Exendin-4 attenuates myocardial ischemia and reperfusion injury by inhibiting high mobility group box 1 protein expression

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Abstract

Background: High mobility group box 1 protein (HMGB1) plays an important role in myocardial ischemia and reperfusion (I/R) injury. Exendin-4 (Ex-4), glucagon-like peptide-1 receptor agonist, has been reported to attenuate myocardial I/R injury. This study was to investigate the potential mechanism by which Ex-4 attenuates myocardial I/R injury in rats.

Methods: Anesthetized male rats were once treated with Ex-4 (5 µg/kg, i.v.) 1 h before ischemia in the absence and/or presence of exendin(9-39) (an antagonist for glucagon-like peptide-1 receptor; 5 µg/kg, i.v.), and then subjected to ischemia for 30 min followed by reperfusion for 4 h. Lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA), superoxide dismutase (SOD) activity and infarct size were measured. HMGB1 expression was assessed by immunoblotting.

Results: The results showed that pretreatment of Ex-4 could significantly decrease the infarct size and the levels of LDH and CK after 4 h reperfusion (all p < 0.05). Ex-4 could also significantly inhibit the increase of the MDA level, the decrease of the SOD level (both p < 0.05). Meanwhile, Ex-4 could significantly inhibit HMGB1 expression induced by I/R. Administration of exendin(9-39) could abolish the protective effect of Ex-4 (all p < 0.05).

Conclusions: The present study suggested that Ex-4 could attenuate myocardial I/R injury which may be associated with inhibiting HMGB1 expression. (Cardiol J 2013; 20, 6: 600–604)

Key words: exendin-4, myocardial ischemia, reperfusion, high mobility group box 1 protein

Introduction

High mobility group box 1 protein (HMGB1), a non-chromosomal nuclear protein that regulates gene transcription and maintains the nucleosome structure, could be negatively released by necrotic cell, apoptotic cell or positively activated innate immune cells (such as macrophages and monocytes) [1, 2]. HMGB1 has been identified as a novel pro-inflammatory cytokine in several cardiovascular diseases [3–6]. Recently, HMGB1 has been found to function as an early pro-inflammatory mediator during myocardial ischemia and reperfusion (I/R), as well as some classical early pro-inflammatory...
cytokine such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and could promote the release of TNF-α and IL-6, whereas HMGB1 A box peptide (a specific HMGB1 antagonist) could protect against myocardial I/R injury and inhibit the release of TNF-α and IL-6 [3], while inflammatory response is considered to be a critical factor of myocardial I/R injury [7, 8]. These results suggested that HMGB1 plays an important role in myocardial I/R injury.

Glucagon-like peptide-1 (GLP-1), a gut incretin hormone secreted from L cells in the intestine in response to food intake, has been considered an attractive agent for the management of type-2 diabetes mellitus [9]. The GLP-1 receptor agonist-exendin-4 (Ex-4), a 39 amino acid peptide, has been shown to activate GLP-1 receptors to increase intracellular cAMP in pancreatic acinar cells and has no effect on VIP receptors, functioning identically to GLP-1. A growing body of evidence shows that GLP-1 and Ex-4 could provide significant beneficial effects for cardiovascular diseases, including myocardial I/R, in both experimental animal models and patients [10–13]. Recently, Cai et al. [14] have found that Ex-4 could inhibit HMGB1 expression induced by high glucose condition in cardiomyocytes. Thus, the hypothesis that Ex-4 may attenuate myocardial I/R injury by inhibiting HMGB1 expression was tested in a rat myocardial I/R model in the present study.

Methods
Animal preparation and experimental designs

The experiment protocol conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, revised in 1996) and was approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250–300 g) were randomly assigned into 4 groups receiving the following treatments:

- Group 1: sham operated control (SO) (n = 10); rats were subjected to surgical manipulation without the induction of myocardial ischemia;
- Group 2: ischemia and reperfusion (I/R) (n = 15): rats were subjected to the left anterior descending coronary artery (LAD) occlusion for 30 min followed by reperfusion for 4 h;
- Group 3: Ex-4 + I/R (Ex-4 – I/R) (n = 15): the rats were once treated with Ex-4 (5 µg/kg, i.v., Sigma, USA) [15, 16] 1 h before LAD occlusion, and subjected to LAD occlusion for 30 min and followed by reperfusion for 4 h. Ex-4 was dissolved in sterile saline;
- Group 4: Ex-4 + exendin(9-39) [Ex(9-39)] + I/R (Ex-4-I/R) (n = 15) the rats were once treated with Ex-4 (5 µg/kg, i.v.) and Ex(9-39) (an antagonist for GLP-1 receptor, 5 µg/kg, i.v., Sigma, USA) [12] 1 h before LAD occlusion, and subjected to LAD occlusion for 30 min and followed by reperfusion for 4 h. Ex(9-39) was dissolved in sterile saline.

After being anesthetized with sodium pentobarbital (45 mg/kg, i.p.), the rats were ventilated artificially with a volume-controlled rodent respirator at 70 strokes per minute. Rats were placed on an electric heating pad to maintain the body temperature at 37°C. Heparin (200 IU/kg, i.v.) was given before ischemia. Lead-II of the electrocardiogram was monitored with subcutaneous stainless steel electrodes. Electrocardiogram was monitored using a computer-based EP system (LEAD2000B, Jinjiang Ltd, China).

A thoracotomy through a left parasternal incision was performed to expose the anterior wall of the left ventricle. A 4–0 silk suture 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 was passed into both ends of the suture, which was then fixed by champing the tube with a mosquito hemostat. A successful myocardial I/R model was confirmed by ST segment elevation in leads-II and regional cyanosis of the myocardial surface. The rats subjected to a 30-min occlusion of the LAD and followed by a 4 h reperfusion.

Assessment of myocardial injury

To assess the lactate dehydrogenase (LDH) and creatine kinase (CK), blood samples were collected and centrifuged. Standard techniques using commercialized assay kits in accordance with the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, China) were used for the analyses. Values were expressed in international units per liter.

Assessment of the infarct size

The infarct size was established by 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described previously [17]. Briefly, after reperfusion the LAD was occluded again and 2 mL of 1.0% Evans blue dye was injected via the femoral vein. Each heart was then sliced horizontally to yield 5 slices. The slices were incubated in 1% TTC for 15 min at 37°C. The infarct area (white) and the area at risk (red and white) from each section were measured using an image analyzer (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD). The infarct size was expressed as...
a percentage of the risk area volume (%, infarct size/risk area).

**MDA and SOD activity assay**

Measurement of myocardium malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activity in myocardial tissue were measured using commercialized assay kits in accordance with the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, China), as described in the previous study [14]. The former were used as indexes of oxygen free radical and the latter as lipid superoxide level in the myocardium, respectively.

**Immunoblotting analysis**

Pulverized frozen ischemia area of left ventricle samples were analyzed by quantitative immunoblotting using HMGB1 antibody (Santa Cruz, USA) as described in the previous study [14]. The expression of protein was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

**Statistical analysis**

Statistical analysis was performed with the SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All values were expressed as mean ± standard deviation. One-way ANOVA or Welch was used for comparisons among groups and the Least-significant Difference or Dunnett T3 was used for post-hoc multiple comparisons. Statistical significance was defined as p < 0.05.

**Results**

**Infarct size**

After 4 h reperfusion, the infarct size induced by I/R was decreased by Ex-4 pretreatment compared with that in I/R group (p < 0.05). Administration of Ex(9-39) could abolish the protective effect of Ex-4 on infarct size (p < 0.05; Fig. 1).

**LDH and CK levels**

After 4 h reperfusion, both LDH and CK levels in I/R group were significantly increased compared to that in SO group (p < 0.05). However, the increase of LDH and CK levels was significantly attenuated by Ex-4 (both p < 0.05). Administration of Ex(9-39) could abolish the protective effect of Ex-4 on myocardial enzyme (p < 0.05) (Fig. 2).

**MDA and SOD levels**

After 4 h reperfusion, the MDA level in I/R group was significantly increased while the SOD level was significantly decreased compared to that in SO group (p < 0.05). Both the increase of the MDA level and the decrease of the SOD level were significantly inhibited by Ex-4 (both p < 0.05). Administration of Ex(9-39) could abolish the protective effect of Ex-4 on oxidative stress (p < 0.05) (Fig. 3).

**HMGB1 expression**

As shown in Figure 4, HMGB1 expression was markedly increased after 4 h reperfusion (p < 0.05), which was significantly inhibited by Ex-4 (p < 0.05). Administration of Ex(9-39) could abolish the protective effect of Ex-4 on HMGB1 expression (p < 0.05).
Previous studies showed that GLP-1 and GLP-1 receptor agonists could attenuate myocardial I/R injury and improve cardiac function [10–12, 18]. In this study, we found that GLP-1 receptor agonists-Ex-4 could attenuate myocardial I/R injury. Meanwhile, we observed that Ex-4 could inhibit HMGB1 expression induced by I/R. While using an antagonist for GLP-1 receptor abolish the protective effect of Ex-4 on myocardial I/R injury and HMGB1 expression. Hu et al. [19] demonstrated that HMGB1 could promote the apoptosis of cardiomyocytes in a dose-dependent manner while apoptosis plays a critical role in myocardial I/R injury accompanied by inflammation [7, 20]. HMGB1 could promote the release of TNF-α, IL-6 during myocardial I/R, whereas a specific HMGB1 antagonist could attenuate myocardial I/R injury and inhibit the release of TNF-α and IL-6 [3], indicating that inhibiting HMGB1 expression could suppress the inflammatory process and myocardial I/R injury. Thus, the above results suggest that Ex-4 could attenuate myocardial I/R injury which may be associated with inhibiting HMGB1 expression. In addition, we found that pretreatment of Ex-4 could decrease the level of MDA (one of reactive oxygen species and as index for oxidative stress) and increase the level of SOD (key antioxidant enzymes). Previous studies indicated that oxidative stress may be involved in the release of HMGB1. Tang et al. [21] reported that hydrogen peroxide, one of reactive oxygen species, could stimulate macrophages and monocytes to actively release HMGB1. Tsung et al. [22] further confirmed that HMGB1 released from cultured hepatocytes was also found to be an active process regulated by reactive oxygen species, indicating that inhibiting reactive oxygen species may decrease the HMGB1 expression. These results suggest that pretreat-

**Discussion**

Previous studies showed that GLP-1 and GLP-1 receptor agonists could attenuate myocardial I/R injury and improve cardiac function [10–12, 18]. In this study, we found that GLP-1 receptor agonists-Ex-4 could attenuate myocardial I/R injury. Meanwhile, we observed that Ex-4 could inhibit HMGB1 expression induced by I/R. While using an antagonist for GLP-1 receptor abolish the protective effect of Ex-4 on myocardial I/R injury and HMGB1 expression. Hu et al. [19] demonstrated that HMGB1 could promote the apoptosis of cardiomyocytes in a dose-dependent manner while apoptosis plays a critical role in myocardial I/R injury accompanied by inflammation [7, 20]. HMGB1 could promote the release of TNF-α, IL-6 during myocardial I/R, whereas a specific HMGB1 antagonist could attenuate myocardial I/R injury and inhibit the release of TNF-α and IL-6 [3], indicating that inhibiting HMGB1 expression could suppress the inflammatory process and myocardial I/R injury. Thus, the above results suggest that Ex-4 could attenuate myocardial I/R injury which may be associated with inhibiting HMGB1 expression. In addition, we found that pretreatment of Ex-4 could decrease the level of MDA (one of reactive oxygen species and as index for oxidative stress) and increase the level of SOD (key antioxidant enzymes). Previous studies indicated that oxidative stress may be involved in the release of HMGB1. Tang et al. [21] reported that hydrogen peroxide, one of reactive oxygen species, could stimulate macrophages and monocytes to actively release HMGB1. Tsung et al. [22] further confirmed that HMGB1 released from cultured hepatocytes was also found to be an active process regulated by reactive oxygen species, indicating that inhibiting reactive oxygen species may decrease the HMGB1 expression. These results suggest that pretreat-
ment of Ex-4 could inhibit HMGB1 expression which may be associated with inhibiting reactive oxygen species induced by myocardial I/R.

Limitations of the study
In the study, we only investigated the effect of Ex-4 on myocardial I/R injury and HMGB1 expression. The precise mechanisms underlying our observation will require future elucidation.

Conclusions
The present study suggested that Ex-4 could attenuate myocardial I/R injury which may be associated with inhibiting HMGB1 expression.

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Conflict of interest: none declared

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