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Original Article

Salvianolic Acid A Protects Neonatal Cardiomyocytes against Hypoxia/Reoxygenation-Induced Injury by Preserving Mitochondrial Function and Activating Akt/GSK-3β Signals*

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ABSTRACT **Objective**: To investigate the effects of salvianolic acid A (SAA) on cardiomyocyte apoptosis and mitochondrial dysfunction in response to hypoxia/reoxygenation (H/R) injury and to determine whether the Akt signaling pathway might play a role. **Methods**: An *in vitro* model of H/R injury was used to study outcomes on primary cultured neonatal rat cardiomyocytes. The cardiomyocytes were treated with 12.5, 25, 50 μg/mL SAA at the beginning of hypoxia and reoxygenation, respectively. Adenosine triphospate (ATP) and reactive oxygen species (ROS) levels were assayed. Cell apoptosis was evaluated by flow cytometry and the expression of cleaved-caspase 3, Bax and Bcl-2 were detected by Western blotting. The effects of SAA on mitochondrial dysfunction were examined by determining the mitochondrial membrane potential (△Ψm) and mitochondrial permeability transition pore (mPTP), followed by the phosphorylation of Akt (p-Akt) and GSK-3β (p-GSK-3β), which were measured by Western blotting. **Results**: SAA significantly preserved ATP levels and reduced ROS production. Importantly, SAA markedly reduced the number of apoptotic cells and decreased cleaved-caspase 3 expression levels, while also reducing the ratio of Bax/Bcl-2. Furthermore, SAA prevented the loss of △Ψm and inhibited the activation of mPTP. Western blotting experiments further revealed that SAA significantly increased the expression of p-Akt and p-GSK-3β, and the increase in p-GSK-3β expression was attenuated after inhibition of the Akt signaling pathway with LY294002. **Conclusion**: SAA has a protective effect on cardiomyocyte H/R injury; the underlying mechanism may be related to the preservation of mitochondrial function and the activation of the Akt/GSK-3β signaling pathway.

KEYWORDS salvianolic acid A, cardiomyocyte, hypoxia/reoxygenation injury, mitochondria, Akt/GSK-3β

Salvianolic acid A (SAA) is the main active constituent of a classical Chinese medicine, *Salvia miltiorrhiza*. SAA has a wide range of pharmacological activities such as prevention against myocardial ischemia and regulation of the immune system.[1,2] Experiments indicated that SAA preserved cardiac function against ischemia/reperfusion (I/R) injury by reducing the myocardial infarct area and myocardial enzyme leakage.[3,4] Using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique, we found that the concentration of SAA was higher in the plasma of dogs administered *Salvia miltiorrhiza* extracts. Pharmacokinetic/pharmacodynamic analyses also identified SAA as one of the major anti-myocardial ischemia components of *Salvia miltiorrhiza*.[5-7] However, the mechanisms underlying the protective effects of SAA against myocardial I/R injury remain unknown. Previous experiment has suggested that SAA regulated the mitochondrial apoptosis pathway, conferring a protective effect.[3] This study used a model of hypoxia/reoxygenation (H/R) damaged primary cardiomyocytes to imitate myocardial I/R injury *in vitro*. Then the mechanisms by which SAA protected against cardiomyocyte H/R injury were investigated.

METHODS

Drugs and Reagents

SAA was purchased from Beijing Fangcheng Biological Technology Co., Ltd. (China, batch No. 120826). LY294002 was from Cell Signaling (USA, batch No. 9901). CellTiter-Glo® luminescent cell

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viability assay kit was purchased from Promega Co. (USA, batch No. G7570). The DCFH-DA probe was obtained from Sigma Chemical Co. (USA, Catalog No. B-6883). The FITC-annexin V/propidium iodide (PI) apoptosis detection kit was from BD Biosciences (USA, Catalog No. 556547). The MitoProbe™ transition pore assay kit (No. M34153) and MitoProbe™ JC-1 assay kit (No. M34152) for flow cytometry were from Molecular Probes (USA). The anti-cleaved-caspase 3 antibody (No. C8487) was from Sigma (USA). The antibodies for anti-Bcl-2 (No. 2870), anti-Bax (No. 2772), anti-Akt (No. 9272), anti-p-Akt (Ser473, No. 4060), anti-GSK-3β (No. 9315), anti-p-GSK-3β (Ser9, No. 5558) and anti-GAPDH (No. 2118) were from Cell Signaling (USA).

Primary Cardiomyocyte Culture

Cardiomyocytes were prepared from neonatal Sprague Dawley (SD) rats (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China), with certification No. SYXK (CQ) 2007–002, as previously described with some modifications.[8] Briefly, the neonatal rats (24-h old) were disinfected with 70% ethanol and the hearts were rapidly removed and placed in cold phosphate buffered saline (PBS). After discarding the atria and aorta, the ventricles were minced into fragments with scissors. These tissue fragments were digested in PBS containing 0.625 g/L trypsin (GIBCO) and 0.5 g/L collagenase Ⅱ (GIBCO) in a tube at 37 ℃. The digesting solution was gently shaken 5–8 times (10 min at a time) until the tissue fragments disappeared. Then, the cell suspension was centrifuged at 1,500 r/min for 10 min and the cell pellet was resuspended in dulbecco minimum essential medium (GIBCO) supplemented with 10% newborn calf serum (TBD21HY, Tianjin, China), penicillin (100 U/mL) and streptomycin (100 μg/mL). The dispersed cells were replated on a 10-cm culture dish at 37 ℃ for 1 h to enrich the culture for cardiomyocytes. The nonadherent cells were collected into culture medium supplemented with 5-bromo-2-deoxyuridine (0.1 mmol/L, Sigma), which was used to inhibit cardiac fibroblast proliferation. The isolated cardiomyocytes were seeded onto gelatin-coated well plates, and then cultured in a humidified incubator with 5% CO_2 at 37 ℃. The culture medium was changed on the following day.

H/R and Drug Treatment of Cardiomyocytes

A H/R-induced cardiomyocyte injury model was established as previously described.[7,8] The newly isolated cardiomyocytes developed a synchronous beating pattern after 48 h and were then starved with DMEM without newborn calf serum for 12 h prior to conducting the experiments. The cardiomyocytes were washed 3 times with glucose-free DMEM and then incubated with glucose-free DMEM which was preflushed with 95% N_2 and 5% CO_2 for 15 min. Cells that were placed in an incubator chamber which was ventilated with 95% N_2 and 5 % CO_2 for 15 min to induce hypoxia, and then placed in a 37 ℃ humidified incubator for 2 h. Reoxygenation was accomplished by opening the chamber and replacing the glucose-free DMEM with fresh normal medium under normoxic conditions. Reoxygenation times varied depending on the experimental objectives: a 0.5-h reoxygenation period was used for detection of mitochondial permeability transition pore (mPTP) opening; a 1-h reoxygenation period was used for assay of reactive oxygen species (ROS) levels and Western blot analysis. Additionally, a 2-h reoxygenation period was used to measure adenosine triphospate (ATP) levels and a 6-h reoxygenation period was used for the flow cytometry assay of cell apoptosis and the mitochondrial membrane potential ($\triangle\Psi m$).

SAA treatments solutions were prepared by dissolving the drug in dimethylsulfoxide (DMSO) and added to the culture medium at a 1:1000 dilution to achieve final concentrations of 12.5, 25 or 50 mg/mL at the start of the hypoxia and reoxygenation periods. For the model group, equivalent volumes of DMSO were added whereas the normal control group received no treatment.

Assay of Cellular ATP Levels

Cellular ATP levels were measured by a CellTiter-Glo® luminescent cell viability assay kit according to the manufacturer's instructions. Briefly, the cells were seeded onto a white wall and transparent bottom 96-well plate. After 2 h reoxygenation, the cardiomyocytes were maintained at room temperature for 30 min, and then CellTiter-Glo® reagent (100 μL) was added to each well. The plate was gently shaken for 2 min, and then incubated at room temperature for 10 min to stabilize the chemiluminescent signal. A Synergy™4 (BioTek, USA) microplate reader was then used to measure luminescence (RLU). Total ATP levels were expressed as luminescent intensity.

Assay of Intracellular ROS Levels

The production of intracellular ROS was determined using the fluorescent probe dichloro-dihydro-

fluorescein diacetate (DCFH-DA). The DCFH-DA itself did not emit a fluorescent signal, but was hydrolyzed to DCFH once it entered the cell and became 'trapped'. The DCFH was then oxidized to DCF by intracellular ROS, which then emited a green fluorescent signal that can be measured. For these experiments, cardiomyocytes were seeded in 96 well culture plates at a density of 10^5 per well. Before hypoxic treatment, the cells were loaded with the DCFH probe in situ by incubating them in serum free culture medium with 10 μmol/L DCFH-DA at 37 ℃ for 30 min. After reoxygenating the cells for 1 h, the fluorescent density of each well was measured using a Synergy™4 (BioTek, USA) microplate reader at excitation and emission wavelengths of 488 and 525 nm, respectively. The ROS levels were indirectly reflected by the intensity of green fluorescence.

Flow Cytometric Detection of Apoptosis

Preliminary experimental results[7] showed that SAA significantly inhibited H/R-induced lactate dehydrogenase (LDH) leakage in cardiomyocytes. SAA at concentrations of 25 and 50 μg/mL was selected in order to determine whether it could prevent H/R-induced apoptosis in cardiomyocytes. Apoptosis was measured using a FITC-annexin V/PI apoptosis detection kit according to the manufacturer's protocol. Briefly, after reoxygenation for 6 h, the cells were washed twice with cold PBS then digested with 0.25% trypsin. The cells were centrifuged at 1,000 r/min for 5 min and then collected; the supernatant was discarded. Cells were resuspended in 1 × annexin V binding buffer at a concentration of 10^6/mL and 100 μL (10^5 cells) was placed in a 5 mL tube followed by the addition of 5 μL FITC annexin V and 5 μL PI. The cells were gently mixed and then incubated for 15 min at room temperature in the dark. The samples were immediately analyzed using a flow cytometer (Elite Epics, Coulter Beckman, USA). Approximately 10,000 cells were counted for each sample and data were analyzed with the Expo32 software.

Measurement of Mitochondrial Membrane Potential

MitoProbe™ JC-1 assay kit was used to measure $\triangle\Psi$m according to the manufacturer's protocol. Briefly, after reoxygenation for 6 h, the cells were harvested after washing twice with PBS then digested with 0.25% trypsin. For the positive control tube, cells were resuspended in PBS with 50 μmol/L 2-(2-(3-chlorophenyl)hydrazinylyidene)propanedinitrile (CCCP) and incubated at 37 ℃ for 5 min. Then the tube was centrifuged at 1,000 r/min for 5 min and the supernatant was discarded. The cells in each group were then resuspended in PBS containing 2 μmol/L JC-1, the cell concentration adjusted to 10^6 cells/mL and incubated at 37 ℃ for 30 min with 5% CO_2. After incubation, the tubes were centrifuged at 1,000 r/min for 5 min, the supernatant was discarded and the cells washed with PBS. After centrifugation at 1,000 r/min for 5 min, the cells were collected, gently resuspended with 500 μL PBS, and subsequently transferred to a new tube. The fluorescent density was recorded using a flow cytometer (Elite Epics, Coulter Beckman, USA) with excitation at 488 nm, emission at 529 nm (monomer form of JC-1, green) and at 590 nm (aggregate form of JC-1, red). The $\triangle\Psi$m of cardiomyocytes in each treatment group was calculated as the fluorescent ratio of red to green.

Detection of mPTP Opening

A MitoProbe™ transition pore assay kit was used to detect mPTP opening of cardiomyocytes as previously described.[9] Briefly, after reoxygenation for 0.5 h, the cells were harvested and resuspended in prewarmed hank's balanced salt solution (HBSS) at a final concentration of 10^6 cells/mL, and then the cells in each remaining groups were divided into 2 tubes. To the first tube, 2 μL of 2 μmol/L calcein AM was added. To the second tube, 2 μL of 2 μmol/L calcein AM along with 2 μL of 80 mmol/L $CoCl_2$ were added. In addition, to a ionomycin control tube, 2 μL of 2 μmol/L calcein AM and 2 μL of 80 mmol/L $CoCl_2$ along with 2 μL of 100 μmol/L ionomycin were added. The samples were then gently mixed and incubated at 37 ℃ for 15 min in the dark. The supernatant was discarded after centrifugation at 1,000 r/min for 5 min, and the cells were washed with HBSS and resuspended in 350 μL PBS. The samples were analyzed using a flow cytometer with 494 nm excitation and 517 nm emission, and the fluorescence intensity in different groups was recorded.

Western Blot Analysis

The expression levels of cleaved-caspase 3, Akt, p-Akt, Bcl-2, Bax, GSK-3β and p-GSK-3β were measured by Western blot. After 1 h reoxygenation, cells were washed with PBS and then lysed at 4 ℃ with ice-cold RIPA lysis buffer (No. C1053, Applygen Technologies Inc., Beijing, China) that contained phosphatase inhibitors for 30 min. The lysates were centrifuged, and protein concentration was determined

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with the bicinchoninic acid (BCA) protein assay kit (Applygen Technologies Inc., Beijing, China).

Samples with equivalent amounts of total protein (20 μg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). The membranes were first blocked in 5% dry milk for 30 min and then incubated overnight at 4 ℃ with primary antibody (rabbit anti-cleaved-caspase 3, rabbit anti-Akt, rabbit anti-p-Akt, rabbit anti-Bcl-2, rabbit anti-Bax, rabbit anti-GSK-3β at a 1:1000 dilution; rabbit anti-p-GSK-3β at a 1:500 dilution and rabbit anti-GAPDH at a 1:2000 dilution). The membranes were washed 5 times in 1×Tris-buffered saline Tween 20 (TBST) buffer and then incubated with horseradish peroxidase-(HRP-) conjugated goat anti-rabbit secondary antibodies (dilution 1:40000) for 90 min at room temperature. The protein bands were detected with an enhanced chemiluminescence (ECL) system (Thermo, USA), visualized with the Chemi Doc XRS+gel documentation system (Bio-Rad, USA) and analyzed by using Image lab 3.0 software (Bio-Rad, USA). The expression levels of GAPDH served as an internal control for protein loading to further assure the same volume for all the samples.

Statistical Analysis

All data are presented as mean ± standard deviation ($\bar{x} \pm s$). The data analyses were performed using one-way ANOVA analysis followed by Bonferroni's multiple comparisons test. In all cases, values of $P<0.05$ were considered statistically significant.

RESULTS

Effect of SAA on ATP Levels in Cardiomyocytes Exposed to H/R

The intracellular ATP levels were significantly decreased after 2 h of hypoxia followed by 2 h reoxygenation compared to the controls ($P<0.01$). In contrast, treatments with 12.5–50 μg/mL of SAA significantly increased ATP levels in a concentration dependent manner relative to the H/R group ($P<0.01$, Figure 1).

SAA Reduced H/R Mediated ROS Production in Cardiomyocytes

The DCF fluorescence intensity of the control group was 72 ± 12 and increased by 397% after 2 h of hypoxia followed by 1 h reoxygenation. These data indicated

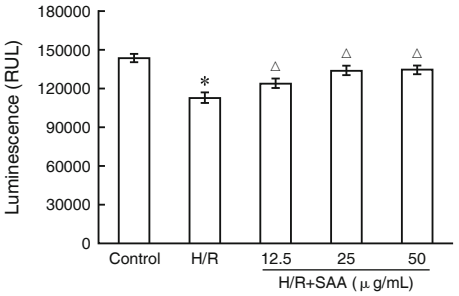

Figure 1. Effects of SAA on ATP Levels in Cardiomyocytes Exposed to 2 h Hypoxia Followed by 2 h Reoxygenation (n=6, $\bar{x} \pm s$)
Notes: $^{*}P<0.01$ *vs*. control group; $^{\triangle}P<0.01$ *vs*. H/R group

that the ROS level significantly increased in the H/R group compared to the control group ($P<0.01$) and this effect was attenuated in cardiomyocytes treated with 12.5, 25 and 50 μg/mL SAA ($P<0.01$). SAA treatments of cardiomyocytes significantly improved H/R-induced ROS generation (Figure 2).

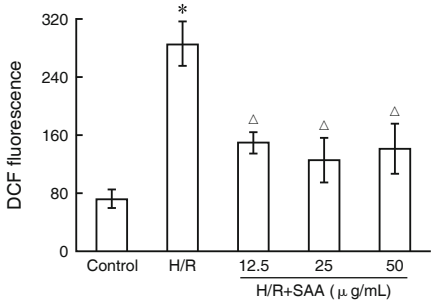

Figure 2. Effects of SAA on ROS Generation in Cardiomyocytes Exposed to 2 h Hypoxia Followed by 1 h Reoxygenation (n=6, $\bar{x} \pm s$)
Notes: $^{*}P<0.01$ *vs*. control group; $^{\triangle}P<0.01$ *vs*. H/R group

SAA Reduced H/R Induced Apoptosis in Cardiomyocytes

The percentage of apoptotic cells was significantly increased in cells after exposure to 2 h hypoxia followed by 6 h reoxygenation compared to the control group ($P<0.01$) and this effect was attenuated in cardiomyocytes treated with 25 and 50 μg/mL SAA ($P<0.01$, Figure 3).

Effect of SAA on H/R Induced Mitochondrial Dysfunction

The JC-1 fluorescent ratio of red/green was significantly decreased following H/R compared to the controls, demonstrating that $\triangle\Psi m$ was significantly reduced in cardiomyocytes exposed to hypoxia for 2 h followed by reoxygenation for 6 h ($P<0.01$). However, treatments with 25 and 50 μg/mL SAA significantly prevented the decrease in $\triangle\Psi m$

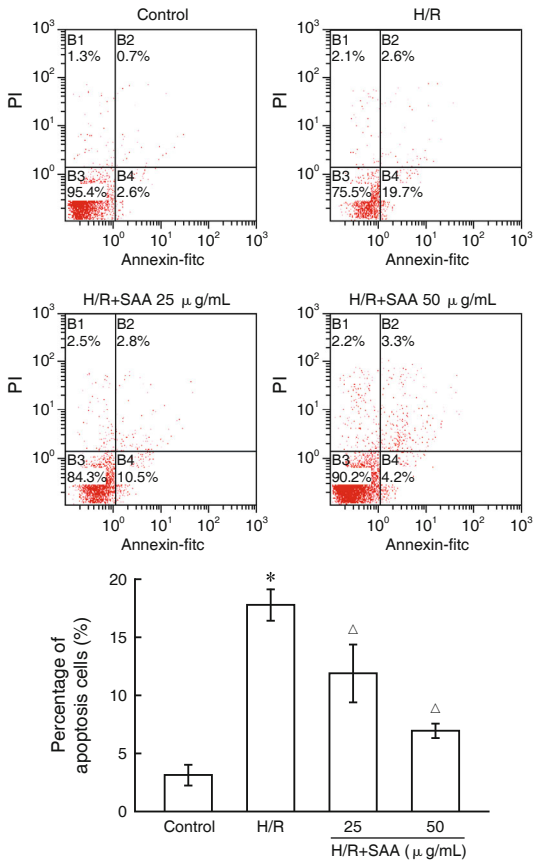

Figure 3. Effects of SAA on H/R-Induced Cardiomyocyte Apoptosis (n=5, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. control group; △P<0.01 *vs*. H/R group

caused by H/R (P<0.01, Figure 4).

The average fluorescence intensity of calcein was significantly decreased in cardiomyocytes exposed to hypoxia for 2 h followed by reoxygenation for 0.5 h, suggesting that the mPTP opening was significantly increased (P<0.01). However, treatment with 50 μg/mL SAA significantly inhibited the decrease in the average fluorescence intensity of calcein (P<0.01) caused by H/R, indicating that the mPTP opening induced by H/R was partially blocked (Figure 5).

Effects of SAA on Expression of Cleaved-Caspase 3, Bcl-2 and Bax

Cleaved-caspase 3 protein expression was significantly increased in cardiomyocytes exposed to hypoxia for 2 h followed by reoxygenation for 1 h compared to controls (P<0.01) and this effect was attenuated in cells treated with 25 and 50 μg/mL SAA (P<0.01). The ratio of Bax to Bcl-2 was significantly higher in cardiomyocytes exposed to hypoxia for 2 h followed by reoxygenation for 1 h compared to controls (P<0.05) and this effect was attenuated in cells treated with 25 and 50 μg/mL SAA (P<0.05, Figure 6).

Effects of SAA on Expression of Akt and p-Akt

There were no significant differences in Akt protein expression among the 4 groups tested. The protein expression of p-Akt decreased, to some extent, in cardiomyocytes exposed to hypoxia for 2 h followed by reoxygenation for 1 h compared to controls (P<0.05). Compared with the H/R group, cells treated with 25 and 50 μg/mL SAA both significantly increased expression of p-Akt protein (P<0.05, Figure 7).

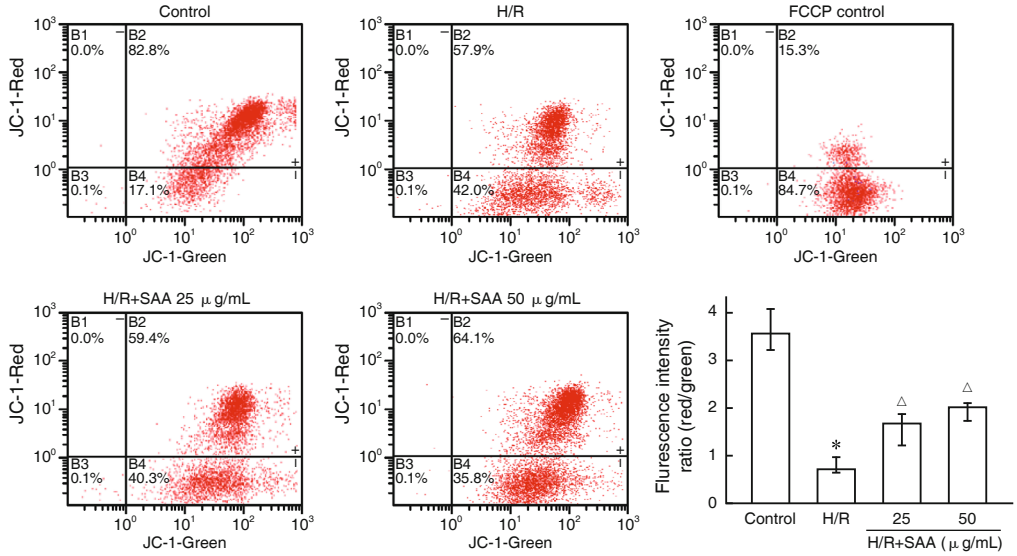

Figure 4. Effects of SAA on △Ψm of Cardiomyocytes Exposed to 2-h Hypoxia Followed by 6-h Reoxygenation (n=3, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. control group; △P<0.01 *vs*. H/R group

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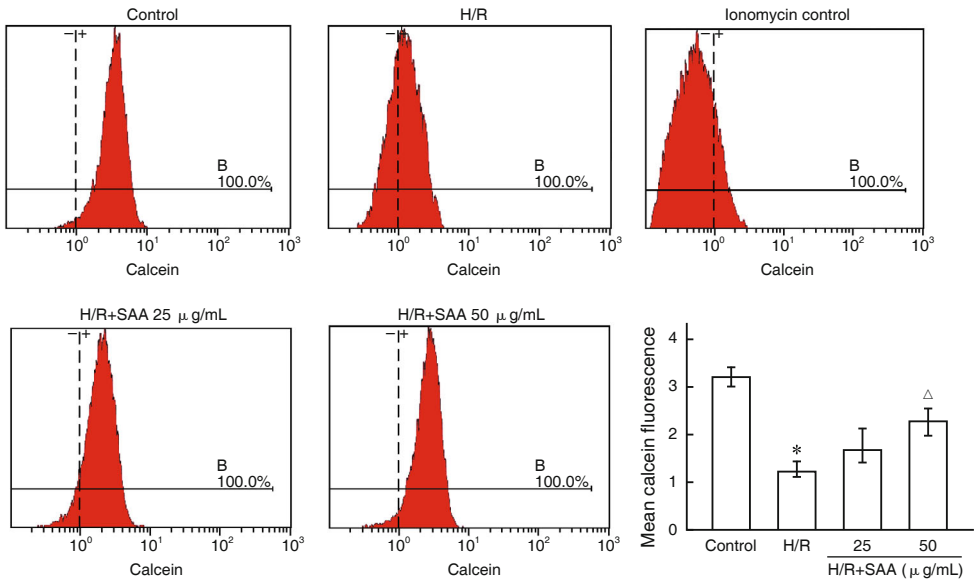

Figure 5. Effects of SAA on the Opening of mPTP in Cardiomyocytes (n=3, $\bar{x} \pm s$)

Notes: $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.01 *vs*. H/R group

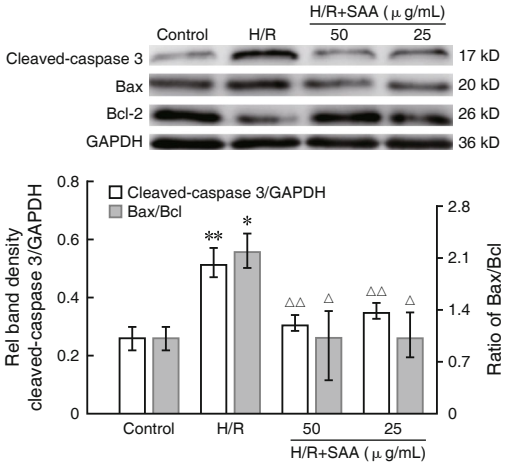

Figure 6. Effects of SAA on Expression of Cleaved-Caspase 3 and Bax/Bcl-2 Ratio in Cardiomyocytes by Western Blot (n=3, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05, $^{**}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. H/R group

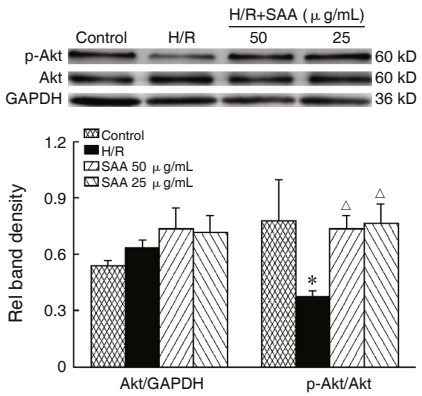

Figure 7. Effects of SAA on Expression Levels of Akt and p-Akt in Cardiomyocytes (n=3, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05 *vs*. control group; $^{\triangle}P$<0.05 *vs*. H/R group

Effects of SAA on Expression of GSK-3β and p-GSK-3β

There were no significant differences in the protein expression of GSK-3β in cardiomyocytes of each group. The expression of p-GSK-3β decreased to some extent in cardiomyocytes exposed to hypoxia for 2 h followed by reoxygenation for 1 h compared to controls (P<0.05). Compared with the H/R group, cells treated with 25 and 50 μg/mL SAA both significantly increased the expression of p-GSK-3β (P<0.05 and P<0.01, respectively, Figure 8).

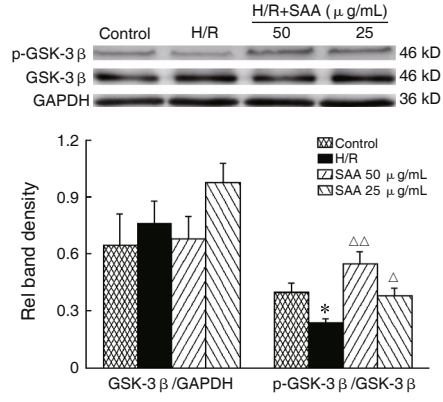

Figure 8. Effects of SAA on Expression Levels of GSK-3β and p-GSK-3β in Cardiomyocytes by Western Blot (n=3, $\bar{x} \pm s$)

Notes: $^{*}P$<0.05 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. H/R group

There were no significant differences in the protein expression of GSK-3β in cardiomyocytes from each group. Cells treated with 50 μg/mL SAA

had a significant increase in the protein expression of p-GSK-3β compared with H/R cells (P<0.05) and this effect was significantly attenuated in cells treated with both 50 μg/mL SAA and LY294002 (P<0.05, Figure 9).

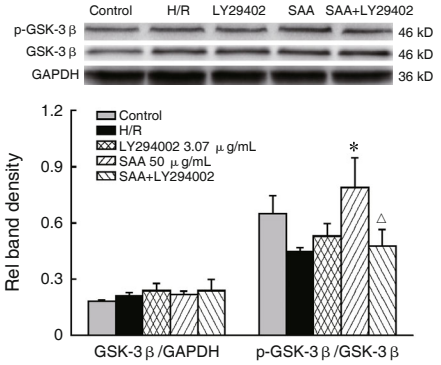

Figure 9. Effects of SAA on Expression Levels of p-GSK-3β in Cardiomyocytes (n=3, $\bar{x} \pm s$)

Notes: *P<0.05 *vs*. H/R group; △P<0.05 *vs*. H/R+SAA 50 μg/mL group

DISCUSSION

Cardiomyocyte necrosis and apoptosis are the major contributors to myocardial I/R injury. A previous study has shown that SAA significantly inhibits H/R injury induced intracellular LDH leakage which is proportional to the extent of membrane damage and cell necrosis.[10] In the present study, we have shown that SAA significantly attenuated cardiomyocyte apoptosis and decreased the expression of cleaved-caspase-3 and the ratio of Bax/Bcl-2 proteins, suggesting that SAA inhibits I/R induced myocardial infarction through the reduction of cell apoptosis.

Recent evidence indicated that mitochondria played an important role in the process of H/R induced cardiomyocyte apoptosis. Ischemia and hypoxia lead to a series of changes in the structure and function of mitochondria, including impaired ATP synthesis, increased mitochondrial proton leakage, and especially the burst of ROS generation at the early stage of reperfusion which resulted in metabolic disorders and oxidative damage. The data presented here showed that H/R injury induced a significant decrease in ATP levels and an increase in ROS generation, but treatments with SAA at the concentration of 12.5–50 μg/mL could significantly increase ATP levels and reduce ROS production, which suggested that SAA could improve the energy and antioxidant state of cardiomyocyte after H/R injury. This is consistent with other results suggesting that SAA has significant protective effects against H_2O_2-induced cardiomyocyte injury.[7]

Furthermore, H/R-induced mitochondrial dysfunction also resulted in decreased respiratory chain enzyme activity and $\triangle\Psi m$,[11,12] and increased opening of the mPTP[13] followed by pro-apoptotic protein release. These events directly lead to energy dissipation and the stimulation of apoptosis in cardiomyocytes. In this study, the results showed that treatments with SAA significantly preserved the $\triangle\Psi m$, thereby enhancing the tolerance of cardiomyocytes to H/R. It is well-known that the mPTP opening leads to an increase in mitochondrial membrane permeability and the release of cytochrome C and apoptosis-inducing factor. Sustained high levels of mPTP opening can directly lead to cardiomyocyte injury; and a certain level of mPTP opening and subsequent closing leads to cell apoptosis.[13] Therefore, mPTP is considered to be an important target for the prevention and treatment of myocardial I/R injury. Inhibiting mPTP opening either through direct intervention with the pore components or indirectly by eliminating the pathological event (such as ROS production, disruption of Ca^{2+} homeostasis or changes in pH) would result in a significant protective effect against myocardial I/R injury.[14,15] We next investigated the extent of mPTP opening induced by H/R and found that treatment with SAA at a concentration of 50 μg/mL significantly reduced mPTP opening, suggesting that the protective effect of SAA on cardiomyocyte was partially due to inhibition of the mPTP opening, which reduced or delayed the process of cell death.

The PI3K/Akt signaling pathway plays a key role in the survival of cardiomyocytes injured by I/R.[16-18] The phosphorylation of Akt at Ser473 represents the final activation of this pathway. To further investigate whether Akt is involved in the protective effects of SAA, we determined the effect of SAA on the protein expression of Akt and p-Akt. GSK-3β has been shown to be a critical target of Akt and the phosphorylation of Akt inhibits GSK-3β activity,[19,20] which is considered to be an important mechanism for the Akt-mediated cardioprotective effects. Our results showed that treatments with SAA significantly increased the expression of p-Akt, and p-GSK-3β proteins, indicating that the protective effect of SAA was related to activation of the Akt signaling pathway and inactivation of GSK-3β, which was closely related to a decrease in H/R injury.[20,21] In order to further investigate whether the inhibitory effect of 50 μg/mL SAA was dependent on activation of the Akt signaling pathway, LY294002, an Akt signaling pathway specific

inhibitor was used in our study, and the effects of SAA on the phosphorylation of GSK-3β were significantly decreased. These results indicated that the SAA mediated cell protective effects were related to the inhibition of GSK-3β activity, which was dependent on the activation of Akt signaling.

In conclusion, the present study showed that SAA had a protective effect on cardiomyocyte H/R injury; the underlying mechanism may be related to the preservation of mitochondrial function and the activation of the Akt/GSK-3β signaling pathway.

Conflict of Interests

The authors declare that there is no conflict of interests.

Author Contributions

Liu JX, Fan JP and Li XL contributed to the study design; Li XL performed research, analyzed the data and wrote the manuscript. Liang LN assisted in manuscript preparation.

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