



Research Article

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Liraglutide directly protects cardiomyocytes against reperfusion injury possibly via modulation of intracellular calcium homeostasis

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Abstract

Background Liraglutide is glucagon-like peptide-1 receptor agonist for treating patients with type 2 diabetes mellitus. Our previous studies have demonstrated that liraglutide protects cardiac function through improving endothelial function in patients with acute myocardial infarction undergoing percutaneous coronary intervention. The present study will investigate whether liraglutide can perform direct protective effects on cardiomyocytes against reperfusion injury. **Methods** *In vitro* experiments were performed using H9C2 cells and neonatal rat ventricular cardiomyocytes undergoing simulative hypoxia/reoxygenation (H/R) induction. Cardiomyocytes apoptosis was detected by fluorescence TUNEL. Mitochondrial membrane potential ($\Delta\Psi_m$) and intracellular reactive oxygen species (ROS) was assessed by JC-1 and DHE, respectively. Fura-2/AM was used to measure intracellular Ca^{2+} concentration and calcium transient. Immunofluorescence staining was used to assess the expression level of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a). *In vivo* experiments, myocardial apoptosis and expression of SERCA2a were detected by colorimetric TUNEL and by immunofluorescence staining, respectively. **Results** *In vitro* liraglutide inhibited cardiomyocytes apoptosis against H/R. $\Delta\Psi_m$ of cardiomyocytes was higher in liraglutide group than H/R group. H/R increased ROS production in H9C2 cells which was attenuated by liraglutide. Liraglutide significantly lowered Ca^{2+} overload and improved calcium transient compared with H/R group. Immunofluorescence staining results showed liraglutide promoted SERCA2a expression which was decreased in H/R group. In ischemia/reperfusion rat hearts, apoptosis was significantly attenuated and SERCA2a expression was increased by liraglutide compared with H/R group. **Conclusions** Liraglutide can directly protect cardiomyocytes against reperfusion injury which is possibly through modulation of intracellular calcium homeostasis.

J Geriatr Cardiol 2017; 14: 57–66. doi:10.11909/j.issn.1671-5411.2017.01.008

Keywords: Calcium overload; Cardiomyocyte; Liraglutide; Reperfusion injury; Sarcoplasmic reticulum Ca^{2+} -ATPase

1 Introduction

Acute myocardial infarction (MI) is a major cause of death and disability worldwide. In patients with ST segment elevation myocardial infarction (STEMI), the treatment for reducing acute myocardial ischemic injury and limiting MI size is timely and effective myocardial reperfusion using either thrombolytic therapy or primary percutaneous coronary intervention (PPCI).^[1,2] The process of restoring coronary blood flow can paradoxically induce myocardial injury and cardiomyocytes death, a phenomenon which has been termed “myocardial reperfusion injury” which has been inferred in both experimental MI models and in patients

with STEMI.^[1,3,4] For patients with STEMI, timely myocardial reperfusion using PPCI is essential to salvage viable myocardium, limit MI size, preserve left ventricular (LV) systolic function, and prevent the onset of heart failure, however, myocardial reperfusion injury weakens the benefit of reperfusion therapy and brings patients larger MI size, more severe heart failure and worse prognosis. Despite of development of PPCI combined with medical therapies, there isn’t effective therapy for myocardial reperfusion injury.^[3,5]

The glucagon-like peptide-1 (GLP-1) is a hormone derived from the transcription product of the proglucagon gene that is secreted from intestinal endocrine L cells in response to nutrients.^[6] Once in the blood stream, GLP-1 exerts incretin-like actions stimulating insulin secretion in a glucose-dependent manner when interacting with its receptor (GLP-1R) on β -pancreatic cells. Because GLP-1 half-life is short, GLP-1 analogues including DPP-4 inhibitors and GLP-1R agonists have been developed for the treatment of patients with type 2 diabetes mellitus.^[5,7] The pleiotropic

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Received: October 20, 2016

Revised: December 21, 2016

Accepted: January 3, 2017

Published online: January 28, 2017

properties of GLP-1 render it specifically suited to protecting tissue against ischemia/reperfusion injury.^[8-14] Liraglutide, a GLP-1 analogue, was reported to reduce cardiac rupture and infarct size and improve cardiac output in normal and diabetic mice.^[15] Our previous clinical trial has showed liraglutide improved heart function in patients with acute myocardial infarction undergoing PCI.^[16] The underlying mechanism isn't completely clear. Previous studies showed liraglutide elicited favorable changes in markers of inflammation and endothelial function,^[16,17] however, the effect of liraglutide on cardiomyocytes isn't elucidative.

Previous studies have showed Ca^{2+} overload and oxidative stress are the primary stimulators to damage mitochondrial function and induce cardiomyocytes apoptosis in hypoxia/reoxygenation (H/R) condition. The cardiac sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) is a key pump responsible for intracellular calcium handling and contractility in cardiac cells. Impaired calcium reuptake resulting from decreased expression and activity of SERCA2a is a hallmark of HF.^[18] So we hypothesized liraglutide implements direct protective effects on cardiomyocyte against reperfusion injury through maintaining intracellular calcium homeostasis which is related to SERCA2a. The study focused on direct effect of liraglutide on cardiomyocytes under H/R condition to make sure how liraglutide can directly protect cardiomyocytes against H/R injury. The results of present study indicated liraglutide directly protect cardiomyocytes against H/R, preserved $\Delta\Psi_m$, reversed reactive oxygen species (ROS) production and calcium overload. Moreover, liraglutide could improve calcium transient and enhance expression of SERCA2a in cardiomyocytes against reperfusion injury.

2 Methods

2.1 Ethics statement

The present study was performed in accordance with the guidelines of the Ethic Committee of Chinese PLA (People's Liberation Army) General Hospital, Beijing, China.

2.2 H9C2 cells culture

H9C2 cells (Chinese Academy of Medical Sciences, Shanghai, China) were maintained in Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12, Thermo Fisher Biochemical Products, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 100 mg/mL penicillin/streptomycin (Beyotime, China).

2.3 Isolation and culture of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes were isolated from 2-day-old Sprague-Dawley rats by enzymatic digestion with 0.05% trypsin and 0.015% collagenase. Cardiac fibroblasts were removed, and neonatal rat ventricular myocytes were plated onto 35-mm dishes at a density of 1×10^6 cells/mL in Dulbecco's modified Eagle's medium containing 10% FBS (Hyclone, Australian), 100 units/mL penicillin/streptomycin, and 0.1 mmol/L 5-bromo- 2-deoxyuridine to inhibit fibroblast proliferation.

2.4 H/R injury induction *in vitro* and liraglutide treatment

Hypoxic conditions were produced using fresh Hanks solution with 95% N_2 and 5% CO_2 . The pH was adjusted to 6.8 with lactate to mimic ischemic conditions. The dishes were put into a hypoxic incubator (*In vivo* 2–400, Ruskinn) that was equilibrated with 95% N_2 and 5% CO_2 and the actual oxygen concentration was zero. Ambient O_2 levels in the hypoxia incubator were monitored by an O_2 analyzer (series-2000, Alpha Omega). After hypoxic treatment, the culture medium was rapidly replaced with fresh DMEM with 1% FBS to initiate reoxygenation. Hypoxia/reoxygenation procedure was achieved by 4 h of hypoxia treatment (anoxia) and 4 h of reoxygenation treatment. For liraglutide treatment, cultured cells were pre-incubated with the optimal concentration of liraglutide (200 nmol/L) for 12 h before hypoxia.

2.5 *In vitro* TUNEL apoptosis assay of cardiomyocytes by confocal microscopy

The apoptosis of cardiomyocytes was examined by TUNEL assay. Briefly, cultured cardiomyocytes were fixed with 4% paraformaldehyde (Millipore, USA) and permeabilized with 1% Triton X-100 (Sigma Aldrich, USA) and in phosphate-buffered saline (PBS) (Invitrogen, USA) for 30 min, followed by 3 times (3 × 10 min) wash of fresh PBS. Then, an Apo-BrdU In Situ DNA Fragmentation Assay Kit (BioVision, USA) was applied for 1 h, followed by incubating the treated plates with 5 μL anti-BrdU FITC antibody. Fifteen minutes of DAPI immunostaining was conducted to identify the nuclei of cardiomyocytes. Images were then taken on an inverted Leica TCS-SP2 AOBS confocal laser-scanning microscope (Leica, Germany). Apoptosis was quantified as the percentage of healthy (no apoptosis) cardiomyocytes, and normalized to the percentage under control condition.

2.6 Colorimetric TUNEL apoptosis assay of myocardium

TUNEL staining was performed on myocardial frozen

sections according to the manufacturer's instructions (Col- orimetric TUNEL Apoptosis Assay Kit, Beyotime, Shanghai, China).^[19] TUNEL was performed to detect cellular apoptosis on terminal ileum tissue using *in situ* cell death detection Kit according to the manufacturer's instructions. Sections were post-fixed in ethanol-acetic acid (2: 1) and rinsed. Then the sections were incubated with proteinase K (100 µg/mL), rinsed, incubated in 3% H₂O₂, and washed with PBS for 10 min followed by washing with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 5 min. After the sections were washed twice with PBS, incubated in TUNEL reaction mixture, and rinsed again. Sections were visualized using converter-POD with 0.02% 3,3'-diaminobenzidine (DAB). Mayer's hematoxylin was used for counter-staining. The sections were finally mounted onto gelatin-coated slides and then they were air dried overnight at room temperature. Yellow stained nucleus was TUNEL positive cells.

2.7 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ of H9C2 cells was measured by cationic probe JC-1 (JC-1 assay Kit, Beyotime, Shanghai, China). JC-1 aggregates red fluorescence in mitochondria of normal cells. JC-1 accrues as green fluorescence monomer in the cytosol of apoptotic cells. $\Delta\Psi_m$ was increased linearly corresponds to the formation of JC-1 aggregates and their fluorescence. Briefly, H9C2 were washed twice with PBS, and then loaded with JC-1 at 37°C for 20 min. After rinse twice in staining buffer, green and red fluorescent intensity was measured by confocal microscopy. The $\Delta\Psi_m$ was calculated as the fluorescence intensity ratio of red to green. In the present study, quantitative analysis of fluorescence intensity was made using Image-Pro software.

2.8 Measurement of intracellular reactive oxygen species production

After treatment, the intracellular ROS and superoxide generation was measured by Dihydroethidium (DHE, Invitrogen, SanDiego, CA, USA) staining according to the manufacturer's instructions. H9C2 were incubated in FBS-free DMEM containing DHE (10 µmol/L) for 30 min in a light-protected humidified chamber at 37°C. Then, fluorescence microscopy (Olympus) was used to examine the fluorescence.

2.9 Detection of intracellular Ca²⁺ concentration

Intracellular Ca²⁺ was measured using the calcium-dependent fluorescent dye Fura-2 according to the manufacturer's instructions. Briefly, H9C2 cultures were trans-

ferred to 1 mL fresh DMEM containing 5 µL Fura-2-acetoxy-methylester (AM; 10 µmol/L; Life Technologies, Carlsbad, CA, USA) and incubated in a CO₂ incubator at 37°C for 1 h. Fura-2-loaded cells were then placed on the stage of a confocal microscopy (Olympus) and viewed using a 60 × oil immersion objective.

2.10 Detection of calcium transient in neonatal cardiomyocytes

The cardiomyocytes were placed on a glass coverslip and were incubated with 1 µm Fura-2/AM in HEPES-buffered physiological saline solution for 30 min at room temperature, and then washed three times with HPSS to remove extracellular dye. The coverslip was fixed in the Warner model RC-26 chamber (Warner Instruments, Hamden, CT, USA) and PH-1 heated platform (Warner Instruments, Hamden, CT, USA) that was mounted on an inverted microscope (Olympus America, Melville, NY, USA). Fura-2 fluorescence was alternately excited at the wavelengths of 340 nm and 380 nm with a monochromator (TILL Photonics, Polychrome V, Munich, Bavaria, Germany) and focused on the cells via a 40 oil objective (NA = 1.35, U/340, Olympus).

2.11 Detection of SERCA2a expression by immunofluorescence staining

After being treated as indicated, the cardiomyocytes cultured on glass coverslips in 24-well plates were washed with PBS for three times, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, and then blocked with 5% BSA for 1 h. Then, cardiomyocytes were incubated overnight at 4°C with monoclonal mouse anti-rat SERCA2a antibody (1: 100; Abcam, Cambridge, USA). After being washed with PBS for three times, cardiomyocytes were incubated with goat anti-mouse polyclonal IgG (1: 400; Abcam, Cambridge, USA) at room temperature in the dark for 2 h. For nuclear counterstaining, cardiomyocytes were incubated with 4,6-diamidino-2-phenylidone (DAPI; Sigma, USA) for 5 min. At last, the immunofluorescence images were obtained by inverted fluorescence microscope (Olympus, Tokyo, Japan). The myocardium was also treated according to instruction for immunofluorescence staining.

2.12 Myocardial infarction and reperfusion model, and liraglutide treatment

Male Sprague-Dawley rats (250 ± 10 g) were purchased from the Experimental Animal Center, Chinese PLA general hospital. All procedures were approved by the Institutional Animal Care and Use Committee of the Chinese PLA

General Hospital. Rats were randomly divided into the following groups: (1) Control group, (2) H/R group, (3) H/R+liraglutide group. Rats were anaesthetized with sodium pentobarbital (30 mg/kg, *i.p.*). The animals were then incubated and ventilated by a volume-regulated respirator during surgery. After a left lateral thoracotomy and pericardectomy, the left coronary artery was identified and gently ligated with a 6.0 prolene suture. Successful AMI was confirmed by the typical ST segment elevation in electrocardiography. Myocardial ischemia lasted for 30 min and reperfusion for 2 h. Freshly prepared liraglutide (Sigma-Aldrich, St. Louis, MO, USA) was administered by cauda vein at a dose of 0.18 mg/kg starting 12 h prior to MI which didn't produce severe side effects in the present study.

2.13 Statistical analysis

Data were described as the mean \pm SD of at least three independent experiments and analyzed by one way ANOVA. The limit of statistical significance between treated and control group was $P < 0.05$.

3 Results

3.1 Liraglutide prevents apoptosis of cardiomyocytes induced by H/R *in vitro*

TUNEL staining demonstrated H/R induce apoptosis of H9C2 cardiomyocytes *in vitro*. Pre-treated with liraglutide (200 nmol/L) decreased H/R-induced apoptosis of H9C2 cells. The results showed percentage of apoptotic cells was obviously higher in H/R group compared to control group, however, which was significantly lower in liraglutide group than H/R group (Figure 1).

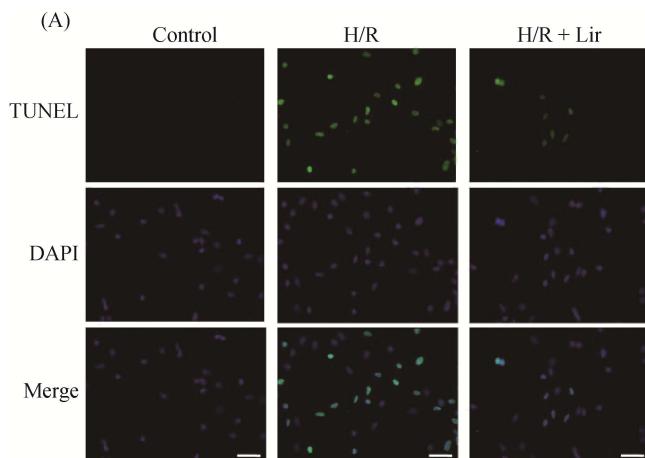


Figure 1. Liraglutide prevents cardiomyocytes apoptosis against H/R *in vitro* using fluorescence TUNEL. Representative colorimetric TUNEL staining images (A) and quantitative analysis in H9C2 cells (B), bar = 30 μ m. All values are presented as the mean \pm SD, $n = 3$; ** $P < 0.01$ vs. control group, ## $P < 0.01$ vs. H/R group. H9C2 cells incubated in normal condition or in simulated H/R condition or in simulated H/R condition plus pretreatment with liraglutide. H/R: hypoxia/reoxygenation; Lir: liraglutide.

3.2 Liraglutide preserves $\Delta\Psi_m$ of cardiomyocytes against H/R

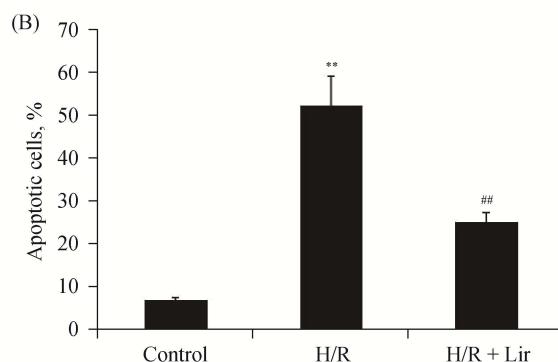
At 4 h after reoxygenation, $\Delta\Psi_m$ was detected by using JC-1 probe and was assessed as the fluorescence intensity ratio of red to green. As shown in Figure 2, lower level of red fluorescence and higher level of green fluorescence were observed in H/R cells, which revealed that H/R decreased $\Delta\Psi_m$ and damaged mitochondria function, but liraglutide increased fluorescence intensity ratio of red to green and preserved $\Delta\Psi_m$ of cardiomyocytes (Figure 2).

3.3 Liraglutide reduces reactive oxygen species production in cardiomyocytes against H/R

At 4 h after reoxygenation, ROS production in H9C2 was measured by fluorescence microscopy. The results showed that H/R significantly induces ROS production in H9C2 cells, and the pre-treatment of liraglutide decreased ROS production in H9C2 cells (Figure 3).

3.4 Liraglutide reduces Ca^{2+} overload in cardiomyocytes against H/R

At 4 h after reoxygenation, we investigated the effect of liraglutide on H/R-induced Ca^{2+} overload in cardiomyocytes using the calcium-dependent fluorescent dye Fura-2. The results showed the fluorescence was stronger in H/R group than in control group. Meanwhile, the fluorescence was decreased in liraglutide group compared with H/R group, which indicated that H/R caused a marked increase of cytosolic Ca^{2+} concentration and liraglutide pretreatment significantly inhibited H/R-induced increase of cytosolic Ca^{2+} concentration (Figure 4).



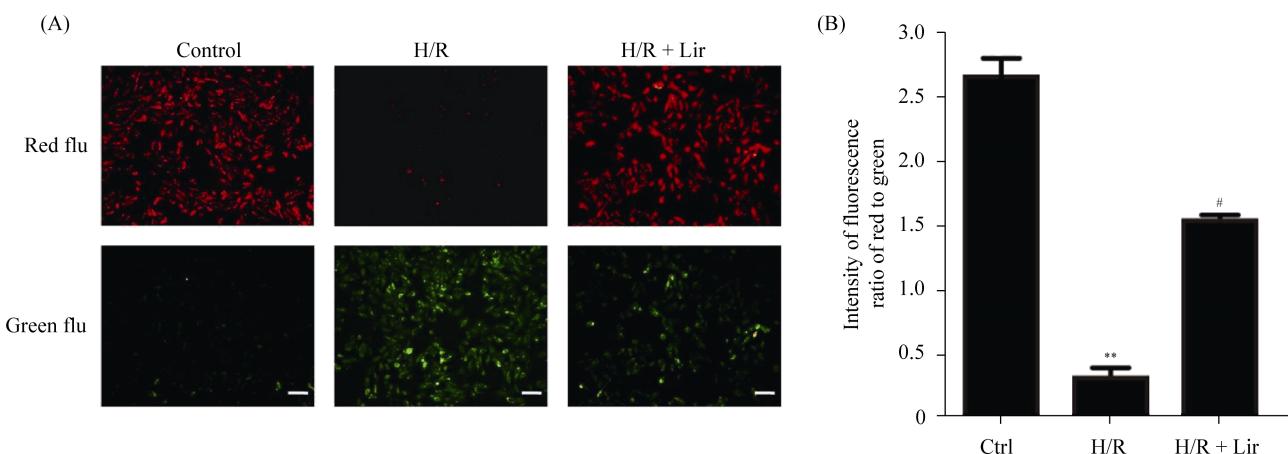


Figure 2. Liraglutide preserves $\Delta\Psi_m$ of cardiomyocytes against H/R. Representative images of JC-1 fluorescence (A) and quantitative analysis of fluorescence intensity in H9C2 cells (B), bar = 30 μ m. All values are presented as the mean \pm SD, $n = 3$; ** $P < 0.01$ vs. control group, # $P < 0.05$ vs. H/R group. $\Delta\Psi_m$ was assessed by fluorescence microscopy using JC-1 probe in H9C2 cells incubated in normal condition or in simulated H/R condition or simulated in H/R condition plus pretreatment with liraglutide. The results showed H/R obviously decreased red fluorescence intensity and increased green fluorescence intensity, and liraglutide reversed the effect. The results indicate liraglutide preserves $\Delta\Psi_m$ of cardiomyocytes and protect mitochondrial function in cardiomyocytes against H/R. Data were normalized as the ratio of red fluorescence to green fluorescence. H/R: hypoxia/reoxygenation; Lir: liraglutide.

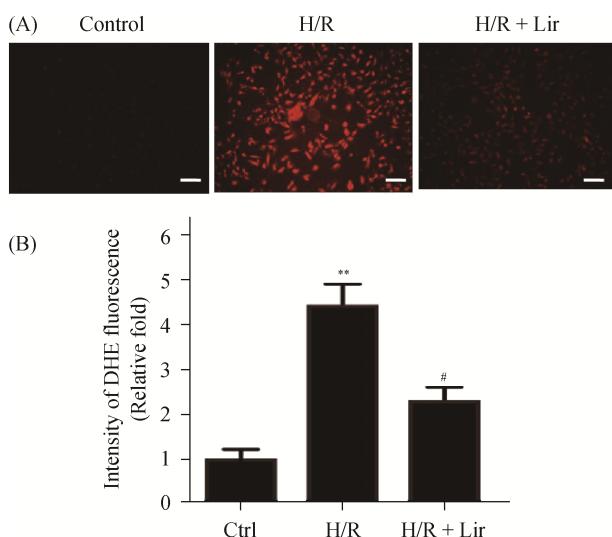


Figure 3. Liraglutide reduces reactive oxygen species production in cardiomyocytes against H/R. Representative images of fluorescence (A) and quantitative analysis of fluorescence intensity in H9C2 cells (B), bar = 30 μ m. All values are presented as the mean \pm SD, $n = 3$; ** $P < 0.01$ vs. control group, # $P < 0.05$ vs. H/R group. ROS was assessed by DHE staining in H9C2 cells incubated in normal condition or in simulated H/R condition or simulated in H/R condition plus pretreatment with liraglutide. The results showed fluorescence intensity is stronger in H/R group than in control group, meanwhile fluorescence intensity is lower in liraglutide group than in H/R group. H/R: hypoxia/reoxygenation; Lir: liraglutide; ROS: reactive oxygen species.

3.5 Liraglutide improved calcium transient in neonatal cardiomyocytes against H/R

At 4 h after reoxygenation, we investigated intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) activities in neonatal cardiomyocytes treated with H/R or liraglutide plus H/R procedure and compared the results with cardiomyocytes cultured in normal condition. The results showed the H/R-induced alterations of basal calcium content, amplitude, frequency, and time to peak of Ca^{2+} transients which were reversed by liraglutide (Figure 5).

3.6 Liraglutide promoted expression of SERCA2a in cardiomyocytes against H/R

At 4 h after reoxygenation, we investigated the effect of liraglutide on expression of SERCA2a in H9C2 cells by immunofluorescence staining. The results demonstrated H/R reduced expression of SERCA2a, but liraglutide reverse the effect. Pre-treatment liraglutide promoted expression of SERCA2a in cardiomyocytes against H/R (Figure 6).

3.7 Liraglutide attenuated apoptosis in reperfused rat hearts

In peri-infarcted site, apoptotic cells were more frequently observed in I/R group compared with control group, however, the apoptotic cells were decreased in liraglutide group. TUNEL staining demonstrated that the percentage of apoptotic cells in liraglutide group was higher than control group, which was significantly lower than I/R group (Figure 7).

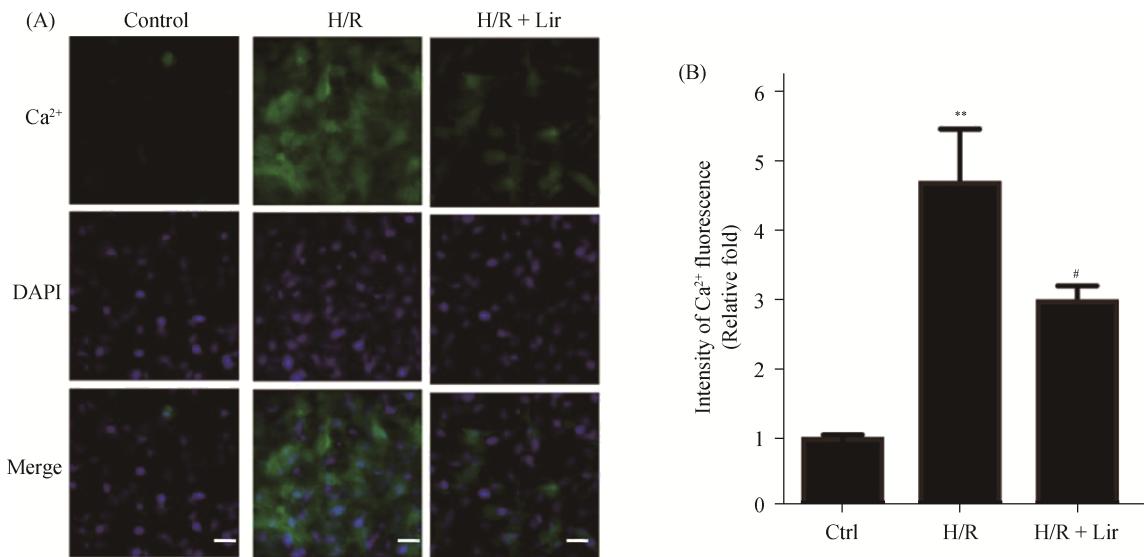


Figure 4. Liraglutide reduces Ca^{2+} overload in cardiomyocytes against H/R. Representative images of fluorescence (A) and quantitative analysis of fluorescence intensity in H9C2 cells (B), bar = 30 μm . All values are presented as the mean \pm SD, $n = 3$; ** $P < 0.01$ vs. control group, # $P < 0.05$ vs. H/R group. Ca^{2+} content was assessed using Fura-3/AM in H9C2 cells incubated in normal condition or in simulated H/R condition or simulated in H/R condition plus pretreatment with liraglutide. The green fluorescence intensity by Fura-3 was obviously stronger in H/R group, and liraglutide reversed the effect. H/R: hypoxia/reoxygenation; Lir: liraglutide.

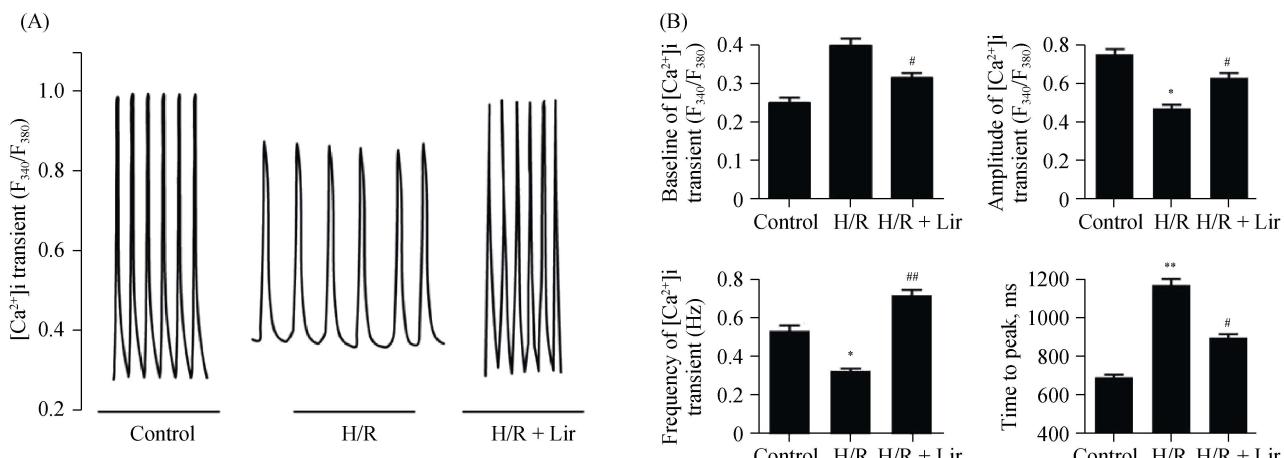


Figure 5. Liraglutide improved calcium transient in neonatal cardiomyocytes against H/R. (A): Representative calcium transient in neonatal cardiomyocytes; (B): increased basal calcium content, decreased amplitude and frequency, and longer time to peak in Ca^{2+} transients which were reversed by liraglutide. All values are presented as mean \pm SD, $n = 3$; * $P < 0.05$ vs. control group, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. H/R group. Calcium transient was assessed using Fura-2/AM in neonatal cardiomyocytes incubated in normal condition or in simulated H/R condition or simulated in H/R condition plus pretreatment with liraglutide. H/R: hypoxia/reoxygenation; Lir: liraglutide.

3.8 Liraglutide promoted expression of SERCA2a against reperfusion injury *in vivo*

In vivo, we investigated the effect of liraglutide on expression of SERCA2a in reperfused rat hearts. The results demonstrated expression of SERCA2a was lower in I/R group compared with control group, but expression of SERCA2a was higher in liraglutide group than I/R group.

Pretreatment of liraglutide improved the expression of SERCA2a in rat hearts against I/R (Figure 8).

4 Discussion

Myocardial reperfusion injury includes four recognized forms: reperfusion-induced arrhythmias, myocardial stunning,

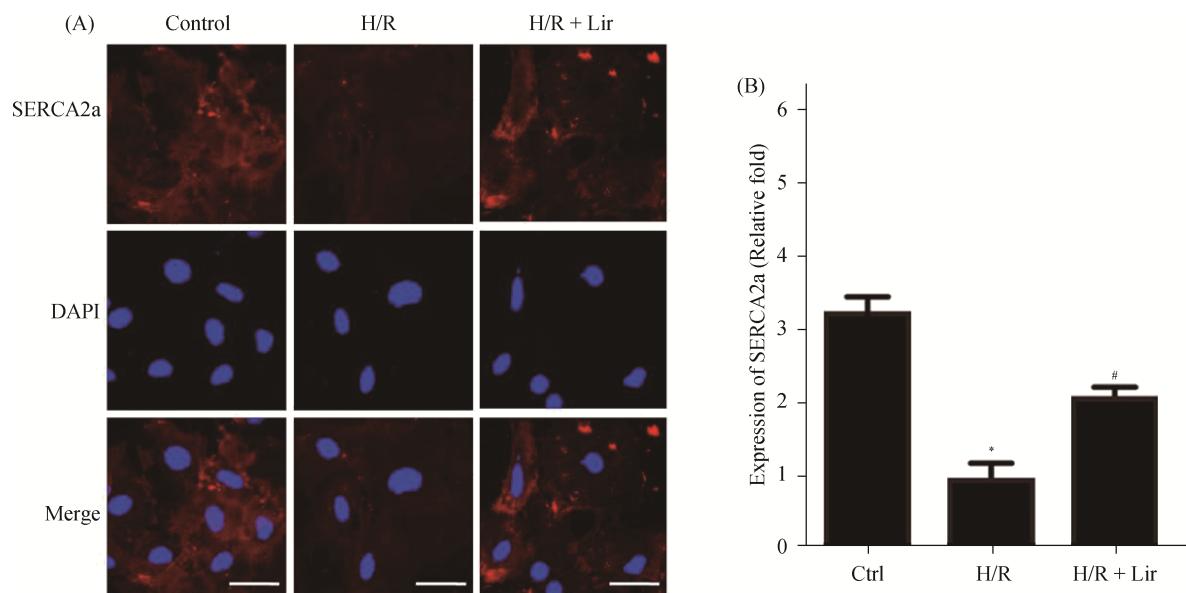


Figure 6. Liraglutide promoted expression of SERCA2a in cardiomyocytes against H/R. Representative images of fluorescence (A) and quantitative analysis of fluorescence intensity in H9C2 cells (B), bar = 30 μ m. All values are presented as the mean \pm SD, $n = 3$; $^*P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. H/R group. SERCA2a was assessed by immunofluorescence staining in H9C2 cells incubated in normal condition or in simulated H/R condition or simulated in H/R condition plus pretreatment with liraglutide. The results showed fluorescence intensity is decreased in H/R group than in control group, but the fluorescence intensity is stronger in liraglutide group compared with H/R group. The results indicated liraglutide promote the expression of SERCA2a. H/R: hypoxia/reoxygenation; Lir: liraglutide.

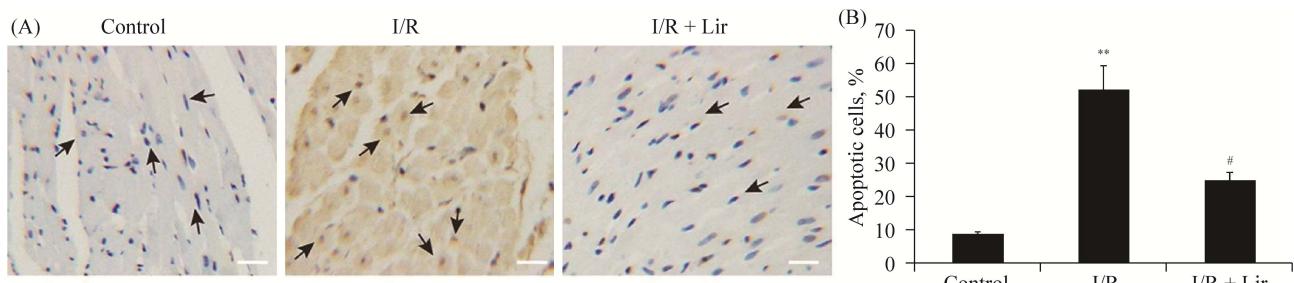


Figure 7. Liraglutide attenuates apoptosis in reperfused rat hearts. Representative colorimetric TUNEL staining images (A) and quantitative analysis in the peri-infarcted zone of heart sections (B), bar = 30 μ m. All values are presented as the mean \pm SD, $n = 3$; $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ vs. I/R group. Apoptotic nuclei were identified as TUNEL positive (brown); total nuclei were counterstained by DAPI (blue); apoptotic cells nuclei were considered as apoptotic cardiomyocytes (black arrow). I/R: ischemia/reperfusion; Lir: liraglutide.

microvascular obstruction and lethal myocardial reperfusion injury. The first two of which are reversible and the second two are irreversible.^[4] The two irreversible play important roles for heart function and clinical outcome so that it is important to prevent the two irreversible forms of reperfusion injury for patients with STEMI treated with PPCI. Microvascular obstruction was referred to the “inability to reperfuse a previously ischemic region” which namely is no reflow.^[20,21] The presence of no reflow is associated with a larger MI size, a lower LV ejection fraction, adverse LV remodeling, and worse clinical outcomes.^[4,21] Vascular endothelial cells oxidative damage resulting from H/R injury

is responsible for microcirculation perfusion disturbances and the progression of cardiac dysfunction.^[17] Our clinical trial showed liraglutide lowered the prevalence of no-reflow in patients with STEMI undergoing PCI.^[16] Previous study demonstrated heart function was improved at three months post PCI in liraglutide group compared with control group.^[16] We previously studied the effects and mechanisms of liraglutide on endothelial cells oxidative damage.^[17] The results indicate liraglutide pretreatment abrogated Ca²⁺-mediated oxidative apoptosis. Liraglutide regulated the rate of IP3R/ SERCA2a gene transcription and conserved SERCA2a-ATPase activity via the maintenance of ATP

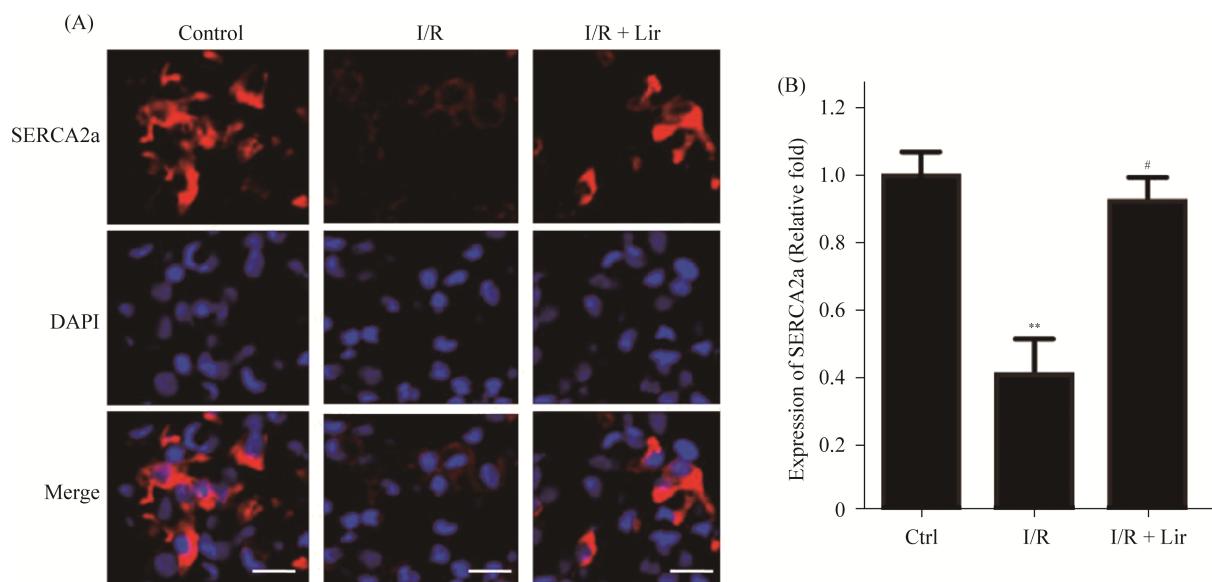


Figure 8. Liraglutide promoted expression of SERCA2a in reperfused rat hearts. Representative images of fluorescence (A) and quantitative analysis of fluorescence intensity in myocardium (B), bar = 30 μ m. All values are presented as the mean \pm SD, $n = 3$; ** $P < 0.01$ vs. control group; # $P < 0.05$ vs. I/R group. SERCA2a was assessed by immunofluorescence staining in reperfused myocardium. The results showed fluorescence intensity is decreased in I/R group than in control group, but the fluorescence intensity is stronger in liraglutide group compared with I/R group. The results indicated liraglutide promote the expression of SERCA2a in reperfused rat hearts *in vivo*. I/R: ischemia/reperfusion; Lir: liraglutide.

production under H/R, which drove excessive Ca^{2+} reflux to the sarcoplasmic reticulum and inhibited Ca^{2+} release from the SR, ultimately restoring Ca^{2+} homeostasis.

Reperfusion-induced death of cardiomyocytes that were viable at the end of the index ischemic event is defined as lethal myocardial reperfusion injury.^[22] The existence of lethal myocardial reperfusion injury has been inferred in both experimental MI models and in patients with STEMI.^[4] The major contributory factors for reperfusion-induced death of cardiomyocytes include oxidative stress, calcium overload, mitochondrial permeability transition pore (mPTP) opening, and hypercontracture, all of which contribute to the activation of apoptosis and necrosis in cardiomyocytes.^[5,23] Though liraglutide can protect cardiac function in patients with STEMI undergoing PPCI through improving endothelial function as previously reported,^[17,24] whether and how liraglutide can directly protect cardiomyocytes against H/R injury remains unclear. In the present study, we investigated the direct effects of liraglutide on cardiomyocytes against H/R injury *in vitro* and *in vivo*. We observed liraglutide showed significant salutary effects on cardiomyocytes against reperfusion injury.

Mitochondria plays critical role in cell life. There has been an increasing body of evidence indicating that the mPTP acts as a key nodal point in mediating cardiac dysfunction and cell death. mPTP opening results in inner

membrane potential ($\Delta\Psi_m$) collapse, respiratory chain uncoupling, halt of mitochondrial ATP synthesis, and eventually mitochondrial swelling, rupture, and cell death.^[25,26] Calcium overload and ROS play important roles during induction of mPTP opening, and previous studies have showed Ca^{2+} overload and oxidative stress are the primary stimulators to damage mitochondrial function and induce cardiomyocytes apoptosis in H/R condition.^[4,27-29] In the present study, *in vitro* results indicated liraglutide prevents cardiomyocytes apoptosis and ROS production against H/R. be decreased by liraglutide. More importantly, H/R-induced calcium overload and loss of $\Delta\Psi_m$ are significantly reversed by liraglutide. Previous studies have demonstrated that the mitochondrial membrane potential has a positive correlation with mPTP opening in cardiomyocytes subjected to oxidative or hormonal stress.^[30-32] So it is rational to infer that liraglutide protect cardiomyocytes against reperfusion injury at least partly through inhibiting mPTP opening via attenuating calcium overload.

The cardiac SERCA2a is a key pump responsible for intracellular calcium handling and contractility in cardiac cells. Impaired calcium reuptake resulting from decreased expression and activity of SERCA2a is a hallmark of HF.^[18] In cardiomyocytes, SERCA2a controls the removal of cytosolic Ca^{2+} and the storage of Ca^{2+} in the sarco/endoplasmic reticulum.^[33] SERCA2a is important for calcium modulation

and increased SERCA2a expression and activity are beneficial for maintaining calcium homeostasis.^[18,33] To further elucidate the effect of liraglutide on calcium modulation, we observed the effects of liraglutide on calcium transient and on expression of SERCA2a. The present study confirmed liraglutide improve calcium transient with lower basal calcium content, larger amplitude of calcium waves, shorter time to peak of calcium waves, and higher frequency compared with H/R group. Improved calcium transient is related to improved calcium reuptake and release. Improved calcium reuptake is critical for reducing calcium overload in cytoplasm and improving cardiomyocytes contraction. SERCA2a is responsible for calcium reuptake from cytoplasm to sarco/endoplasmic reticulum.^[18,33] The results showed SERCA2a expression is decreased in H/R group compared with control group, but liraglutide promotes SERCA2a expression in cardiomyocytes. Moreover, liraglutide can inhibit oxidative stress and cardiomyocytes apoptosis via reducing calcium overload against H/R. Meantime, liraglutide can preserve function of cardiomyocytes through improving calcium transient. The present results showed liraglutide could protect cardiomyocytes against H/R injury through inhibiting calcium overload via affecting SERCA2a expression in cardiomyocytes.

In reperfused rat hearts, the results confirmed liraglutide significantly attenuated cardiomyocytes apoptosis *in vivo*. Myocardial SERCA2a expression was also significantly increased in liraglutide group. Liraglutide with the dose used in the study didn't show obvious side effects compared with other groups. The *in vivo* results further confirmed the protective effect of liraglutide on cardiac function against reperfusion injury is at least partly related to SERCA2a.

In summary, liraglutide can directly protect cardiomyocytes against reperfusion injury possibly through SERCA2a to attenuate calcium overload and improve calcium transient. Improved calcium homeostasis followed by reduced ROS production and preserved mitochondrial function can decrease cardiomyocytes apoptosis and improve cardiomyocytes function. The present study provide more evidence for liraglutide to be used to protect cardiac function in patients with STEMI undergoing PPCI, however, it need further study to elucidate the molecular mechanism underlying the protective effect of liraglutide on modulation of calcium homeostasis in cardiomyocytes against reperfusion injury.

Acknowledgements

This work is supported by grants from National Natural Science Foundation of China (No. 81102079) and China

Postdoctoral Science Foundation (No. 201003776). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors report no conflict of interest. The authors are responsible for the content and writing of the paper.

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