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Cyclosporin A Protected Cardiomyocytes Against Oxidative Stress Injury by Inhibition of NF-κB Signaling Pathway

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Abstract

Purpose—This study aims to investigate the effects and the molecular mechanism of cyclosporin A (CsA) against oxidative stress injury in cultured neonatal rat cardiomyocytes.

Methods—Bax/Bcl-2, cl-casp-9/casp-9, cl-casp-3/casp-3, and iNOS/β-actin ratios and p-IκB and IκB levels were analyzed by western blot. IL-1β and TNF-α levels were analyzed by ELISA.

Results—CsA effectively improved the cell viability and reduced the extracellular lactate dehydrogenase release in cardiomyocytes after H_2O_2-induced oxidative damage. CsA significantly increased the superoxide dismutase activity, glutathione production, and catalase activity but decreased the malonaldehyde level. CsA treatment considerably reduced the H_2O_2-induced intracellular generation of reactive oxygen species, mitochondrial dysfunction, and release of cytochrome *c*. CsA could act against H_2O_2-induced ATP reduction, TCA cycle enzymes, mitochondrial complex I enzyme, and complex V enzyme in cardiomyocytes. CsA significantly decreased the Bax/Bcl-2 ratio, cl-casp-9/casp-9, and cl-casp-3/casp-3 in a concentration-dependent manner. CsA also remarkably reduced the cleaved PARP level and DNA fragmentation. NF-κB was closely related to oxidative stress injury. CsA inhibited NF-κB activation, thereby preventing the upregulation of IL-1β, TNF-α, iNOS, and intracellular NO release.

Conclusions—CsA protected cardiomyocytes against H_2O_2-induced cell injury. Hence, CsA may be developed as a candidate drug to prevent or treat myocardial ischemia reperfusion injury.

Keywords—CsA, Cardiomyocytes, H_2O_2, Oxidative stress, NF-κB.

INTRODUCTION

Myocardial ischemia reperfusion (IR) injury is central to the pathology of major cardiovascular diseases.[5] Reperfusion therapy of ischemic heart is inevitably associated with reperfusion injury.[22] Oxidative stress and mitochondrial dysfunction are widely accepted to be the major contributors to myocardial IR injury.[31]

Increased reactive oxygen species levels have been associated with defects in the mitochondrial dysfunction due to the activation of the NF-κB signaling pathway.[2] NF-κB is closely related to myocardial IR injury. NF-κB plays a pivotal role in myocardial IR injury and is involved in cell survival and cardiac inflammation.[13]

Cyclosporin A (CsA) is a powerful immunosuppressive drug. Our previous experiments demonstrated that CsA could protect H9c2 cells against $CoCl_2$-induced hypoxic injury and is a potential therapeutic agent for cardiac hypoxic injury.[38] CsA is a free radical scavenger, but its regulatory mechanism remains unclear.[6]

This study aims to investigate whether CsA prevents oxidative stress and mitochondrial dysfunction elicited by the exposure of cultured neonatal rat cardiomyocytes to oxidative stress injury and determine the underlying mechanism. In this experiment, we estab-

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lished the culture of primary cardiomyocytes. Cardiomyocytes were treated with CsA and subjected to oxidative stress injury. Results demonstrated that CsA can attenuate the oxidative damage of cardiomyocytes due to antioxidant action.

EXPERIMENTAL PROCEDURES

Source of CsA

CsA was purchased from Sigma.

Animals

Animal use in the experiment was performed according to the guidelines established by the Guide for the Care and Use of Laboratory Animals of Taishan Medical University. The procedure was approved by the Local Animal Ethics Committee. Neonatal Wistar rats, 1 or 2 day old, were purchased from the Animal Center of Shandong University (Jinan, China).

Neonatal Rat Primary Cardiomyocyte Culture

The hearts of 1 or 2 day old neonatal rats were collected and washed to remove the surrounding blood. The hearts were minced and trypsinized with 0.125% trypsin. The suspension was incubated in DMEM at 37 °C under an atmosphere of 5% CO_2 and 95% air for 90 min to exclude nonmyocyte cells that can rapidly attach to the surface. The cardiomyocytes were cultured in DMEM supplemented with 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was replaced with a new culture medium 48 h after culture. After 72 h, the cardiomyocytes were in the state of confluence and beat synchronously. Cardiomyocytes at this time were used in subsequent experiments.

Cells were cultured to 70–80% confluence, treated with 150 μM H_2O_2 for 3 h, and cultured in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. After inducing oxidative stress injury, the cells were maintained in complete medium with 10, 30, and 50 ng/mL CsA.

Cell Viability Assay

Cell viability assay was performed using CCK-8 kit according to the manufacturer's instruction (Dojindo). The cardiomyocytes were plated in 96-well plates at a density of 5000 cells/well. When the cells were grown to 70–80% confluence, H_2O_2-induced death was performed using the protocol for cardiomyocytes. Each well was added with CCK-8 solution at a dilution of 1/10 culture medium, and the cells were incubated for 4 h at 37°C. Absorbance at 450 nm was recorded with a microplate reader (Biotek, MQX200).

$$\text{Cell viability } (\%) = \left(OD_{\text{treatment group}} - OD_{\text{blank}}\right) / \left(OD_{\text{control group}} - OD_{\text{blank}}\right) \times 100$$

Lactate Dehydrogenase, Superoxide Dismutase, Glutathione, Catalase, and Malonaldehyde Assays

Lactate dehydrogenase activity was detected in the cell culture supernatant. In brief, the culture medium was collected to measure lactate dehydrogenase activity according to the manufacturer's instruction given that lactate dehydrogenase can catalyze the conversion of lactate into pyruvate (NJJC Bio). The formed pyruvate can react with 2,4-dinitro-phenylhydrazine to form pyruvate-dinitro-phenylhydrazone, which is colored maroon in alkaline solution. Absorbance at 450 nm was recorded to reflect the lactate dehydrogenase activity of the medium.

Superoxide dismutase activity in cardiomyocytes was evaluated according to the manufacturer's instructions (NJJC Bio). In this colorimetry-based assay, superoxide ions are generated from the conversion of xanthine and oxygen into uric acid and hydrogen peroxide by xanthine oxidase. The superoxide anion then converts WST-1(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to WST-1 formazan, a colored product that absorbs light at 450 nm.

The antioxidant status of cardiomyocytes was evaluated based on the glutathione level by using commercial assay kits (NJJC Bio). The biological sample was first deproteinized with 5% 5-Sulfosalicylic Acid Solution, centrifuged to remove the precipitated protein, and assayed for glutathione. Glutathione level was measured using a kinetic assay. In the assay, catalytic amounts of glutathione continuously reduce 5,5-dithiobis (2-nitrobenzoic acid) into 5-thio-2-nitrobenzoic acid, and the glutathione disulfide formed is recycled by glutathione reductase and NADPH. The yellow product, 5-thio-2-nitrobenzoic acid, was evaluated spectrophotometrically at 412 nm.

Catalase activity in cardiomyocytes was assessed according to the manufacturer's recommendations (NJJC Bio). The catalase assay kit uses the peroxidatic function of catalase to determine enzyme activity. In brief, 25 μL of the sample was added with 50 μL of the reaction buffer (500 mM potassium phosphate buffer, pH 7.0) and 25 μL of 200 mM H_2O_2 Solution. The rate of optical density change was kinetically monitored at 520 nm.

Malonaldehyde level in the supernatant was determined by measuring the amount of thiobarbituric acid reactive substances. Cardiomyocytes in each group were added to the reaction mixture consisting of 1.5 mL of 0.8% thiobarbituric acid, 200 μL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), and 600 μL of distilled H_2O. The mixture was heated for 45 min at 90 °C. After cooling to room temperature, the samples were centrifuged at 10,000×*g* for 10 min. Absorbance of the supernatant was recorded at 532 nm.

Hoechst33342/PI Assay

Cell apoptosis was analyzed by staining with Hoechst33342 and PI. Cardiomyocytes were collected, washed in Phosphate Buffered Saline (PBS) and resuspended in 0.8–1 mL of cell staining buffer containing 5 μL of Hoechst33342 and 5 μL of PI. The cardiomyocytes were then incubated for 20–30 min at 4 °C in the dark, washed in PBS, and smeared. Red and blue fluorescence was observed under the fluorescence microscope.

Measurement of Intracellular Reactive Oxygen Species

DCF-DA method was used to detect the level of intracellular reactive oxygen species. Cardiomyocytes were seeded on culture slides (5×10^5 cells/well) for 24 h after inducing oxidative stress injury. After 24 h, the cardiomyocytes were incubated with CsA (10, 30, and 50 ng/mL) for 24 h at 37 °C. After the addition of 25 mM of DCF-DA solution for 10 min, the fluorescence of DCF was detected using FACS.

Assay for Cellular Adenosine 5′-triphosphate (ATP) Levels (Luciferase-based assay)

In brief, 10,000 cardiomyocytes per well were seeded into a clear 96-well plate at 200 μL per well one day prior to assay. The medium was removed from the plate. The cells were treated with 25 μL of the solution containing 10 μM of each compound and incubated at 37 °C for 10 min. The cells were then added with 25 μL of 10 μM amyloid beta solution. The cells were incubated at 37 °C for 7 h and washed twice with PBS. The cells were lysed using 1% Triton-X 100 in TBST buffer solution. Protein concentrations in each well was determined using BCA protein determination kit (Pierce). Equal amount of cell lysates from each well were plated into a white 96-well plate. ATP level in each sample was determined using ATP determination kit (Invitrogen).

Measurement of Mitochondrial Membrane Potential

The fluorescent, lipophilic, and cationic probe, namely, JC-1 (Beyotime), was employed to measure the mitochondrial membrane potential ($\Delta\psi$m) of cardiomyocytes according to the manufacturer's instructions. The cardiomyocytes were plated in 96-well plates at a density of 5000 cells/well. When the cells were grown to 70–80% confluence, they were treated with 150 μM H_2O_2 for 3 h, and cultured in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. The cells were then incubated with JC-1 staining solution for 20 min at 37 °C. Fluorescence was detected with a FACS caliber (Becton–Dickinson). The wavelengths of excitation and emission were set at 490 and 535 nm for detection of the monomeric form of JC-1. Wavelengths of 525 and 590 nm were used to detect the aggregation of JC-1. The ratio of 'red' to 'green' fluorescence represents the $\Delta\psi$m of cardiomyocytes.

Quantification of Enzyme Activities

The activities of the enzyme aconitase were evaluated using commercial kits in accordance with the instructions of the manufacturer (Abcam). In brief, 1×10^6 cells were harvested for each assay, washed with cold PBS, and resuspended in 100 μL of cold Assay Buffer. Subsequently, 100 μL of cysteine HCL was added to 100 μL of $(NH_4)_2Fe(SO_4)_2$ to prepare fresh activation solution.The solution was incubated on ice for 1 h to activate aconitase. Each well was added with 50 μL of appropriate Reaction Mix and incubated at 25 °C for 30–60 min. The well was then added with 10 μL of developer solution and incubated at 25 °C for 10 min. Optical density at 450 nm was recorded.

The activities of the enzymes α-ketoglutarate dehydrogenase (α-KGDH) were assessed using commercial kits in accordance with the manufacturer's instructions (Abcam). In brief, 1×10^6 cells were added to 100 μL of ice-cold KGDH Assay Buffe on ice. The solution was centrifuged at 10,000×*g* for 5 min, and the supernatant was collected in a fresh tube. Each well was added with 50 μL of the reaction mix to each well. Absorbance was recorded at 450 nm.

The activities of the enzyme succinate dehydrogenase (SDH) were evaluated using commercial kits in accordance with the manufacturer's instructions (Abcam). In brief, cells were harvested and washed with ice-cold PBS. The cells (1×10^6) were rapidly homogenized in 100 μL of ice-cold SDH Assay Buffer and centrifuge at 10,000×*g* for 5 min. The supernatant was transferred to a fresh tube. Each sample well was mixed with 50 μL of SDH reaction mix. Absorbance was recorded at 600 nm.

The activity of the enzyme complex I was measured using commercial kits based on the manufacturer's instructions (Abcam). The suspension cells were harvested by centrifugation and washed twice with PBS. Proteins were extracted from the sample by adding 10× Detergent solution to a final dilution of 1/10. The tube was incubated on ice for 30 min to allow solubilization. The supernatant was collected, loaded on the plate, and incubated for 3 h at 25 °C. The wells were washed with 300 μL of 1× Buffer solution. Absorbance was recorded at 450 nm.

The activity of complex V was measured using commercial kits in accordance with the manufacturer's instructions (Abcam). In brief, the provided tube containing bovine heart mitochondria (BHM) was added with 40 μL of detergent and incubated on ice for 30 min. The supernatant containing solubilized BHM was collected and added with 5 mL of 1× Mito Buffer. About 50 μL of BHM was added to each well of the precoated 96-well microplate, and the plate was covered. Each well was added with 300 μL of 1× Wash Solution, and the wells were emptied. Each well was then added with 40 μL of Phospholipids, 200 μL of Complex V Activity Solution, and test compounds. Complex V activity was determined based on decrease in absorbance at OD 340 nm.

Western Blot Analysis

Cells were homogenized in RIPA lysis buffer containing protease inhibitor PMSF. Mitochondrial and cytosolic proteins were isolated using the Mitochondria/Cytosol Fractionation kit according to the manufacturer's protocol (BioVision). Total protein was measured by BCA (Pierce) and size separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences). The blots were incubated with antibodies against cytochrome *c* (cyto-c) (abcam), Bax (abcam), Bcl-2 (abcam), cleaved caspase-9 (Cell Signaling), caspase-9 (abcam), cleaved caspase-3 (Cell Signaling), caspase-3 (abcam), inducible nitric oxide synthase (iNOS) (abcam), p-IκB (abcam), IκB (abcam), and β-actin (abcam). The blots were added with goat anti-rabbit IgG and goat anti-mouse IgG (abcam) and developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Quantification of Cytokines Interleukin-1β (IL-1β) and Tumor Necrosis Factor (TNF-α)

Lysates of cardiomyocytes were obtained through a protein extraction buffer containing protease inhibitor. Cytokines IL-1β and TNF-α were determined using their corresponding ELISA kit (Abcam). In brief, 100 μL of the sample was added to the precoated plate and incubated overnight at 4 °C. Each well of the precoated plate was washed with washing buffer and added with 100 μL of the labeled antibody solution. The mixture was incubated for 1 h at 4 °C in the dark. The well was washed again and added with chromogen. The mixture was incubated for 30 min at room temperature in the dark. The resulting color was assayed at 450 nm by using a microplate absorbance reader after adding the stop solution.

Measurement of PARP

The levels of cleaved poly (ADP-ribose) polymerase (PARP) were measured by colorimetry according to the manufacturer's instructions (Trevigen). The PARP Universal Colorimetric Assay kit measures the incorporation of biotinylated poly (ADP-ribose) onto histone proteins in a 96-well plate. The kit has sensitivity as low as 0.01 units of PARP per well. Absorbance was recorded at 450 nm.

Measurement of DNA Fragmentation

DNA fragmentation in cell lysates was determined using the Cellular DNA Fragmentation ELISA kit (Roche), which is based on the quantitative detection of BrdU-labeled DNA fragments. After exposure to BrdU for 18 h, the cells were re-seeded in 96-well culture plates at 1.5×10^4 cell/well and incubated in DMEM as control medium or DMEM with CsA at concentrations of 10, 30, and 50 ng/mL after H_2O_2 treatment. After 24 h, DNA fragmentation was determined according to the Manufacturer's instructions. Absorbance was recorded spectrophotometrically at 450 nm.

Measurement of Intracellular Nitric Oxide (NO)

NO production was detected by evaluating nitrite accumulation in the culture medium through the Griess reaction. The cells were treated with H_2O_2 alone or with CsA after H_2O_2. The culture supernatant was collected and mixed with an equal volume of Griess reagent and incubated at room temperature for 20 min. Absorbance was recorded at 540 nm.

Statistical Analyses

All results are expressed as mean ± SEM. For multiple comparisons, the statistical analysis was performed by using one-way ANOVA followed by the Tukey's multiple comparison tests. Results were considered significant when $p < 0.05$.

RESULTS

Attenuation of H_2O_2-Induced Death of Cardiomyocytes by Using CsA

After oxidative stress injury, the viability of cardiomyocytes was assessed by CCK-8 assay. Figure 1a shows that CsA treatment prevented the loss of cell viability induced by oxidative stress injury in cardiomyocytes in a dose-dependent manner. CsA significantly decreased the number of apoptotic cells in a concentration-dependent manner (Figs. 1b and 1c). Lactate dehydrogenase, a stable cytoplasmic enzyme in all cells, is rapidly released in a culture medium when the plasma membrane is damaged. Thus, increased lactate dehydrogenase activity in a culture medium corresponds to the degree of cell necrosis. The results suggested that CsA effectively reduced lactate dehydrogenase leakage induced by oxidative stress injury (Fig. 1d). Thus, CsA could protect cardiomyocytes against H_2O_2-induced cytotoxicity.

Effects of CsA on Superoxide Dismutase Activity, Glutathione Production, Catalase Activity, and Malonaldehyde Concentration in Cardiomyocytes

Treatment of cardiomyocytes with oxidative stress injury significantly reduced the superoxide dismutase activity, glutathione production, and catalase activity but increased the malonaldehyde concentration in the mitochondrial membranes. CsA increased the superoxide dismutase activity (Fig. 2a), glutathione production (Fig. 2b), and catalase activity (Fig. 2c) and prevented malonaldehyde concentration (Fig. 2d) in the mitochondrial membranes of cardiomyocytes. These phenomena are clear manifestations of the amelioration of oxidative abnormalities in cardiomy-

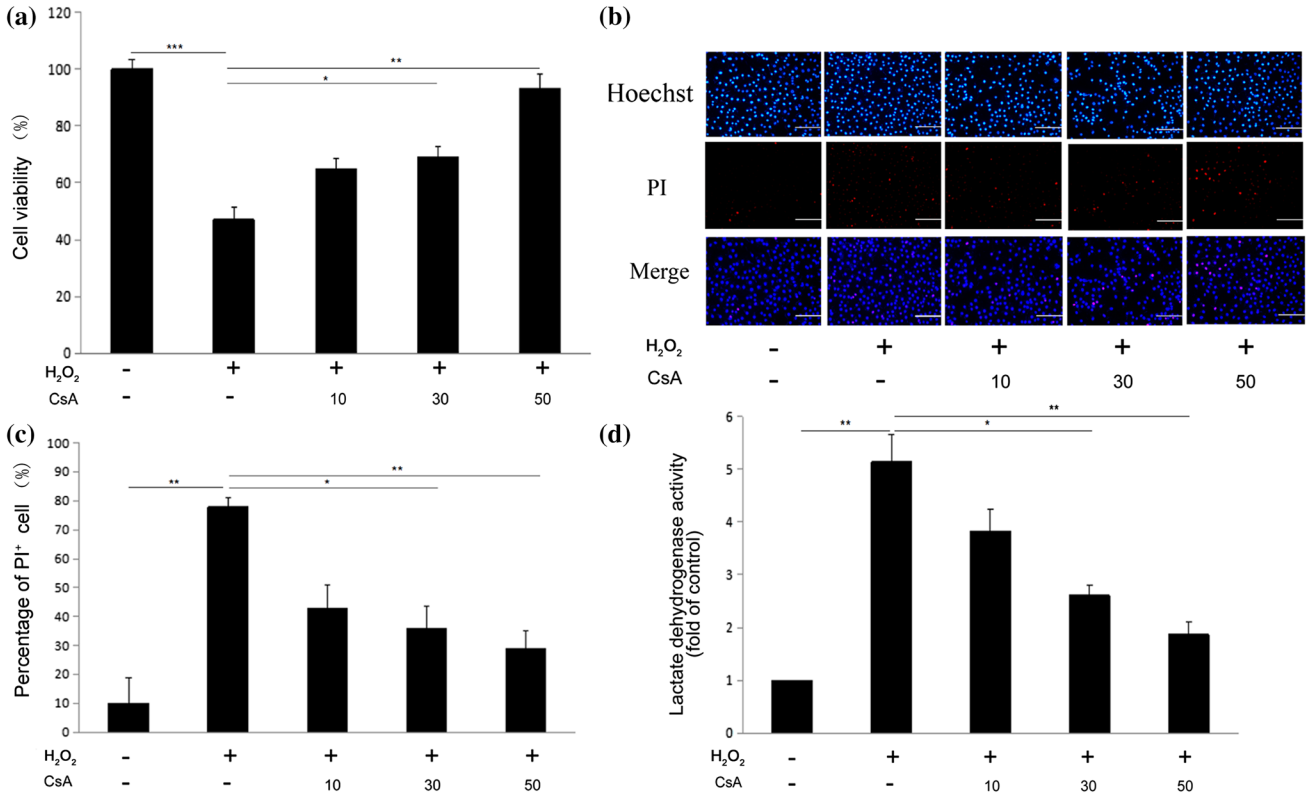

FIGURE 1. Effects of CsA on cell viability, cell apoptosis, and lactate dehydrogenase in cardiomyocytes exposed to H_2O_2 injury. (a) After H_2O_2 injury, the viability of cardiomyocytes was significantly decreased. Treatment with CsA (10, 30, and 50 ng/mL) for 24 h considerably increased the cell viability. (b) CsA (10, 30, and 50 ng/mL) protected cardiomyocytes from cell apoptosis. Determination of cell apoptotic rate by using a fluorescence microscope after staining with Hoechst33342/PI. Scale bar = 100 μm. (c) Representative percentages of PI^+ are shown. (d) After H_2O_2 injury, the level of lactate dehydrogenase released remarkably increased. Treatment with CsA (10, 30, and 50 ng/mL) for 24 h significantly decreased the lactate dehydrogenase level. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

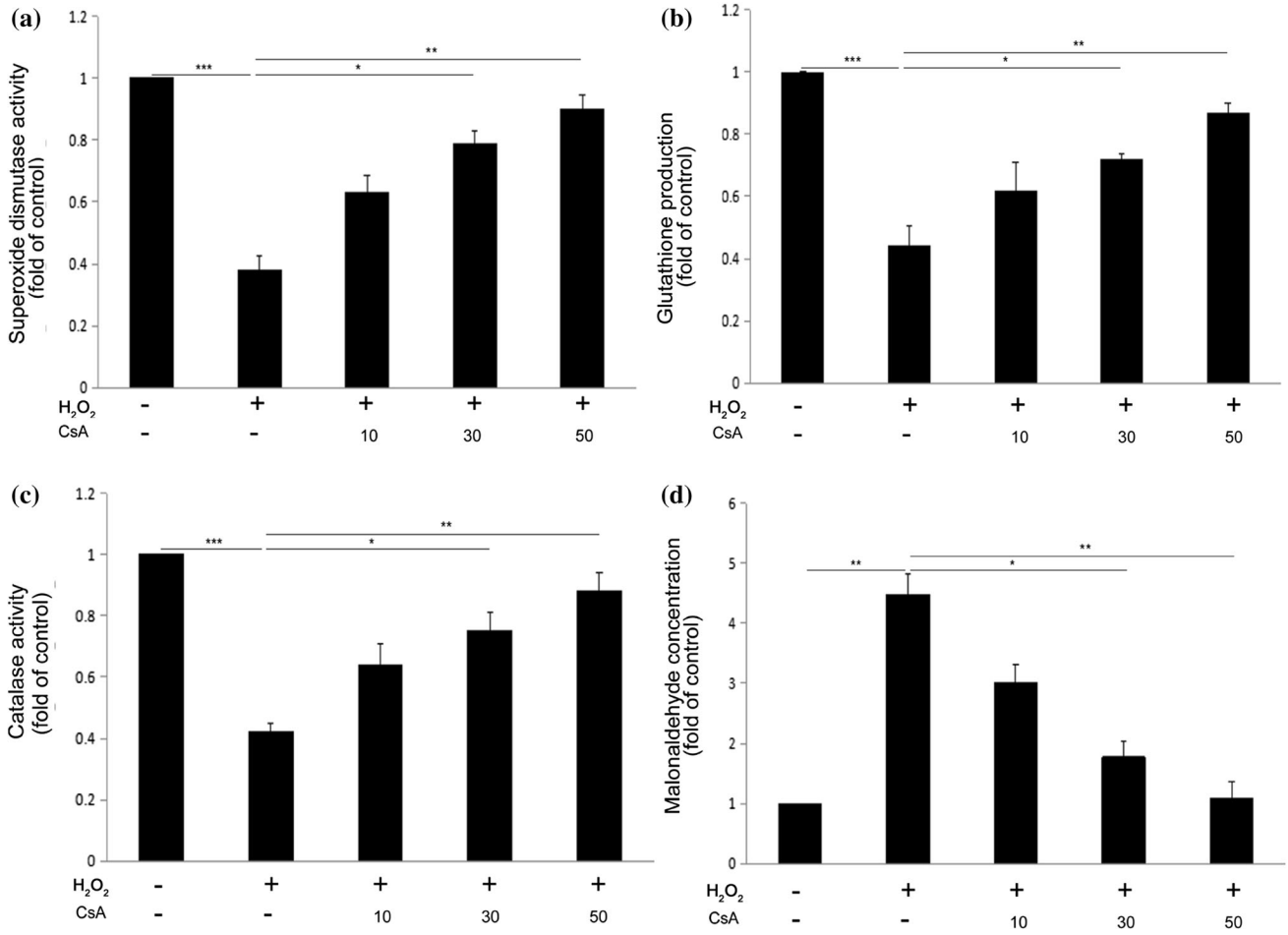

FIGURE 2. Effects of CsA on the levels of superoxide dismutase, glutathione, catalase, and malonaldehyde in cardiomyocytes exposed to H_2O_2. The oxidative abnormalities were obviously ameliorated by CsA treatment, as indicated by the significant increase in the superoxide dismutase activity, glutathione production, and catalase activity and decrease in the malonaldehyde concentration. (a) Superoxide dismutase assay. (b) Glutathione assay. (c) Catalase assay. (d) Malonaldehyde assay. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ocytes exposed to oxidative stress injury. These results are consistent with those of the lactate dehydrogenase assay.

Effect of CsA on H_2O_2-Induced Generation of Reactive Oxygen Species in Cardiomyocytes

The involvement of reactive oxygen species in the H_2O_2-induced apoptosis of cardiomyocytes was evaluated by measuring the level of reactive oxygen species produced. As a general index of reactive oxygen species production in cultured cells, DCF fluorescence analysis revealed that CsA decreased the amount of reactive oxygen species produced in cardiomyocytes exposed to oxidative stress injury (Figs. 3a and 3b).

Effect of CsA on $\Delta\psi m$ in Cardiomyocytes

CsA blocking of activities against H_2O_2-induced mitochondrial permeability transition pore (mPTP) opening was evaluated by JC-1 assay, which measures the change in the mitochondrial membrane potential ($\Delta\psi m$). Figure 4a demonstrates that CsA suppressed the H_2O_2-induced mitochondrial depolarization and maintained mitochondrial function in a dose-dependent manner.

Effect of CsA on H_2O_2-Induced Mitochondrial Abnormalities

After oxidative stress induced mitochondrial abnormalities, cyto-c was released into the cytosol

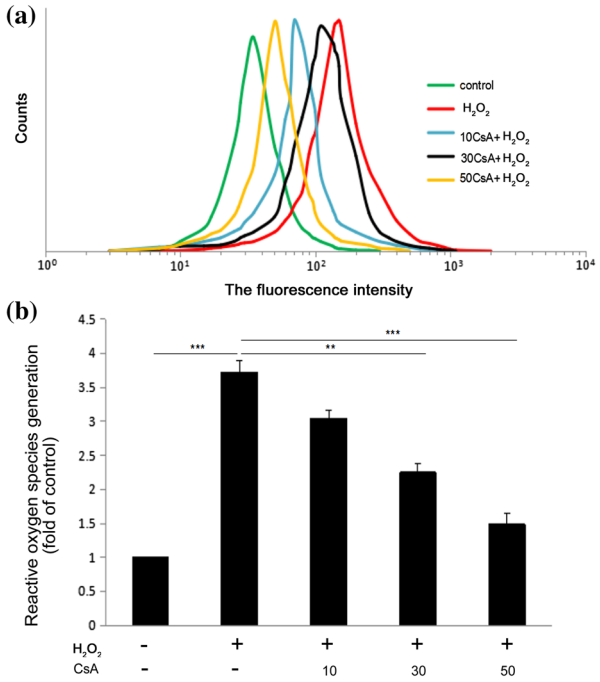

FIGURE 3. Effect of CsA on reactive oxygen species in cardiomyocytes exposed to H_2O_2. CsA inhibited the production of reactive oxygen species in a dose-dependent manner after H_2O_2 injury. (a) Flow cytometric analysis of reactive oxygen species fluorescence in each group. (b) Determination of reactive oxygen species generation by using the DCFH-DA fluorescence probe. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

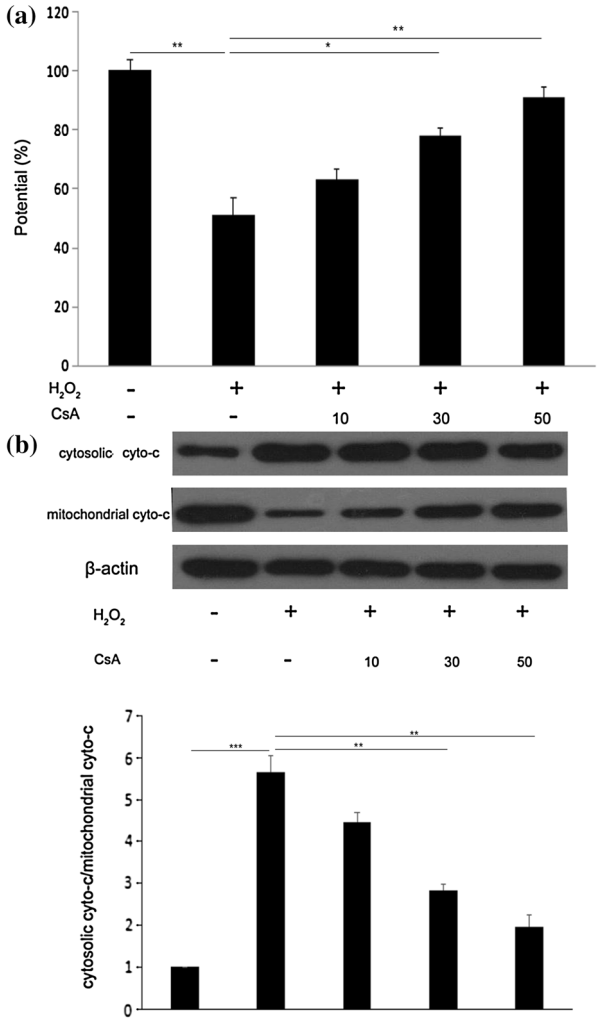

FIGURE 4. Effect of CsA on mitochondrial function in cardiomyocytes exposed to H_2O_2. (a) Determination of $\Delta\psi m$ by FACS. CsA attenuated the dissipation of $\Delta\psi m$ caused by H_2O_2 injury. (b) Determination of cyto-c by western blot. CsA reduced the release of cyto-c from the mitochondria. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

from the mitochondria. CsA suppressed the release in a dose-dependent manner. Moreover, CsA inhibited the H_2O_2-dependent loss of mitochondrial cyto-c in a dose-dependent manner. CsA also reduced the ratio of cyto-c between the cytosol and mitochondria (Fig. 4b).

Suppression of H_2O_2-Induced Effects on Tricarboxylic Acid Cycle (TCA) Enzymes in Cardiomyocytes by CsA

The mitochondrion plays the main role in ATP production. Therefore, decreased ATP viability corresponds to the degree of mitochondrial abnormalities. A luciferase-based assay for detection of cellular ATP levels was performed to validate the effect of CsA on the mitochondrial function. In the present study, CsA protected cardiomyocytes against H_2O_2-induced impairment of ATP production (Fig. 5a). CsA blocked the detrimental H_2O_2-induced mPTP opening and eventually repaired mitochondrial dysfunction by promoting ATP generation.

CsA exerted bioenergetic-related protective effects on the mitochondria isolated from cardiomyocytes exposed to oxidative stress injury. CsA prevented the H_2O_2-induced inhibition of the TCA cycle enzymes such as aconitase (Fig. 5b), α-ketoglutarate dehydrogenase (Fig. 5c), and succinate dehydrogenase (Fig. 5d).

CsA also rescued the activities of mitochondrial complex I enzyme (Fig. 6a) and complex V enzymes (Fig. 6b) in cardiomyocytes challenged with oxidative stress injury.

Effect of CsA on H_2O_2-Induced Cell Apoptosis in Cardiomyocytes

We analyzed the levels of several mitochondrion-related apoptotic parameters in cardiomyocytes exposed to oxidative stress injury to determine whether CsA could inhibit H_2O_2-triggered cell apoptosis. The western blot results showed that treatment with

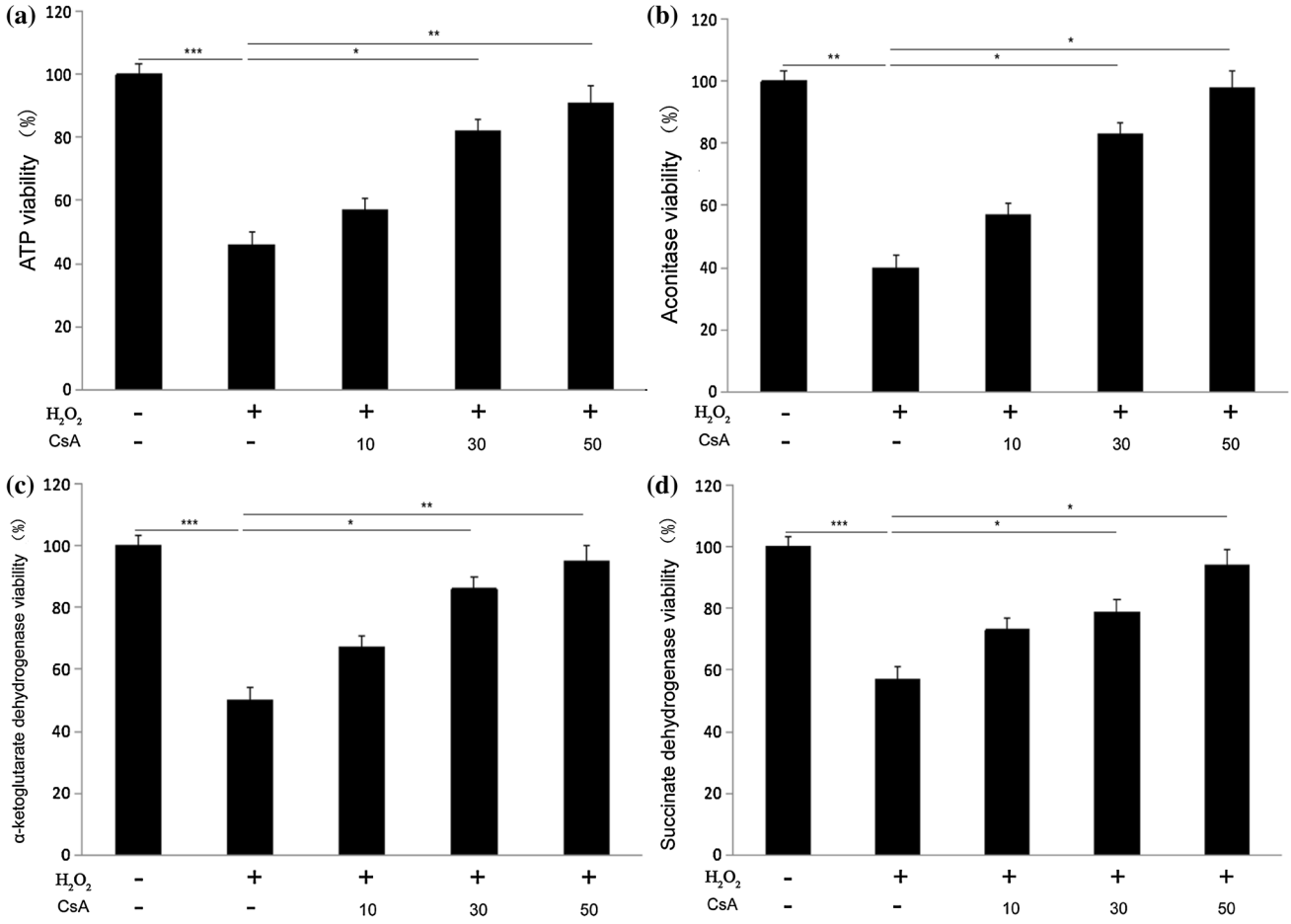

FIGURE 5. Effects of CsA on ATP and the activities of the mitochondrial enzymes such as aconitase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase in cardiomyocytes exposed to oxidative stress injury. (a) ATP assay. CsA reversed the down-regulation of ATP in cardiomyocytes exposed to H_2O_2. (b) Aconitase assay. CsA increased the aconitase level in cardiomyocytes after oxidative stress injury. (c) α-ketoglutarate dehydrogenase assay. CsA reversed the down-regulation of α-ketoglutarate dehydrogenase caused by oxidative stress injury. (d) Succinate dehydrogenase assay. CsA attenuated the effect of oxidative stress injury on succinate dehydrogenase. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

oxidative stress injury markedly upregulated the Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3 ratios in cardiomyocytes. Importantly, CsA significantly inhibited the increased ratios of Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3 (Fig. 7). CsA also significantly reduced the levels of cleaved PARP (Fig. 8a) and DNA fragmentation (Fig. 8b), which are hallmarks of apoptosis.

Anti-Inflammatory Effects of CsA on H_2O_2-Treated Cardiomyocytes

Figure 9a illustrates that CsA treatment attenuated the effects of oxidative stress injury on the secretion of IL-1β in cardiomyocytes. Moreover, CsA prevented the H_2O_2-elicited increase in the secretion of TNF-α in a concentration-dependent manner (Fig. 9b).

Oxidative stress injury induced the upregulation of iNOS, as shown by the western blot results. However, such up-regulation was dose-dependently prevented by CsA (Fig. 7). Similar effects were observed regarding NO release, as shown in Fig. 10. Oxidative stress injury also increased the NO level, but such increase was reduced by CsA.

Inhibition of H_2O_2-Induced Cell Injury in Cardiomyocytes by CsA Through the Suppression of the NF-κB Signaling Pathway

Oxidative stress injury induced the up-regulation of the p-IκB protein and the down-regulation of the IκB protein in cardiomyocytes (Fig. 11). These changes were significantly reversed by CsA. Thus, CsA pre-

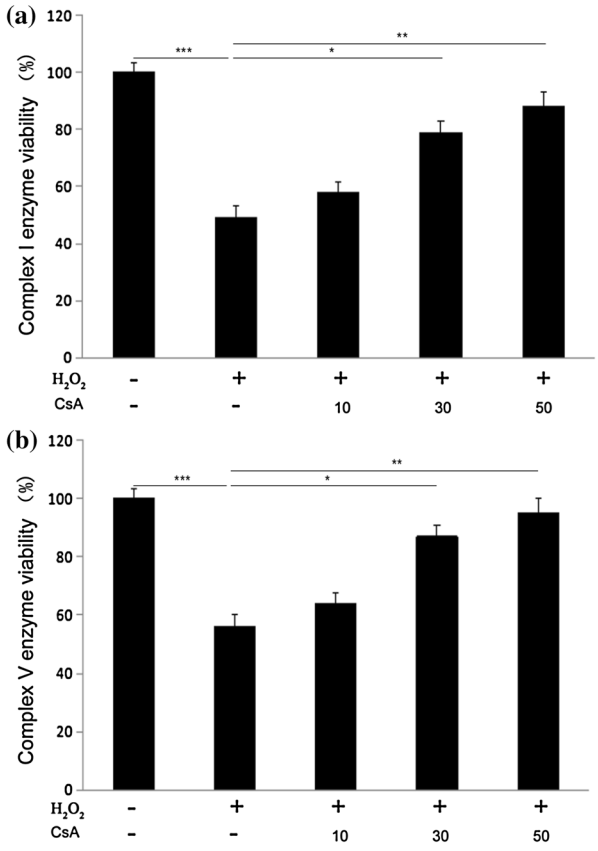

FIGURE 6. Effects of CsA on the activities of complex I enzyme and complex V enzyme in cardiomyocytes exposed to oxidative stress injury. (a) Complex I enzyme assay. CsA markedly increased the complex I enzyme level after H_2O_2 injury. (b) Complex V enzyme assay. CsA significantly increased the complex V enzyme caused by H_2O_2 injury in a dose-dependent manner. Each bar represents mean ± SEM. n = 3; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

vented H_2O_2-induced cell injury on cardiomyocytes by suppressing the NF-κB signaling pathway.

DISCUSSION

H_2O_2-induced death in cardiomyocytes is one of the most frequently applied experimental cell models of myocardial oxidative stress injury.[41] This model was used to further investigate the mechanisms of the cardioprotective effects of CsA in the present study. We have demonstrated that CsA protected the mitochondria and prevented the cell death of cardiomyocytes exposed to oxidative stress injury by a mechanism associated with the inhibition of NF-κB activation.

In this regard, CCK-8 assay was performed to evaluate the viability of cardiomyocytes after oxidative stress injury. CsA prevented H_2O_2-induced loss of cell viability in cardiomyocytes. CsA exhibited cardioprotective actions by suppressing H_2O_2-induced lactate dehydrogenase leakage in cardiomyocytes.

The mitochondria are important in biomedical studies, and mitochondrial dysfunction downregulates the mitochondrial levels of antioxidant capacities.[23,36] CsA exhibited antioxidant capacities against H_2O_2-induced death in cardiomyocytes. CsA suppressed the H_2O_2-induced inhibition of anti-oxidant defense systems, such as superoxide dismutase activity, glutathione production, and catalase activity and increased the malonaldehyde levels in the mitochondrial membranes of cardiomyocytes.

Pro-oxidant agents lead to mitochondrial dysfunction by triggering damage in the components of the mitochondrial membranes and matrix.[3,11] These structures play multiple roles, such as in reactive oxygen species generation and scavenging, ATP formation, cytochrome *c*, $\Delta\psi$m, and cell death.[4,30] Oxidative stress is a major contributor to the pathogenesis and prognosis of myocardial IR injury.[1,28] Thus, an important strategy for myocardial IR injury is to prevent the accumulation of reactive oxygen species. CsA suppressed the pro-oxidant effects of oxidative stress injury in cardiomyocytes.

Oxidative stress induced by reactive oxygen species could further impair the mitochondrial function for myocardial IR injury.[10,42] The mitochondria are susceptible to redox impairment, which has been associated with oxidative stress injury.[8,26] CsA attenuated redox impairment in mitochondrial membranes. CsA also prevented H_2O_2-induced loss of mitochondrial depolarization in cardiomyocytes.

We assessed the oxidative stress and mitochondrial function in cardiomyocytes. CsA significantly decreased the intracellular generation of reactive oxygen species and mitochondrial dysfunctions, release of cytochrome *c*, and $\Delta\psi$m. Thus, CsA may be a potential mPTP blocker to maintain mitochondrial function. H_2O_2-induced intracellular generation of reactive oxygen species, mitochondrial dysfunctions, and release of cytochrome *c* were considerably attenuated by CsA treatment.

The mitochondria are the major producers of ATP in mammalian cells because of the oxidative phosphorylation system.[12,17] Mitochondrial function plays a central role in myocardial IR injury, and the mitochondria participate in the modulation of cell death, which presents an interface with the redox biology of the cell.[16,37] In this study, CsA exhibited a capacity to interact directly or indirectly with the mitochondria. Moreover, mitochondria-related signaling is of clinical interest in preventing or treating mitochondrion-related diseases, such as myocardial IR injury. Consequently, CsA reversed the effect of oxidative stress injury on the ATP levels in cardiomyocytes and

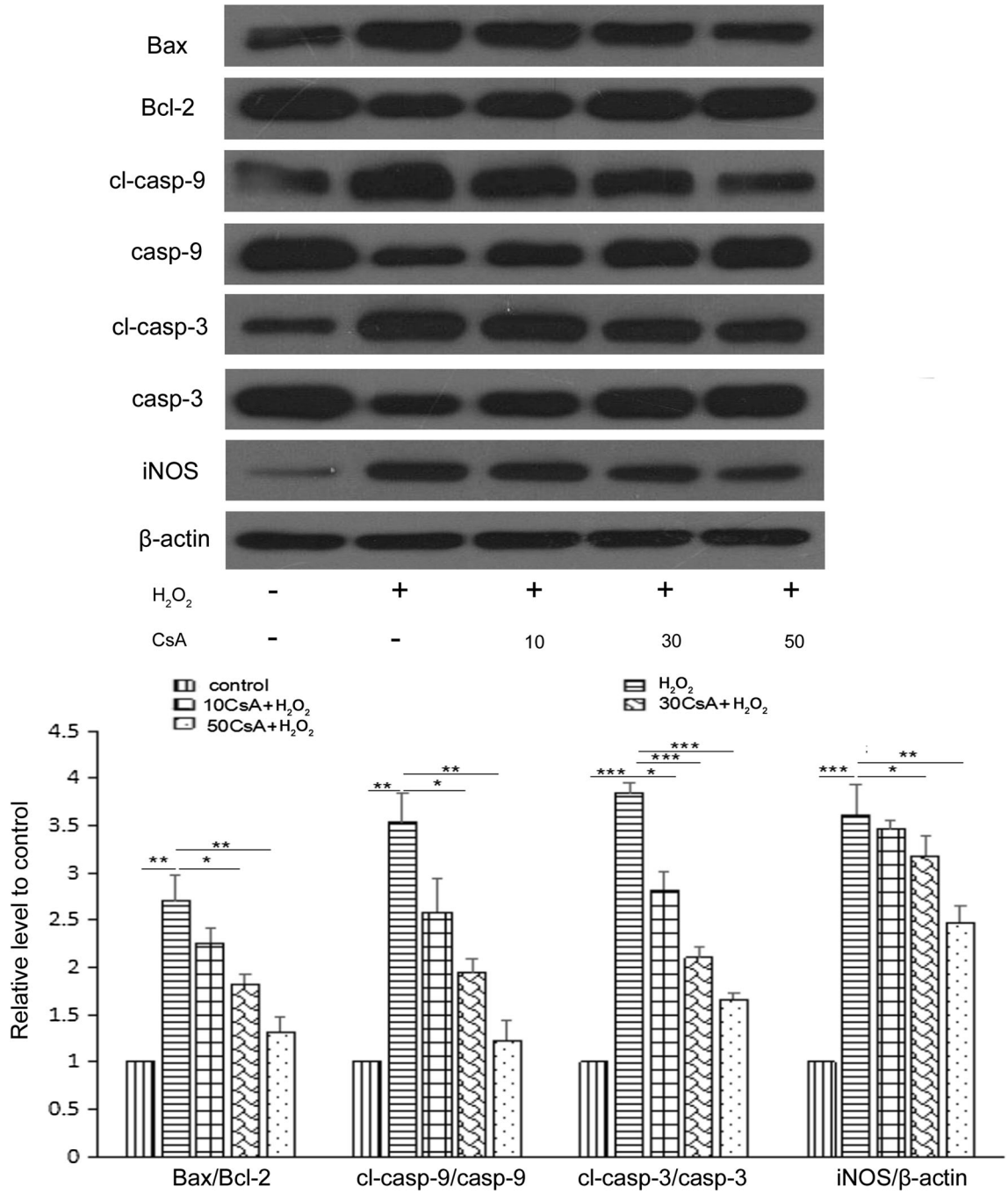

FIGURE 7. Effects of CsA on Bax/Bcl-2, cl-casp-9/casp-9, cl-casp-3/casp-3, and iNOS/β-actin ratios in cardiomyocytes exposed to H_2O_2. CsA significantly reduced the ratios of Bax/Bcl-2, cl-casp-9/casp-9, cl-casp-3/casp-3, and iNOS/β-actin compared with that in the H_2O_2 injury group. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

inhibited the activities of TCA cycle enzymes (aconitase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase) and the activities of mitochondrial complex I and complex V enzymes of cardiomyocytes challenged with oxidative stress injury.

Mitochondrial damage and cell death could be due to excessive generation of reactive oxygen species, which is a vicious cycle in myocardial IR injury.[21,34] The two former processes increased the formation of pro-apoptotic proteins and reduced the formation of anti-apoptotic proteins, thereby increasing the levels of reactive oxygen species.[19,29] The activities of pro-apoptotic proteins such as Bax, cleaved caspase-9, and cleaved caspase-3 were upregulated but were down-regulated by oxidative stress injury in cardiomyocytes.[9,18] CsA reversed these effects. The results

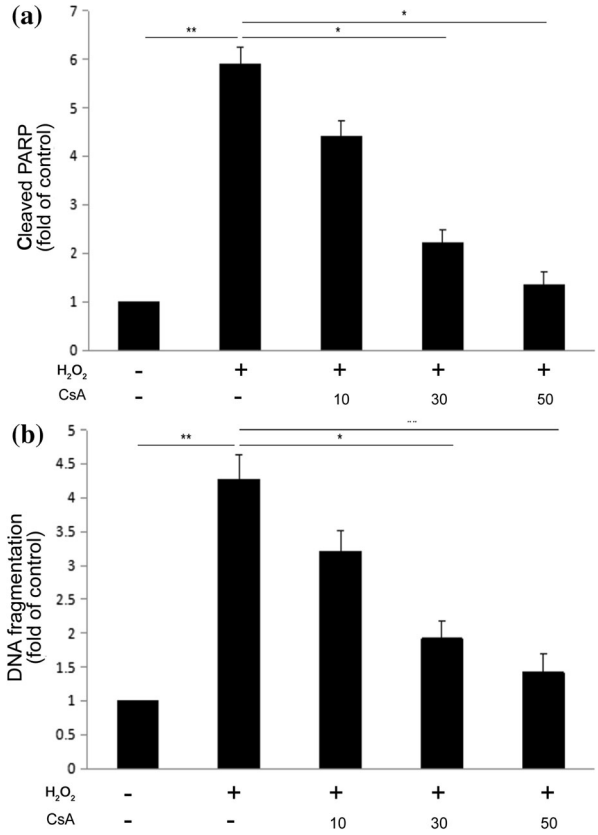

FIGURE 8. Effects of CsA on cleaved PARP and DNA fragmentation in cardiomyocytes exposed to H_2O_2. (a) Cleaved PARP. CsA reversed the level of cleaved PARP in cardiomyocytes after H_2O_2 injury. (b) DNA fragmentation. CsA significantly decreased the DNA fragmentation in cardiomyocytes after H_2O_2 injury. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

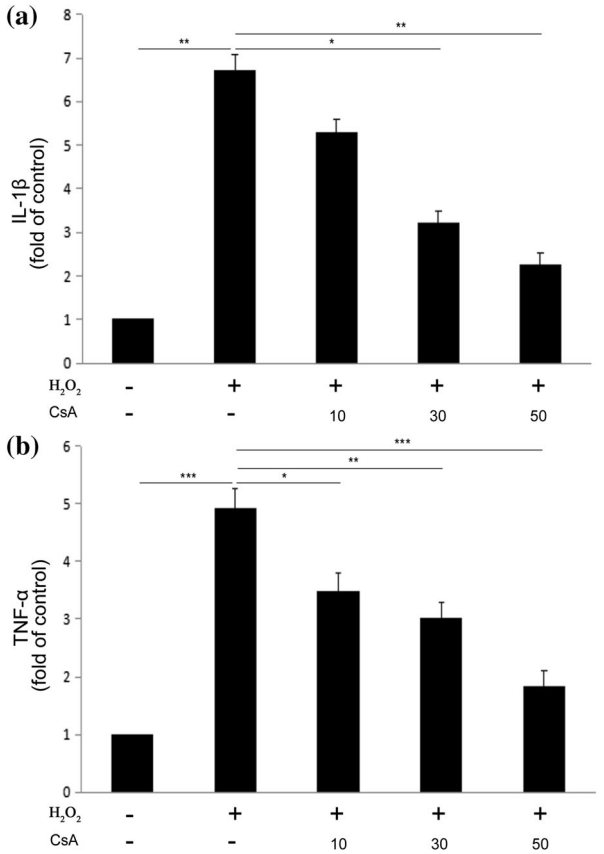

FIGURE 9. Effect of CsA on IL-1β and TNF-α in cardiomyocytes exposed to H_2O_2. (a) IL-1β was analyzed by ELISA. CsA significantly reduced the H_2O_2-induced increase in IL-1β. (b) TNF-α was analyzed by ELISA. CsA significantly reduced TNF-α caused by H_2O_2 injury. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

demonstrated that CsA significantly decreased Bax/Bcl-2 ratio, cleaved caspase-9/caspase-9, and cleaved caspase-3/caspase-3 in a concentration-dependent manner. In addition, CsA reduced the effect of the oxidative stress injury challenge on the levels of cleaved PARP and DNA fragmentation in cardiomyocytes.

Mitochondrial dysfunction and bioenergetics impairment have been observed in the pathogenesis of myocardial IR injury.[25,40] In this study, CsA was discovered as a new mPTP modulator that efficiently protects the mitochondria against H_2O_2-induced mitochondrial membrane depolarization.

NF-κB is the major modulator of the redox biology in mammalian cells.[32,33] NF-κB plays an important role in coordinating mitochondrion-related aspects and cell death, such as mitochondrion-related redox biology and Bax/Bcl-2 ratio, cleaved caspase-9/caspase-9, and cleaved caspase-3/caspase-3 in several experimental models.[24,39] H_2O_2-induced injury in cardiomyocytes could elevate NF-κB activity, which could be reversed by CsA treatment. CsA plays a role

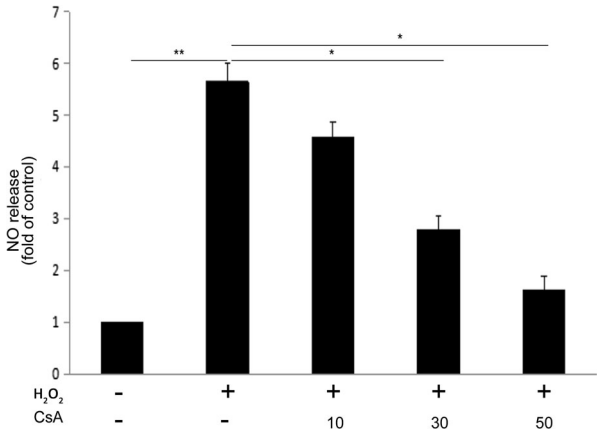

FIGURE 10. Effect of CsA on NO in cardiomyocytes exposed to H_2O_2. CsA remarkably inhibited NO release in a dose-dependent manner. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

in cytoprotection against oxidative stress injury that induced mitochondrion-related oxidative stress and cell death in cardiomyocytes. CsA protected the

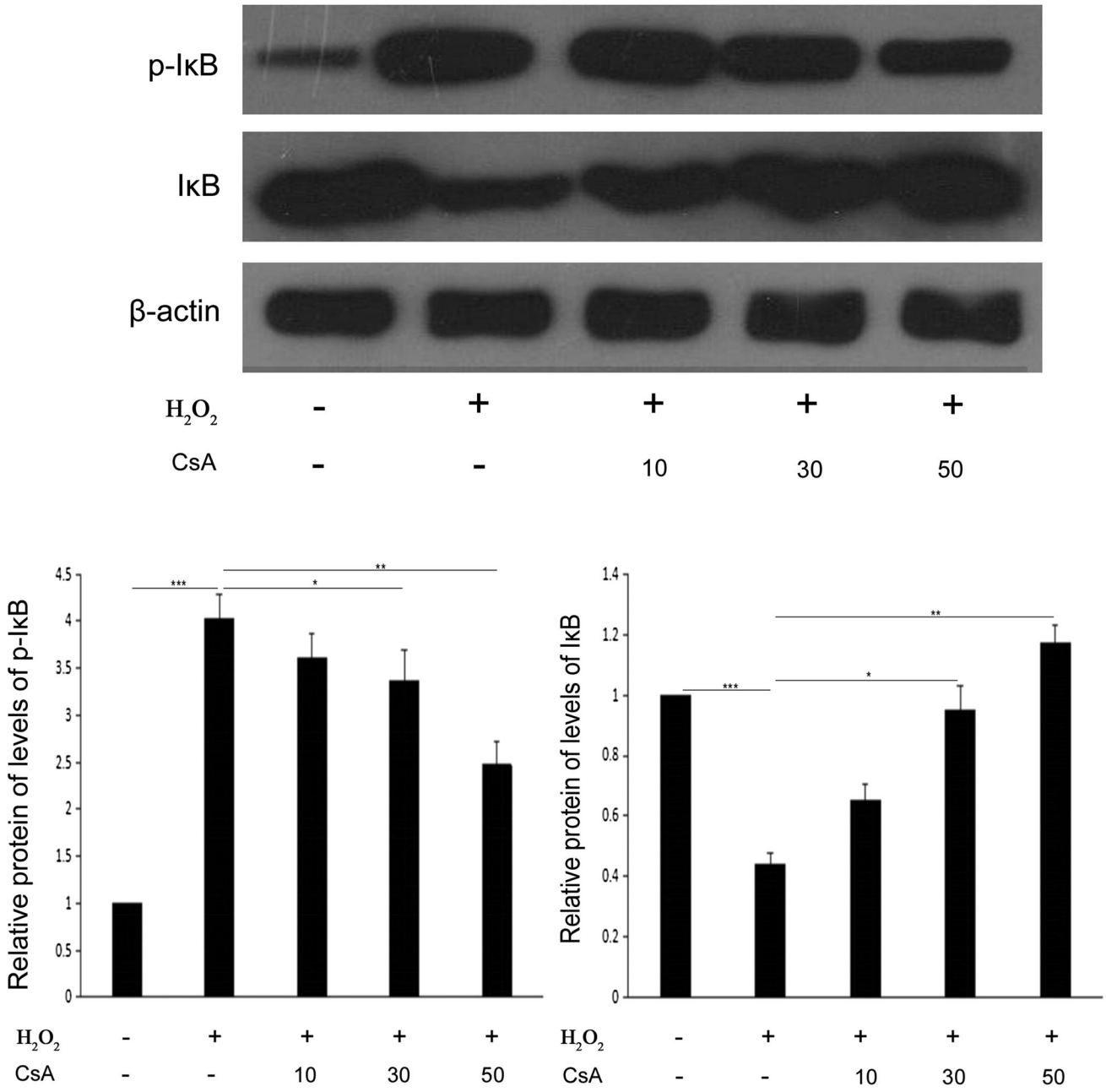

FIGURE 11. CsA significantly inhibited H_2O_2-induced activation of the NF-κB signaling pathway by reducing p-IκB expression and increasing IκB expression. Each bar represents mean ± SEM. $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

mitochondrial function by a mechanism related to the NF-κB signaling pathway. The inhibition of the NF-κB signaling pathway by CsA attenuated the effects of oxidative stress injury on the redox state of the mitochondrial membranes and also on the function of the TCA cycle and complexes I and V of the mitochondria. CsA protected cardiomyocytes by inhibiting the NF-κB signaling pathway, which was activated by exposure to oxidative stress injury. The cardioprotective effect of CsA was associated with the downregulated NF-κB signaling. Our findings imply that CsA may possess potential therapeutic benefits for myocardial IR injury.

Disruption of the inner mitochondrial membrane enhances the generation of reactive species and cell apoptosis by the organelles. This process causes inflammatory damages in myocardial IR injury.[7,27] As pro-inflammatory cytokines, IL-1β and TNF-α exhibited pro-inflammatory effects by networking with particular receptors in cell membranes and regulating the NF-κB pathway.[20,35] NO is regulated by the NF-κB pathway, and iNOS has attracted

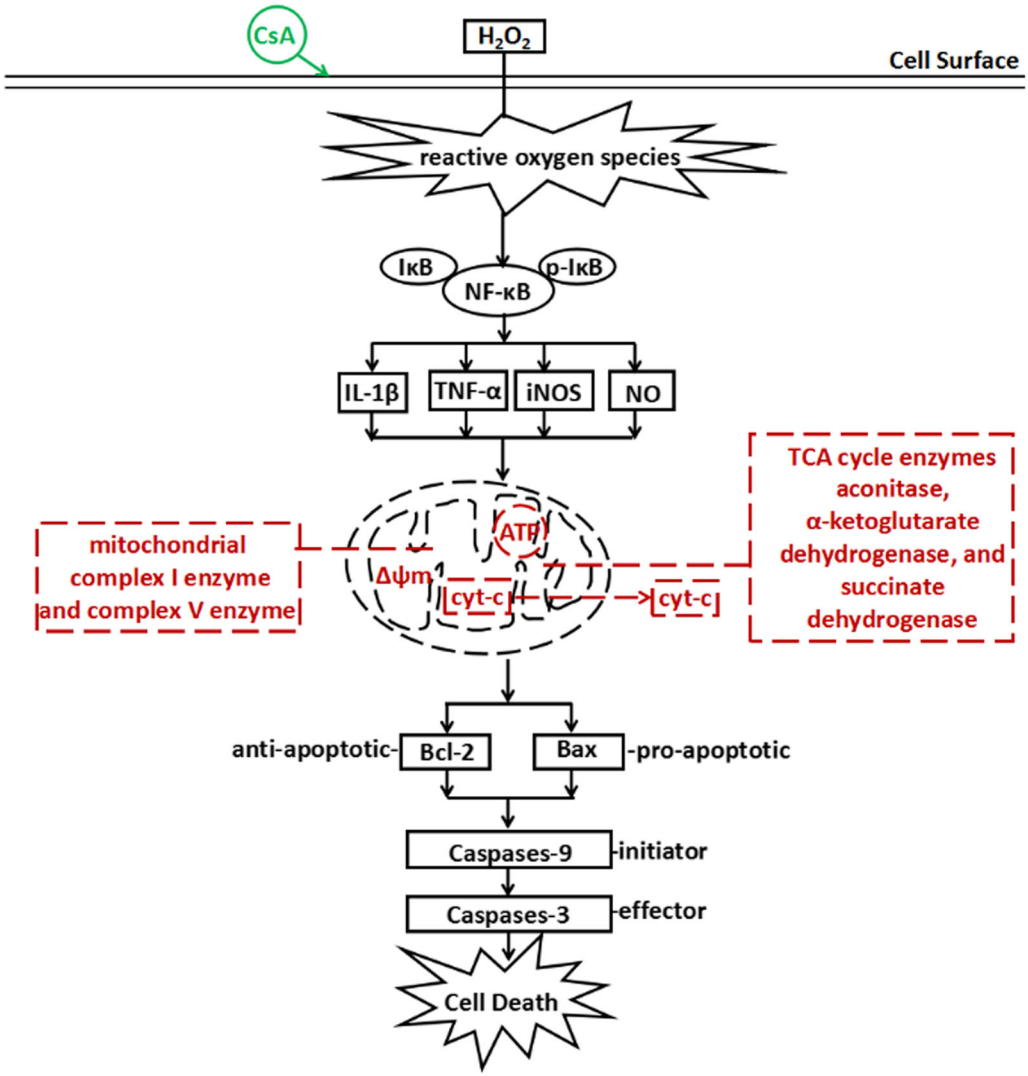

FIGURE 12. CsA affected the H_2O_2-induced death in cardiomyocytes by inhibiting the NF-κB signaling pathway. CsA attenuated intracellular reactive oxygen species generation induced by H_2O_2 injury and suppressed the downstream NF-κB signaling pathway. CsA reduced the pro-apoptotic Bax expression and increased the anti-apoptotic Bcl-2 expression by regulating the function of the mitochondria. CsA inhibited cytochrome *c* release, activation of caspase-9 and caspase-3, ATP reduction, and activation of the TCA cycle enzymes and mitochondrial complex I and complex V enzymes. CsA suppressed the protein expression of IL-1β, TNF-α, and iNOS and decreased the NO release. These results are related to the downregulation of NF-κB activation.

attention due to its ability to modulate redox and inflammatory aspects in myocardial IR injury.[14,15] Furthermore, CsA induced anti-inflammatory effects by abolishing the H_2O_2-dependent activation of the NF-κB and the upregulation of IL-1β, TNF-α, iNOS, and NO. CsA inhibited the H_2O_2-induced expression of iNOS and the production of reactive oxygen species and NO. CsA suppressed the activation of NF-κB by inhibiting p-IκB degradation and the promotion of IκB evolution. CsA-triggered anti-inflammatory effects are dependent on the NF-κB signaling pathway. Hence, CsA inhibited H_2O_2-induced inflammatory mechanisms by inhibiting NF-κB activation.

Our previous study suggested that CsA could reduce $CoCl_2$-induced hypoxic injury in H9c2 cells by inhibiting the p38 and ERK MAPK signaling pathways. This finding is partly consistent with our present work. In this study, CsA could protect oxidative stress-induced cardiomyocyte apoptosis by inhibiting the activation of the NF-κB signaling pathway. We will conduct further studies to determine the upstream and downstream relationship between the p38 and ERK MAPK signaling pathways and the NF-κB signaling pathway. We provided clear evidence that CsA treatment can protect against oxidative stress injury in cardiomyocytes by suppressing mitochondrion-related apoptosis and inducing anti-inflammatory ef-

fects (Fig. 12). In conclusion, CsA is a good candidate for further development of new therapeutics for myocardial IR injury.

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CONFLICT OF INTEREST

Author Meng Ma declares that he has no conflict of interest. Author Xiaohui Ma declares that she has no conflict of interest. Author Jie Cui declares that she has no conflict of interest. Author Yifeng Guo declares that she has no conflict of interest. Author Xiuqin Tang declares that she has no conflict of interest. Author Chuanmin Chen declares that he has no conflict of interest. Author Ying Zhu declares that she has no conflict of interest. Author Chao Cui declares that she has no conflict of interest. Author Gang Wang declares that he has no conflict of interest.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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