Am J Transl Res 2016;8(6):2597-2608
www.ajtr.org /ISSN:1943-8141/AJTR0029879

*Original Article*

# Isoflurane reduces oxygen-glucose deprivation-induced oxidative, inflammatory, and apoptotic responses in H9c2 cardiomyocytes

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Received April 5, 2016; Accepted June 13, 2016; Epub June 15, 2016; Published June 30, 2016

**Abstract:** Isoflurane (ISO) protects the heart from hypoxia-reperfusion injury. However, the molecular mechanisms of ISO in oxygen-glucose deprivation (OGD)-induced H9c2 cardiomyocyte injury is yet to be understood. Using H9c2 cells cultured in vitro, we examined the cytotoxicity of different doses of ISO (0.7%, 1.4%, and 2.1%) to H9c2 cells and found that 2.1% ISO had significant toxicity to the cell. Thus, 1.4% ISO was selected for the subsequent experiments. ISO notably ameliorated cell viability loss, lactate dehydrogenase release, and creatine kinase activity of H9c2 cells that were treated with OGD. ISO suppressed OGD-induced pro-inflammatory tumor necrosis factor-α, interleukin (IL)-1β, IL-6, IL-8 production, and nuclear factor (NF)-κB activation in H9c2 cells. ISO reduced the reactive oxygen species and malondialdehyde generation, but it enhanced the superoxide dismutase activity in OGD-stimulated H9c2 cells. In addition, diminished OGD-induced cell apoptosis and preserved mitochondrial membrane potential were observed in ISO-treated H9c2 cells. ISO markedly up-regulated the anti-apoptotic Bcl-2expression but inhibited the pro-apoptotic expressions of Bax, procaspase-3, cleaved caspase-3, and caspase-3 activity. Mechanistically, the cardioprotective effects of ISO on OGD-induced H9c2 cell injury were mediated by the Akt signaling pathway. These findings suggest that ISO alleviates OGD-induced H9c2 cell injury and may therefore be used to prevent and treat ischemic heart diseases.

**Keywords:** Isoflurane, H9c2 cardiomyocyte, inflammation, apoptosis, oxygen-glucose deprivation

## Introduction

Cardiovascular disease (CVD), such as the ischemic heart disease, poses a major threat to human health and is one of the leading causes of disability and death worldwide [1]. Despite rapid progresses in the treatment of CVD and improved life quality of patients, no effective therapy is currently available for preventing CVD [2]. Thus, investigating its pathogenesis and finding the possible prevention strategies are hot topics in the medical research field.

Oxygen-glucose deprivation (OGD) has been reported to create the cellular model of ischemic heart injury using H9c2 cardiomyocytes [3-5]. OGD-induced myocardial injuries are a complex pathophysiological process that includes reactive oxygen species (ROS) generation, calcium overloading, and mitochondrial permeability transition pore opening, and they ultimately lead to cell death or apoptosis [6]. Many pro-inflammatory cytokines are released during the processes of ischemic heart injury, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 [7, 8]. NF-κB is a nuclear transcription factor that regulates the gene expression involved in inflammation and apoptosis in various diseases, such as ischemic heart injury [9]. Reportedly, ROS can provoke NF-κB activation in lipopolysaccharide-induced H9c2 cardiomyocyte injury [10]. Therefore, the inhibition of inflammation, oxidative stress, and apoptosis may attenuate OGD-induced ischemic heart injury.

Many studies have shown that both preconditioning and postconditioning of isoflurane (ISO) can protect against reperfusion injury after

myocardial ischemia in vitro and in vivo [11-14]. ISO preconditioning can inhibit cardiac myocyte apoptosis during oxidative and inflammatory stress by activating Akt and enhancing Bcl-2 expression [14]. The cardioprotective mechanisms of ISO postconditioning have been shown to involve the activation of phosphoinositide 3-kinase (PI3K)/Akt signaling [15]. In addition, ISO postconditioning has been reported to exert anti-inflammatory, anti-oxidative, and anti-apoptotic effects on experimental lung injury [16]. However, the cardioprotective effects and underlying mechanisms of ISO on OGD-induced H9c2 cell injury need further investigation.

Here, we evaluated the cardioprotective effects of ISO on OGD-stimulated H9c2 cells. We found that 1.4% ISO had no significant toxicity to the cells and was thus selected for the subsequent experiments. ISO promoted cell survival and inhibited lactate dehydrogenase (LDH) release and creatine kinase (CK) activity of the OGD-treated H9c2 cells. ISO reduced the levels of pro-inflammatory cytokines (TNF-α, IL-1b, IL-6, and IL-8) and inhibited the activation of NF-κB in H9c2 cells exposed to OGD. ISO also diminished OGD-induced ROS burst and increased anti-oxidative defense in H9c2 cells. In addition, the apoptotic H9c2 cells resulted from OGD stimulation was decreased by ISO treatment. Mechanistically, the cardioprotective effects of ISO were mediated by PI3K/Akt signaling in H9c2 cells subjected to OGD.

## Materials and methods

### *Cell culture*

The rat cardiomyocyte cell line H9c2 was cultured in Dulbecco's modified Eagle medium (DMEM)/F12 medium (Gibco, BRL, UK), which was supplemented with 10% fetal bovine serum (Gibco), penicillin G (100 U/mL), streptomycin (100 mg/mL), and glutamine (2 mM; all from Sigma-Aldrich, St. Louis, MO, USA), at 37°C in a humidified incubator with 5% $CO_2$.

### *OGD and ISO treatment*

For OGD stimulation, H9c2 cells were cultured in glucose-free DMEM medium and placed in an anaerobic chamber with $N_2/CO_2$ (95%/5%) at 37°C for 3 h. Then, the cells were cultured in normal DMEM medium in a normal cell incubator (normal conditions). The cells cultured under normal conditions all the time were used as control (Ctrl). For ISO treatment, at 3 h after OGD treatment, the cells were exposed to 0.7%, 1.4%, or 2.1% of ISO (Baxter Healthcare Corporation, Deerfield, IL, USA) for 0.5 h in a metabolic chamber (Columbus Instruments, Columbus, OH, USA) at 2 L/min. During exposure, the concentration of ISO was monitored with a Datex Capnomac (SOMA Technology Inc., Cheshire, CT, USA). Then, the cells were cultured under normal conditions. The cells without ISO treatment were exposed to room air (RA) as the control. To investigate the inhibitory effects of PI3K inhibitors LY294002 and wortmannin (both obtained from Calbiochem, LaJolla, CA, USA), H9c2 cells were pretreated with or without LY294002 (1 μM) or wortmannin (10 μM) for 1 h, washed out, and subjected to OGD for 3 h, followed by 0.5 h of ISO exposure.

### *Cell viability assay*

H9c2 cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Briefly, the cells were seeded into 96-well culture plates at a density of $5 \times 10^3$ cells/well and treated as described in the section on OGD and ISO treatment. Twenty-four hours after the OGD stimulation, MTT (20 μL; 5 mg/mL) solution was added to each well. After 4 h of incubation, the solution was removed, and the produced formazan was solubilized in dimethyl sulfoxide (150 μL) at 37°C for 30 min. The optical density was determined at 570 nm using a Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

### *Measurement of LDH and CK levels*

The release of LDH and CK, two indicators of cytotoxicity, was detected using colorimetric assay. In brief, 24 h after H9c2 cells were treated with OGD, the supernatants were collected for the measurement of LDH and CK levels. The activities of LDH and CK were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) in accordance with the protocols of the manufacturers. Absorbance values were read at 490 nm for LDH and at 660 nm for CK on a Microplate Reader (Molecular Devices).

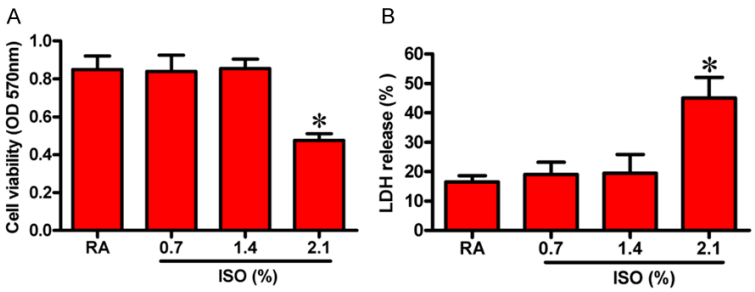


**Figure 1.** Effects of ISO on the viability and LDH release of H9c2 cells. H9c2 cells were exposed to various concentrations of ISO (0, 0.7%, 1.4%, or 2.1%) for 0.5 h. The cells were continuously cultured in normal conditions for 24 h. A. MTT assay was performed to assess the effect of ISO on cell viability. B. LDH release of H9c2 cells was measured in the medium. Data were expressed as means ± SD from three independent experiments. *P < 0.05 vs. room air (RA) group.

*Quantitative real-time polymerase chain reaction (qPCR)*

After 24 h of exposure to OGD, the total RNA was extracted from the H9c2 cells with different treatments using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and then reverse transcribed and synthesized into cDNA using SuperScript First-Strand Synthesis system (Invitrogen). qPCR was performed with Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). The threshold cycle (Ct) was obtained from triplicate samples and averaged. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and standardized byglycerinaldehyde-3-phosphate-dehydrogenase (GAPDH). Primers used for PCR amplification were: for TNF-α, 5'-CCTGCCCCAATCCCTTTATT-3' (forward), and 5'-CCAATTCTCTTTTTGAGCCAGAA-3' (reverse); for IL-1β, 5'-CAACCAACAAGTGATATTCTCCATG-3' (forward), and 5'-GATCCACACTCTCCAGCTG-CA-3' (reverse); for IL-6, 5'-CTGCGCAGCTTTA-AGGAGTTC-3' (forward), and 5'-TCTGAGGTGCC-CATGCTACA-3' (reverse); for IL-8, 5'-GGCAGC-CTTCCTGATTTCTG-3' (forward), and 5'-CTTGG-CAAAACTGCACCTTCA-3' (reverse); and for GA-PDH, 5'-AACGACCCCTTCATTGAC-3' (forward), and 5'-TCCACGACATACTCAGCAC-3' (reverse).

*Enzyme-linked immunosorbent assay (ELISA)*

After 24 h of being exposed to OGD, the levels of cytokines in the supernatants of the H9c2 cells with different treatments were measured using commercially available ELISA kits. Rat TNF-α, IL-1β, IL-6, and IL-8 ELISA kits were obtained from R&D systems (Minneapolis, MN, USA). All of the experiments were performed as instructed by the manufacturers.

*Western blot analysis*

The nuclear and cytoplasmic proteins of H9c2 cells with different treatments were prepared using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Grand Island, NY, USA). The two ultimate extracts were measured by using a standard Western blot technique. Equal amounts of protein from each sample were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel and then were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) for immunoblotting. The membranes were blocked with 5% non-fat milk in TBST buffer (50 mM Tris, pH 7.4; 250 mM NaCl; 0.1% Tween 20) and probed with rabbit anti-rat antibodies against NF-κB p65, IκB-a (both obtained from Abcam, Cambridge, UK), Bax, Bcl-2, procaspase-3, cleaved caspase-3 (all from Santa Cruz, Dallas, Texas, USA), phosphorylated (p)-Akt (Ser473), Akt, lamin B, and β-actin (all from Cell Signaling Technology Danvers, MA, USA) overnight at 4°C. Then, the membranes were exposed to the horseradish-peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescent detection reagents (Pierce, Rockford, IL, USA). Band intensities were quantified and normalized to loading controls, and the gray values were analyzed using QuantityOne software (Bio-Rad, USA).

*ROS detection*

The intracellular ROS production of H9c2 cells with different treatments was measured using a fluorescent probe, namely, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, the cells were incubated with 5 μM DCFH-DA for 30 min at 37°C in the dark. The fluorescent dye was then discarded, and the cells were washed twice with PBS. The cells were harvest-

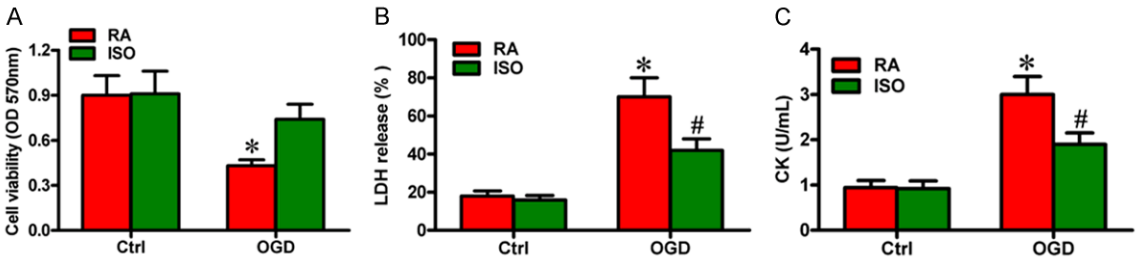


**Figure 2.** ISO inhibited OGD-induced H9c2 cell injury. After 3 h of OGD stimulation, H9c2 cells were exposed to 1.4% ISO for 0.5 h and subsequently cultured in normal conditions. (A) Cell viability was assessed by MTT assay. The levels of LDH (B) and CK (C) were determined in the medium. Data were expressed as means ± SD from three independent experiments. *P < 0.05 vs. control (Ctrl) groups. #P < 0.05 vs. OGD + RA group.

ed for fluorescence intensity (excitation wavelength 484 nm and emission wavelength 501 nm) detection using a BD FACSCalibur (San Jose, CA, USA). The relative levels of ROS were quantitatively determined based on the mean fluorescence intensity.

*Measurement of MDA and SOD*

MDA level and SOD activity in supernatants of the H9c2 cells with different treatments were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the instructions of the manufacturers. Briefly, the cells were collected and centrifuged at 250 *g* for 5 min, and the supernatants were used for measuring the MDA level and SOD activity.

*Flow cytometry assay of cell apoptosis*

The apoptosis of H9c2 cells was analyzed by flow cytometry using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis kit (BD Bioscience, MA, USA). Briefly, after the indicated treatments, the cells were collected, centrifuged, and resuspended in 500 μL of binding buffer. Then, the cells were incubated with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI; Sigma) for 15 min at room temperature in the dark and detected by flow cytometry with BD FACSCalibur. The results were analyzed by CellQuest software (BD Bioscience).

*Mitochondrial membrane potential (ΔΨm) assay*

ΔΨm was determined by the J-aggregate forming lipophilic cationic probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. JC-1 stains the mitochondria in cells with a high ΔΨm by forming red fluorescence J-aggregates, whereas JC-1 is present as a green fluorescent monomer, in cells with depolarized mitochondria. In this way, mitochondrial depolarization can be determined by a decreased ratio of red-to-green fluorescence intensity. Briefly, after the indicated treatments, JC-1 (0.5 μg/mL) was added to the H9c2 cells and then incubated for 10 min at 37°C. Subsequently, the cells were washed twice with PBS, incubated in 1 mL of culture medium, and analyzed under a fluorescence microscope (Olympus, Tokyo, Japan). To measure red fluorescence, an excitation wavelength of 550 nm and an emission wavelength of 600 nm were used; to measure green fluorescence, an excitation wavelength of 488 nm and an emission wavelength of 610 nm were used. The ratio of fluorescence intensity of red to green in cells was used as a marker of ΔΨm.

*Caspase-3 activity assay*

Caspase-3 activity was measured with a Caspase Activity Kit from Beyotime Institute of Biotechnology. The colorimetric assay was based on the hydrolysis of the amino acid chain acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), thus resulting in the release of the pNA. Briefly, after the indicated treatments, the H9c2 cells were lysed, and the supernatant was mixed with buffer containing the substrate peptides for the caspase attached to pNA. The release of pNA was quantified by determining the absorbance with a Microplate Reader (Molecular Devices) at 405 nm. Caspase-3 activity was expressed as the fold change compared with the control value.

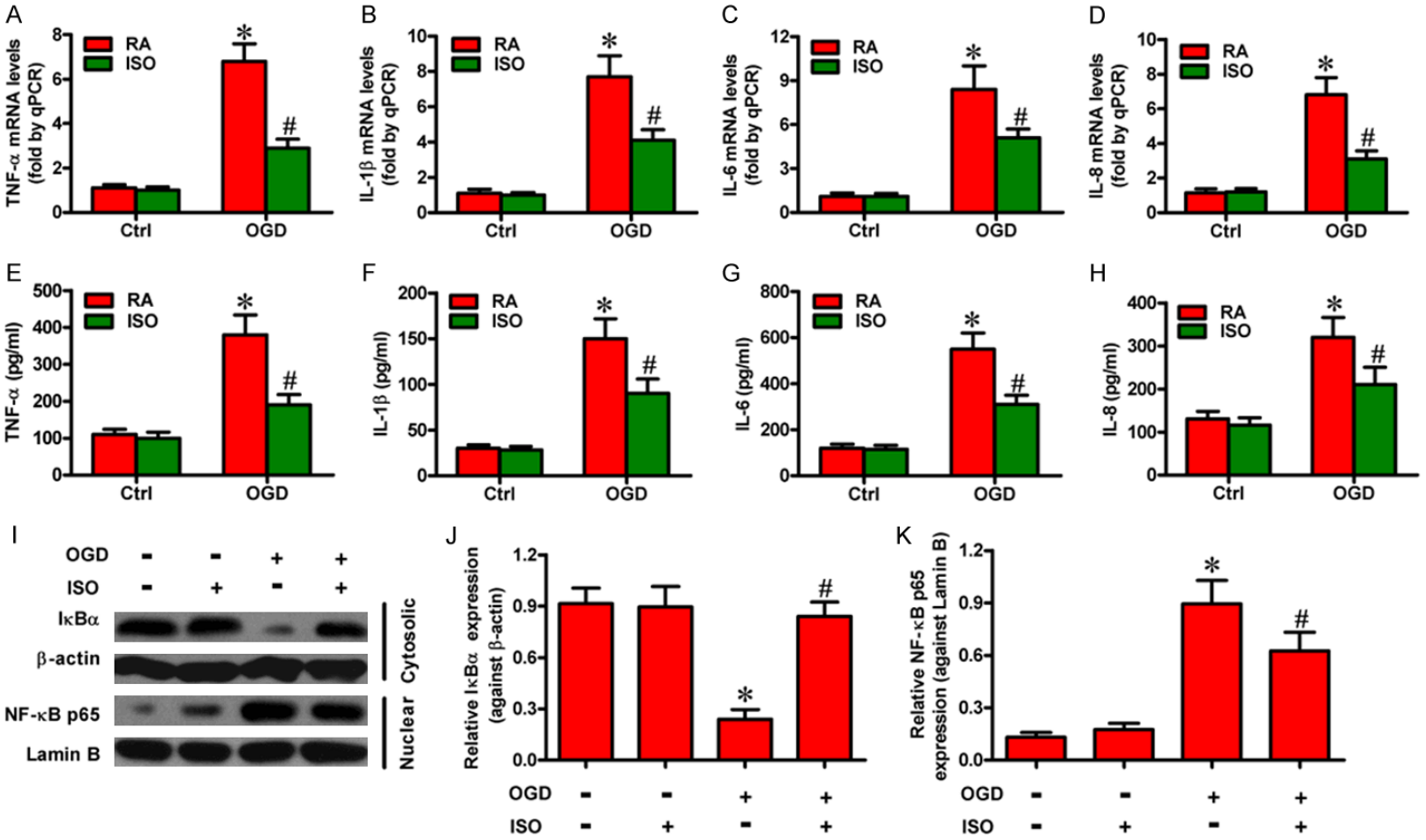


**Figure 3.** ISO inhibited OGD-induced pro-inflammatory cytokine mRNA expression and release and NF-κB activation in H9c2 cells. After 3 h of OGD stimulation, H9c2 cells were exposed to 1.4% ISO for 0.5 h and subsequently cultured in normal conditions. (A-D) qPCR assay was performed to evaluate the mRNA expression of TNF-α (A), IL-1b (B), IL-6 (C), and IL-8 (D). GAPDH was used as the endogenous control. (E-H) The release of TNF-α (E), IL-1β (F), IL-6 (G), and IL-8 (H) in the supernatants of the cells were assessed by ELISA. I. Representative Western blot results of IkB-a in the cytosol and NF-kB p65 in the nucleus. (J and K) Quantitative expressions of IkB-a normalized against b-actin (J) and NF-kB p65 normalized against lamin B (K). Data were expressed as means ± SD from three independent experiments. *P < 0.05 vs. Ctrl groups. #P < 0.05 vs. OGD + RA group.

*Statistical analysis*

All data are expressed as mean ± standard derivation (SD). Student's t-test was used to compare the difference between two groups and one-way ANOVA. Then, Dunnett's post hoc test was applied for more than two groups. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

## Results

*Cytotoxic effects of ISO on H9c2 cells*

We first investigated the cytotoxicity of ISO to H9c2 cells using MTT and LDH assays. MTT assay showed that the two lower doses of ISO (0.7% and 1.4%) exhibited no significant inhibitory effects on the viability of H9c2 cells. However, 2.1% ISO led to a notable reduction in the number of survivor cells (**Figure 1A**). The release of LDH was much higher in 2.1% ISO-treated cells than that in 0.7% or 1.4% ISO-treated cells (**Figure 1B**). These data suggested that 2.1% ISO conferred cytotoxicity to H9c2 cells. Thus, 1.4% ISO was selected for the subsequent experiments.

*ISO protected H9c2 cells against OGD-induced injury*

The results of MTT assay showed that OGD exposure significantly inhibited H9c2 cell viability, which was alleviated by ISO treatment (**Figure 2A**). Consistently, the release of LDH in H9c2 cells with OGD stimulation was significantly higher than that of the control group. However, ISO treatment markedly attenuated the increase in the release of LDH in OGD-treated H9c2 cells (**Figure 2B**). Similarly, the increased level of CK induced by OGD was obviously inhibited by ISO exposure (**Figure 2C**). These results demonstrated that ISO effectively inhibited OGD-induced H9c2 cell injury.

*ISO reduced OGD-induced pro-inflammatory cytokine production and NF-κB activation in H9c2 cells*

To investigate the anti-inflammatory effects of ISO on OGD-treated H9c2 cells, we performed qPCR and ELISA assays to assess the mRNA expression and the release of TNF-α, IL-1β, IL-6, and IL-8. As shown in **Figure 3A-D**, OGD insult resulted in significant increases in mRNA expressions of TNF-α (**Figure 3A**), IL-1β (**Figure 3B**), IL-6 (**Figure 3C**), and IL-8 (**Figure 3D**). However, the treatment of ISO reduced these increases from H9c2 cells that underwent OGD. Consistently, the enhanced release of TNF-α (**Figure 3E**), IL-1β (**Figure 3F**), IL-6 (**Figure 3G**), and IL-8 (**Figure 3H**) induced by OGD was considerably decreased by ISO treatment. To confirm whether or not NF-κB was involved in the anti-inflammatory effects of ISO on H9c2 cells with OGD exposure, Western blot was performed to evaluate the expression of IκB-α in the cytosol and NF-κB p65 in the nucleus of OGD-stimulated H9c2 cells. OGD significantly induced the down-regulation of IκB-α (**Figure 3I** and **3J**) in the cytosol and up-regulation of NF-κB p65 (**Figure 3I** and **3K**) in the nucleus of H9c2 cells. However, ISO treatment reversed the changes in these two proteins (**Figure 3I-K**). These results indicated that ISO inhibited OGD-induced inflammatory cytokine production and NF-κB activation in H9c2 cells.

*ISO inhibited OGD-induced oxidative stress and increased anti-oxidative defense in H9c2 cells*

Oxidative stress plays an essential role in the pathogenesis of ischemic heart disease. To confirm the anti-oxidative effect of ISO, we first examined ROS production in H9c2 cells with OGD exposure followed by ISO treatment. As shown in **Figure 4A**, H9c2 cells with OGD stimulation produced extensive ROS production, whereas ISO reduced the increase in ROS generation. In addition, the increased production of MDA, a biomarker of oxidative stress, induced by OGD was significantly inhibited by ISO (**Figure 4B**). By contrast, the reduced activity of SOD, an important anti-oxidative enzyme, in H9c2 cells exposed to OGD was significantly enhanced by the ISO treatment (**Figure 4C**). These results suggested that ISO attenuated OGD-induced oxidative stress and enhanced anti-oxidative defense in H9c2 cells.

*ISO inhibited OGD-induced apoptosis of H9c2 cells*

To further confirm the protective effect of ISO on cardiomyocyte injury, we examined the apoptosis of H9c2 cells subjected to OGD. The results of flow cytometry showed that the percentage of apoptotic H9c2 cells in the OGD-

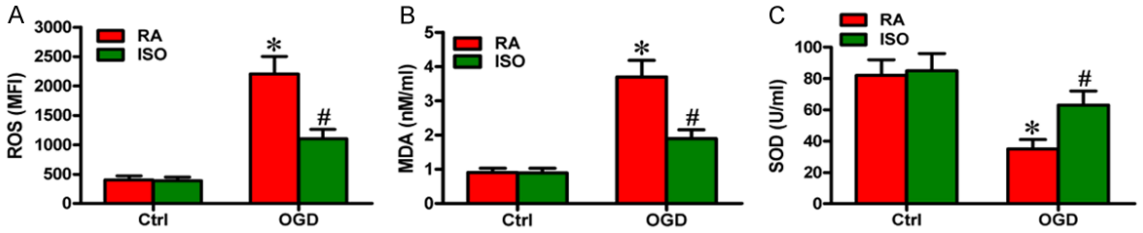


**Figure 4.** ISO inhibited oxidative stress and enhanced anti-oxidative defense in OGD-exposed H9c2 cells. After 3 h of OGD stimulation, H9c2 cells were exposed to 1.4% ISO for 0.5 h and subsequently cultured in normal conditions. (A) ROS production was assessed using DCFH-DA fluorescent probe and analyzed by flow cytometry. (B and C) MDA production (B) and SOD activity (C) in the supernatants of the cells with indicated treatments were measured by commercial kits. Data were expressed as means ± SD from three independent experiments. *P < 0.05, **P < 0.01 vs. Ctrl groups. #P < 0.05 vs. OGD + RA group.

treated group was higher than that in the normal control group (**Figure 5A** and **5B**); the percentage of apoptotic H9c2 cells in the ISO-treated group significantly decreased compared with that in the OGD-stimulated group (**Figure 5A** and **5B**). The anti-apoptotic effects of ISO on OGD-induced H9c2 cell apoptosis were also confirmed through the mitochondrial membrane potential analysis (**Figure 5C**). To investigate the underlying mechanisms of anti-apoptotic effects of ISO involved in OGD-treated H9c2 cells, we detected the activity of caspase-3 and the expressions of apoptosis-associated proteins, including procaspase-3, cleaved-caspase-3, Bax, and Bcl-2. As shown in **Figure 5D**, caspase-3 activity was significantly attenuated by the ISO treatment in H9c2 cells treated with OGD. In addition, the expressions of the pro-apoptotic proteins (procaspase-3, cleaved-caspase-3, and Bax) were up-regulated but that of the anti-apoptotic Bcl-2 was down-regulated in H9c2 cells with OGD exposure. However, ISO reversed all changes in the expressions of the proteins mentioned above (**Figure 5E** and **5F**). The results demonstrated that ISO effectively prevented the increase in apoptosis observed in H9c2 cells upon OGD.

*The protective effects of ISO on H9c2cells with OGD-induced injury were dependent on the PI3K/Akt pathway*

PI3K/Akt signal cascade plays an important role in both apoptosis and survival in cardiomyocytes. Here, we investigated whether or not Akt was involved in ISO-mediated protective effects in OGD-stimulated H9c2 cells. As shown in **Figure 6A** and **6B**, OGD had no significant effect on the overall expression of Akt in the H9c2 cells, but it significantly reduced the expression of p-Akt in the cells. ISO significantly increased the expression of p-Akt in OGD-treated cells compared with those in the control group. Conversely, the PI3K inhibitor LY-294002 and wortmannin decreased the level of p-Akt in the cells with ISO treatment. More importantly, ISO-mediated pro-survival and anti-apoptotic effects against OGD in H9c2 cells were also alleviated by LY294002 and wortmannin (**Figure 6C** and **6D**). These results indicated that ISO-mediated protective effects against OGD were dependent on Akt activation in H9c2 cells.

## Discussion

In the present study, we found that ISO protects H9c2 cardiomyocytes against OGD-induced injury by inhibiting inflammation, oxidative stress, and apoptosis. The key findings are as follows: first, 1.4% ISO had no significant toxic effects on H9c2 cells and therefore was selected for the subsequent experiments. Second, ISO reduced the cytotoxicity induced by OGD in H9c2 cells. Third, OGD-induced pro-inflammatory cytokine production and NF-κB activation in H9c2 cells were significantly attenuated by ISO. Fourth, ISO inhibited OGD-induced ROS and MDA production and enhanced SOD activity in H9c2 cells. Fifth, ISO prevented OGD-induced H9c2 cell apoptosis. Lastly, ISO-mediated cardioprotective effects on OGD-induced H9c2 cell injury depended on PI3K/Akt signaling.

H9c2 cardiomyocyte is widely used for investigating the protective effects of agents on OGD-induced ischemic heart injury [3-5]. Hypoxia is

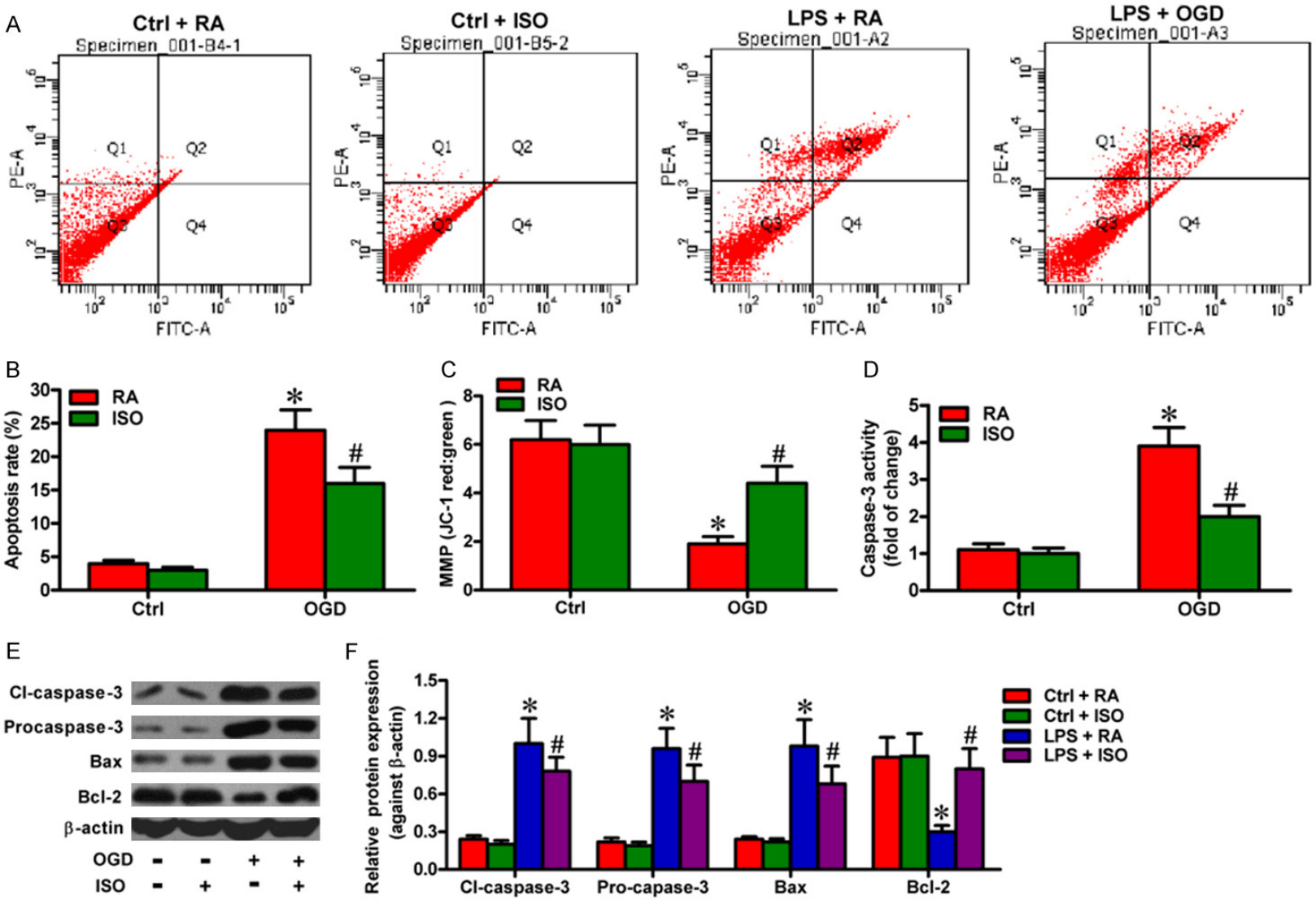


**Figure 5.** ISO prevented H9c2 from OGD-induced apoptosis. After 3 h of OGD stimulation, H9c2 cells were exposed to 1.4% ISO for 0.5 h and subsequently cultured in normal conditions. (A and B) Cell apoptosis was evaluated by Annexin V-FITC and PI staining and analyzed by flow cytometry. Representative images (A) and statistical analysis (B) were shown. (C) JC-1 staining was performed to determine the changes in the mitochondrial membrane potential. (D) Caspase-3 activity was used to assess the apoptosis of H9c2 cells. (E) The expression of procaspase-3, cleaved-caspase-3, Bax, and Bcl-2 was analyzed by Western blot. β-actin was used as

internal control. (F) Quantitative expressions of procaspase-3, cleaved-caspase-3, Bax, and Bcl-2 were normalized against b-actin. Data were expressed as means ± SD from three independent experiments. *P < 0.05, **P < 0.01 vs. Ctrl groups. #P < 0.05 vs. OGD + RA group.

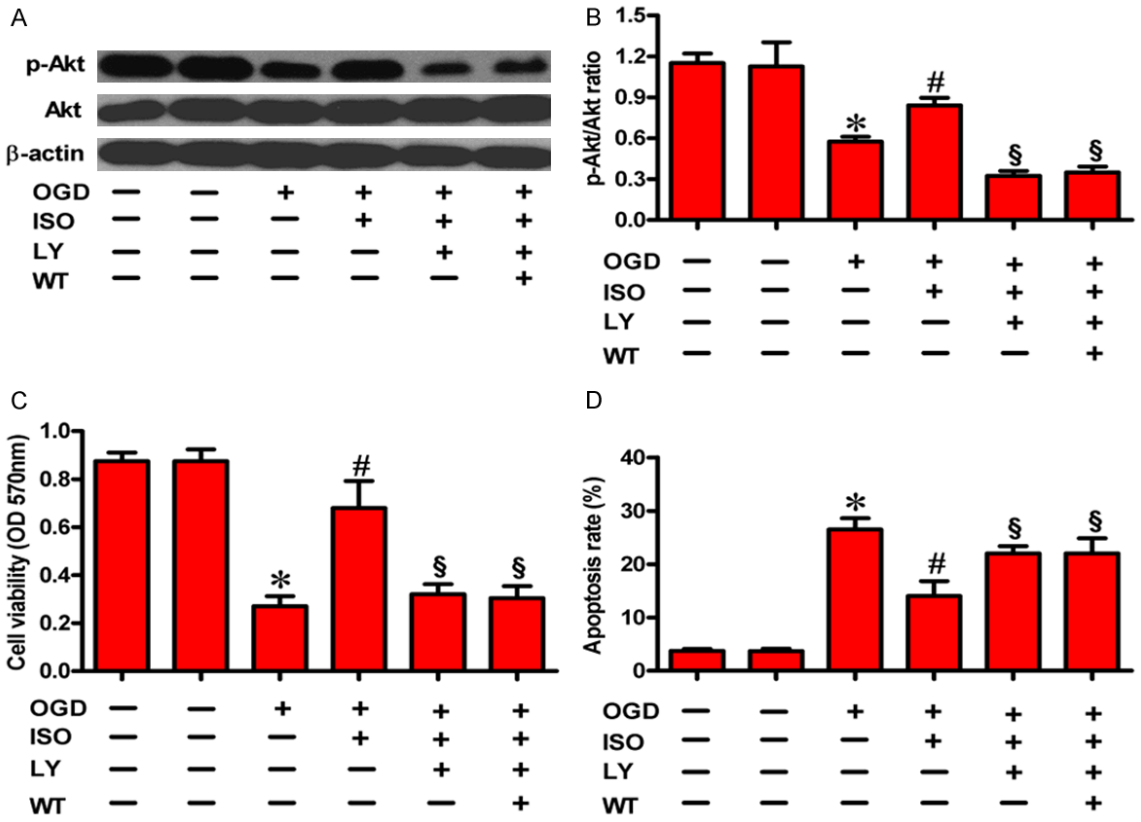


**Figure 6.** ISO-mediated protective effects against OGD-induced H9c2 cell injury were dependent on PI3K/Akt signaling. H9c2 cells were pre-treated with LY294002 (LY, 1 μM) and wortmannin (WT, 10 μM) for 1 h, subjected to OGD for 3 h, and exposed to 0.5 h of ISO (1.4%). Then, the cells were cultured in normal conditions. (A) Western blots showing the expressions of p-Akt and Akt in H9c2 cells. β-actin was used as the loading control. (B) Relative expressions of p-Akt/Akt in each group of H9c2 cells. (C and D) Cell viability and apoptosis were measured by MTT assay (D) and flow cytometry assay (E), respectively. Data were expressed as means ± SD from three independent experiments. *P < 0.05, vs. Ctrl groups. #P < 0.05 vs. OGD + RA group.

an important physiological stimulus associated with ischemia, which causes cardiomyocyte injury. Inflammation is an important signature in ischemic heart disease. Prolonged inflammatory response promotes cytotoxicity in cardiomyocytes [17], and this cytotoxicity is the major source of inflammatory cytokines during injury [18]. Inflammatory cytokines are early predictors of organ dysfunction and are essential for the pathogenesis of myocardial injury [19, 20]. Thus, agents that reduce the levels of pro-inflammatory cytokines may be considered potential therapeutic strategies for ischemic heart injury. ISO has been shown to exert anti-inflammatory effects on several diseases [21-23]. In the present study, the results indicate that ISO reduces the pro-inflammatory cytokine production from OGD-stimulated H9c2 cells. NF-κB is a critical transcription factor in the pathogenesis of cardiac dysfunction [24]. NF-κB is suggested to transcribe many genes involved in inflammation, such as TNF-α, IL-1β, IL-6, and IL-8 [8, 25]. The inhibitor of NF-κB, BAY 11-7082, significantly reduces inflammation and apoptosis in a rat cardiac ischemia-reperfusion injury model [26]. In the present study, ISO inhibited OGD-induced IκB degradation and NF-κB p65 nuclear translocation in H9c2 cells. Therefore, the inhibition of pro-inflammatory cytokine production in OGD-exposed H9c2 cells mediated by ISO may be involved in the suppression of NF-κB activation.

Oxidative stress has been validated to be involved in the process of ischemia-reperfusion heart injury [27, 28]. The balance of ROS production and removal is essential for the redox state and homeostasis in the heart [29]. Ischemia and hypoxia can trigger ROS generation, which plays an important role in the initiation and development of heart failure [30]. ROS may attack polyunsaturated fatty acid within the cell membrane and result in lipid peroxides production, such as MDA. MDA is a product of lipid oxidation and is considered a significant hallmark of oxidative stress [31]. In our study, the production of both ROS and MDA increased in H9c2 cells that underwent OGD, whereas ISO reversed these changes. These findings suggest that ISO protects H9c2 cells from OGD-induced injury by suppressing oxidative stress. In addition, the levels of anti-oxidative enzymes decrease in the cardiomyocytes of injured heart; thereby inhibiting defenses against oxidative stress [32]. SOD is one of the major anti-oxidative enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. SOD activity is found to significantly decrease in H9c2 cells with ischemia-reperfusion injury [8]. Our data showed that ISO could significantly increase the activity of SOD in H9c2 cells that were subjected to OGD. Taken together, these findings suggest that the cardioprotective effects of ISO on OGD-challenged H9c2 cells are partially dependent on its anti-oxidant activity.

Many studies have shown that ischemia-reperfusion injury can induce myocardial cell apoptosis through oxidative stress [4, 5, 8]. Oxidative stress is a major apoptotic stimulus in ischemic heart disease [33]. Mitochondrial damage results in increased production of ROS. ROS-induced oxidative stress in turn induces rapid depolarization of mitochondrial membrane potential [34]. In the current study, we found that the number of apoptotic H9c2 cells significantly increased in the cells with OGD exposure, but ISO reduced the apoptotic cells. A previous study showed that OGD-induced myocardial apoptosis is a mitochondrial-dependent process [35]. The relative ratio of pro-apoptotic and anti-apoptotic members of the Bcl-2 family, which is the mitochondrial-related death switch, determines cell fate [36]. Previous studies showed that ROS could trigger myocyte apoptosis by up-regulating pro-apoptotic members (e.g., Bax, Bak, Bid, and Bim) and by down-regulating anti-apoptotic members (e.g., Bcl-2) of the Bcl-2 family [33, 37]. Bax plays a dominant role in initiating cell death by “making holes” in the outer mitochondrial membrane. It disrupts the integrity of the mitochondrial membrane and hastening the release of cytochrome c from mitochondria [38], thus ultimately leading to caspase-9/caspase-3 activation and DNA fragmentation, but Bcl-2 inhibits it. A previous study reported that ISO inhibits cardiac myocyte apoptosis during oxidative and inflammatory stress by activating Akt and enhancing Bcl-2 expression [14]. Consistently, in the present study, increased levels of pro-apoptotic proteins (procaspase-3, cleaved-caspase-3, and Bax) and caspase-3 activity and decreased anti-apoptotic Bcl-2 were found in H9c2 cells that were subjected to OGD. By contrast, ISO reversed these changes induced by OGD. These results demonstrate that ISO can dramatically inhibit OGD-induced H9c2 cell apoptosis partly by blocking mitochondrial-dependent apoptosis signaling.

Akt plays a major role in cell survival signaling pathways by activating endothelial nitric oxide synthase, phosphorylating and inactivating pro-apoptotic proteins (e.g., Bid and Bax), and inhibiting caspase formation [39]. Many studies have demonstrated that the PI3K/Akt signaling pathway is involved in the protection against myocardial ischemia-reperfusion injury [5, 14, 35]. The preservation of mitochondrial integrity and the attenuation of cardiomyocyte necrotic and apoptotic death have been shown to be involved in Akt-mediated protection [40]. In the present study, we found that OGD had no significant effect on the overall expression of Akt in the H9c2 cells, but it significantly reduced the phosphorylation of Akt. ISO significantly increased the phosphorylation of Akt in cells with OGD exposure. However, pretreatment with LY294002 or wortmannin, two inhibitors of PI3K, decreased the levels of Akt phosphorylation. Moreover, the pro-survival and anti-apoptotic effects of ISO on OGD-treated H9c2 cells were counteracted by LY294002 or wortmannin pretreatment. These data indicate that the mechanism of ISO-mediated cardioprotection against OGD-induced injury maybe related to the PI3K/Akt pathway.

In conclusion, we found that ISO exerted protective effects against OGD-induced H9c2 cell injury and demonstrated that this action occurs

by reducing pro-inflammatory cytokine production, eliminating oxidative stress, improving SOD activity, and inhibiting cell apoptosis. Mechanistically, the cardioprotective effects mediated by ISO may be related to the PI3K/Akt signaling pathway.

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