Stimulation of glucagon-like peptide-1 receptor through exendin-4 preserves myocardial performance and prevents cardiac remodeling in infarcted myocardium

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DeNicola M, Du J, Wang Z, Yano N, Zhang L, Wang Y, Qin G, Zhuang S, Zhao TC. Stimulation of glucagon-like peptide-1 receptor through exendin-4 preserves myocardial performance and prevents cardiac remodeling in infarcted myocardium. Am J Physiol Endocrinol Metab 307: E630–E643, 2014. First published August 12, 2014; doi:10.1152/ajpendo.00109.2014.—We have demonstrated that GLP-1 improved myocardial functional recovery in acute myocardial ischemic injury. However, whether stimulation of the GLP-1 receptor (GLP-1R) with exendin-4, a selective GLP-1R agonist, could initiate a protective effect in the heart remains to be determined. Mouse myocardial infarction (MI) was created by ligation of the left descending artery. After 48 h of MI, animals were divided into the following groups (n = 5–7/group): 1 sham (animals that underwent thoracotomy without ligation), 2 MI [animals that underwent MI and received a daily dose of intraperitoneal injection (ip) of saline]; and 3) MI + exendin-4 [infarcted mice that received injections of exendin-4 (0.1 mg/kg ip)]. Two weeks later, cardiac function was assessed by echocardiography and an isovolumetrically perfused heart. Compared with control MI hearts, stimulation of GLP-1R improved cardiac function, which was associated with attenuation of myocardial hypertrophy, the mitigation of interstitial fibrosis, and an improvement in survival rate in post-MI hearts. Furthermore, H9c2 cardiomyoblasts were preconditioned with exendin-4 at a dose of 100 nmol/l and then subjected to hydrogen peroxide exposure at concentrations of 50 and 100 μmol/l. The exendin-4 treatment decreased lactate dehydrogenase leakage and increased cell survival. Notably, this event was also associated with the reduction of cleaved caspase-3 and caspase-9 and attenuation of reactive oxygen species production. Exendin-4 treatments improved mitochondrial respiration and suppressed the opening of mitochondrial permeability transition pore and protected mitochondria function. Our results indicate that GLP-1R serves as a novel approach to eliciting cardioprotection and mitigating oxidative stress-induced injury.

glucagon-like peptide-1 receptor; exendin-4; heart; infarction; oxidant stress

MYOCARDIAL INFARCTION CONtributes SIGNIFICANTLY to deaths related to coronary artery disease (14, 31). Because diabetes mellitus has threatened to become a global health crisis, the treatment of diabetes and its implications constitutes a major health care expenditure (19). A significant proportion of diabetic patients are known to develop diabetic cardiomyopathy, with a high incidence of congestive heart failure (33). In the ischemic myocardium, an increase in glucose uptake and subsequent ATP generated through glycolysis helps to sustain myocardial electric and mechanical performance, maintains cellular ultrastructure, promotes myocardial recovery, and slows mitochondrial injury. Based on these premises, the mechanism of enhancing myocardial energetic efficiency by increasing glucose availability and utilization has led to a vigorous pursuit of therapeutic approaches designed to augment glucose uptake and oxidation or reduce fatty acid uptake and oxidation as cardioprotective strategies in myocardial infarction and other clinical states of left ventricular systolic dysfunction (43).

Glucagon-like peptide-1 (GLP-1) is an incretin hormone produced in the small intestine that has been thought to have a variety of insulinotropic effects. It is a major target for research for type 2 diabetes because it stimulates pancreatic insulin release based upon glucose concentration in the bloodstream. During myocardial ischemia, glucose is the favored energy source for the heart since it does not require oxygen, so an increase in glucose uptake before ischemia by GLP-1 and exendin-4 should elicit protective effects (27, 28). We have further shown that GLP-1 protects the heart against ischemic injury (44). Like GLP-1, exendin-4 stimulates the production of insulin as a direct response to blood glucose levels while also having a longer half life, making it a more favorable target than GLP-1 for a therapeutic approach (13). Exendin-4 is a mimetic agonist of the GLP-1 receptor (GLP-1R) containing 39 amino acids (12). Exendin-4, also called exenatide, is a GLP-1 receptor agonist now available for treating type 2 diabetes mellitus. Exendin-4 does not possess a dipeptidyl peptidase-IV recognition site and is also a potent insulinotropic agent (11). Additionally, although exendin-4 has been shown to have protective effects against oxidative stresses of pancreatic β-cells, endothelial cells, and islets (15, 18, 37), it remains unknown whether continual delivery of exendin-4 would preserve cardiac performance and prevent myocardial remodeling. To demonstrate the effect that exendin-4 can have on cardiomyocytes and overall heart function, we took both in vitro and in vivo approaches to test the effect of exendin-4 on H9c2

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cardiomyoblasts exposed to oxidative stress and post-myocardial infarction (MI). Our results indicated that the stimulation of GLP-1R with exendin-4 produced beneficial effects of improving cardiac function recovery, attenuating myocardial hypertrophy, reversing cardiac remodeling, and improving survival rate in post-MI animals. Furthermore, the treatment of H9c2 cardiomyoblasts with exendin-4 led to a profound protective phenotype against reactive oxidant stress, as indicated by the reduction of lactate dehydrogenase (LDH) release, the increase in cell survival rate, the decrease in production of reactive oxygen species (ROS), the protection of mitochondrial function, and the mitigation of apoptosis. Notably, the cellular protective effect of stimulation of GLP-1R is associated with the improvement in mitochondrial respiration capacity and suppression of mitochondrial permeability transition pore (mPTP) opening. Taken together, the results suggest that exendin-4 elicits a potent protective signal pathway against myocardial dysfunction in the ischemic heart, and this provides new insight into developing new therapeutic strategies for heart disease.

MATERIALS AND METHODS

Chemical supplies and animals. Exendin-4 and perfusion chemicals were obtained from Sigma-Aldrich (St. Louis, MO). 5-(and-6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) was obtained from Invitrogen. The MitoCapture mitochondrial apoptosis detection kit was obtained from BioVision (Tokyo, Japan). All chemicals for the heart perfusion were purchased from Sigma. The reagents for Western blot were purchased from Bio-Rad (Hercules, CA). Intracellular GSH assay kit was purchased from AbCam (Cambridge, MA). Calcein AM was purchased from Life Technologies (Grand Island, NY).

Animals. Adult male imprinting control region mice were supplied by Charles River Laboratories (Wilmington, MA). All animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Roger Williams Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

In vivo MI. The mouse MI model was created following thoracotomy by applying permanent ligation of the left coronary artery, as described previously (36, 40, 41). Briefly, mice were anesthetized with an intraperitoneal (ip) injection of pentobarbital sodium at a dose of 50 mg/kg; additional doses of pentobarbital were given as needed during the procedure to maintain an anesthetized state. Mice were placed in a supine position and intubated with an endotracheal tube. Ventilation was achieved with a Mini rodent ventilator (Harvard Apparatus, Holliston, MA). Thoracotomy was performed with tenotomy scissors. A 7-0 nylon suture was passed with a tapered needle under the left anterior descending coronary artery. The suture was tied to create coronary occlusion. Upon completion of ligation, the chest was closed in a layered fashion, and air was evacuated to prevent pneumothorax. Mice in the sham group were anesthetized and underwent thoracotomy without coronary ligation. The post-MI animals received an ip injection of either exendin-4 or vehicle on a daily basis starting 48 h after MI. Animals were divided into three groups: 1) sham [animals underwent thoracotomy only and the placement of suture around the left coronary artery without ligation; MI control animals received an intraperitoneal injection of saline (0.1 ml)], 2) MI (animals underwent the ligation of left coronary artery), and 3) MI + exendin-4 [MI animals received exendin-4 treatments (0.1 mg/kg (Sigma, St. Louis, MO), which was injected ip at 48 h following surgical operation on a daily basis for a 2 consecutive wk after MI)]. Following the cardiac procedure, animals were then extubated and allowed to recover until they regained full alertness and started to move around. Buprenorphine (0.03 mg/kg) was given by a subcutaneous injection 2 h before surgery and a second time on day 2 posturgery to provide adequate analgesia. Postoperative animals were observed on a daily basis to evaluate the survival rate.

Echocardiographic assessment of cardiac performance. Mice were anesthetized with 1.5% isoflurane, and temperature was maintained at 37°C. Nair lotion (Church & Dwight Canada, Mississauga, ON, Canada) was applied on the precardial region for 3 min to cleanly remove the hair, and the region was covered with prewarmed ultrasound transmission gel (Aquasonic; Parker Laboratory, Fairfield, NJ). Transthoracic echocardiography was performed using an Acuson Sequoia C512 system with a 15L8 linear array probe. All images were acquired at a depth setting of 25 mm. Two-dimensional B-mode and M-mode echocardiographic images were obtained at the level of the papillary muscles from the parasternal short-axis view. Wall thickness and chamber dimension were determined from M-mode tracings using cardiac calcs software. All left ventricle (LV) dimensions are presented as the average of measurements using three to five consecutive selected beats.

Perfused isovolumetrically contracting heart. The methodology of Langendorff’s perfused heart preparation and measurement of left ventricular (LV) function has been described previously in detail (40, 42). Briefly, mice were anesthetized with a lethal ip injection of pentobarbital sodium (120 mg/kg). Hearts were rapidly excised and arrested in ice-cold Krebs-Henseleit buffer. They were then cannulated via the ascending aorta for retrograde perfusion by the Langendorff method using Krebs-Henseleit buffer containing (in mM): 110 NaCl, 4.7 KCl, 1.2 MgSO4 7H2O, 2.5 CaCl2 2H2O, 11 glucose, 1.2 KH2PO4, 25 NaHCO3, and 0.5 EDTA. The buffer, aerated with 95% O2–5% CO2 to give a pH of 7.4 at 37°C, was perfused at a constant pressure of 55 mmHg. A water-filled latex balloon inserted into the left ventricle and attached to the tip of polyethylene tubing was then inflated sufficiently to provide a left ventricular (LV) end-diastolic pressure (LVEDP) of ~5–10 mmHg measured by means of a disposable Gould pressure transducer. LV functional analysis was recorded using software and a computer-based recording system (BIOPAC, Goleta, CA). These parameters included LV systolic pressure, LVEDP, heart rate (HR), and LV-developed pressure (dp/dt), where LV dp/dt max is LV systolic pressure minus LVEDP. LV dP/dt min was continuously recorded. At the end of the experiments, myocardial samples were collected for histological analysis.

Histological analysis. Sections (10 μm) were prepared from paraffin-embedded tissues with sections from apex, mid-left ventricle, and base. Images of the sections from the base to the apex of the left ventricle were taken using an Olympus BX51 microscope with Spot Advanced software. Myocyte cross-sectional area was measured from images captured from the sections obtained mid-distance from the base to the apex. Wheat germ agglutinin staining was carried out using immunofluorescent staining to measure cell size. Suitable cross-sections were defined as having nearly circular-to-oval myocyte sections. The outline of myocytes was traced in the LV of each animal, using NIH ImageJ software to determine myocyte cross-sectional area. A value of six to eight fields in each section was captured, and three sections from each heart were calculated by the measurements of ~400–600 cells in remote area from infarction of an individual heart. The mean area was calculated for the LV in each heart, and the group mean was calculated. Cardiac interstitial fibrosis was quantified by picrosirius red staining of three sections from each heart. Computer-assisted image analysis (ImageJ) was used to quantify the red-stained area of each section.

In vitro H9c2 cardiomyoblast culture and oxidant stress. H9c2 cardiomyoblasts, a clonal line derived from rat heart (ATCC, Rockville, MD), were grown in DMEM with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2. Monolayers were passed by trypsinization at 70–80% confluence. H9c2 myoblasts were exposed

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with 100 µmol/l hydrogen peroxide for 2 h and then resumed with normal differentiation medium for 1 h. After stress, cytotoxicity and cell viability were measured according to manufacturers’ instructions, with minor modifications.

Measurements of LDH leakages. Loss of plasma membrane integrity (cell necrosis) was assessed by measurement of the activity of LDH in the supernatant. A 50µl of supernatant was transferred into a 96-well plate. The release of LDH in the culture medium from H9c2 cardiomyoblasts at the end of the experiments was determined as an indicator of cell necrosis, using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) as per the manufacturer’s protocol. The optical density was determined spectrophotometrically at a wavelength of 490 nm, and the values are expressed as percentage of control values (10).

Estimation of cell viability (MTT assay). In a subset of experiments, cell viability was measured by MTT assay; 0.25 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; St. Louis, MO) was added to 1 ml of cell suspension, and cells were subsequently incubated for 4 h at 37°C. After three washes of cells with PBS, the insoluble formazan product was dissolved in buffer containing 1% HCl in isopropanol and 0.1% Triton X-100 (50:1). The optical density of each culture well was measured at a wavelength of 550 nm, and the values are expressed as percentages of control values (46).

Measurement of ROS. The contents of intracellular ROS in H9c2 cardiomyoblasts were assessed by using CM-H2DCFDA. CM-H2DCFDA is nonfluorescent until the removal of the acetate groups by intracellular esterases, and then oxidation occurs within the cell (20, 24). H9c2 cardiomyoblasts were treated with exendin-4 for 2 h and followed by incubation with H2O2 for 2 h, and then cells were washed with phosphate-buffered saline. Cells were then incubated with 10 µmol/l of CM-H2DCFDA in the culture medium for 1 h; cardiomyoblasts were trypsinized, harvested, washed with PBS, and then centrifuged at 300 g for 5 min. The cell pellets were resuspended with PBS. The fluorescent intensity of the oxidized CM-H2DCFDA produced from cells was determined using a Cyan flow cytometer (Beckman Coulter, Fullerton, CA) at channel one (excitation: 488 nm; emission: 515–545 nm). The graphs were generated by using the FlowJo_V10 software.

Immunofluorescent staining. Immunohistochemical staining was performed as described before (36, 41). At the end of the experiment, cells were washed in PBS, fixed via immersion in 4% paraformaldehyde for 15 min, and permeabilized by incubation in PBS containing 0.1% Triton X-100 for 10 min at room temperature. Cells were then rinsed three times with PBS, blocked with 1% BSA in PBS for 1 h, and then incubated with polyclonal anti-active caspase 3 antibody (Abcam, Cambridge, MA) at a dilution of 1:100 overnight at 4°C. After three washes with PBS, cells were incubated with goat anti-rabbit Alexa Fluor 555 secondary antibody (Life Technologies) in PBS for 1 h at room temperature. Cells were then counterstained with 4',6-diamidino-2-phenylindole to visualize the nuclei. Active caspase-3-positive cells were observed using confocal laser scanning microscopy (LSM 700; Carl Zeiss). The percentage of apoptotic positive cells was determined in five randomly chosen fields and was normalized with the total number of stained nuclei.

Assessment of mitochondrial function. Cardiomyoblasts’ mitochondrial damage was assessed by examining mitochondrial membrane depolarization, which was indicated by the ratio of red and green signals in the micrographs (32). Mitochondrial membrane depolarization was assessed by using the MitoCapture mitochondrial apoptosis detection kit according to protocols provided by the manufacturer (BioVision, Tokyo, Japan). MitoCapture is a cationic dye that aggregates in the mitochondria and gives off a bright red fluorescence in healthy cells. In the mitochondrial damaged cells, MitoCapture fails to

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**A**

In vivo mouse myocardial infarction

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<tr>
<th></th>
<th>Sham</th>
<th>MI</th>
<th>Exendin-4 + MI</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>0.9% saline, i.p. on daily basis</td>
<td>Vehicle (0.9% saline, i.p. on daily basis)</td>
<td>Exendin-4 (0.1mg/kg i.p. on daily basis)</td>
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Measurements

1. Cardiac function: Echocardiography and isovolumetric heart.
2. Animal survival rate
3. Histological analyses: Myocyte sizes, interstitial fibrosis and scar size.
4. Western blots: p38, Akt-1, caspase 3 and caspase 9

**B**

In vitro cell culture experiments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Oxidant stress</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td>1 hour</td>
<td>± H₂O₂ (50,100 µmol/L)</td>
<td>1 hour</td>
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<tr>
<td>2 hours</td>
<td>± Exendin-4 (100 nmol/L)</td>
<td>Normal medium</td>
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Measurements

1. Cell cytotoxicity and survivals: LDH and MTT assay
2. ROS productions
3. Apoptosis and apoptotic mitochondria
4. Mitochondrial respiration
5. Mitochondrial permeability transition pore
aggregate in the mitochondria as a result of altered mitochondrial transmembrane potential and remains in the cytoplasm in its monomeric form, which exhibits green fluorescence. Briefly, after the cardiomyoblasts were treated with H2O2 and exendin-4 as described above, the cells were incubated in 1 ml of incubation buffer containing 1 µl of MitoCapture for 15 min at 37°C in an incubator containing 5% CO2. After staining, the fluorescent signals were measured with a microscope. The red fluorescent signals were excited at 530 nm and detected at 630 nm, and the green fluorescence was excited at 488 nm and detected at 530 nm.

**Western blot analysis.** We prepared samples from cardiomyoblasts and myocardium and performed immunoblotting analysis. Proteins were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 1× Tris-buffered saline containing 0.5% Tween 20 for 1 h. The blots were incubated with their respective polyclonal antibodies, rabbit-p38 ployclonal, rabbit-Akt-1 polyclonal, caspase-3, and caspase-9 ployclonal antibodies (1:1,000), for 2 h and visualized by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h and developed with ECL chemiluminescence detection reagent (Amersham Pharmacia Biotech).

**Mitochondrial oxygen consumption.** Oxygen consumption rates (OCR) were measured using an XF24 Extracellular Analyzer (Sea-horse Bioscience). Cardiomyoblasts were plated onto an XF24 cell culture plate at a density of 60,000 cells. All reagents were calibrated to pH 7.4 before injection. Cardiomyoblasts were treated with or without exendin-4 prior to being subjected to 50 µmol/l H2O2 according to the protocol above. Measurement of oxygen consumption was carried out under basal conditions, in the presence of the mitochondrial inhibitors oligomycin (10 µmol/l) or antimycin A (10 µmol/l), or in the presence of the mitochondrial uncoupler carbonyl-cyanide-p-trifluoromethoxy-phenylhydrazone (2.5 µmol/l) to assess maximal mitochondrial respiration. The Seahorse XF-24 software calculated oxygen consumption rate automatically. Approximately six independent experiments were performed in each group.

**Measurement of intracellular GSH.** GSH was measured using an intracellular GSH kit according to the manufacturer’s protocol. Briefly, after cardiomyoblasts were treated with or without exendin-4 in cardiomyoblasts exposed to 50 µmol/l H2O2 as described the protocol above, the cells were loaded with green dye (50 nmol/l) for 30 min. The fluorescence intensity of green dye was detected with a flow cytometer using the FL1 channel.

**mPTP.** mPTP opening was determined as reported previously (1). Briefly, after cardiomyoblasts were treated with or without exendin-4 prior to being subjected to 50 µmol/l H2O2, the cells were washed with Hanks’ balanced salt solution-10 mM HEPES (pH 7.2) before staining with 1 µmol/l calcein-AM (Molecular Probes) in the presence of 8 mmol/l cobalt chloride (CoCl2) at room temperature for 20 min in dark conditions. CoCl2 was added to quench the cytoplasmic staining so that only the fluorescence mitochondria were imaged. Change in fluorescence intensity is an index of PTP opening.
Results

Exendin-4 improves cardiac function in the post-MI heart. A mouse MI model was used to determine whether administration of exendin-4 protected hearts from myocardial infarction (Fig. 1A). After 2 wk of MI, echocardiographic assessment showed that, in MI control mice, LV internal dimension (LVID) was increased from 4.3 mm in sham to 5.3 mm in MI at diastolic stage and from 3.1 mm in sham to 4.5 mm in MI at systolic stage, suggesting a dilated LV (Fig. 2, A and B). Ejection fraction (EF) was reduced to 43.5% in MI from 61% in sham (Fig. 2C), and fractional shortening (FS) was reduced from 28.1% in sham to 17.2% in MI (Fig. 2D), indicating the systolic dysfunctions following myocardial infarction. Exendin-4 treatment prevented an increase in LVID in infarcted mice, as evidenced by the reduced LVID in both diastolic and systolic stages, and preserved the EF and FS, respectively. Cardiac contractility and function were further evaluated using an isovolumetric isolated heart. As shown in Fig. 3A, exendin-4 treatment dramatically improved the recovery of post-infarction rate pressure product and LVDP compared with infarcted mice that received vehicle treatments. HRs were increased by exendin-4 treatment. In addition, both LV-dP/dt_max and LV-dP/dt_min demonstrated marked improvements after 2 wk of MI compared with MI only (Fig. 3B). Coronary effluent is another way of measuring heart function; coronary flow increased in MI heart receiving exendin-4 treatment compared with MI mice.

Exendin-4 attenuates cardiac hypertrophy and increases survival rate in post-MI animals. Myocardial hypertrophy is an important pathological response of MI. As shown in Fig. 4, both heart weight/tibia length (TL) and lung weight/TL were increased in the MI mice. Mice treated with exendin-4 showed a decreased heart/body weight ratio and heart/tibia length ratio, suggesting that exendin-4 exhibits an anti-hypertrophic effect (Fig. 4, A and B). Exendin-4 treatment significantly improved survival rate in post-MI animals compared with control post-MI animals (Fig. 4C). Additionally, the increase in cross-sectional areas of myocytes in the untreated MI group did not occur in the MI group treated with exendin-4 (Fig. 4D). We also estimated interstitial fibrosis, as shown in Fig. 4E, and exendin-4 attenuated the content of cardiac interstitial fibrosis in the infarcted hearts. In addition, exendin-4 treatment also reduced scar sizes in MI hearts (Fig. 4F). Because we have shown previously that GLP-1 treatment increased p38 phosphorylation, which was associated with an improvement of myocardial protection, we examined the status of p38 and Akt-1 in exendin-4-treated MI hearts following stimulation of GLP-1R with exendin-4. Likewise, as shown in Fig. 5, A and
Exendin-4 reduced cell cytotoxicities in H9c2 cardiomyoblasts exposed to oxidative stress. Cell cytotoxicity and survival rate in cells exposed to H$_2$O$_2$ were determined using the experimental protocol described in Fig. 1B. As shown in Fig. 6A, compared with that in the vehicle, LDH release increased 19 and 42% in H9c2 cardiomyoblasts subjected to oxidative stress (50 and 100 μmol/l H$_2$O$_2$, respectively). With exendin-4 treatment, LDH leakage decreased significantly to 13.2 and 32.8% of oxidant stress-treated groups (50 and 100 μmol/l H$_2$O$_2$, respectively). As shown in Fig. 6B, pretreatment of cardiomyoblasts with exendin-4 increased viability in cardiomyoblasts exposed to 50 μmol/l H$_2$O$_2$ treatment compared with the control group. Likewise, cardiomyoblasts subjected to 100 μmol/l H$_2$O$_2$ oxidative stress demonstrated a reduction of viability to 23% of the control group. However, exendin-4 treatment dramatically improved cell viability in cardiomyoblasts exposed to oxidative stress (50 and 100 μmol/l H$_2$O$_2$).

**Exendin-4 attenuates the production of ROS in H9c2 cardiomyoblasts.** As shown in Fig. 7, the levels of intracellular ROS were determined 1 h following cell exposure to H$_2$O$_2$ treatment. A remarkable reduction of ROS ($P < 0.001$) was observed when H9c2 cardiomyoblasts were pretreated with exendin-4.
exendin-4. After H9c2 cardiomyoblasts were exposed to 1 µM of H2O2, the intensity of CM-H2DCFDA fluorescence showed only a mild increase. However, when cardiomyoblasts were exposed to the series concentration of H2O2 treatment, CM-H2DCFDA fluorescence intensity increased significantly compared with control (Fig. 7A). Pretreatment of exendin-4 attenuated the CM-H2DCFDA fluorescence intensity in H9c2 cardiomyoblasts exposed to 50 µmol/l H2O2 compared with that of control H9c2 cardiomyoblasts (Fig. 7A). Exendin-4 treatment reduced the CM-H2DCFDA fluorescence-positive cells to 7% in exendin-4-treated cells compared with non-exendin-4 treatment (14%) in cardiomyoblasts exposed to 50 µmol/l H2O2 (Fig. 7B). These results indicate that exendin-4 effectively mitigates the generation of ROS in H9c2 cardiomyoblasts exposed to oxidant stress.

**Exendin-4 protects against H2O2-induced mitochondrial damage.** Mitochondrial transmembrane potential was evaluated by using a cationic dye in living cells, MitoCapture (BioVison), according to the manufacturer’s instructions. Alterations in the mitochondrial membrane potential represent an early transition in the induction of apoptosis. The mitocapture dye accumulates in mitochondria when the mitochondrial membrane is intact and emits a red signal in the control or exendin-4-treated H9c2 cardiomyoblasts (Fig. 8). When H9c2 cardiomyoblasts were subjected to 10 µmol/l H2O2 treatment, the mitochondrial membrane potential began to decrease. In H9c2 cardiomyoblasts exposed to 100 µmol/l H2O2 treatment, almost all H9c2 cardiomyoblasts lost the red fluorescent signal. As shown in Fig. 8, H2O2 caused significant mitochondrial damage in a dose-dependent manner. However, following treatment with exendin-4, the H2O2-elicited membrane potential loss was prevented.

**Exendin-4 reduces apoptotic frequencies in H9c2 cardiomyoblasts from oxidative stress.** To assess whether exendin-4 reduced the rate of apoptosis in H9c2 cardiomyoblasts...
following oxidative stress, an immunofluorescent staining assay was performed by detecting activated caspase-3 in nuclei. A representational image is shown in Fig. 9A. Active caspase-3-positive nuclei were exhibited in the H9c2 cardiomyoblasts exposed to oxidant stress compared with the control group. However, pretreatment of exendin-4 resulted in a significant decrease in active caspase-3-positive nuclei. As shown in Fig. 9B, there are 43 and 71.4% active caspase-3-positive nuclei in cardiomyoblasts subjected to 50 and 100 μmol/l H2O2, respectively. However, when the cells were pretreated with exendin-4, the rate of apoptotic cells reduced to 27.3 and 44.1%, respectively, suggesting that exendin-4 treatment reduced apoptosis from reactive oxidant stress.

Exendin-4 attenuated the expression of active caspase-3 and -9. We further determined whether exendin-4 treatment could lead to the reduction of active caspase-3 and active caspase-9 in the cells exposed to oxidant stress. As shown in Fig. 10A, when cardiomyoblasts were exposed to 50 and 100 μmol/l H2O2 treatments, oxidative stress resulted in abundant increases in cleaved caspase-3 and cleaved caspase-9 compared with normal cells. However, exendin-4 treatment caused a decrease in cleaved caspase-3 and cleaved caspase-9 compared with the nontreatment control group. Desitometric analysis demonstrated that exendin-4 treatment resulted in a significant difference in cleaved caspase-3/procaspase-3 and cleaved caspase-9/procaspase-9 between the exendin-4 treatment and control groups (Fig. 10B).
Exendin-4 enhanced mitochondrial respiration capacities. To test directly the effect of exendin-4 on respiration capacity of cardiomyoblasts exposed to H$_2$O$_2$, OCR was assessed. As shown in Fig. 11, cardiomyoblasts exhibited a significant reduction in OCR in response to H$_2$O$_2$ compared with control group. Treatment of cells with exendin-4 prevented the reduction of OCR in both basal (Fig. 11A) and maximal respiratory capacities (Fig. 11B). As shown in Fig. 11, C and D, following the injection of oligomycin and antimycin A, the respiration capacities were severely damaged in cardiomyoblasts exposed to H$_2$O$_2$. However, exendin-4 prevented the decline in the respiration capacity following injection of oligomycin and antimycin A.

Exendin-4 increased GSH levels in cardiomyoblasts exposed to oxidative stress. Hydrogen peroxide can be converted to HO, which is removed by intracellular enzymes such as glutathione peroxidase (GPx). GPx catalyzes the elimination reaction of hydrogen peroxide using reduced glutathione (GSH). As shown in Fig. 12, A and B, cardiomyoblasts lost intracellular GSH levels significantly by treatment with H$_2$O$_2$ (50 µmol/l), as demonstrated by a 23% reduction in GSH levels in cardiomyoblasts exposed to H$_2$O$_2$ treatment compared with the vehicle treatment group. Strikingly, the decline in GSH level was prevented effectively by treatment of cells with exendin-4.

Exendin-4 inhibited the mPTP opening. Opening of the mitochondrial PT pore is often associated with both apoptotic and necrotic cell death. The status of the mPTP can be determined with the membrane-permeating fluorescent pore calcein-AM, which freely enters mitochondria but cannot exit except through an open mPTP following processing by cellular esterase. Utilizing CoCl$_2$ quenching of cytosolic fluorescence, the release of calcein from mitochondria was analyzed by epifluorescent microscope and quantitative analysis. As shown in Fig. 13, A and B, oxidative stress resulted in a significant reduction in mitochondrial green fluorescence in cells exposed to H$_2$O$_2$, a finding indicative of initiating mPTP opening. However, the treatment of cardiomyoblasts with exendin-4 attenuated the mPTP opening in cardiomyoblasts exposed to oxidative stress, establishing that stimulation of GLP-1R is sufficient to suppress mitochondrial defect.

### DISCUSSION

Salient findings and perspectives. Using an in vivo mouse model, we demonstrated that the delivery of exendin-4 to activate the GLP-1R effectively improved cardiac functional recovery, increased animal survival rate, reduced hypertrophic response, and reversed cardiac fibrosis following myocardial infarction. Furthermore, in an in vitro cardiomyoblast culture model, our observations demonstrated that exendin-4 protected the cells from oxidative stress by increasing cell survival rate, decreasing cytotoxicities, mitigating rate of apoptosis, attenuating ROS production, and protecting the mitochondria. The cellular-protective effect of GLP-1R is associated with the improvement in mitochondrial respiration and inhibition of mPTP. Taken together, the results provide new insights into understanding the unique function of exendin-4 in myocardial protection and hold promise in potentially developing a new therapeutic strategy in treating heart failure and infarction.

Both GLP-1(7–36) amide and the GLP-1R agonist exendin-4 have been shown to increase heart rate and blood pressure in both anesthetized and consciously restrained rats, although the mechanisms are controversial (3, 39). Furthermore, in a pacing-induced heart failure model, the stimulation of GLP signaling with GLP-1 has also been demonstrated to improve cardiac performance in conscious dogs with dilated cardiomyo-
In this study, when we employed a myocardial infarction model, echocardiographic analysis reveals that stimulation of GLP-1R prevented cardiac dysfunction in post-myocardial infarction animals, which is consistent with the observation that GLP-1 improves functional recovery (44). This is supported by the recent observation in both experimental animals and human subjects that exendin-4 exerts protective effects in acute myocardial ischemia and diabetic hearts, respectively (2, 22). Additionally, it is noted in this observation that the protective effect induced by exendin-4 results from a long-term treatment (22). It will be very interesting to assess the effects of short-term exendin-4 treatment in protecting infarcted hearts. The results using the isovolumetrically perfused heart also indicate that activation of GLP-1R signifi-

Fig. 10. Ex-4 treatment reduces active caspase-3 and caspase-9 proteins in cardiomyoblasts exposed to oxidant stress. A: representative gels showing cleaved and procaspase-9 and caspase-3 proteins in cardiomyoblasts exposed to oxidant stress (50 and 100 μmol/l H2O2, respectively). B: densitometric analysis of ratios of cleaved/procaspase-9 and procaspase-3 proteins. Values represent means ± SE (n = 3/group). *P < 0.05.
Fig. 11. Effects of Ex-4 on mitochondrial respiration capacities. A: basal oxygen respiration of H9c2 cells. B: maximal oxygen respiration capacity of cells through uncoupling of oxidative phosphorylation by FCCP. C: oxygen respiration capacity of cells after inhibiting ATP synthase with oligomycin. D: oxygen respiration capacity of cells after inhibiting complex III of mitochondria with antimycin A. Values are shown as means ± SE (n = 6/group). *P < 0.05; **P < 0.01; ***P < 0.001.

Significantly mitigated myocardial injury, as indicated by an improvement in recovery of developed pressure as well as rate pressure products. In agreement with our studies, evidence from the observations of Bose et al. (4) suggests that GLP-1, when added prior to ischemia, induced a significant reduction in infarct size compared with the control group. A very similar observation was obtained in a mouse cerebral ischemia model in which it was shown that injections of exendin-4 following ischemia were able to reduce infarct size and improve function by protecting the tissue from ischemic reperfusion injury (34). Kavianipour et al. (16) reported that GLP-1(7–36) was not found to alter myocardial glucose utilization; hemodynamic variables and consequent infarction changed in porcine myocardium. These discrepancies of GLP-1 function in these studies definitely vary upon the model (in vivo/in vitro), species, and the duration of GLP-1 (short/sustained half-life). Native GLP-1 is rapidly degraded by dipeptidyl peptidase-IV in the bloodstream. Accordingly, the direct cardiovascular response of GLP-1 may be masked by the effective fragments under in vivo conditions (25).

The mitogen-activated protein kinases play a central role in the transmission of signals from cell surface receptors and environmental cues to the transcriptional machinery in the nucleus and are involved in cell growth, differentiation, transformation, and protection (6, 23, 29, 30, 45). Our observation indicates that stimulation of GLP-1R also activated p38 and Akt-1 signaling, which were identified to involve GLP-1-induced cardioprotection. However, it will be interesting to explore the functional role of p38 and Akt-1 in the modulation of GLP-1 and GLP-1R-elicted protection in the setting of acute and chronic myocardial ischemia in the future, which might provide new insight into the understanding of functions of GLP-1 and GLP-1R in the ischemic heart.

Coronary effluent was increased by the administration of the GLP-1R agonist compared with that of control myocardial infarction, suggesting that an increase in coronary effluent might be associated with functional improvement after the administration of exendin-4. In addition, an increase in coronary effluents might also be related to vascular dilation. It is also likely that sufficient PO2 in the perfusate contributes to the increase in coronary effluents. We have observed previously that the cardiac-protective effect is associated with the increase in coronary effluent as well as increased angiogenesis (40, 42). Future studies also need to define whether GLP-1R activation stimulates vascular growth in the infarcted myocardium.

Cellular cytotoxicities were closely associated with the production of ROS. Our results indicated that oxidant stress elicits the production of ROS, which was prevented by the administration of exendin-4. This is well supported by an observation in which exenatide triggers a protective effect against both oxidant stress-induced and hypoxia/reoxygenation injuries (7–9). The mechanism for how exendin-4 mitigates ROS remains unknown. However, in line with our observations, a previous report indicated that exendin-4 decreases endogenous ROS production and increases ATP production in diabetic Goto Kakizaki rat islets through suppression of Src activation (26). It is reported that the reduced rate of mitochondrial respiration capacity contributes to impairment of mitochondrial function and cardiac function (5). The present study shows that cardiomyoblasts exhibited the reduced mitochondrial respiration capacity in response to oxidative stress. However, mitochondrial respiration was improved by stimulation of GLP-1R, indicating that GLP-1R stimulation could ameliorate the impairment of mitochondria respiration and dysfunction. Perturbations to mitochondria from the opening of a large multiprotein conductance channel referred to as mPTP have been reported. Earlier observations demonstrated that hypoxia triggers mitochondrial perturbations and cell deaths of adult and neonatal ventricular myocytes (1). Our observation indicates that stimulation of GLP-1R suppressed the mPTP in car-
diomyoblasts exposed to oxidative stress, suggesting that inhibition of mPTP is critical for GLP-1R-elicited protective effects. Interestingly, our studies reveal that exendin-4 treatment also protects mitochondria from oxidative stress-induced injuries. This was supported by another observation in which the GLP-1(28–36) amide nonapeptide rapidly enters isolated mouse hepatocytes and targets the mitochondria, where it inhibits oxidative stress (35). In addition, it is known that GSH is important for maintaining redox levels of cells and can be used to detect cell death (9). Our results indicate that GLP-1R stimulation prevented the decrease in GSH levels in cells exposed to oxidative stress, implying the role of exendin-4 in attenuating oxidative stress-induced injury. It will be interesting to decipher the specific signaling pathway by which exendin-4 reduces the rate of apoptosis following oxidative stress in cells and the diabetic rat (17, 38). Therefore, this evidence indicates that exendin-4 is essential for initiating a protective signal for the survival of cells and organs during oxidative stress or injuries.

Conclusions. In this observation, we observed that in vivo delivery of exendin-4 into post-myocardial infarction animals showed that stimulation of GLP-1R resulted in a marked improvement in cardiac function, attenuation of cardiac hypertrophy and cardiac fibrosis, and an improvement in animal survival rate. Coronary effluent in the post-myocardial infarction heart was also increased by administration of exendin-4, suggesting the regulatory effect of GLP-1R on coronary perfusion. Furthermore, we have demonstrated that stimulation of GLP-1R showed a remarkable protective effect against reactive oxidant stress, as indicated by the decrease in lactate dehydrogenase, the increase in cell survival, mitigation of ROS production, protection of mitochondria, the increase in mitochon-
drial respiration capacity, the suppression of mPTP opening, and the inhibition of apoptosis. Taken together, our results indicate that GLP-IR plays an essential role in reducing cardiac injury and remodeling and holds promise in the development of a novel therapeutic strategy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
T.C.Z., M.D., and J.D. performed experiments; T.C.Z., S.Q., L.Z., and Y.W. wrote the manuscript; T.C.Z., M.D., and J.D. edited and revised manuscript; T.C.Z., M.D., J.D., Z.W., L.Z., Y.W., Q.G., and S.Z. approved final version of manuscript; M.D., J.D., Z.W., N.Y., and L.Z. analyzed data; T.C.Z., M.D., and N.Y. prepared figures.

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