Real-Time PCR Detection System. Gene expression was determined as the relative optical density ratio between target gene and β-actin. The $\Delta\Delta C_q$ method was used to quantify gene expression (21).

Immunofluorescence. HL-1 cardiomyocytes in the 3 groups were treated as described above and cultured in 24-well plates for 24 h at 37°C with cover slips. Cells adhered to the cover slips were fixed in 4% paraformaldehyde (room temperature,

10 min), blocked with bovine serum albumin (Biowest USA, Riverside, MO, USA) at room temperature for 30 min, and incubated with primary antibodies against MAS1, p-eNOS, and PI3K (all antibodies: 1:500) at 4°C overnight. Following overnight incubation, cover slips were washed with PBS and incubated in the dark with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:1,000; Abcam; ab6717) at room temperature for 1 h. Cover slides were subsequently washed with PBS (3 times, 30 sec/wash) and stained with 4',6-diamidino-2-phenylindole for 5 min at room temperature. Slides were prepared with Mounting Media and Antifades (Invitrogen; Thermo Fisher Scientific, Inc.) and observed using a fluorescence microscope. The IOD, calculated by multiplying the area (size) and average density of fluorescence, was evaluated using Image-Pro Plus 7 software (20).

Statistical analysis. Experimental results were presented as the mean + standard error of the mean. One-way analysis of variance was used to compare differences among the 3 groups, followed by a Bonferroni post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Primers	Direction	Sequences, 5'-3'
Adcy1	F	GTCGGATGGATAGCACTGGG
	R	TAGCATCTCTCCCTTGCCCT
Adcy2	F	GGAGTGCATTCTGGGAACG
	R	AGAGATGCTGAGGACTCCGT
Adcy3	F	AAAAGGCAGCGCCATGAGA
	R	TGATGTCCAACACCAGACCG
Adcy4	F	TACCTGGCCCGAGAGATGAA
	R	AGCACACTCACTCCTTGGTG
Adcy5	F	CCATCACCTGGTGTTCCTC
	R	TTGAAGGCCGACTCCATCAC
Adcy6	F	GGAGCAGTGCATTGAGACCT
	R	TAGAGTCCTTGGTCCGGGAG
Adcy7	F	CACAGTCAATGTTGCCAGCC
	R	CCCCTTTGCCTTTGACGTTG
Adcy8	F	CCTGGGGGACTGCTACTACT
	R	CTCCCAGGGATTCCTCCAGA
Adcy9	F	TCTCAGGTGAGGTCCAGGAG
	R	GTTCTTGGGACTGGAGGTGG
Adrb1	F	CTACAACGACCCCAAGTGCT
	R	CTCGCAGCTGTCGATCTTCT
Akt1	F	ATAACGGACTTCGGGCTGTG
	R	CCTGGTTGTAGAAGGGCAGG
Atp1b2	F	TTGAGGAGTGGAAAGGAGTTCGTG
	R	CGTGAGGAAACCATAGAAGACGA
Cacna2d2	F	TGCTTTTACCGCTGCTCACC
	R	TCAAACAGGTTCCGATTGTCCT
Cacna2d3	F	CCGCTTTTGGTGGGGAGAT
	R	TTGGAGACCGTCGATTTCTTCA
Cacng8	F	CCAACCTCACAGCAGGTGAT
	R	CAACCCTTCCAGGCAGCATA
Cox6a2	F	CGCCTCCTGACCTTTGTGCT
	R	CGGATGCGGAGGTGGTGATA
Fgfr4	F	GGAGCAGCAAGAGCAGGTGTT
	R	CAGGAAGGAAGCTGGCGATCT
Fgf8	F	CTCATCCGGACCTACCAGCTCT
	R	GGGTCTCCGTCTTCTGCCAT
Fgf12	F	GGCGATAGCCAGCTCCTTGA
	R	TTCAGCTGGGGTTCTGGTCTC
Gnas	F	TCTGTGGGAGGATGAGGGAG
	R	TGGTCACTTGGCACGTAGTC
Gsk3b	F	CCGAGGAGAGCCCAATGTTT
	R	CTGATCCACACCACTGTCCC
Mapk11	F	AAACAGTATGGGAGGTGCCG
	R	AGCTCACGGTATGTCCTCCT
Mapk12	F	GTGGTAACCCGGTGGTATCG
	R	AGGGAGGCCTTCCATGTAGT
Mapk13	F	GACAAGATGCTGGAGCTGGA
	R	GTTTCCATTCGTCCACGCTG
Mapk14	F	AACAGGATGCCAAGCCATGA
	R	GGGTCTGTGTAAGTACTGAGCAA
Map4k1	F	GACCAGTGCAGGTGATCCAA
	R	CAGAGCAGACGGAAGGTGAG

Table I. Continued.

Primers	Direction	Sequences, 5'-3'
Mas1	F	TCTGCTGACAGCCATCAGTG
	R	ACCAAGAAGCTGAGGATGGC
Nos3	F	CTGTATGGCTCTGAGACTGGC
	R	GCAGGAGACACTGTTGAATCGGA
Pik3ca	F	GCAGGGTTTTCTGTCCCCTC
	R	CCAGATGTTCTCCATGATTCCGA
Pla2g4a	F	GTTTCAGCATGGCACTGTGTG
	R	GAACCCAAAATGGCCACCAC
Rapgef4	F	ATAAAAAGGCCGTTGGAGCGA
	R	GACTCTCCAAAGGCTGTCCC
Ryr2	F	ATCTGCACCTTTGTGCTGGAGC
	R	TCCATAGAGGAGTGTCCGGTGG
β-actin	F	AGAAAATCTGGCACCCACACC
	R	AGAGGGTACAGGGATAGCA

F, forward; R, reverse; Adcy, adenylyl cyclase; Adrb1, adrenoceptor β1; Atp1b2, ATPase Na⁺/K⁺ transporting subunit β2; Cacna2d, calcium voltage-gated channel auxiliary subunit α2δ; Cacng8, calcium channel voltage-dependent γ subunit 8; Cox6a2, cytochrome C oxidase subunit 6A2; Pla2g4a, phospholipase A2 group 4A; Ryr2, ryanodine receptor 2; Fgfr, fibroblast growth factor receptor; Gsk3b, glycogen synthase kinase 3β; Mapk, mitogen-activated protein kinase; Map4k1, mitogen-activated protein kinase kinase kinase 1; Pik3ca, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α; Nos3, nitric oxide synthase 3; Rapgef4, rap guanine nucleotide exchange factor 4.

Results

SX treatment attenuates decreases in mitochondrial membrane potential induced by ischemic injury. HL-1 cardiomyocytes in the control, ischemic injury and ischemic injury + SX groups were stained with JC-1 dye and observed under a fluorescence microscope. The area and average density of fluorescence, as indicated by IOD, were evaluated. A red signal indicated a positive (polarized) mitochondrial membrane potential. Results indicated that the mitochondrial membrane potential decreased significantly in the ischemic injury group when compared with the control group ($P < 0.001$; Fig. 2). In turn, treatment with SX significantly reversed the decrease in mitochondrial membrane potential in ischemic HL-1 cardiomyocytes ($P < 0.001$; Fig. 2).

SX treatment attenuates ischemic injury-induced elevations in Adcy2, 3 and 8 mRNA, and decreases the levels of Adcy1 and 6 mRNA. mRNA expression in HL-1 cardiomyocytes of the control, ischemic injury and ischemic injury + SX groups was measured by RT-qPCR. Following ischemic injury, significantly increased levels of Adcy2 ($P < 0.05$), 3 ($P < 0.01$) and 8 ($P < 0.05$) mRNA were observed in HL-1 cardiomyocytes (Fig. 3A-C); however, the levels of Adcy1 and 8 mRNA did not differ significantly when compared with the control group (Fig. D-E). Treatment with SX significantly attenuated the elevated levels of Adcy2, 3 and 8 mRNA in ischemic cardiomyocytes ($P < 0.01$),

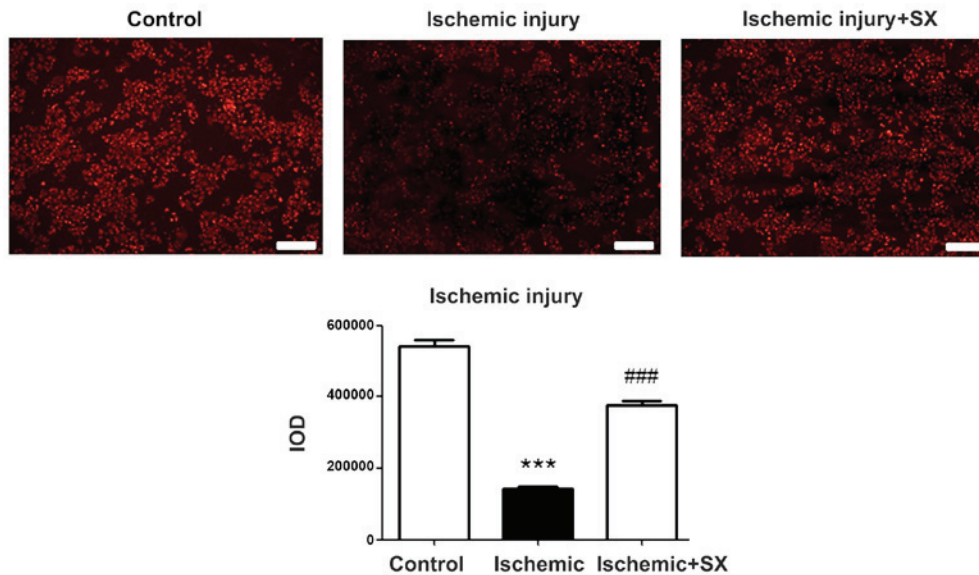


Figure 2. SX treatment attenuated the decrease in mitochondrial membrane potential induced by ischemic injury. HL-1 cardiomyocytes in 3 experimental groups were stained with JC-1 dye (red) and observed under a fluorescence microscope (magnification, x100). Data were presented as the mean + standard error of the mean (n=3 wells/group). Red signal indicated positive (polarized) mitochondrial membrane potential. The mitochondrial membrane potential decreased significantly in the ischemic injury group when compared with the control group, and SX treatment significantly attenuated the decrease in mitochondrial membrane potential in ischemic HL-1 cardiomyocytes. ***P<0.001 vs. control group; ###P<0.001 vs. ischemic injury group. Scale bars=100 μ m. SX, Suxiao Jiuxin Pill; IOD, integrated optical density.

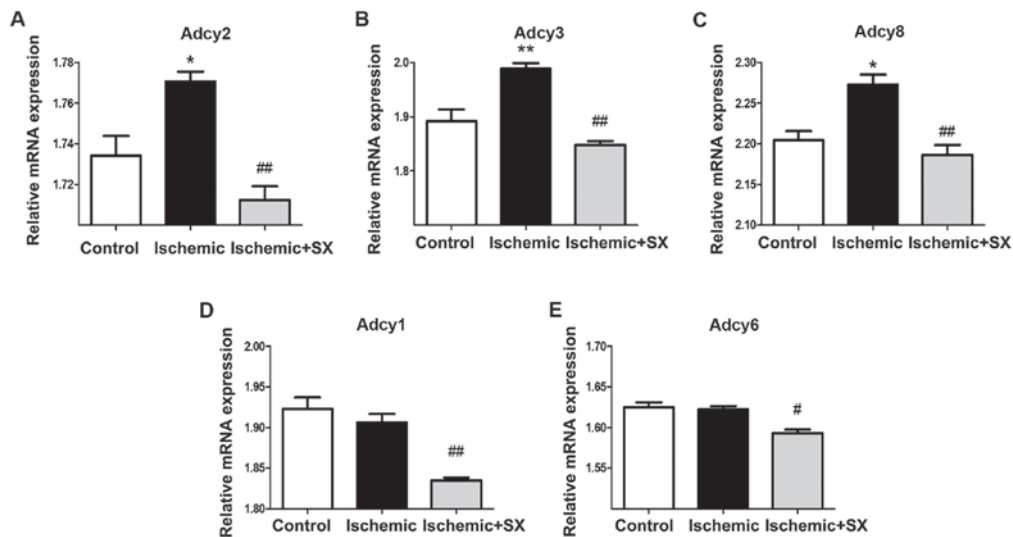


Figure 3. SX treatment attenuated the increase in levels of Adcy2, 3 and 8 mRNA, and decreased the levels of Adcy1 and 6 mRNA during ischemic injury. mRNA expression in the 3 experimental groups was detected by reverse transcription-quantitative polymerase chain reaction. The levels of (A) Adcy2, (B) 3 and (C) 8 mRNA in HL-1 cardiomyocytes were significantly increased following ischemic injury, while the levels of (D) Adcy1 and (E) 6 mRNA did not differ significantly following ischemia. SX treatment attenuated the increases in Adcy2, 3 and 8 mRNA, and significantly decreased Adcy1 and 6 mRNA in the cell culture model of ischemic injury. Data were represented as the mean + standard error of the mean (n=3 wells/group). *P<0.05 and **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. ischemic injury group. SX, Suxiao Jiuxin Pill.

and also decreased the levels of Adcy1 (P<0.01) and 6 (P<0.05) mRNA in ischemic cells (Fig. 3).

SX treatment attenuates ischemic injury-induced reductions in Cox6a2, Gsk3b and Pik3ca mRNA. Significantly decreased levels of Cox6a2 (P<0.001), Pik3ca (P<0.001) and Gsk3b (P<0.01) mRNA were observed in the ischemic injury group when compared with the control group. In turn, treatment with SX significantly reversed the decreases in Cox6a2 (P<0.05), Pik3ca (P<0.001) and Gsk3b (P<0.01) mRNA (Fig. 4).

SX treatment does not alter the levels of Rapgef4, Mapk11, Mapk13, Mapk14 and Fgf8 mRNA in the ischemic injury model. The levels of Rapgef4, Mapk11, Mapk13, Mapk14 and Fgf8 mRNA were analyzed in the 3 groups. Significantly increased levels of Rapgef4 (P<0.05), Mapk11 (P<0.05), Mapk13 (P<0.01) and Mapk14 (P<0.01), and significantly decreased levels of Fgf8 mRNA (P<0.05) were observed in the ischemic injury group when compared with the control group (Fig. 5). However, treatment with SX had no significant effect on the expression of these genes in ischemic

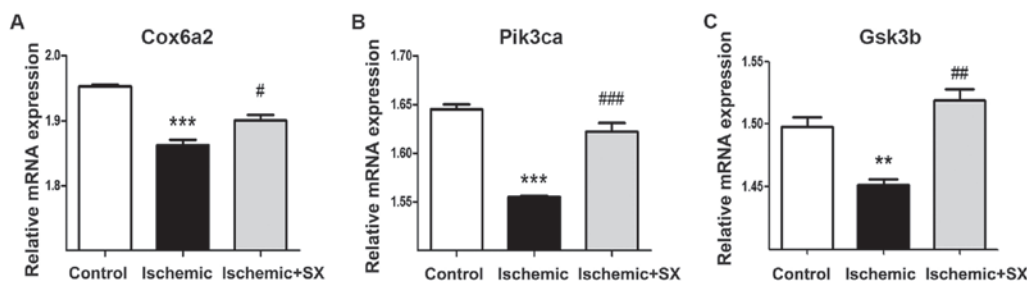


Figure 4. SX treatment attenuated the decrease in Cox6a2, Gsk3b and Pik3ca mRNA in the ischemic injury model. The levels of (A) Cox6a2, (B) Pik3ca and (C) Gsk3b mRNA were significantly decreased in the ischemic injury group when compared with the control group. The decreases in Cox6a2, Gsk3b and Pik3ca mRNA were significantly attenuated by SX treatment. Data were represented as the mean + standard error of the mean (n=3 wells/group). **P<0.01 and ***P<0.001 vs. the control group; #P<0.05, ##P<0.01 and ###P<0.001 vs. the ischemic injury group. SX, Suxiao Jiuxin Pill.

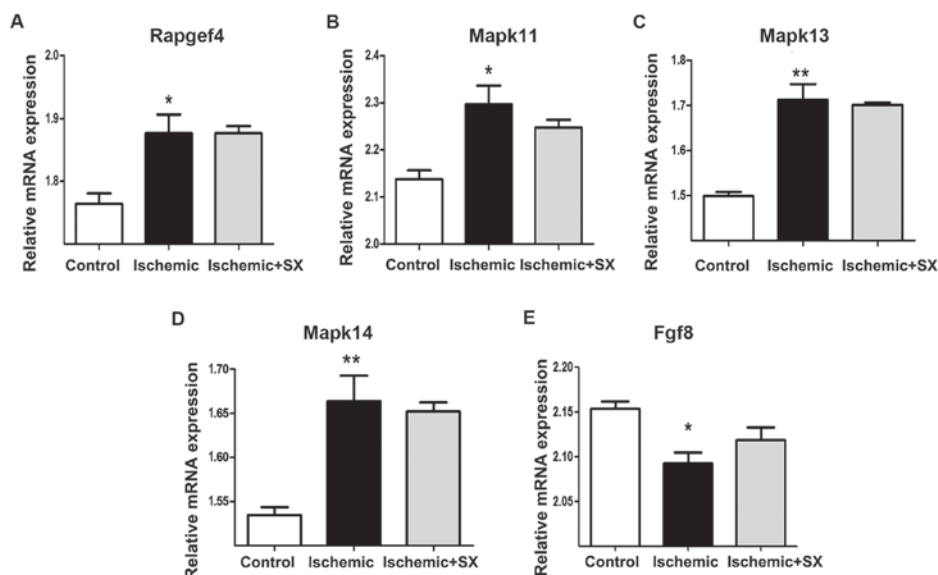


Figure 5. Levels of Rapgef4, Mapk11, Mapk13, Mapk14 and Fgf8 mRNA were not altered by SX treatment in the ischemic injury model. The levels of (A) Rapgef4, (B) Mapk11, (C) Mapk13 and (D) Mapk14 mRNA were significantly increased, and the level of (E) Fgf8 mRNA was significantly decreased in the ischemic injury group when compared with the control group. SX treatment had no significant effect on the expression of these genes in ischemic cardiomyocytes. Data were represented as the mean ± standard error of the mean (n=3 wells/group). *P<0.05 and **P<0.01 vs. the control group. SX, Suxiao Jiuxin Pill.

cardiomyocytes (Fig. 5). Therefore, the statistical differences among 3 groups calculated by ANOVA were caused by differences between ischemic injury and control groups, or differences between ischemic injury + SX and control groups. In addition, no statistical differences were observed in the mRNA expression levels of Adcy4, Adcy5, Adcy7, Adcy9, Adrb1, Akt1, Atp1b2, Cacna2d2, Cacna2d3, Cacng8, Fgfr4, Fgf12, Gnas, Mapk12, Map4k1, Mas1, Nos3, Pla2g4a and Ryr2 among the 3 experimental groups (data not shown).

SX treatment attenuates ischemic injury-induced reductions in PI3K, MAS1, and p-eNOS protein expression. The levels of PI3K, MAS1 and p-eNOS protein expression in the 3 experimental groups were examined by immunofluorescence staining. The area and average density of fluorescence, as indicated by IOD, were evaluated. Target proteins were stained green. The protein expression of PI3K was significantly decreased in the ischemic injury group when compared with the control group (P<0.001; Fig. 6), and treatment with SX significantly restored the expression of PI3K to normal levels (P<0.001; Fig. 6). The levels of MAS1

and p-eNOS did not differ significantly among the 3 groups (Fig. 7).

Discussion

The present study demonstrated that SX protected cardiomyocytes against mitochondrial injury and attenuated alterations in the expressions of Adcy2, 3 and 8, Cox6a2, Gsk3b and Pik3ca during ischemic injury.

IHD is a significant cause of morbidity and mortality (22). Although therapeutic interventions, including thrombolysis and percutaneous coronary intervention, have proven efficacious in the treatment of ischemic injury, the underlying pathological process of IHD suggests that further protection may be possible (23). The traditional Chinese medicine SX has been implicated as a protective agent against cardiovascular diseases, including IHD and non-IHD diseases (7), and has been reported to inhibit vasoconstriction of human arteries through endothelium-dependent and -independent vasorelaxation (24). Additionally, SX has been indicated to lower blood lipid levels, inhibit the development of atherosclerosis and

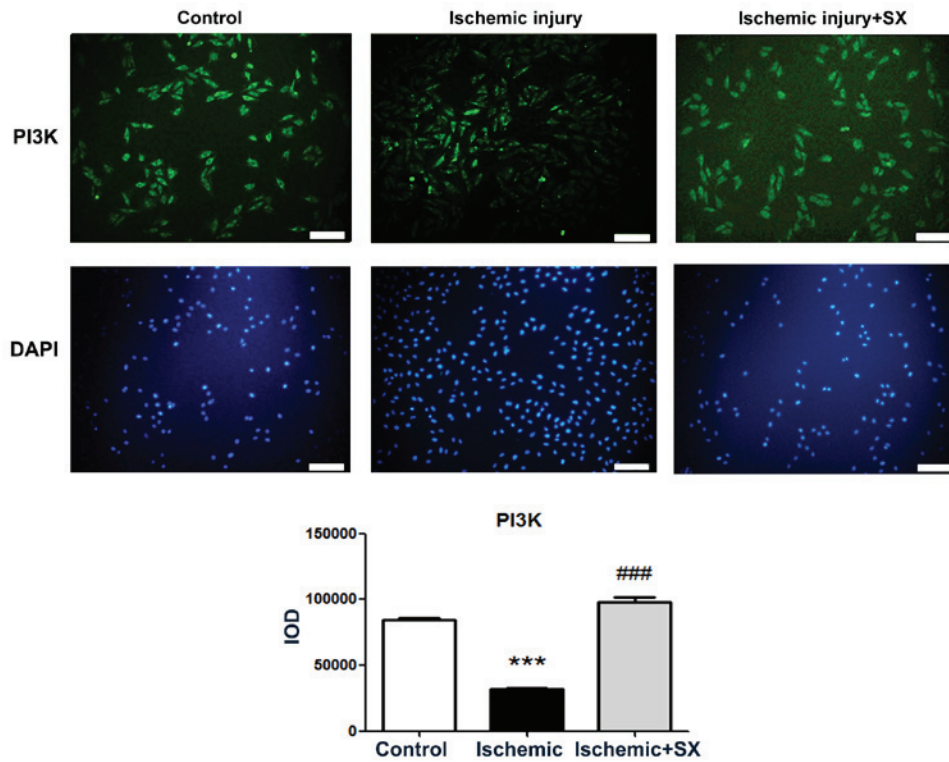


Figure 6. Reduced protein expression of PI3K was attenuated by SX treatment in the ischemic injury model. The levels of PI3K protein expression in the 3 experimental groups were examined by immunofluorescence staining (magnification, x100). PI3K protein was stained with fluorescein isothiocyanate (green) and cells were counterstained with 4',6-diamidino-2-phenylindole (blue). Data were represented as the mean + standard error of the mean (n=3 wells/group). The expression levels of PI3K were significantly decreased in the ischemic injury group compared with the control group, whereas SX treatment significantly restored the expression of PI3K to normal levels during ischemic injury. ***P<0.001 vs. the control group and ###P<0.001 vs. the ischemic injury group. Scale bars=100 μ m. SX, Suxiao Jiuxin Pill; IOD, integrated optical density.

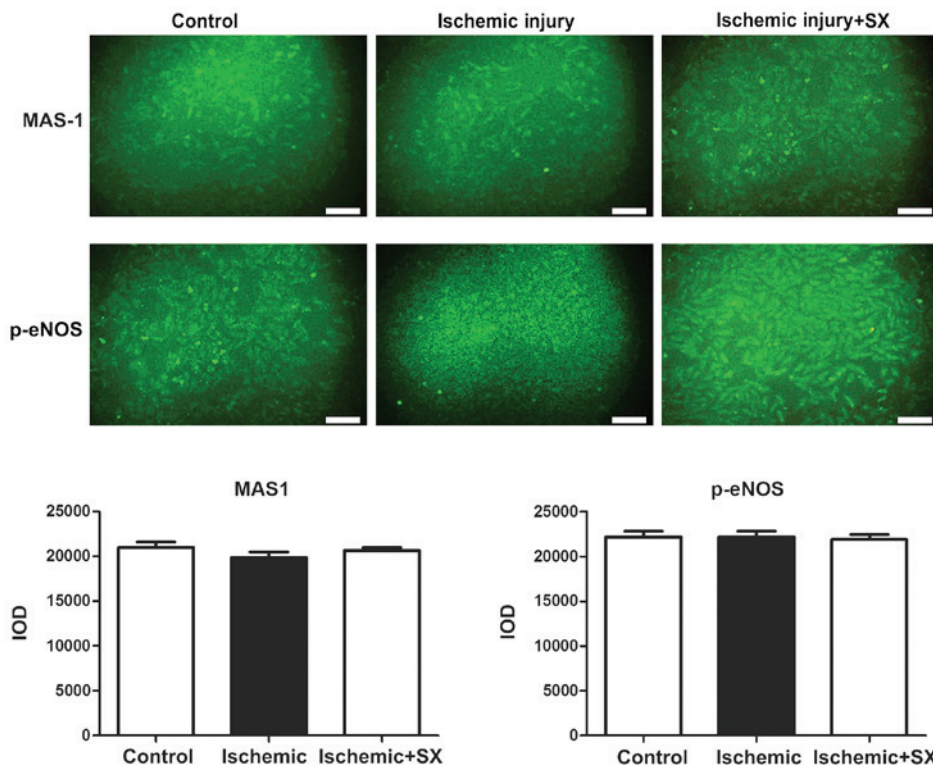


Figure 7. Protein expression of MAS1 and p-eNOS was similar in the 3 experimental groups. The protein expression levels of MAS-1 and p-eNOS in the 3 experimental groups were examined by immunofluorescence staining. The target proteins were stained with fluorescein isothiocyanate (green). No statistical differences in the expression levels of MAS1 and p-eNOS among the 3 groups were identified. Scale bar=100 μ m. Data were represented as the mean \pm standard error of the mean (n=3 wells/group). SX, Suxiao Jiuxin Pill; IOD, integrated optical density; eNOS, endothelial nitric oxide synthase; p-, phosphorylated.

suppress inflammatory responses and oxidative stress in rat models of atherosclerosis (25,26).

SX contains tetramethylpyrazine and borneol as its primary components (7). Pretreatment with tetramethylpyrazine has been reported to significantly increase coronary arterial diameter and suppress vasoconstriction mediated by endothelin-1 in rabbits (27). In addition, injection of tetramethylpyrazine induced a significant decrease in plasma endothelin-1 levels (27). A previous study demonstrated that the direct positive inotropic effect of tetramethylpyrazine in rat cardiac myocytes may be associated with production of NO (28). Furthermore, pretreatment with tetramethylpyrazine suppressed vasopressin-induced depression of the S-wave level in a rat model of angina, which was not observed following pretreatment with vehicle alone (saline) (29). This suggested that tetramethylpyrazine ameliorated vasopressin-induced subendocardial ischemia *in vivo*. However, studies into the effects of borneol on ischemic heart disease are limited, and further research efforts are required to identify the properties of borneol in ischemic injuries.

SX may alter gene or protein expression levels in blood vessels in the absence of ischemic injuries (6). Pretreatment of human internal mammary arteries *ex vivo* with plasma concentrations of SX (1 mg/ml) significantly depressed maximal contraction to KCl and U46619, and when 10 mg/ml SX was applied, the subsequent contraction was abolished (6). However, it is unclear whether SX alone alters gene and protein expression in cardiomyocytes in the absence of ischemic injuries, and further studies are required to investigate this.

Mitochondria in the heart serve two roles that are essential for cell survival: ATP synthesis and maintenance of Ca²⁺ homeostasis (30). Although mitochondria do not regulate cytosolic Ca²⁺ under aerobic physiologic conditions (31), mitochondrial Ca²⁺ overload alters intracellular Ca²⁺ homeostasis and causes an overload cycle that result in irreversible cell damage during IHD (31). Activated soluble guanylate cyclase has been demonstrated to protect against post-ischemic mitochondrial inflammation in mice by reducing phosphorylated cyclophilin D and the formation of mitochondrial permeability transition pores (32). In addition, fatty acid nitroalkenes have been reported to induce resistance to ischemic cardiac injury in rat heart mitochondria, by suppressing superoxide formation and reversibly inhibiting complex II-linked respiration in a pH-dependent manner (33). Furthermore, the mitochondrial K_{ATP} channel serves a critical role in cardioprotection during cardiac ischemia (34). In the present study, it was observed that SX treatment protected against damage to the mitochondrial membrane potential in an ischemic injury model. Further research is now required to fully elucidate the mechanisms underlying this protection, which may involve mitochondrial permeability transition pores, mitochondrial complexes, the K_{ATP} channel and reactive oxygen species.

In the present study, the increased levels of Adcy2, 3 and 8 mRNA in the ischemic injury model were significantly attenuated by SX treatment, and the levels of Adcy1 and 6 mRNA were significantly decreased following SX treatment. Adcy proteins (encoded by the Adcy gene) catalyze the conversion of ATP to cyclic adenosine monophosphate (cAMP) (35). Adcys are activated or inhibited by guanine nucleotide binding proteins (G proteins), which are coupled to membrane receptors that respond to hormonal stimulations, among others (35).

cAMP, as a second messenger, serves as a regulatory signal through its interactions with cAMP-binding proteins, including protein kinase A, ion channels, transcription factors and enzymes (36,37).

ADCY2 protein, encoded by the Adcy2 gene, accelerates phospho-acidification and the synthesis and breakdown of glycogen (35). Abnormal ADCY2 protein has been implicated in chronic obstructive pulmonary disease and bipolar disorder (38,39). Upregulation of the ADCY3 protein (encoded by the Adcy3 gene) has been indicated to increase the tumorigenic potential of cells by activating the cAMP response element binding protein pathway (40). The calmodulin-stimulated ADCY8 protein (encoded by the Adcy8 gene) has been reported to regulate the sensitivity of zebrafish retinal axons to midline repellents, and is required for normal midline crossing (41). Furthermore, ADCY8 is associated with mouse avoidance behavior and human mood disorder (42). Mutations in ADCY1 (encoded by the Adcy1 gene) have been reported to cause recessive hearing impairment in humans and defects in hair cell function and hearing in zebrafish (43), and mutations in contactin associated protein 1 and ADCY6 (encoded by the Adcy6 gene) may be responsible for severe arthrogyriposis multiplex congenita with axoglial defects (44). To the best of our knowledge, the present study demonstrated for the first time that the mRNA expressions of Adcy2, 3 and 8 were significantly increased during ischemic injury, which was significantly attenuated by SX treatment. In addition, the expressions of Adcy1 and 6 were significantly decreased following SX treatment. These findings suggest potential molecular mechanisms involved in ischemic injury and indicate the possible pharmaceutical targets of SX treatment.

The present study also demonstrated that the significant decreases in Gsk3b and Pik3ca mRNA in ischemic injury were significantly attenuated by SX treatment. In Fig. 6, although the cell number appeared larger in ischemic injury group, IOD of PI3K (encoded by the Pik3ca gene) was lower in ischemic injury group when compared with control and SX treatment groups. Alterations in the protein expression of PI3K following SX treatment were also investigated by immunofluorescence. COX6A2 (encoded by the Cox6a2 gene) is the last enzyme in the respiratory electron transport chain of the mitochondrial membrane (45). GSK3β (encoded by the Gsk3b gene) phosphorylates glycogen synthase and is active in a number of intracellular signaling pathways, including cell proliferation, migration and glucose regulation (46). The PI3K protein (encoded by the Pik3ca gene) is involved in cell differentiation, motility, survival and intracellular trafficking (47). The significant decrease in Cox6a2 mRNA in cardiomyocytes indicated that the mitochondrial membrane was damaged during ischemic injury. Furthermore, the decrease in Gsk3b mRNA indicated that the energy storage and proliferative capacities of cell were impeded during ischemic injury. In addition, the significant decrease in Pik3ca mRNA suggested that cell differentiation and survival was impaired during ischemic injury. The present study demonstrated that SX treatment significantly alleviated the decreases in Cox6a2, Gsk3b and Pik3ca mRNA during ischemic injury. These findings may in part explain the protective effects of SX in the treatment of ischemic cardiac injury.

Treatment with SX had no apparent effect on the expression of genes associated with membrane structures and signals, with the exceptions of *Adcy2*, 3 and 8 and *Cox6a2*. *ADRβ1* (encoded by the *Adrb1* gene) is a G-protein-coupled receptor expressed predominantly in cardiac tissue (48). G proteins (encoded by the *Gnas* gene) are important signal transducing molecules in cells (49). Na^+/K^+ -ATPase (encoded by the *Atp1b2* gene) maintain resting potential and effective transport, and regulate cellular volume (50). Furthermore, *CACNA2δ* channels (encoded by the *Cacna2d2*, *Cacna2d3* and *Cacng8* genes) are present in the membrane of excitable cells, including that of cardiac muscle (51). Cardiac RYR2 (encoded by the *Ryr2* gene) mediates the sarcoplasmic release of stored calcium ions during cardiac calcium-induced calcium release (52). *PLA2* (encoded by the *Pla2g4a* gene) hydrolyzes phospholipids into arachidonic acid and mediates inflammation (53). In the present study, SX treatment exhibited no significant effect on the aforementioned structural proteins.

Treatment with SX also had no apparent effect on the expression of genes associated with cell proliferation, with the exceptions of *Gsk3b* and *Pik3ca*. The serine/threonine kinase AKT (encoded by the *Akt1* gene) phosphorylates and inactivates components involved in apoptosis (54). FGFRs (encoded by the *Fgfr4*, 8 and 12 genes) regulate key biological processes, including cell proliferation and differentiation during development and tissue repair (55). MAPK (encoded by the *Mapk11*, 12, 13 and 14 genes) and MAP4K1 (encoded by the *Map4k1* gene) regulate cell functions, including proliferation, mitosis, cell survival and apoptosis (56). *MAS1* (encoded by the *Mas1* gene) modulates the growth-regulating pathway when activated (57). In the present study, SX treatment exhibited no significant effect on the aforementioned proteins involved in cell proliferation.

Treatment with SX did not alter the expression of genes associated with vasodilation and muscle relaxation in the present study. The eNOS protein (encoded by the *Nos3* gene) is responsible for the generation of NO in the vascular endothelium (58), and *RAPGEF4* (encoded by the *Rapgef4* gene) is associated with glucagon-like peptide-stimulated secretion of atrial natriuretic peptide in the heart (59). In the present study, the aforementioned proteins were not altered significantly by SX treatment.

In conclusion, SX protected cardiomyocytes against mitochondrial injury, and attenuated alterations in the expressions of *Adcy2*, 3 and 8, *Cox6a2*, *Gsk3b* and *Pik3ca* during ischemic injury.

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