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Original Article

Levosimendan reduces myocardial ischemia-reperfusion injury by regulating the expression of HIF-1α

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Abstract: Objective: Levosimendan (Levo) is a novel calcium ion sensitizer that enhances myocardial contractile function and participates in the mediation of ischemia-reperfusion (I-R). Hypoxia inducible factor-1α (HIF-1α) is involved in ischemia and hypoxia injury. This study aims to investigate the effect and mechanism of Levo on myocardial I-R injury. Methods: Myocardial I-R injury model rats were divided into IR group and I-R+Levo group followed by measuring HIF-1α and HO-1 level, contents of caspase-3, MDA and SOD as well as ROS content and apoptosis by flow cytometry. Results: Compared with sham group, MDA content, caspase-3 activity, HIF-1α and HO-1 levels in I-R model rats were significantly elevated, along with reduction of SOD activity. Among I-R model rats, Levo can further significantly upregulate HIF-1α and HO-1 levels, while reduced MDA, ROS content, SOD, caspase-3 activity and cell apoptosis. Conclusion: Levo ameliorates myocardial I-R injury through upregulating HIF-1α and reducing cell apoptosis, which provides new insights for the further therapy against ischemia-reperfusion in clinical practice.

Keywords: I-R injury, oxidative stress, apoptosis, levosimendan, HIF-1α

Introduction

The re-supply of ischemic myocardium inevitably leads to ischemia-reperfusion (IR) and becomes the main factor to the therapeutic effect of acute myocardial infarction (AMI) [1, 2].

Levosimendan (Levo) emerges as a novel calcium ion sensitizer that enhances myocardial contractility [3, 4], diastolic coronary vessels [5], and plays a therapeutic role in coronary heart disease, heart failure and myocardial infarction [6-8]. In addition, extensive studies have confirmed that levosimendan can ameliorate myocardial IR injury [9-12].

HIF-1α is a nuclear protein produced under hypoxic conditions and promotes the expressions of downstream target genes, which is involved in ischemia and hypoxia injury of organ tissues and its repair process [13-17]. At present, it has been confirmed that levosimendan regulates HIF-1α expression and the relevant function [18-20]. In this study, we investigate the role of levosimendan in the regulation of myocardial I-R injury by using rat model as well as illustrate the possible mechanism.

Materials and methods

Main reagents and materials

Male SD rats (6 weeks, weighted 250±20 g) were purchased from Beijing Weitong Lihua. Rabbit anti-rat β-actin, HIF-1α, HO-1 polyclonal antibodies were bought from American Abcam. HRP-conjugated IgG was obtained from Jackson ImmunoResearch. PrimeScriptTM RT reagent Kit was collected from Dalian Takara. Mass peroxidation product malondialdehyde (MDA) and superoxide dismutase (SOD) detection kit were acquired from Jiangsu Biyuntian. DCFH-DA and type II collagenase were provided from Sigma in the United States. Annexin V/PI apoptosis detection kit was from Beijing Suo Labao Bio.

Establishment of rat myocardial I-R model

The rats were intraperitoneally injected with 10% chloral hydrate. After anesthesia, they

were placed on the operating table. Chest was opened and heart was exposed followed by ligation to the left anterior descending coronary artery. The ECG monitoring showed the ST segment on the Q lead. The back of the arch was lifted by 0.1 mV or the T wave was high, the color of the myocardium became pale, and the beat was weakened, indicating that the AMI model was successfully established. After 60 min, the ligature was released and the blood supply to the rat myocardium was restored. The apical redness indicated successful reperfusion. A sham operation group (sham group) with thoracotomy without ligation was used as a control. Rats were sacrificed at 12 h post operation, myocardial tissue was collected, and the expression of related genes and proteins was detected. Myocardial homogenate was used to detect the content of MDA and SOD. All operations and protocols on animals were approved by the Laboratory Animal Ethics Committee of Affiliated Hangzhou First People's Hospital.

Levo treatment of I-R model rats

In the Levo treatment study, rats with IR injury were randomly separated into IR group (IR), in which intravenous saline was administered to the tail vein 10 min before reperfusion, and I-R+Levo group, in which Levo was injected at a dose of 0.1 μmol/Kg through tail vein 10 min before reperfusion. Myocardial tissues were collected at 12 h after reperfusion.

Detection of caspase-3 enzyme activity in rat myocardial tissue

Through serial dilution, pNA standard was prepared for quantification by using standard curve and absorbance value at 405 nm was detected. The myocardial tissue was collected and the homogenate was prepared. Homogenate was lysed and centrifuged to collect the supernatant in which protein concentration was detected by BCA kit followed by addition of 65 μL Assay buffer, 25 μL lysate supernatant, 10 μLc-DEVD-pNA (2 mM) for 2 h incubation and subsequent detection of A405 value when the color changes. Experimental group A405/ The control group A405×100% was defined as the relative enzyme activity unit.

MDA and SOD detection

The rat myocardial tissue was cut into pieces, and the tissue homogenate was prepared, centrifuged to collect supernatant. According to the kit instructions, the contents of MDA and SOD in the myocardial tissue were determined by ultraviolet-visible spectrophotometry.

Apoptosis detection

The rat myocardial tissue was collected. The type II collagenase was digested for 60 min to acquire cell suspension followed by washing and addition of 5 μL Annexin V-FITC and 5 μL of PI Solution to measure cell apoptosis by FC500MCL.

ROS content detection

The rat myocardial tissue was collected, and the tissue was excised for 6 min to acquire cell suspension which was incubated with 0.1% DCFH-DA probe for 30 min followed by detecting ROS level by FC500MCL flow cytometry.

qRT-PCR detection

Total RNA was extracted followed by cDNA synthesis using the PrimeScript™ RT reagent Kit according to the kit instructions. cDNA was set as a template, a down-step PCR amplification reaction was carried out via Taq DNA polymerase in a total of 10 μL of reaction system, including 5.0 μL of 2× SYBR Green Mixture, 0.5 μL of 5 μm/L forward and reverse primers, 1 μL of cDNA, and ddH_2O with conditions: 95°C 5 min, 40 cycles of 95°C 15 sec, 60°C 1 min.

Western blot

100 μL of RIPA lysate was added to every 50 mg of tissue to extract protein which was quantified by BCA assay and separated on SDS-PAGE for western blot using antibodies (HIF-1α, HO-1, β-actin Dilution ratios were 1:800, 1:2000, 1:10000). After addition of ECL, the membrane was exposed and developed.

Statistical process

SPSS 18.0 software was utilized for analyzing data which were displayed as mean ± standard deviation (SD) and assessed by student t test. $P<0.05$ indicates a significance [7].

Results

Oxidative stress injury and apoptosis in I-R model rat

I-R model rats showed significantly elevated MDA content (**Figure 1A**), reduced SOD activity

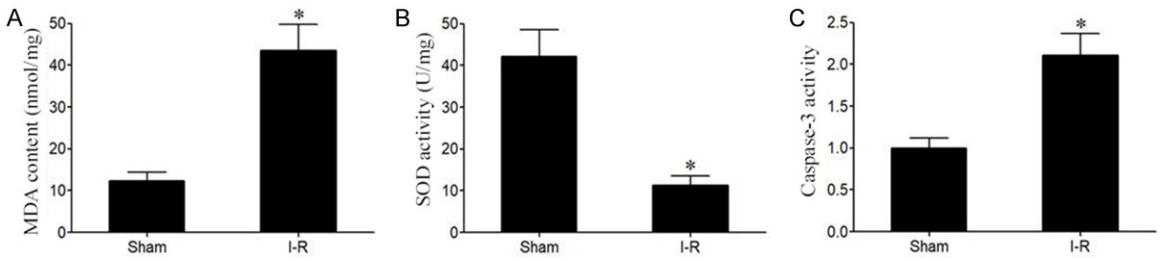

Figure 1. Oxidative stress injury and apoptosis in I-R model rat myocardial tissue. A. The kit detects the MDA content in rat myocardial tissue; B. The kit detects SOD enzyme activity in rat myocardial tissue; C. The kit detects caspase-3 enzyme activity in rat myocardial tissue. * represents P<0.05 compared with the Sham group.

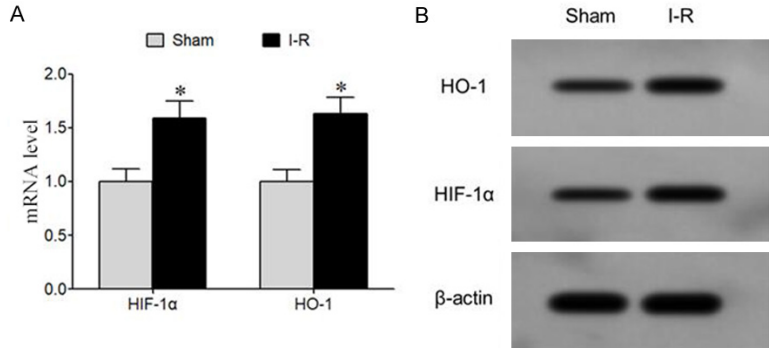

Figure 2. Up-regulation of HIF-1α and HO-1 expression in myocardial tissue of I-R model rats. A. qRT-PCR was used to detect the expression of HIF-1α and HO-1 mRNA in rat myocardial tissue; B. Western blot was used to detect the expression of HIF-1α and HO-1 protein in rat myocardial tissue. * represents P<0.05 compared with the Sham group.

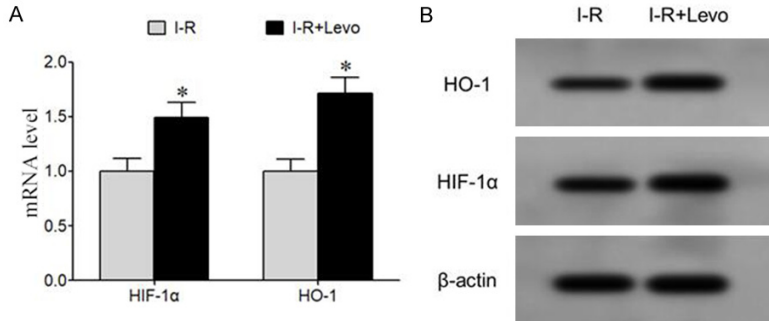

Figure 3. Levo up-regulates the expression of HIF-1α and HO-1 in rat myocardial tissue. A. qRT-PCR was used to detect the expression of HIF-1α and HO-1 mRNA in rat myocardial tissue; B. Western blot was used to detect the expression of HIF-1α and HO-1 protein in rat myocardial tissue. * represents a comparison with the I-R group, P<0.05.

(**Figure 1B**) and enhanced caspase-3 activity (**Figure 1C**) compared to sham rats, indicating the model was successfully established.

Up-regulation of HIF-1α and HO-1 in I-R model rats

Our result showed that HIF-1α and HO-1 mRNA (**Figure 2A**) and protein (**Figure 2B**) in I-R model rats were significantly upregulated in comparison with those in sham rats.

Levo upregulates HIF-1α and HO-1 in rat myocardial tissue

We noted that Levo treatment further significantly upregulated HIF-1α and HO-1 mRNA (**Figure 3A**) and protein (**Figure 3B**) compared to I-R group rats.

Levo reduces oxidative stress, apoptosis, and alleviates I-R injury

Compared with I-R group alone, Levo significantly decreased MDA content, SOD activity, caspase-3 enzyme activity and and ROS content in I-R model rats (**Figure 4**). Meanwhile, the treatment of Levo significantly reduced cell apoptosis (**Figure 4**).

Discussion

Levosimendan (Levo) is a novel calcium ion sensitizer that up-regulates Ca^{2+} sensitivity in cardiomyocytes without increasing oxygen consumption. In a calcium-dependent manner, it binds to cardiac troponin C to exert positive inotropic effects, enhance myocardial contractility, but it does not affect ventricular diastolic, thereby improving cardiac function [3, 4]. Levosimendan can produce vasodilatation by activating the ATP-sensitive K+ channel (KATP). It therefore improves coronary blood flow via

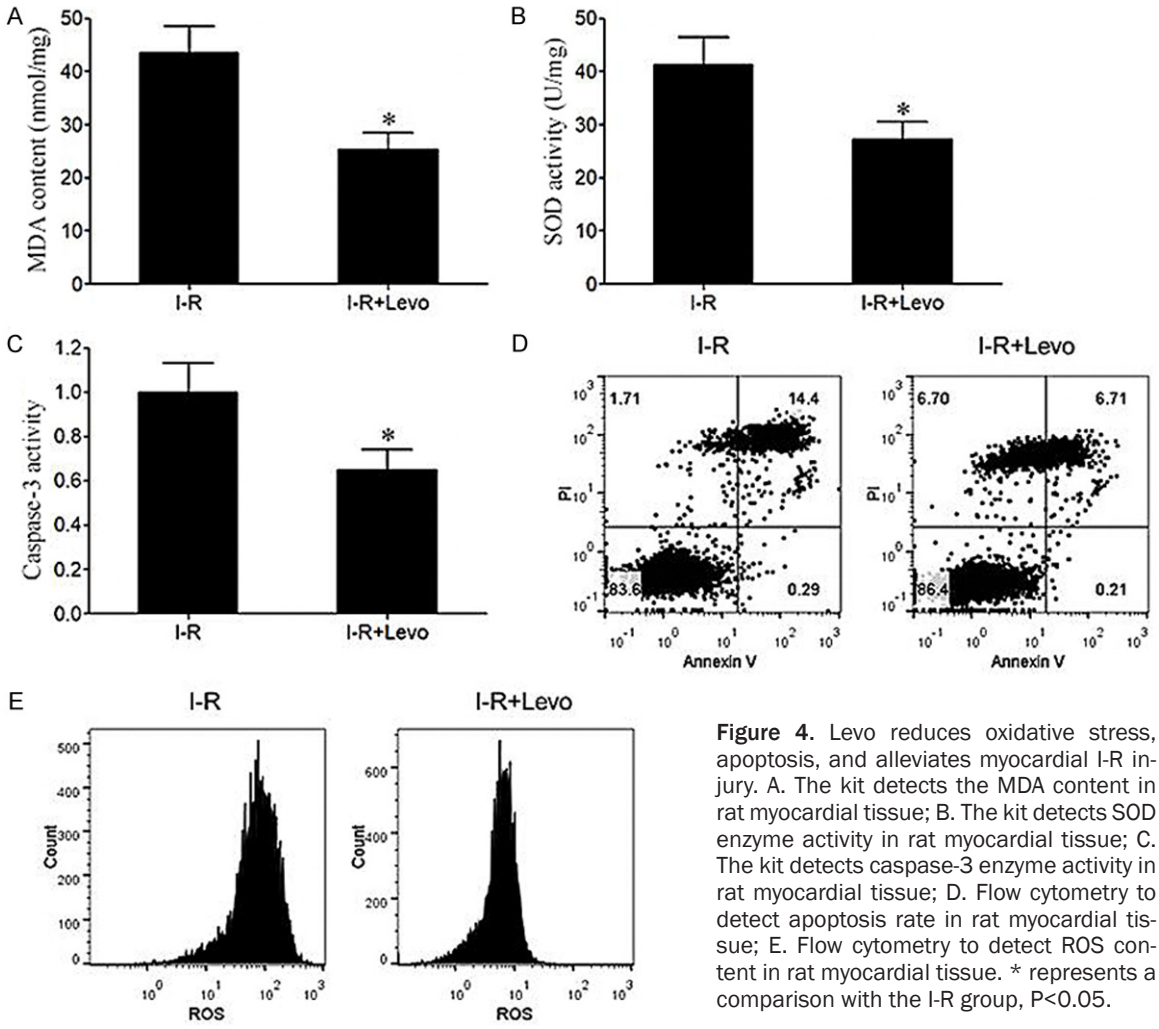

Figure 4. Levo reduces oxidative stress, apoptosis, and alleviates myocardial I-R injury. A. The kit detects the MDA content in rat myocardial tissue; B. The kit detects SOD enzyme activity in rat myocardial tissue; C. The kit detects caspase-3 enzyme activity in rat myocardial tissue; D. Flow cytometry to detect apoptosis rate in rat myocardial tissue; E. Flow cytometry to detect ROS content in rat myocardial tissue. * represents a comparison with the I-R group, $P<0.05$.

altering coronary artery resistance vessels and venous volume. In patients with heart failure, the positive inotropic and vasodilating effects of levosimendan can increase myocardial contractility and reduce the anterior and posterior loads without affecting their diastolic function [5]. Clinically, levosimendan is mainly used for treating coronary heart disease and heart failure. However, an increasing number of studies have confirmed that levosimendan can ameliorate myocardial IR injury [9-12].

HIF-1 is a nuclear protein produced under hypoxic conditions and can maintain oxygen in hypoxic tissue cells. Oxygen-regulated transcription factors that are stable under hypoxic conditions can participate in the adaptive response to hypoxic microenvironment and regulate cellular hypoxia [21, 22]. HIF-1 consists of α subunit and β subunit and is constitutively expressed in cells. The expression of the β subunit (ARNT) is independent of oxygen, and the stability of the protein expressed by the alpha subunit is regulated by the intracellular oxygen concentration [21, 23]. Under normal oxygen conditions, HIF-1α is degraded by proteasome under proline hydroxylase (PHD), and HIF-1α is found without DNA binding activity [21, 22, 24]. HIF-1α is a transcriptional regulator induced by hypoxia. Under hypoxic conditions, the level of ubiquitination of HIF-1α is significantly reduced, and post-translational modification is attenuated, resulting in up-regulation of protein stability and HIF-1α activation. PHD activity is inhibited so that HIF-1α can not be degraded, causing the accumulation of HIF-1α in cytoplasm. Hypoxia can also upregulate of HIF-1α and form a stable dimeric structure with HIF-1β. Furthermore, it enters the nucleus and binds with the hypoxic response element to

promote transcription, resulting in elevated expression of heme oxygenase (HO-1) and vascular endothelial growth factor (VEGF), thus participating in the repair of organ tissues [13-17]. At present, studies have confirmed that levosimendan regulates HIF-1α expression and function [18-20].

We showed elevated MDA content and caspase-3 activity and reduced SOD activity in IR model rats, indicating that oxidative stress was induced in rat myocardium after I-R modeling. The severity is significantly increased as the apoptosis is increased. The detection of gene and protein expression showed upregulated HIF-1α, HO-1 gene and protein in I-R model group. The results revealed that in the myocardial tissue of IR model rats, expression of HIF-1α was up-regulated, which increased expression of downstream anti-oxidative stress factor HO-1, and initiated the repair mechanism of ischemia-anoxia injury. Treatment with Levo in rats with I-R model significantly reduced MDA content and caspase-3 activity in rat myocardium, which resulted in decreased apoptosis, ROS content and SOD activity. Our data showed upregulated HIF-1α, HO-1 gene and protein in myocardial tissue was further increased by injection of Levo in I-R model rats. The results indicated that Levo treatment can promote the repair mechanism of endogenous hypoxic-ischemic injury in myocardial tissue, further increase the expression of HIF-1α and HO-1, improve the anti-oxidative stress of rats, and ameliorate myocardial IR damage. Goetzenich et al [18] showed that pretreatment of Levo before hypoxic treatment of rat cardiomyocytes significantly reduced the damage of rat cardiomyocytes and increased cell viability. Moreover, HIF-1α in cardiomyocytes was significantly upregulated, indicating that Levo can enhance myocardial cell damage induced by hypoxia-induced cardiomyocytes through upregulating HIF-1α, which is consistent with our study. Goetzenich et al [18] used a cell model in vitro to study the anti-hypoxia injury of Levo. Differently, our study established a rat myocardial IR injury model which was treated with Levo. We have shown that Levo can upregulate HIF-1α, increase t HO-1 level, improve the damage repair mechanism of rat myocardium, and reduce IR damage of myocardium. Recent evidence unraveled that Sevoflurane can significantly improve myocardial injury caused by I/R in rats, and its mechanism might be related to the activation of the Akt/HIF-1α/VEGF signaling pathway [25]. Previous data illustrated the effect of Levosimendan on isoproterenol-induced myocardial injury in high-fat-fed rats involves modulation of PI3K/Akt/mTOR signaling pathway [26]. Our study further investigated the mechanism of Levo as well as the implication to Akt/HIF-1α pathway.

Conclusion

In conclusion, Levo can increase HIF-1α and HO-1 levels, which can ameliorate the oxidative stress and apoptosis in rat myocardium, thus reducing myocardial I-R injury, which may open a new insight for further therapy against ischemia-reperfusion.

Disclosure of conflict of interest

None.

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