

Isoproterenol-mediated heme oxygenase-1 induction inhibits high mobility group box 1 protein release and protects against rat myocardial ischemia/reperfusion injury *in vivo*

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Abstract. Isoproterenol (ISO) has been reported to inhibit high mobility group box 1 (HMGB1) protein release via heme oxygenase-1 (HO-1) induction in lipopolysaccharide (LPS)-activated RAW 264.7 cells and increase the survival rate of cecal ligation and puncture (CLP)-induced septic mice. Therefore, it was examined whether ISO-mediated HO-1 induction inhibits HMGB1 release in cardiac myocytes and attenuates myocardial ischemia/reperfusion (I/R) injury in rats. Anesthetized male rats were pretreated with ISO [intraperitoneal (i.p.) injection of 10 mg/kg] prior to ischemia in the absence and/or presence of zinc protoporphyrin IX (ZnPPiX, i.p., 10 mg/kg), which is an inhibitor of HO-1, and then subjected to ischemia for 30 min followed by reperfusion for 24 h. The myocardial I/R injury and oxidative stress were assessed. In addition, the HO-1 protein and HMGB1 expression were measured by western blot analysis. ISO significantly attenuated the myocardial I/R injury, reduced oxidative stress,

and induced HO-1 and reduced HMGB1 release. However, all these effects caused by ISO were significantly reversed in the presence of ZnPPiX. These results suggested that ISO has a pivotal role in the protective effects on myocardial I/R injury. This protection mechanism is possibly due to the inhibition of HMGB1 release via the induction of HO-1.

Introduction

High-mobility group box 1 (HMGB1) protein, which was previously thought to function only as a nuclear factor that enhances transcription, was recently identified to be a crucial cytokine that mediates the response to infection, injury and inflammation (1). Myocardial ischemia/reperfusion (I/R) injury has been found to involve a complex pathophysiological process (2-3), and HMGB1 has been reported to act as a representative and novel proinflammatory cytokine that contributes to the pathophysiological progress of myocardial I/R injury (4). Therefore, anti-HMGB1 release may become a novel therapeutic target for myocardial I/R injury (5).

Heme oxygenase-1 (HO-1), an inducible isoform of heme oxygenase (HO) enzymes, has been reported to be anti-inflammatory, anti-apoptotic and decrease proliferation in several cell types, including cardiac myocytes (6,7). Hydroxysafflor yellow A has been demonstrated to provide a protective effect against I/R injury in H9c2 cardiac myocytes by upregulating the expression and activity of HO-1 (8). Moreover, Hwa *et al* (7) further reported that 2-methoxycinnamaldehyde protects against myocardial I/R injury via anti-oxidant and anti-inflammatory action due to HO-1 induction.

Isoproterenol (ISO) is a β -adrenergic receptor (AR) agonist and has been reported to mediate HO-1 induction via the phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (p38 MAPK) pathways in RAW 264.7 cells. This was demonstrated to result in the inhibition of HMGB1 release in lipopolysaccharide (LPS)-activated RAW 264.7 cells and increase the survival rate of cecal ligation and puncture (CLP)-induced septic mice (9). Therefore, it was hypothesized that ISO may also have a pivotal role in the protective effects on myocardial I/R injury in rats. This protection mechanism is possibly due to the inhibition of HMGB1 release via the induction of HO-1 during rat myocardial I/R injury *in vivo*.

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Abbreviations: ISO, isoproterenol; HO-1, heme oxygenase-1; HMGB1, high mobility group box 1 protein; I/R, ischemia/reperfusion; LPS, lipopolysaccharide; CLP, cecal ligation and puncture; ZnPPiX, zinc protoporphyrin IX; PI3K, phosphatidylinositol 3-kinase; p38 MAPK, p38 mitogen-activated protein kinase; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; LDH, lactate dehydrogenase; CK, creatine kinase; MDA, malondialdehyde; SOD, superoxide dismutase; LVEF, left ventricular ejection fraction; MAP, mean artery pressure; HR, heart rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TTC, 2,3,5-triphenyltetrazoliumchloride; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species

Key words: isoproterenol, heme oxygenase-1, high mobility group box 1 protein, myocardial ischemia and reperfusion

Materials and methods

Materials. ISO hydrochloride and zinc protoporphyrin IX (ZnPPiX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Colorimetric lactate dehydrogenase (LDH), creatine kinase (CK), myocardium malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The rat tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Wuhan Elabscience Biotechnology Co., Ltd. (Wuhan, China). The antibodies used to recognize HO-1 (mouse monoclonal anti-HO-1 antibody against HO-1) and HMGB1 (mouse monoclonal anti-HMGB1 antibody against HMGB-1) were purchased from Sigma-Aldrich.

Animal preparation and experimental design. The experimental protocol conformed to the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, revised 1996) and was approved by the National Animal Care and Use Expert Committee (China).

Male Sprague-Dawley (SD) rats (specific pathogen free; weight, 250-300 g) were purchased from the Hunan Slac Jingda Laboratory Animal Co., Ltd. (Hunan, China). All animals were kept in an environmentally controlled breeding room (temperature, 23 \pm 2°C; humidity, 60 \pm 5%; 12 h dark/light cycle). Rats had access to water and commercial pellet feed *ad libitum* for one week and were randomly divided into seven groups receiving the following treatments: Group 1: The sham operated control group (SO, n=12), rats received saline via intraperitoneal (i.p.) injection and were subjected to surgical manipulation without the induction of myocardial ischemia; group 2: The I/R group (I/R, n=12), the rats were injected with saline via i.p. after 1 h left anterior descending coronary artery (LAD) occlusion was performed for 30 min followed by reperfusion for 24 h; group 3: The ISO and I/R group (ISO-I/R, n=12), rats were pretreated with ISO hydrochloride solution (i.p., 10 mg/kg) (9), after 1 h LAD occlusion was performed for 30 min followed by reperfusion for 24 h; Group 4: The ZnPPiX, ISO and I/R group (ZnPPiX-ISO-I/R; n=9), the rats were pretreated with ZnPPiX (an inhibitor of HO-1, i.p., 10 mg/kg) (9) plus ISO hydrochloride solution (i.p., 10 mg/kg), after 1 h, LAD occlusion was performed for 30 min followed by reperfusion for 24 h.

Subsequent to administration of sodium pentobarbital (45 mg/kg, i.p.; Sigma-Aldrich) anesthetic, the rats were ventilated artificially with a volume-controlled rodent respirator (Jinjiang Ltd., Chengdu, China) at 70 strokes/min. The rats were placed on an electric heating pad (Jinjiang Ltd.) to maintain their body temperature at 37.8°C. Heparin (200 IU/kg) was administered intravenously prior to ischemia. Lead-II of the electrocardiogram (Jinjiang Ltd.) was monitored with subcutaneous stainless steel electrodes. The electrocardiogram was monitored using a computer-based EP system (LEAD2000B; Jinjiang Ltd.).

A thoracotomy through the left parasternal incision was performed. The pericardium was incised and the anterior wall of the left ventricle was exposed. A 4-0 silk suture (Nanjing Jiancheng Bioengineering Institute) on a small curved needle

was passed through the myocardium beneath the middle segment of the LAD branch coursing down the middle of the anterior wall of the left ventricle. A small vinyl flake (Nanjing Jiancheng Bioengineering Institute) was passed into the two ends of the suture, which was then fixed by clamping the tube with a mosquito hemostat (Jinjiang Ltd.). A successful myocardial I/R model was confirmed by changes of ST segment elevation in Lead-II and regional cyanosis of the myocardial surface. The rats underwent a 30 min LAD occlusion followed by 24 h of reperfusion.

Hemodynamic measurements. After being anesthetized with sodium pentobarbital (45 mg/kg, i.p.), the right common carotid artery of the rat was exposed and cannulated with a 2 F Millar Catheter (Millar Instruments, Inc., Houston, TX, USA) into the left ventricle through the ascending aorta. Heart function was monitored and the related hemodynamic parameters, such as the left ventricular ejection fraction (LVEF), heart rate (HR) and mean artery pressure (MAP) in each group were recorded as described previously (10).

Assessment of myocardial injury. To measure the LDH and CK levels, blood samples were collected, centrifuged at 1,358.37 x g for 15 min (Jinjiang Ltd.) and maintained at -20°C until analysis. Standard techniques using commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute) were applied. Values were expressed in international units per liter.

Assessment of the infarct size. After 24 h of reperfusion, the LAD was occluded again and 2 ml of 1.5% Evans blue dye was injected via the femoral vein. The risk area was analyzed by negative staining with Evans blue. The rats were then sacrificed by heart excision and frozen overnight immediately. The atria and right ventricle were removed and the left ventricle was cut into transverse slices (2-mm thick) from the apex to the base. The risk area was separated from the colored non-ischemic area (blue) and then incubated with a 1% solution of 2,3,5-triphenyltetrazoliumchloride (TTC, in 0.2 M Tris-buffer, pH 7.4) stain for 20 min at 37°C, viable myocardium was stained red by TTC, whereas necrotic myocardium did not stain red. In each slice, the infarct size and the risk area (left ventricular areas) were determined by a computer-assisted image analysis system (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA) and multiplied by the thickness of the slice to calculate volumes of risk area. The infarct size was expressed as a percentage of the risk area volume (% infarct size/risk area).

Measurement of MDA and SOD activity. MDA concentration and SOD activity in myocardial tissues were measured using colorimetric myocardium malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute) as described previously (11). The concentrations were used as indexes of oxygen free radical and lipid superoxide levels in the myocardium, respectively.

Measurement of inflammatory cytokine expression (TNF- α and IL-6) in myocardial tissues of rats. The titres of TNF- α

and IL-6 in cardiac muscle samples were measured using ELISA and the detailed manipulation process was performed according to the manufacturer's recommendations (Wuhan Elabscience Biotechnology Co., Ltd.).

Western blot analysis for the expression of HO-1 and HMGB1. The protein extracts were prepared from frozen and pulverized samples of the ischemia area of the left ventricle as previously reported (12,13). Western blot analysis was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, 50 μ g of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Nanjing Jiancheng Bioengineering Institute). Nonspecific binding sites were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween. The membrane was subsequently probed with primary antibodies (anti-HO-1 antibody, diluted 1:400; and anti-HMGB1 antibody, diluted 1:500, respectively) and incubated in horseradish peroxidase-conjugated secondary antibody (diluted 1:50,000). The protein bands were visualized by an enhanced chemiluminescence system (Jinjiang Ltd.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to correct the variations of different samples. The expression of HO-1 and HMGB1 were normalized to GAPDH expression.

Presentation of data and statistical analysis. All values were expressed as the mean \pm standard deviation. Student's t-test was used for between-group comparisons. One-way analysis of variance or Welch was used for comparisons among groups and the Student-Neuman-Keuls or Dunnett's T3 tests were used for post hoc multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of ISO on functional recovery of a I/R heart. Cardiac function was assessed prior to LAD occlusion and following drug administration (ISO or ZnPPiX). As shown in Table I, no significant difference in LVEF, HR and MAP was identified between the groups ($P > 0.05$).

Subsequent to 24 h of reperfusion, compared with that of the I/R group, the LVEF was significantly improved in the ISO-treated group ($P < 0.05$ versus the I/R group), but no significant difference in HR and MAP was found between the I/R and ISO-treated groups ($P > 0.05$ versus the I/R group). However, compared with that of the ISO-I/R group, the effects of ISO on the LVEF was significantly reversed by the presence of ZnPPiX ($P < 0.05$ versus the ISO-I/R group; Table II).

Effects of ISO on the infarct size in I/R rats. Subsequent to 24 h of reperfusion, compared with that of the I/R group, pretreatment with ISO significantly reduced the infarct size ($P < 0.05$ versus the I/R group). However, the decreased infarct size induced by ISO was significantly reversed by the presence of ZnPPiX ($P < 0.05$ versus the ISO-I/R group; Fig. 1).

Effects of ISO on LDH and CK levels in I/R rats. After 24 h of reperfusion, compared with that of the SO group, the levels of LDH and CK in the I/R group were significantly increased ($P < 0.05$ versus the SO group). However, compared with that

Table I. Summary of hemodynamic measurements prior to myocardial I/R and following drug treatment.

	LVEF (%)	HR (bpm)	MAP (mmHg)
SO	79.0 \pm 2.6	296 \pm 10	83 \pm 3
I/R	77.8 \pm 3.6	285 \pm 11	81 \pm 3
ISO-I/R	82.3 \pm 2.2	306 \pm 8	84 \pm 4
ZnPPiX-ISO-I/R	81.3 \pm 1.7	302 \pm 9	82 \pm 4

All data are expressed as the mean \pm standard deviation. No significant difference in LVEF, HR and MAP was identified between all groups ($P > 0.05$). SO, sham operated; ISO, isoproterenol; I/R, ischemia/reperfusion; ZnPPiX, zinc protoporphyrin IX; LVEF, left ventricular ejection fraction; HR, heart rate; MAP, mean artery pressure.

Table II. Summary of hemodynamic measurements following 24 h of reperfusion.

	LVEF (%)	HR (bpm)	MAP (mmHg)
SO	79.8 \pm 1.7	291 \pm 9	78 \pm 3
I/R	64.8 \pm 3.4 ^a	274 \pm 5	74 \pm 5
ISO-I/R	73.8 \pm 2.1 ^b	269 \pm 9	73 \pm 5
ZnPPiX-ISO-I/R	66.5 \pm 1.3 ^c	270 \pm 9	74 \pm 3

All data are expressed as the mean \pm standard deviation. ^a $P < 0.05$ vs. the SO group; ^b $P < 0.05$ vs. the I/R group; ^c $P < 0.05$ vs. the ISO-I/R. SO, sham operated; I/R, ischemia/reperfusion; ISO, isoproterenol; ZnPPiX, zinc protoporphyrin IX; LVEF, left ventricular ejection fraction; HR, heart rate; MAP, mean artery pressure.

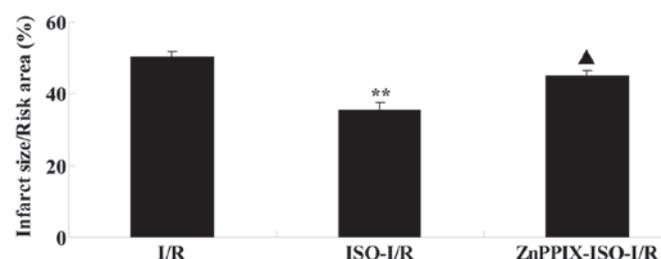


Figure 1. Effects of isoproterenol on infarct size during I/R. (n=9 or 12 for each group). All data are expressed as the mean \pm standard deviation. ^{**} $P < 0.01$ vs. the I/R group; [▲] $P < 0.05$ versus the ISO-I/R group. I/R, ischemia/reperfusion; ISO, isoproterenol; ZnPPiX, Zinc protoporphyrin IX.

of the I/R group, ISO significantly inhibited the increase of LDH and CK levels ($P < 0.05$ versus the I/R group). Moreover, compared with that of the ISO-I/R group, the reduction of LDH and CK release induced by ISO were significantly inhibited by the presence of ZnPPiX ($P < 0.05$ versus the ISO-I/R group; Fig. 2).

Effects of ISO on the production of TNF- α and IL-6 in I/R rats. After 24 h of reperfusion, compared with that of the SO group, TNF- α and IL-6 levels were significantly increased

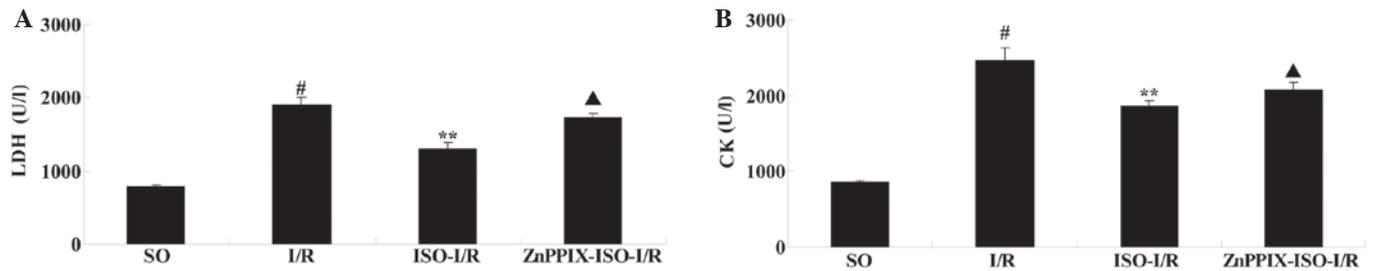


Figure 2. Effects of ISO on LDH (A) and CK (B) expression levels during I/R (n=9 or 12 for each group). All data are expressed as the mean \pm standard deviation. #P<0.05 vs. the SO group; **P<0.01 vs. the I/R group; ▲P<0.05 versus the ISO-I/R group. SO, sham operated; I/R, ischemia/reperfusion; ISO, isoproterenol; ZnPPiX, Zinc protoporphyrin IX; LDH, lactate dehydrogenase; CK, creatine kinase.

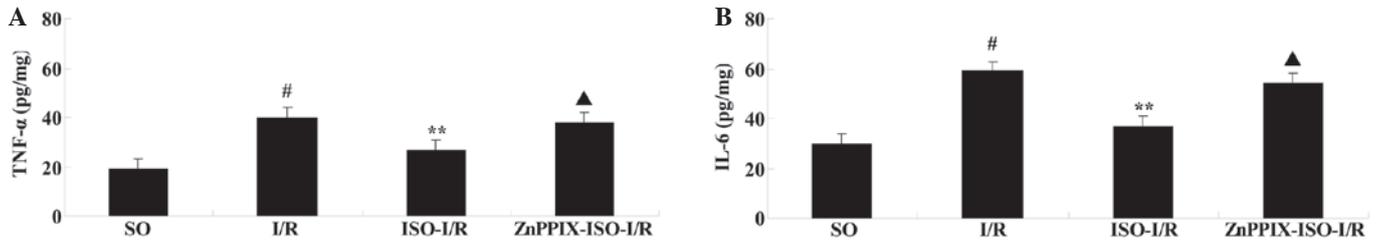


Figure 3. Effects of ISO on TNF- α (A) and IL-6 (B) during I/R. (n=9 or 12 for each group). All data are expressed as the mean \pm standard deviation. #P<0.05 vs. the SO group; **P<0.01 vs. the I/R group; ▲P<0.05 vs. the ISO-I/R group. ISO, isoproterenol; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; SO, sham operated; I/R, ischemia/reperfusion; ZnPPiX, Zinc protoporphyrin IX.

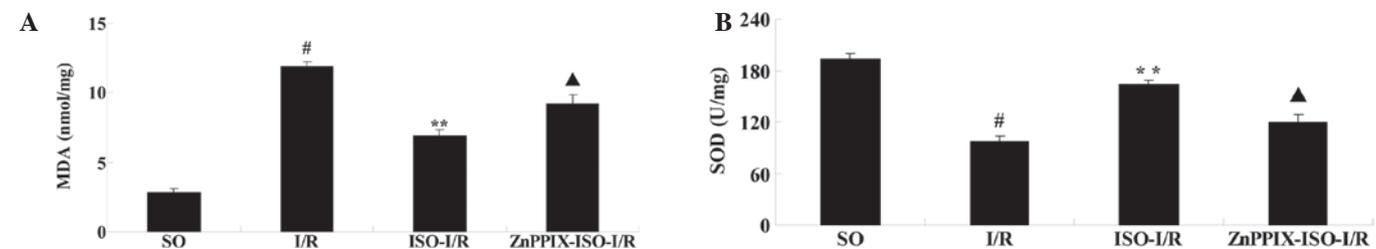


Figure 4. Effects of ISO on MDA (A) and SOD (B) activity assay during I/R. (n=9 or 12 for each group). All data are expressed as the mean \pm standard deviation. #P<0.05 vs. the SO group; **P<0.01 vs. the I/R group; ▲P<0.05 vs. the ISO-I/R group. SO, sham operated; I/R, ischemia/reperfusion; ISO, isoproterenol; ZnPPiX, zinc protoporphyrin IX; MDA, malondialdehyde; SOD, superoxide dismutase.

in the I/R group (P<0.05 versus the SO group). However, compared with that of the I/R group, ISO resulted in a statistically significant decrease in the production of TNF- α and IL-6 (P<0.05 versus the I/R group). Compared with that of the ISO-I/R group, the effects of ISO on TNF- α and IL-6 production were inhibited by the presence of ZnPPiX (P<0.05 versus the ISO-I/R group; Fig. 3).

Effects of ISO on MDA and SOD activity assay in I/R rats.

After 24 h of reperfusion, compared with that of the SO group, the levels of MDA significantly increased and the levels of SOD significantly decreased in the I/R group (P<0.05 versus the SO group). However, compared with that of the I/R group, ISO significantly inhibited the increase of MDA and the decrease of SOD (P<0.05 versus the I/R group). Conversely, compared with that of the ISO-I/R group, the effects of ISO were significantly reversed by the presence of ZnPPiX (P<0.05 versus the ISO-I/R group; Fig. 4).

Effects of ISO on HO-1 and HMGB1 expression levels in I/R rats. Following 24 h of reperfusion, compared with that

of the SO group, HMGB1 and HO-1 expression levels were markedly increased in the I/R group (P<0.05 versus the SO group). However, compared with that of the I/R group, ISO significantly mediated HO-1 induction and HMGB1 inhibition (P<0.05 versus the I/R group). However, the effects of ISO were significantly reversed by the presence of ZnPPiX (P<0.05 versus the ISO-I/R group) (Fig. 5).

Discussion

The present study demonstrated that ISO significantly attenuated myocardial I/R injury and oxidative stress. Furthermore, the induction of HO-1 and inhibition of HMGB1 were shown to be involved in the effects of ISO on attenuating myocardial I/R injury in rats.

HMGB1, once released from necrotic cells and macrophages, may significantly upregulate IL-1, IL-6, TNF- α , C-reactive protein and macrophage inflammatory proteins (MIP-1 α and MIP-1 β) (14-16). Furthermore, it has been reported to act as a novel proinflammatory mediator that contributes to the progression of myocardial I/R injury (3).

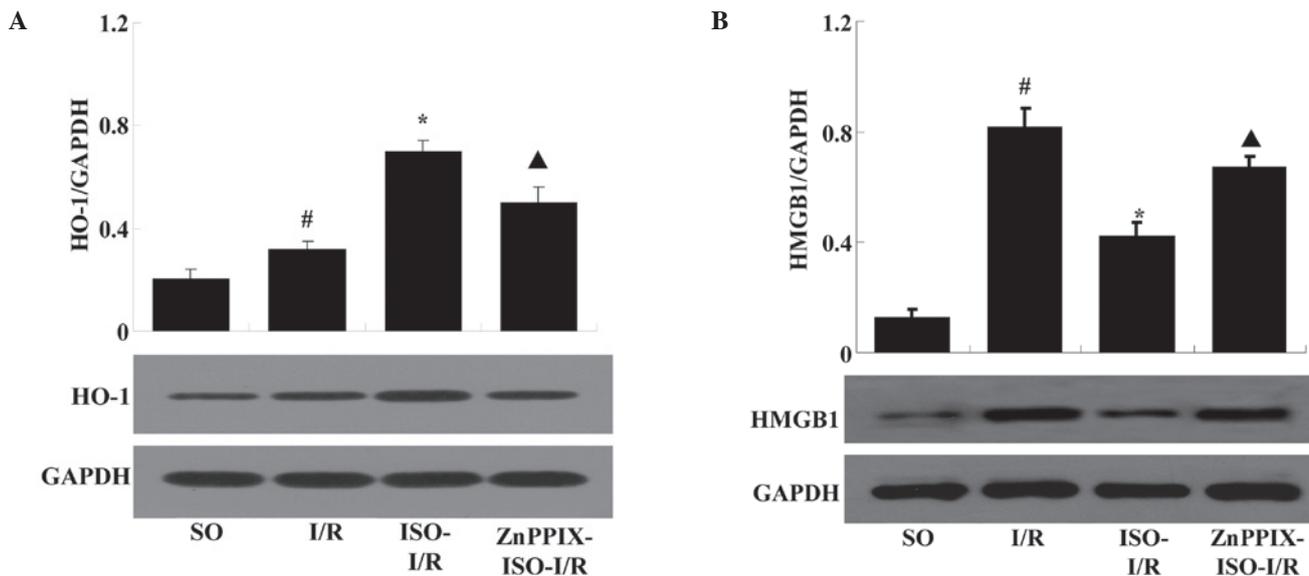


Figure 5. Effects of ISO on HO-1 (A) and HMGB1 (B) expression. (n=9 or 12 for each group). All data are expressed as the mean \pm standard deviation. #P<0.05 vs. the SO group; *P<0.05 vs. the I/R group; ▲P<0.05 vs. the ISO-I/R group. SO, sham operated; I/R, ischemia/reperfusion; ISO, isoproterenol; ZnPPiX, zinc protoporphyrin IX; HO-1, heme oxygenase-1; HMGB1, high mobility group box 1 protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Moreover, in addition to its anti-inflammatory and anti-apoptotic effects and its ability to decrease proliferation, HO-1 is induced and responds to a variety of stimuli and injury, such as oxidative stress and myocardial I/R injury in several organs and cell types (5,6). Furthermore, Takamiya *et al* (17) demonstrated that the expression levels of HMGB1 were higher in HO-1^{-/-} mice than that of HO-1^{+/+} mice. Additionally, the induction of HO-1 has been shown to prevent the release of HMGB1 in endotoxin-activated macrophages *in vitro* and septic animals *in vivo* (18), which is further supported in the rat myocardial I/R injury model in the present study.

Furthermore, there are a number of reports on β -AR-mediated modulation of inflammatory effects (TNF- α and IL-6) (19-21). ISO, as a β -AR agonist, has been reported to increase cluster of differentiation (CD)14 expression and live *E. coli* phagocytosis in macrophages, which may result from the increase in cAMP/PKA signaling (22,23). Macrophages have been shown to exhibit accelerated LPS internalization and detoxification through increased surface CD14 expression or synthesis and release of higher levels of soluble CD14 at inflammatory foci, thus limiting the biological toxicity of LPS (24). These studies suggest the potential impact of ISO on innate immune functions. Furthermore, as ISO has been reported to mediate HO-1 induction in RAW 264.7 cells, which results in the inhibition of HMGB1 release in LPS-activated RAW 264.7 cells and increase in the survival rate of CLP-induced septic mice (9), ISO-mediated HO-1 induction during rat myocardial I/R injury *in vivo* was investigated. In the present study, it was demonstrated that ISO significantly attenuated myocardial I/R injury and inhibited HMGB1 release. These effects were significantly reversed by ZnPPiX, an inhibitor of HO-1, which further confirmed that HO-1 activity is important for ISO to significantly inhibit HMGB1 release and attenuate myocardial I/R injury in rats.

Notably, in the present study, it was also observed that pretreatment with ISO significantly decreased the levels of

MDA, a reactive oxygen species (ROS), and increased the levels of SOD, a key antioxidant enzyme. Hydrogen peroxide has been demonstrated to stimulate cardiac myocytes to cause the release of HMGB1, which suggested that ROS may be involved in the release of HMGB1 (25,26). In addition, HO-1 has been reported to occur in the lung in response to oxidative stress associated with infection, altered oxygen tension and inflammatory diseases. HO-1 remains widely regarded as protective against oxidative tissue injury and as a beneficial molecule in protecting against the oxidative stress induced by numerous stimuli (27,28). Therefore, ISO reduced oxidative stress during myocardial I/R injury in rats in the present study, which may also be associated with the actions of HO-1 induction and inhibition of HMGB1 release.

The present study demonstrated that ISO attenuated myocardial I/R injury in rats, which may be due to its inhibitory effects on HMGB1 release via the induction of HO-1 in cardiac myocytes.

There are a number of limitations to the present study. This study only demonstrated that ISO mediated the induction of HO-1, inhibited HMGB1 release and attenuated rat myocardial I/R injury *in vivo*. However, the associated signaling pathways, such as PI3K/p38 MAPK and other factors, such as nuclear factor erythroid 2-related factor 2 translocation, may have contributed to these cardioprotective effects in cardiac myocytes and should be considered in a future study (29-32). Additionally, the present study only observed that ISO reduced oxidative stress during myocardial I/R injury in rats; the precise mechanisms require further elucidation.

In conclusion, the present study demonstrated that ISO mediated the inhibition of HMGB1 release via the induction of HO-1 in cardiac myocytes and attenuated rat myocardial I/R injury *in vivo*, which may provide an important therapeutic approach for protection against myocardial ischemia and reperfusion injury.

Acknowledgements

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