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Original Article

Anti-apoptotic effect of Suxiao Jiuxin Pills against hypoxia-induced injury through PI3K/Akt/GSK3β pathway in HL-1 cardiomyocytes

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Abstract

Background: Suxiao Jiuxin Pill (SX), Chinese traditional medicine primarily consisting of tetramethylpyrazine and borneol, has been shown to protect against ischemic heart diseases. Nevertheless, the involved mechanism still remains unclear. The following study aimed to investigate the potential protective effect and molecular mechanisms of SX on apoptosis in HL-1 cardiomyocytes.

Methods: Simulated hypoxia was established by culturing HL-1 cardiomyocytes in DMEM with no glucose or serum in a hypoxic chamber with 95% N_2 and 5% CO_2 for 24 h. HL-1 cardiomyocytes were divided into 5 groups: control, hypoxic injury, hypoxic injury + insulin (PI3K agonist, 10 μM), hypoxic injury + SX (100 μg/mL), and hypoxic injury + SX + LY294002 (PI3K inhibitor, 10 μM) (n = 3 wells/group). The anti-apoptotic effect of SX was evaluated by Annexin V/PI analysis. Mitochondrial membrane potential ($\Delta\Psi m$) was detected by JC-1 assay. The protein expression of PI3K, phosphorylated PI3K (p-PI3K), Akt, phosphorylated Akt (p-Akt), GSK3β and phosphorylated GSK3β (p-GSK3β) were detected by western blot.

Results: SX exhibited anti-apoptotic effect in HL-1 cardiomyocytes; nonetheless, the effect was blocked by PI3K inhibitor LY294002. Also, the anti-apoptotic effect of SX was mediated by increased mitochondrial membrane potential ($\Delta\Psi m$). Furthermore, p-PI3K, p-Akt, and p-GSK3β expressions were significantly increased after SX treatment, while they were all reduced after administration of LY294002.

Conclusion: SX protects HL-1 cardiomyocytes from apoptosis induced by hypoxia, partly through enhancing the phosphorylation of PI3K/Akt/GSK3β signaling pathway.

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Keywords: Apoptosis; HL-1 cardiomyocytes; Hypoxia-induced injury; Mitochondrial membrane potential; PI3K/Akt/GSK3β pathway; Suxiao Jiuxin Pill

1. Introduction

Ischemic heart disease (IHD) is the leading cause of death and disability worldwide.[1,2] Ischemic injury of the heart is associated with activation of multiple signal cascades that initiate intracellular ionic and chemical changes, thus causing cardiomyocyte death.[3,4] Growing evidence reveals that apoptosis is one of the major mechanisms which leads to cell death after heart ischemia injury.[5,6] Apoptosis is an active process which requires the activation of associated genes. The phosphoinositide 3-kinases (PI3K) is a conserved family of signal transduction enzymes essential for regulating cell survival. Akt, a serine/threoine kinase, is the central downstream effector molecule of PI3K.[7,8] Activated Akt phosphorylates several downstream targets, including glycogen synthase kinase (GSK3β) which is phosphorylated by Akt at serine 9.[9,10]

Suxiao Jiuxin Pill (SX), Chinese traditional medicine compound that mainly consists of tetramethylpyrazine and borneol, is commonly prescribed in China for treatment of

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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IHD. Among patients with acute coronary syndrome (ACS) who underwent early percutaneous coronary intervention, the incidence of perioperative myocardial infarction was significantly lower in patients who received SX treatment compared to the placebo group.[11] In addition, SX improved hemodynamic and myocardial oxygen metabolism, and reduced the degree and scope of myocardial ischemia in coronary artery occlusion dog model.[12] Although SX has been shown to protect against IHD in both clinical and preclinical testing, its action mechanism remains unclear.

It is important to explore the action mechanism of SX as a novel therapeutic approach for IHD. Our previous study has indicated that SX protects cardiomyocytes against mitochondrial injury and decreases the alterations in the Gsk3b and Pik3ca gene expression during ischemic injury.[13] Therefore, we hypothesized that SX might inhibit cell apoptosis caused by hypoxia in HL-1 cardiomyocytes by activating PI3K/Akt/GSK3β signaling pathway.

2. Methods

2.1. Cell culture

HL-1 mouse cardiomyocytes cell line (Novobio Inc., Shanghai, China) was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM norepinephrine, 2 mM glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 mg/mL amphotericin B in a humidified atmosphere containing 5%CO_2/95% air at 37 °C.

2.2. Chemicals

SX (40 mg/tablet; Tianjin Zhongxin Pharmaceutical Inc., Tianjin, China) was principally composed of Chuan Xiong (Rhizoma Chuanxiong) and Bing Pian (Borneolum). SX was grinded into powder, and dissolved in phosphate buffered saline (PBS) to make final concentration of 100 μg/mL. All the other reagents were purchased as follows: Insulin (St. Louis, Shanghai, China); LY294002 (St. Louis, Shanghai, China); mitochondrion membrane potential kit (Beyotime Inc., Shanghai, China); all antibodies (Abcam Inc., Shanghai, China).

2.3. Experimental design and ischemic injury cell model

Simulated hypoxia was established by culturing HL-1 cardiomyocytes in DMEM with no glucose or serum in a hypoxic chamber with 95% N_2 and 5% CO_2 for 24 h. HL-1 cardiomyocytes were divided into 5 groups: control, hypoxic injury, hypoxic injury + insulin (PI3K agonist, 10 μM), hypoxic injury + SX (100 μg/mL), and hypoxic injury + SX + LY294002 (PI3K inhibitor, 10 μM) ($n = 3$ wells/group). Insulin (10 μM), SX (100 μg/mL) and LY294002 (10 μM) were added into cell culture medium at the beginning of hypoxia. Cells in control group were cultured with DMEM containing glucose and serum in a normoxic chamber with 5% CO_2 for 24 h.

2.4. Cell proliferation assays

Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, Shanghai, China). Briefly, 100 μl of the cell number was seeded in a 96-well plate and placed at 37 °C in a 5% CO_2 saturated humidity incubator. 10 μL CCK-8 solution was added into each well and incubated for 2 h. Absorbance was measured with a Thermo Scientifc Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm to assess the number of viable cells

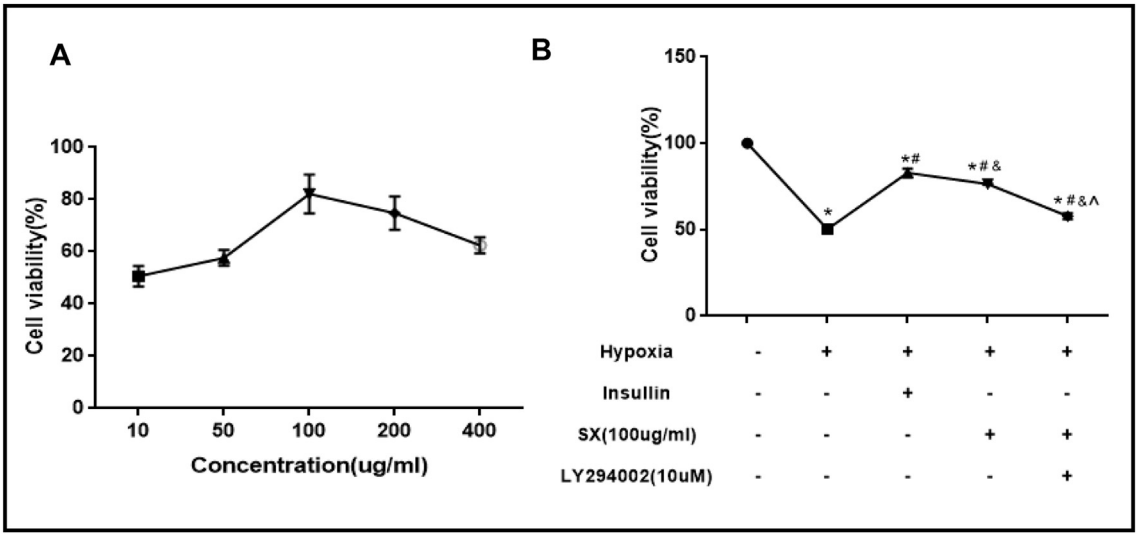

Fig. 1. Effect of SX on HL-1 cardiomyocytes proliferation. (A) Determination of the optimal dosage of SX by CKK-8 assay. (B) Myocardial cells survival rate in each group. Data are mean ± SEM (n = 3). Magnification, ×100; scale bar = 100 μm * $p < 0.05$ compared to hypoxic group; # $p < 0.05$ compared to hypoxia injury group; & $p < 0.05$ compared to hypoxic injury + insulin group; ^ $p < 0.05$ compared to hypoxic injury + SX group (one-way ANOVA). SX, Suxiao Jiuxin Pill.

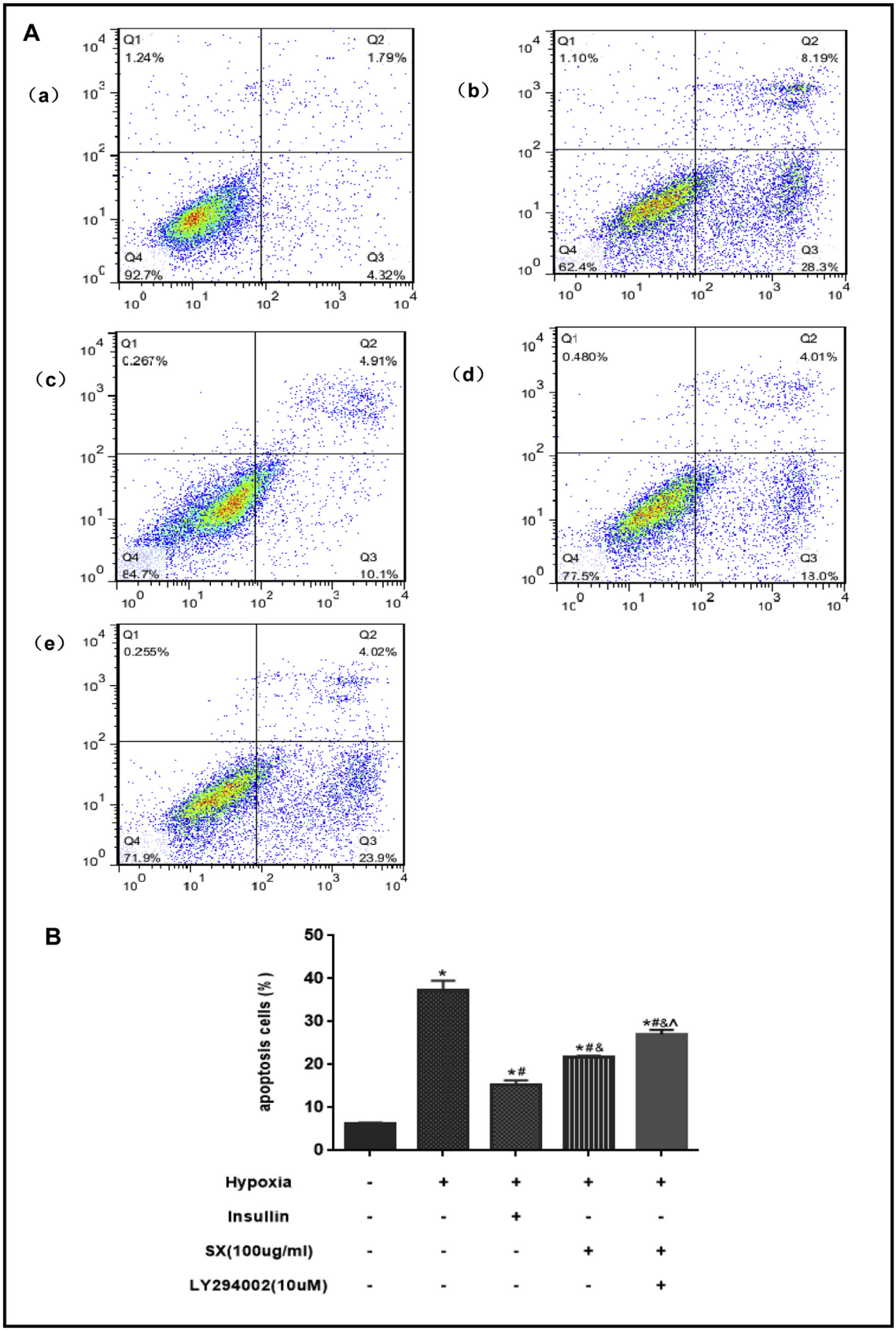

Fig. 2. Effect of SX on HL-1 cardiomyocytes apoptosis. (A) Annexin V/PI assay results. (a) control group; (b) hypoxic injury group; (c) hypoxic injury + Insulin group; (d) hypoxic injury + SX group; (e) hypoxic injury + SX + LY294002 group. Dead cells were labeled with Annexin V (−) propidium iodide (PI) (+) and are shown in the Q1 area; late apoptotic cells were labeled with Annexin V (+) PI (+) and are shown in the Q2 area; early apoptotic cells were labeled with

(background absorbance was measured at 630 nm). Results were obtained from at least three independent experiments. The cell viability ratio was calculated by the following formula:

$$\text{Cell viability ratio (\%)} = (\text{OD}_{\text{treated}}/\text{OD}_{\text{control}}) \times 100\%$$

2.5. Flow cytometric analysis of cell apoptosis

The following 5 groups mentioned above (see "Experimental design and ischemic injury cell Model" section) were under investigation. Apoptotic rates were examined 48 h post-treatment. In accordance with the Annexin V/propidium iodide (PI) apoptosis kit (BioVision, San Francisco, CA, USA), 5×10^5 cells were collected in each tube and 1 mL Annexin V binding buffer was added followed by thorough mixing. Subsequently, 5 μL Annexin V- fluorescein isothiocyanate and 10 μL PI were added. After mixing, the tube was incubated in the dark at 37 °C for 15 min. For the early apoptotic cells, membrane phosphatidylserine was exposed and combined with Annexin V, without PI. For the late apoptotic cells, the membranes were permeable to PI and the cells were stained with Annexin V and PI. The dead cells were stained only with PI. The samples were analyzed using a FACScan flow cytometer (BD Biosciences, USA) within 1 h. Flow cytometry (BD FACSAria; BD Biosciences, Franklin Lakes, NJ, USA) was performed to detect cell apoptosis.

2.6. JC-1 stain for mitochondrial membrane potential ($\Delta\Psi m$)

5 groups of HL-1 cardiomyocytes were cultured in 24-well plates, and treated as mentioned above. Cells were washed with medium once, and 0.25 mL fresh medium was then added into each well. Consequently, JC-1 dye (0.25 mL) was added to each well, and mixed. Cells were then incubated at 37 °C for 20 min. After incubation, culture supernatant was discarded, and cells were washed. Cell were then resuspended with fresh medium (0.5 mL), and cells were observed under fluorescence microscope. Integrated optical density (IOD), calculated by multiplying area and average density of the fluorescence, were evaluated by Image-Pro Plus 7 software (Media Cybernetics Inc., Rockville, MD, USA).

2.7. Western blot assays

The primary antibodies to PI3K, phospho-PI3K, Akt, phospho-Akt (Ser473), GSK3β and phospho-GSK3β (Ser9) and horseradish peroxidase-labeled second antibodies of anti-mouse IgG or anti-rabbit IgG (1:5000 dilution, ProteinTech Group, shanghai, China) were used. β-actin was used as a loading control. Signals were quantified by scanning densitometry and computer-assisted image analysis.

2.8. Statistical analysis

Experimental results were demonstrated as mean ± SEM. One-way analysis of variance (ANOVA) was utilized to compare differences among 3 groups, followed by Bonferroni post hoc testing for multiple comparisons. p value < 0.05 was considered statistically significant. Figures and statistical analysis were made utilizing GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Effect of SX on HL-1 cardiomyocytes proliferation

To determine the optimal SX dosage, SX (10, 50, 100, 200 and 400 μg/mL, respectively) was added to cell culture medium when hypoxia started. Cell morphology was observed using light microscopy. Optimal cell growth was detected following treatment with 100 μg/mL of SX, which indicated that 100 μg/mL SX exerted the greatest protective effects against hypoxia injury. Therefore, 100 μg/mL SX was used for subsequent experiments (Fig. 1A).

Myocardial cells survival rate in each group was detected by CKK-8 assay (Fig. 1B). Serious cell damage and significantly reduced survival rate was observed in hypoxic injury group (50.29 ± 1.05) compared to control group ($p < 0.05$); while the survival rates in hypoxic injury + insulin group (80.99 ± 1.41) and hypoxic injury + SX group (76.38 ± 0.16) were significantly higher compared to hypoxic injury group ($p < 0.05$). Significant difference between in hypoxic injury + insulin groups and hypoxic injury + SX groups were observed ($p < 0.05$). Moreover, cells survival rate in hypoxic injury + SX + LY294002 group (57.81 ± 1.00) was significantly lower compared to SX group ($p < 0.05$).

3.2. Effect of SX on HL-1 cardiomyocytes apoptosis

Flow cytometer and Annexin V/PI were used determined cells apoptotic level (Fig. 2). Significantly higher apoptosis was observed in hypoxic injury group (37.35% ± 1.25) compared to control group (6.14% ± 0.23%) ($p < 0.05$), hypoxic injury + insulin group (15.16% ± 0.67%) ($p < 0.05$) and hypoxic injury + SX group (21.71% ± 0.22%) ($p < 0.05$) (Fig. 2b). Significant difference between in hypoxic injury + insulin groups

Annexin V (+) PI (−) and are shown in the Q3 area; live cells were labeled with Annexin V (−) PI (−) and are shown in the Q4 area. (B) The percentage of cells labeled as Annexin V (+) PI (−), Annexin V (+) PI (+) and Annexin V (−) PI (+) was investigated. Summarized data for TMRE fluorescence intensity measured with confocal microscopy. Data are mean ± SEM for 3 independent experiments performed in duplicate. * $p < 0.05$ compared to hypoxic group; # $p < 0.05$ compared to hypoxia injury group; & $p < 0.05$ compared to hypoxic injury + insulin group; ^ $p < 0.05$ compared to hypoxic injury + SX group (one-way ANOVA). SX, Suxiao Jiuxin Pill.

and hypoxic injury + SX groups were observed ($p < 0.05$) (Fig. 2c–d). Furthermore, the apoptotic fraction of hypoxic injury + SX + LY294002 group (27.03% ± 0.63%) increased compared to SX group ($p < 0.05$) (Fig. 2e).

3.3. Effect of SX on mitochondrial membrane potential ($\Delta\Psi m$) in HL-1 cardiomyocytes

Differently treated HL-1 cardiomyocytes groups were stained with JC-1 dye and then analyzed under fluorescence microscope. Area and average density of the fluorescence, as indicated by IOD, were evaluated using Image-Pro Plus 7 software. Red signal indicated good mitochondrial membrane potential. Mitochondrial membrane potential significantly decreased in the hypoxic injury group compared to control group ($p < 0.05$, Fig. 3b). Furthermore, insulin and SX treatment dramatically increased (normalized) the mitochondrial membrane potential in HL-1 cardiomyocytes compared to hypoxic injury group ($p < 0.05$) (Fig. 3c–d), while treating cells with PI3K inhibitor LY294002 decreased hypoxic injury + SX groups' potential (Fig. 3e).

3.4. Effect of SX on t-PI3K, p-PI3K, t-Akt, p-Akt, t-GSK3β and p-GSK3β expression

The PI3K/Akt pathway is essential for cell survival. GSK3β is one of the downstream targets of Akt, which mediates cell apoptosis. In this study, t-PI3K, p-PI3K, t-Akt, p-Akt, t-GSK3β and p-GSK3β expression were analyzed by western blot. The expression of t-PI3K, t-Akt and t-GSK3β protein were not changed by SX treatment, but the phosphorylation level of PI3K, Akt and GSK3β were significantly altered. Thus, data from p-PI3K/t-PI3K, p-Akt/t-Akt and p-GSK3β/t-GSK3β were further analyzed.

The level of p-PI3K/t-PI3K, p-Akt/t-Akt and p-GSK3β/t-GSK3β decreased significantly in the analyzed cell model compared to control group ($p < 0.05$ Fig. 4A–C). In addition, compared to hypoxic injury group, SX treatment dramatically adversed the decrease in HL-1 cardiomyocytes ($p < 0.05$ Fig. 4A–C). To further examine whether SX produces anti-apoptotic effect by activating PI3K/Akt/GSK3β signaling pathway, the specific PI3K inhibitor LY294002 was added in hypoxic injury + SX group. Nevertheless, the PI3K inhibitor LY294002 abolished the beneficial effect of SX in p-Akt/t-Akt and p-GSK3β/t-GSK3β level ($p < 0.05$ Fig. 4B–C). Moreover, significant difference between hypoxic injury + SX + LY294002 groups and hypoxic injury groups in p-Akt/t-Akt and p-GSK3β/t-GSK3β levels were found ($p < 0.05$ Fig. 4B–C).

4. Discussion

Apoptosis is known as programmed cell death that is different from traditional necrosis. There have been numerous studies documenting the role of apoptosis in myocardial ischemic injury[14–16] reporting how increase in apoptosis augments MI, while reduced apoptosis protects the heart.[17] A recent study reported that brief ischemia could produce delayed cardiac troponin I (cTnI) release which is associated with irreversible myocyte injury from focal apoptosis.[18] Thus, exploring anti-apoptotic agents is a novel therapeutic opportunity for IHD. SX have been used in clinic for treatment of cardiovascular diseases in China for decades. However, there are few documents about the anti-apoptotic effect of SX on myocardial ischemia.

In the present study, we found that with the SX at concentration of 100 μg/mL, the protective effect of myocardial cells was optimal. Also, we demonstrated that SX protected cardiomyocytes against apoptosis induced by hypoxia *in vitro* model. A variety of apoptotic stimuli can induce the decrease in mitochondrial transmembrane potential. Moreover, they can stimulate release of cytochrome C from mitochondria to cytoplasm, thus starting apoptosis cascade reaction mediated by caspase, and leading to apoptosis quick completion.[19] Mitochondria play a pivotal role in the process of cell apoptosis and necrosis.[20] The opening of mitochondrial permeability transition pore (mPTP) is a key determinant of myocardial cell survival. Decrease in $\Delta\Psi m$ is related to the opening of mPTP, while $\Delta\Psi m$ decline or disappearance is an early event of myocardial cell apoptosis.[21]

Based on the above results, we suggested that by inhibiting the loss of $\Delta\Psi m$, SX prevented against apoptosis of myocardial cells, and consequently protected the ischemic heart from injury.

PI3K/Akt/GSK3β signal pathway is the most important signal transduction pathway in myocardial ischemic pretreatment mechanism. Mocanu et al.[22] have thought that the protective effect of ischemic pretreatment on myocardium is mainly due to the activation of PI3K/Akt signal pathway. Meanwhile, Chen et al.[23] have proved that Panax notoginseng could protect myocardial cells from apoptosis induced by ischemia through activating PI3K/Akt signaling pathway. GSK3β plays an important role in pathological conditions of myocardial ischemic injury, and Akt may regulate the activity of GSK3β during ischemia. Ischemia alone significantly reduces S9 phosphorylation of GSK3β,[24] which is consistent with our study. Zhai et al.[24] have suggested that inhibition of GSK3β (inactivation of GSK3β through phosphorylation) exacerbates ischemic injury in prolonged ischemia rats model (ischemia for 2 h), which is at odds with our findings in the HL-1 cardiomyocytes, and is also difficult to reconcile. However, Woulfe et al.[25] have reported that deletion of GSK3β specifically in cardiomyocytes is protective in the setting of permanent MI. Thus, it is believed that GSK3β-mediated regulation of ventricular function in ischemic heart is complex, since overexpression/activation is detrimental, while sustained systemic inhibition could be detrimental too.[26] In our study SX revealed a protective effect by enhancing the moderate phosphorylation of the GSK3β.

In the present study, the specific inhibitor of PI3K could significantly reverse the anti-apoptotic effect of SX in HL-1 cells, indicating that the anti-apoptotic effect of SX is PI3K-dependent. Insulin could prevent cardiomyocytes from apoptosis through activation of PI3K,[27] which was

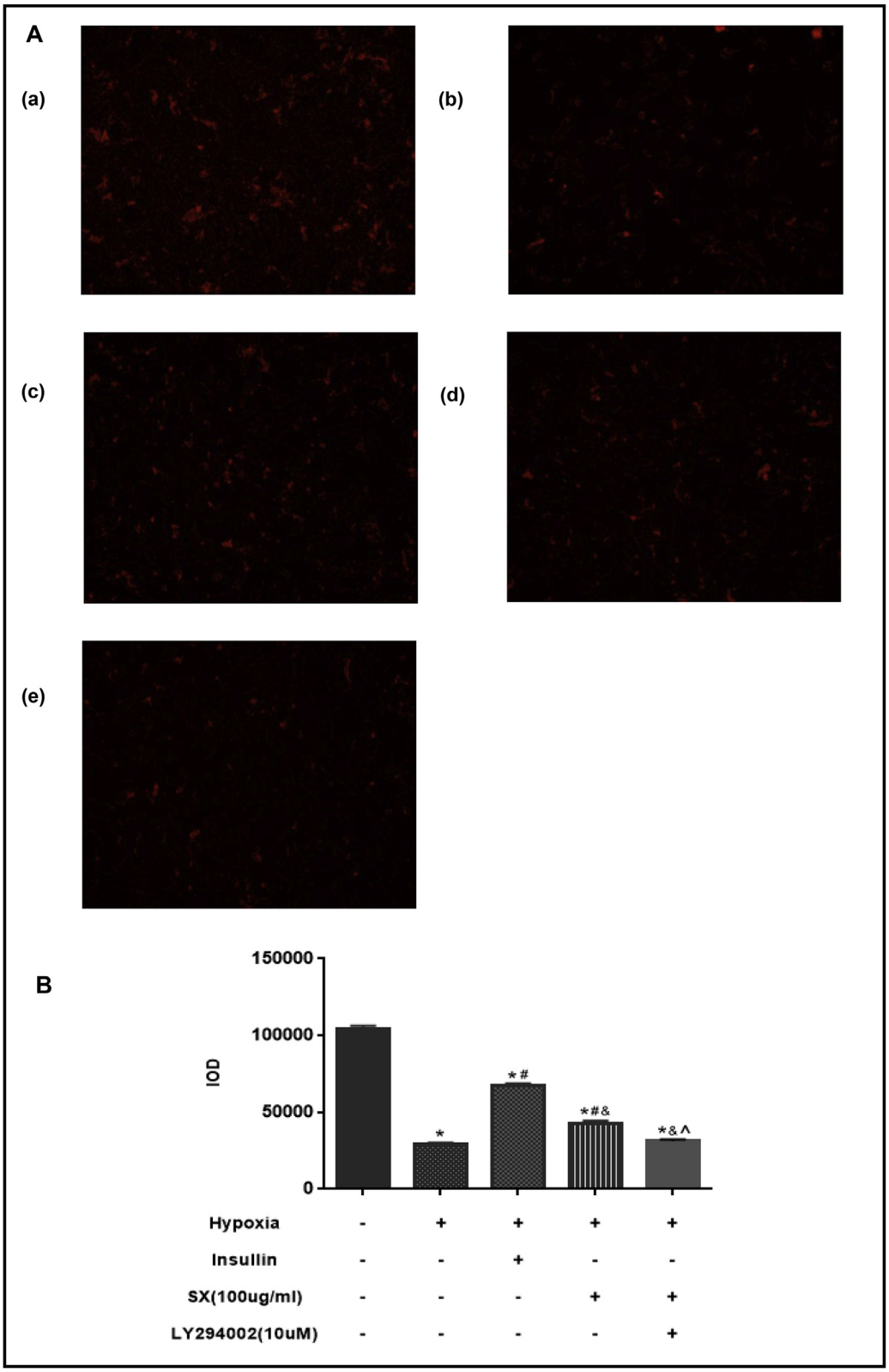

Fig. 3. Effect of SX on mitochondrial membrane potential ($\Delta\Psi m$) in HL-1 cardiomyocytes. (A) HL-1 cardiomyocytes in 5 experimental groups were stained with JC-1 dye (red) and then observed under fluorescence microscope. Area and average density of the fluorescence, as indicated by IOD, were evaluated by Image-Pro Plus 7 software (n = 3/group; mean ± SEM). (a) control group; (b) hypoxic injury group; (c) hypoxic injury + Insulin group; (d) hypoxic injury + SX group; (e) hypoxic injury + SX + LY294002 group. Decreased red fluorescence intensity indicating decreased amount of JC-1 polymer caused by decreased mitochondrial membrane potential. (B) Comparison of mitochondrial membrane potential between groups. * $p < 0.05$ compared to hypoxic group; $^{\#}$ $p < 0.05$ compared to hypoxic injury group; $^{\&}$ $p < 0.05$ compared to hypoxic injury + insulin group; $^{\wedge}p < 0.05$ compared to hypoxic injury + SX group (one-way ANOVA). SX, Suxiao Jiuxin Pill.

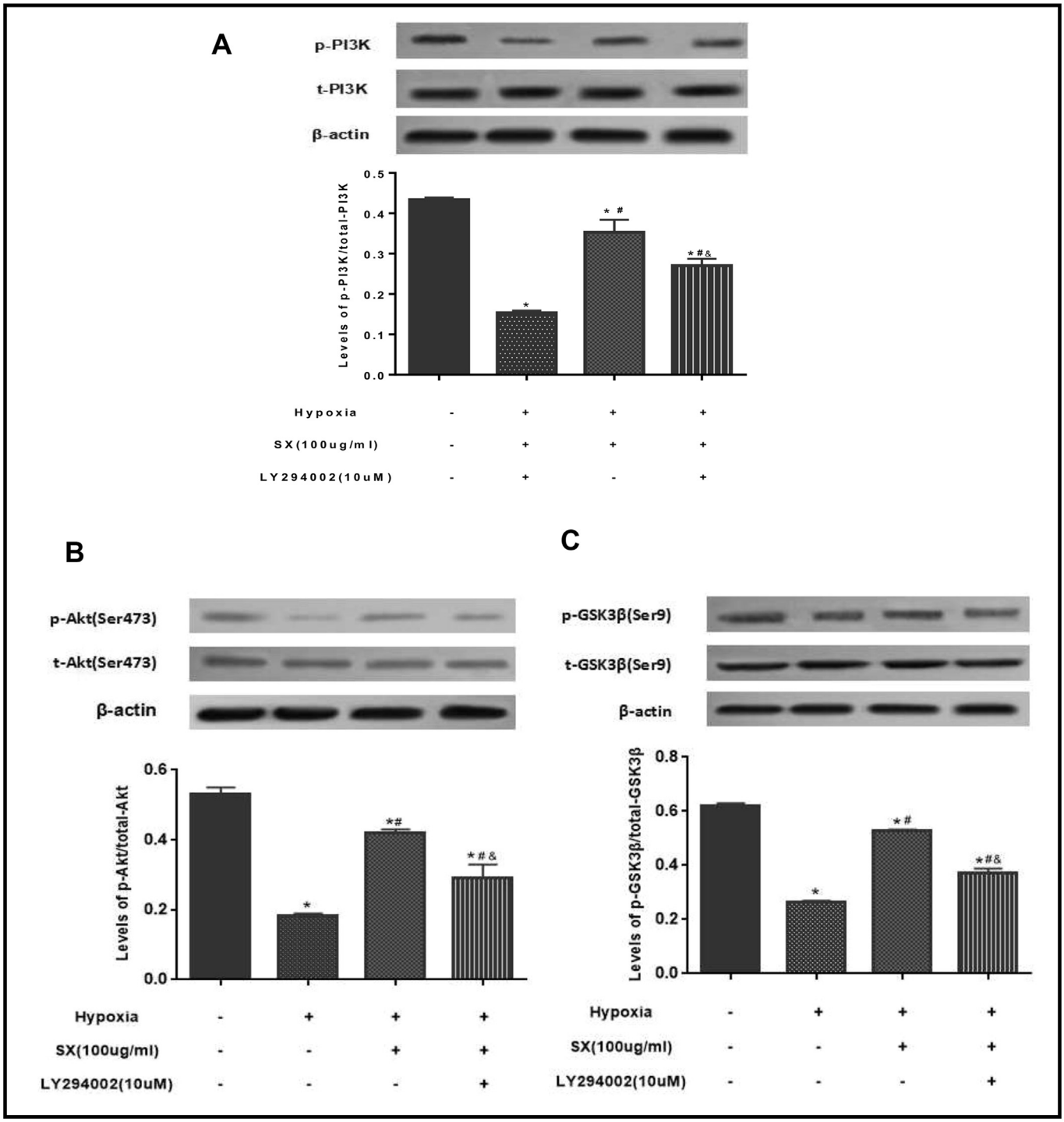

Fig. 4. Effect of SX on t-PI3K, p-PI3K, t-Akt, p-Akt, t-GSK3β and p-GSK3β expression in HL-1 cardiomyocytes. (A) Effect of SX on the expression of p-PI3K/t-PI3K. (B) Effect of SX on the expression of p-Akt/t-Akt. (C) Effect of SX on the expression of p-GSK3β/t-GSK3β. Data are mean ± SEM for 3 independent experiments performed in duplicate. * $p < 0.05$ compared to control group; # $p < 0.05$ compared to hypoxic injury group; & $p < 0.05$ compared to hypoxic injury + SX group (one-way ANOVA). SX, Suxiao Jiuxin Pill.

consistent with our experimental results. Insulin showed a better effect than SX on cell proliferation, apoptosis and mitochondrial membrane potential. Moreover, our data exhibited that SX significantly enhanced the phosphorylation of PI3K, Akt and GSK3β. We observed decreased protein expression of phospho-Akt and phospho-GSK3β following treatment with LY294002, which suggested that PI3K/Akt/GSK3β signaling pathway was involved in the anti-apoptotic effect of SX. GSK3β is related to the opening of mPTP in mycardial reperfusion phase.[28] Thus far, no researches have reported that inhibition of GSK3β prevents opening of mPTP during ischemia. Thus, SX inhibited the loss of ΔΨm through PI3K/Akt pathway, which might not be related to GSK3β (Fig. 5).

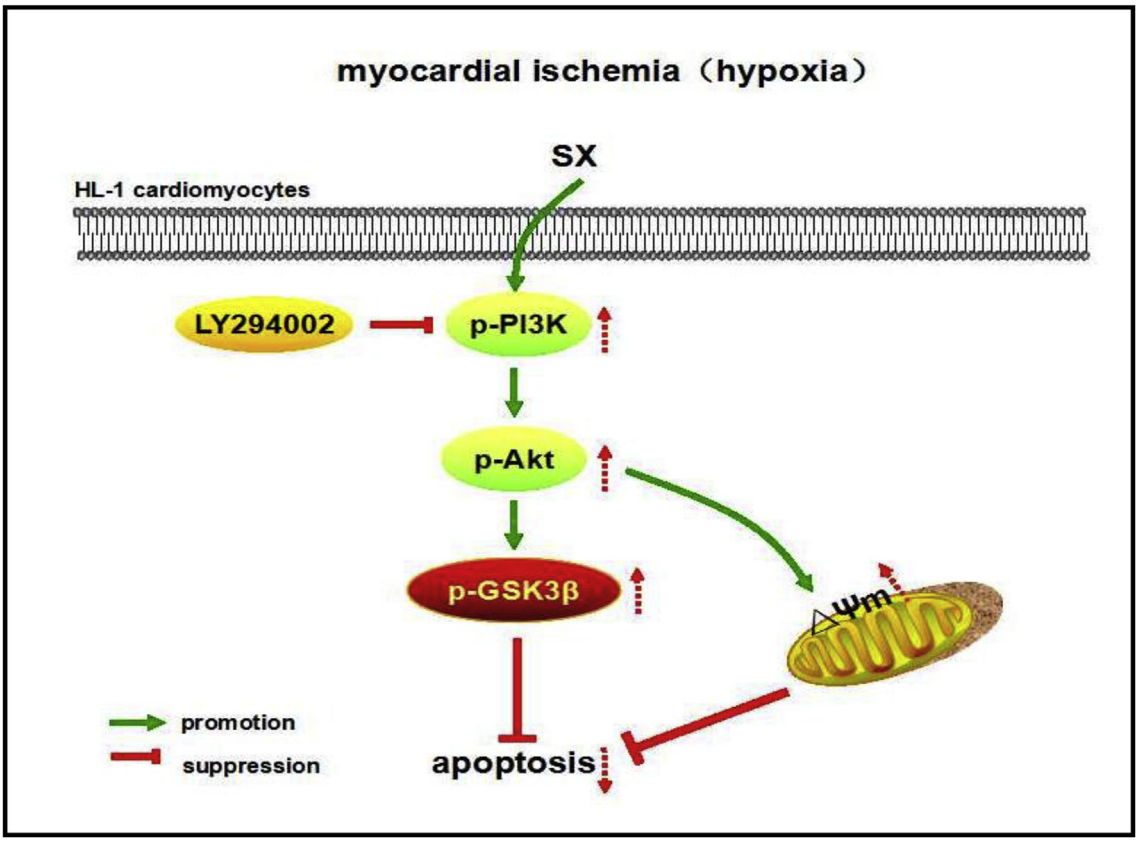

Fig. 5. The diagram of the cell signaling pathway which was involved in cardioprotection of SX against hypoxia-induced injury in HL-1 cardiomyocytes. Myocardial hypoxic injury in HL-1 cardiomyocytes induced dysfunction of PI3K/Akt/GSK3β pathway. However, SX treatment inhibited apoptosis and increased ΔΨm and the expression levels of p-PI3K, p-Akt, and p-GSK3β. The PI3K/Akt inhibition by LY294002 led to decreased phosphorylation of Akt, and GSK3β degradation, resulting in apoptosis. Above all, all the observations in the study indicated that SX may exert its cardioprotection partly through the activation of PI3K/Akt/GSK3β signaling pathway in HL-1 cardiomyocytes. SX, Suxiao Jiuxin Pill.

The total protein level of Akt and GSK3β did not change in cells treated with SX or LY294002. This indicates that SX inhibits a post-translational modification of Akt and produces a protective effect by enhancing the phosphorylation of the relevant target protein in the PI3K/Akt/GSK3β signaling pathway. However, protein expression of phospho-Akt and phospho-GSK3β were not totally abolished after adding LY294002 in ischemic injury + SX group, which suggested that there could be other mechanisms involved. SX is composed of many traditional Chinese medicines, which potentially have many targets. One of the major active components in SX, tetramethylpyrazine, has been reported to ameliorate hypoxia-induced myocardial cell apoptosis via HIF-1α/JNK/p38 and IGFBP3/BNIP3 inhibition to upregulate PI3K/Akt survival signaling.[29] Therefore, the same action mechanisms may happened in the effects of SX. The upstream targets for SX to upregulate PI3K/Akt signaling is worth studying in the future.

In conclusion, firstly, the present study demonstrated that SX protected cardiomyocytes from the hypoxia-mediated injury by reducing apoptosis. Moreover, SX exerted its anti-apoptotic effect through PI3K/Akt/GSK3β pathway *in vitro*, which could contribute to better understanding of the molecular mechanisms governing cardiac protection of SX.

Acknowledgments

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