Chinese Journal of Physiology 60(2): xxx-xxx, 2017
DOI: 10.4077/CJP.2017.BAF434

Attenuation of High Glucose-Induced Rat Cardiomyocyte Apoptosis by Exendin-4 *via* Intervention of HO-1/Nrf-2 and the PI3K/AKT Signaling Pathway

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Abstract

Exendin-4, a glucagon-like peptide-1 receptor agonist, demonstrated cytoprotective actions beyond glycemic control in recent studies. The aims of the present study were to investigate the effects of exendin-4 on high glucose (HG)-induced cardiomyocyte apoptosis and the possible mechanisms. Rat cardiomyocytes were divided into 3 groups: normal glucose group (NG group), high glucose group (HG Group) and HG + exendin-4 group (HG+Ex Group). Cardiomyocyte apoptosis was evaluated by double-staining with annexin V-FITC/PI and flow cytometry. Intracellular reactive oxygen species (ROS) production was detected by DCFH-DA incubation and fluorescence microscopy. LY294002, a PI3K pathway inhibitor, was added to the medium of the HG+Ex+LY Group for further western blot analysis. The proteins analyzed involved oxidative stress-associated proteins, HO-1 and Nrf-2, and apoptosis-associated proteins, caspase-3, Bax/Bcl-2 and p-AKT/AKT. HG treatment induced cardiomyocyte apoptosis ($P = 0.00$) and clearly upregulated ROS production ($P = 0.00$); exendin-4 co-incubation also ameliorated cardiomyocyte apoptosis ($P = 0.004$) and decreased ROS ($P = 0.00$) level significantly. HO-1 and Nrf-2 protein expression levels decreased significantly in the HG group ($P < 0.05$), but the levels were elevated by exendin-4 intervention ($P < 0.05$). Furthermore, exendin-4 attenuated HG-induced higher protein expression, including cleaved caspase-3 and Bax, increased the expression of Bcl-2 protein ($P < 0.05$). However, these impacts of exendin-4 were counteracted significantly by co-incubation with LY294002. In addition, exendin-4 ameliorated HG-induced p-AKT/AKT lower expression, and this impact was also suppressed by LY294002. Exendin-4 ameliorates HG-induced cardiomyocyte apoptosis, and the mechanisms may involve anti-oxidative stress *via* the HO-1/Nrf-2 system, as well as intervention of the PI3K/AKT signaling pathway.

Key Words: cardiomyocyte apoptosis, exendin-4, high glucose, HO-1/Nrf-2 system, PI3K/AKT pathway

Introduction

With the high prevalence of diabetes, more attention has been paid to diabetic cardiomyopathy. Diabetes-associated myocardial impairment was initially recognized by Rubler *et al*. (17) in diabetic patients, who exhibited defects in both electrical and mechanical properties, and increased morbidity and mortality. Myocardial contraction and relaxation in diabetic models are markedly impaired, and the cardiac dysfunction, and even congestive heart failure, may result from a variety of metabolic and biochemical abnormalities. Exploration of the mechanisms and intervention strategies, involving oxidative stress and alteration in Ca^{2+} homeostasis, has been always the hot topics in this field (16, 29).

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Received: April 8, 2016; Revised: July 24, 2016; Accepted: September 8, 2016.
 ISSN : 0304-4920. http://www.cps.org.tw

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by the small intestine in a nutrient-dependent manner, which stimulates insulin secretion and inhibits glucagon secretion and gastric empty (2, 12). GLP-1 has wide-range effects on glucose metabolism, and the GLP-1 pathway has been a therapeutic target in the treatment of diabetes (4, 10). However, GLP-1 has a short half-life of <2 min in the circulation, and it is degraded rapidly by the enzyme dipeptidyl peptidase-4 (DPP-4), limiting the biological efficacy in clinical practice. Current treatment *via* the GLP-1 pathway focuses on increasing the level of GLP-1 in the blood, including the use of either compounds to inhibit DPP-4 activities, or application of GLP-1 receptor agonists. Exendin-4, a GLP-1 receptor agonist, is a potential insulin secretagogue, and has been approved for the treatment of type 2 diabetes (4, 10). Furthermore, exendin-4 has a therapeutic advantage in its resistance to degradation by DPP-4, resulting in a longer pharmacological half-life (4, 10).

Beyond glycemic control, cytoprotective actions of the GLP-1 pathway involves protection of pancreatic beta cells (23) and neurons (5). A variety of cardiovascular benefits of the GLP-1 pathway has been reported, including protection against post-myocardial infarction remodeling (15), improvement of cardiac function in mice over-expressing monocyte chemoattractant protein-1 in cardiomyocytes (26), and amelioration of cardiac ischemia/reperfusion injury (22). Although these cardioprotective effects *via* the GLP-1 pathway have been reported, the related mechanisms of these effects are still unclear.

It is well known that oxidative stress is responsible for the induction of cardiomyocytes apoptosis *via* high glucose. Mangmool, S. *et al*. (11) demonstrated that stimulation of the GLP-1 receptor with exendin-4 attenuated H_2O_2-induced reactive oxygen species (ROS) production and increased the synthesis of antioxidant enzymes. Hence, we hypothesized that exendin-4 may protect diabetic myocardium and ameliorate cardiomyocyte apoptosis, and the mechanisms may involve oxidative stress and apoptosis signaling pathway. The aims of the present study were to investigate the effects of exendin-4 on high glucose-induced cardiomyocyte apoptosis, and the roles of exendin-4 in the intervention of oxidative stress and the PI3K/AKT signal pathways in these pathologic processes.

Materials and Methods

Cell Isolation and Culture

The animal experimental protocols were approved by the Beijing Friendship Hospital Animal Care and Research Committee. All surgery was performed under anesthesia, and all efforts were made to minimize sufferings. Cardiomyocytes were prepared by enzymatic disassociation of 1- to 2-day old Sprague-Dawley rats (n = 8-10) as described previously (9). Briefly, the rats were sacrificed by opening the pleura under ether anesthesia and aseptic conditions. The hearts of the rats were removed rapidly and cleaned in iced-cold D-Hanks balanced salt solution. The minced ventricular myocardium was digested with trypsin (0.07%) and collagenase II (0.04%) at 37°C. Cells were extracted by repeated tissue digestion and the whole-cell suspensions were centrifuged at 1,000 rpm for 10 min. After the removal of supernatants, the cell pellets were re-suspended in DMEM, which contained 15% fetal bovine serum and 1% penicillin-streptomycin. The cells were plated onto 10-cm culture dishes at 37°C, 5% CO_2 for 90 min. When the non-myocytes attached to the dishes, the cardiomyocytes remained in the suspension and were harvested and seeded in six-well culture plates coated with laminin. After incubation at 37°C, 5% CO_2 for 24 h, the cardiomyocytes were identified by α-actinin staining, and were divided into 3 groups: normal glucose group (NG Group: DMEM containing glucose 5.5 mM; Hyclone, MA, USA), high-glucose group (HG Group: DMEM containing glucose 25 mM), high-glucose + exendin-4 group (HG+Ex Group) (exendin-4 purchased from Sigma, St. Louis, MO, USA). The vehicle for exendin-4 was phosphate buffer saline (PBS). Cardiomyocytes were cultured in serum-free DMEM for 12 h before experiments. Cells in different groups were incubated at 37°C, 5% CO_2 for 24 h before further study assessments.

Cell Survival Analysis

Cell Counting Kit-8 (CCK-8, DOJINDO, Japan) was used to evaluate the effects of high-glucose and exendin-4 on the cell viability, as required by manufacturer's protocol. Cardiomyocytes were seeded into 96-well dishes, and the density was adjusted to 5×10^4 cells/ml. Cells were incubated in culture media with normal glucose (5.5 mM), high-glucose (25 mM) or high-glucose + exendin-4 at 10 nM, 20 nM, 30 nM or 40 nM at 37°C, 5% CO_2 for 24 h. CCK-8 (10 μl) was then added to each well and incubated for another 4 h. The absorbance at 450 nm was measured using a microplate reader (BioTek, Winooski, VT, USA).

Determination of Cells Apoptosis by Flow Cytometry

Cells apoptosis was detected by double-stained with annexin V-FITC/PI (BD Pharmingen™, USA), using flow cytometry as described previously (25). In brief, cells in different groups were washed with PBS and detached with trypsin, then re-suspended in binding buffer (1×10^5 cells/ml). Annexin V-FITC (5 μl) and 5 μl propidium iodide (PI) were added and the solution

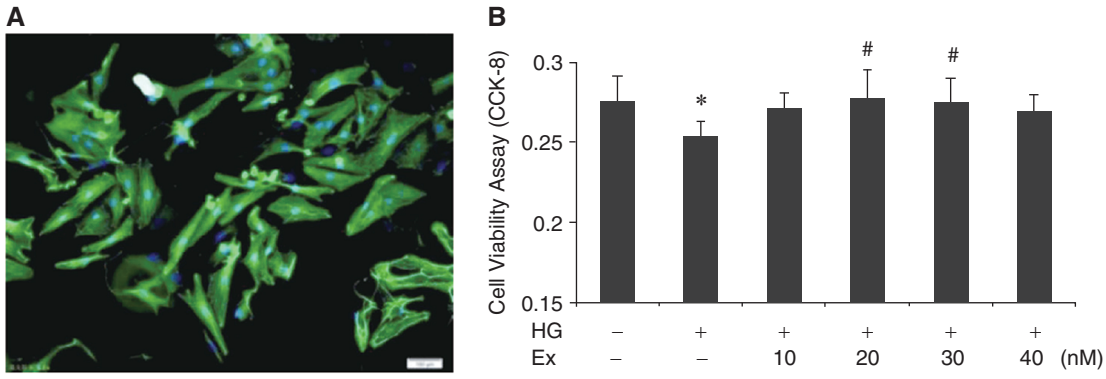

Fig. 1. Cell identification and cell viability assay. (A) α-actinin staining for cell identification. (B) CCK-8 expression levels for the evaluation of cell viability under different cells intervention. *$P < 0.05$, compared with the NG group; $^{\#}P < 0.05$, compared with the HG group.

was incubated for 15 min in dark at room temperature. The percentages of cells apoptosis were analyzed using flow cytometry (LSRFortessa, BD Bioscience, San Jose, CA, USA).

Measurement of Intracellular ROS

Reactive oxygen species (ROS) production was measured using an intracellular ROS assay kit (Biyuntian company, China) and fluorescence microscopy. The cells in different groups were seeded in 24-well plates at 2.0×10^5/well, and cultured for 24 h. 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) was added to each well, which can be oxidized by intracellular ROS. DCHF-DA stock solution (20 mM) was diluted with the medium to a final concentration of 0.1 mM. The cells were the incubated at 37°C for 1 h, and washed three times with PBS. Fluorescent compounds, corresponding to intracellular ROS levels, were detected by a fluorescence microscope (ZEISS, Observer A1, Germany) with excitation and emission of 488 nm and 530 nm, respectively. Results were shown by three independent experiments.

Western Blot Analysis

Western blot analyses were conducted to assess the expression levels of the oxidative stress-associated proteins heme oxygenase-1 (HO-1), nuclear factor E2-related factor 2 (Nrf-2), and the apoptosis-associated proteins bcl-2/bax, cleaved caspase-3, p-AKT and AKT. A PI3K pathway inhibitor, LY294002 (5 μM) (Sigma), was added to the cells incubated with high-glucose and exendin-4, as the HG+Ex+LY Group, for 24 h. In brief, total protein was quantified using a BCA protein assay kit (cwBioTech 02912E, Beijing, PRC) as instructed after lysis of the cardiomyocytes. Equal amounts of proteins (12 μg) were separated by SDS-PAGE. After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane and blocked with Tris buffered saline containing 5% skim milk powder and 0.05% Tween 20. The corresponding primary antibodies, HO-1 (1:1000, Abcam, Cambridge, MA, USA), Nrf-2 (1:1000, CST, USA), bcl-2/bax (1:1000, CST) cleaved caspase-3 (1:1000, CST) and p-AKT/AKT (1:1000, CST), were added in series. The membranes were incubated with the diluted antibody preparations overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) antibodies ($1:10^4$; Jackson, West Grove, PA, USA) for 40 min at room temperature. The blots were visualized using an enhanced chemiluminessence detection kit (Millipore, Boston, MA, USA). Target proteins were quantified and normalized relative to β-actin (1:1000, Zhongshan, Beijing, PRC).

Statistical Analysis

Data was analyzed by one-way ANOVA, expressed as mean ± SD. When a significant difference was identified by ANOVA, a *post-hoc* analysis was performed using the Student-Newman-Keuls test. Data were determined using SPSS 13.0 software, and a *P*-value <0.05 was considered significant.

Results

Cell Identification and Viability

Observed under an inverted microscope, the cardiomyocytes grew adhering to the wall and showed fusiform or polygon shape, with extension of pseudopodia and spontaneous beating. α-Actinin staining showed that the cardiomyocyte cell purity reached 90-95% (Fig. 1A). Compared with the NG medium incubation, CCK-8 assay showed that HG incubation reduced cells viability significantly (0.254 ± 0.009 *vs.*

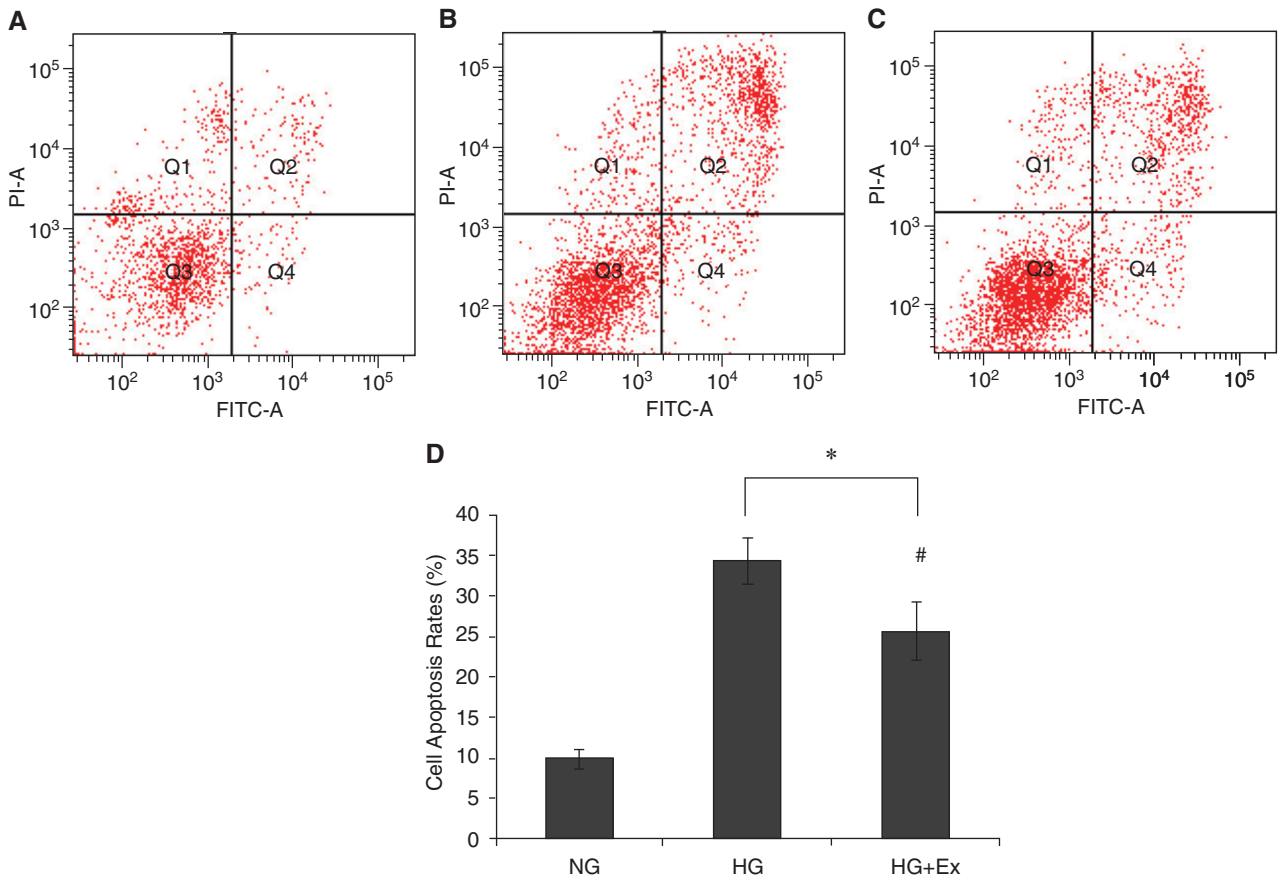

Fig. 2. Flow cytometry evaluation of cells apoptosis in different groups. (A) NG group. (B) HG group. (C) HG+Ex group. *$P < 0.05$, compared with NG group; #$P < 0.05$, compared with HG group.

0.276 ± 0.015, $P = 0.021$). When exendin-4 was added to high-glucose medium, cells viability improved. Compared to high-glucose medium alone, co-incubation with exendin-4 at 20 nM or 30 nM significantly ameliorated cells viability (0.254 ± 0.009, $P = 0.010$ *vs.* 0.278 ± 0.017 and 0.275 ± 0.015, $P = 0.044$, respectively) (Fig. 1B). Incubation with 20 nM exendin-4 for 24 h significantly improved high glucose-induced cell injury. Hence, 20 nM exendin-4 was used in further studies.

Effects of Exendin-4 on Cells Apoptosis Analyzed by Flow Cytometry

Flow cytometry analysis demonstrated the effect of exendin-4 on the inhibition of cardiomyocyte apoptosis incubated in HG medium (Fig. 2). Compared with the NG Group, cardiomyocyte apoptosis increased significantly in the HG Group ($P = 0.00$) (Fig. 2D). Although the cell apoptosis rate in the HG+Ex Group was still higher than that in the NG Group, co-incubation with exendin-4 significantly improved high glucose-induced cell apoptosis in the HG+Ex Group ($P = 0.004$) (Fig. 2D).

Effects of Exendin-4 on ROS Production

Intracellular ROS production was stained with DCHF-DA and determined by fluorescence microscopy. A small amount of ROS was detected in NG the Group (Figs. 3, A and D). Incubated in high-glucose medium, the intracellular ROS level was significantly higher in the HG Group than that in the NG Group ($P = 0.00$) (Figs. 3, B and D). When co-incubated with high glucose and exendin-4 (HG+Ex Group), the ROS level showed a significantly decrease (Figs. 3, C and D) compared with the level in the HG Group ($P = 0.00$), although it was still higher than that in the NG Group ($P = 0.002$).

Expression Levels of HO-1 and Nrf-2

HO-1 is a rate-limiting enzyme for oxidative degradation of cellular heme, and also a potent antioxidant enzyme. Nrf-2 is a regulator of numerous genes, encoding antioxidant and cytoprotective factors, such as HO-1. After intervention of high glucose for 24 h, HO-1 and Nrf-2 protein expression levels were decreased in the HG Group (Fig. 4), as compared with the NG group ($P <$

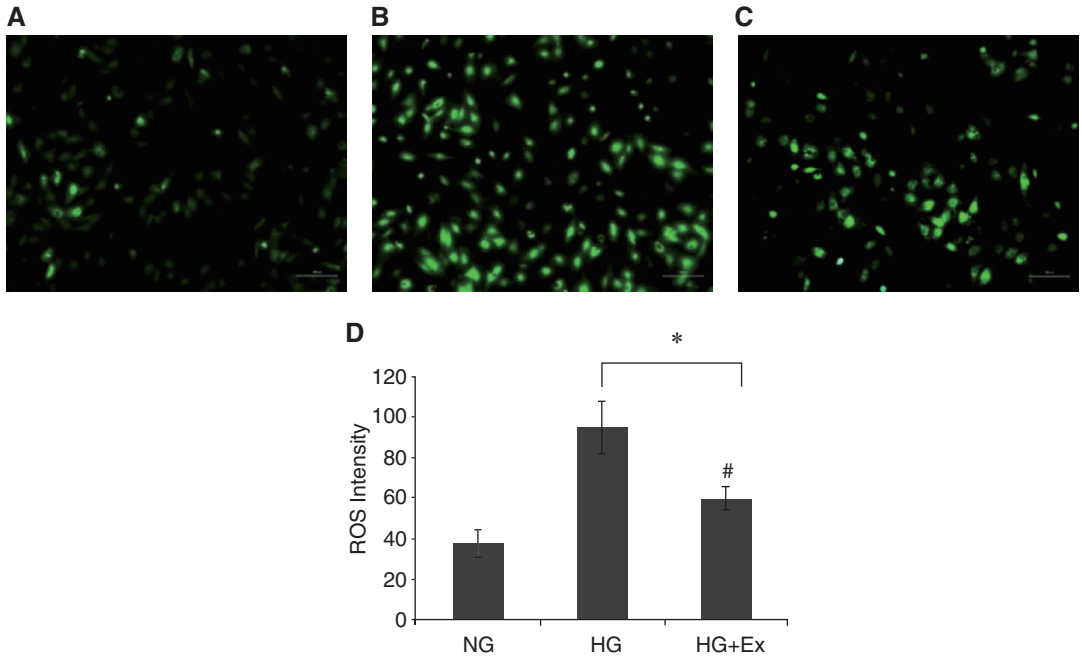

Fig. 3. Intracellular ROS production assay in different groups. (A) NG group. (B) HG group. (C) HG+Ex group. *$P < 0.05$, compared with the NG group; #$P < 0.05$, compared with the HG group.

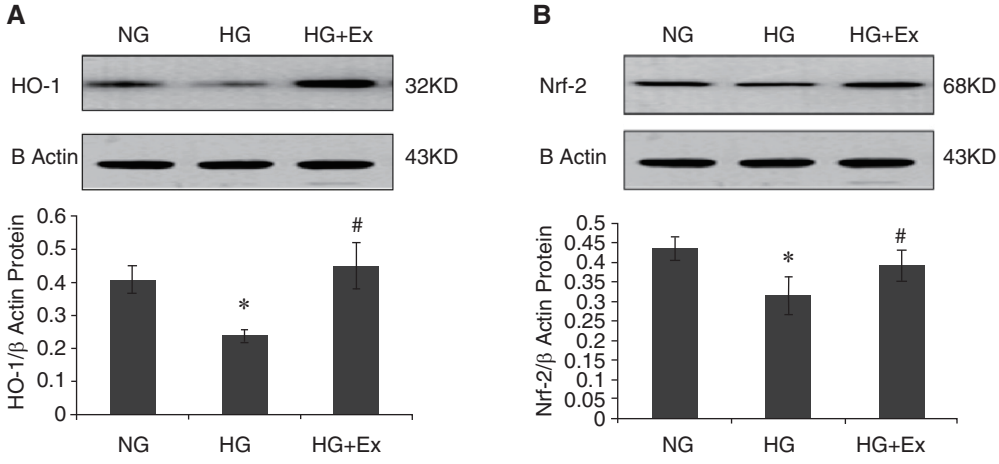

Fig. 4. Western blot analysis of expression of oxidative stress-associated proteins. Expression of HO-1 (A) and Nrf-2 protein (B) in different groups. *$P < 0.05$, compared with the NG group; #$P < 0.05$, compared with the HG group.

0.05). But when co-incubated with HG and exendin-4, expression levels of both proteins increased significantly in the HG+Ex Group, when compared with those in the HG Group ($P < 0.05$) (Fig. 4).

Expression Levels of Apoptosis-Associated Proteins

In the HG Group, increased levels of both Bax and cleaved caspase-3 proteins were detected, and Bcl-2 protein expression showed a marked decrease, as compared with the expression levels in the NG Group ($P < 0.05$) (Figs. 5, B, C and D). When co-incubated with exendin-4 in HG medium, Bax and cleaved caspase-3 significantly declined in expression, and Bcl-2 protein expression displayed an marked elevation, compared with the expression levels in the HG Group ($P < 0.05$). Treatment with LY294002, a PI3K pathway inhibitor, weakened the effects of exendin-4 on the expression of apoptosis-associated proteins in the HG+Ex Group. Bax and cleaved caspase-3 protein levels increased again, and Bcl-2 expression decreased significantly, as compared with the levels in the HG+Ex Group ($P < 0.05$) (Figs. 5, B, C and D). Moreover, p-AKT/AKT level declined significantly in the HG Group ($P < 0.05$), as compared with that in the NG Group. After intervention with exendin-4, p-AKT/AKT level increased

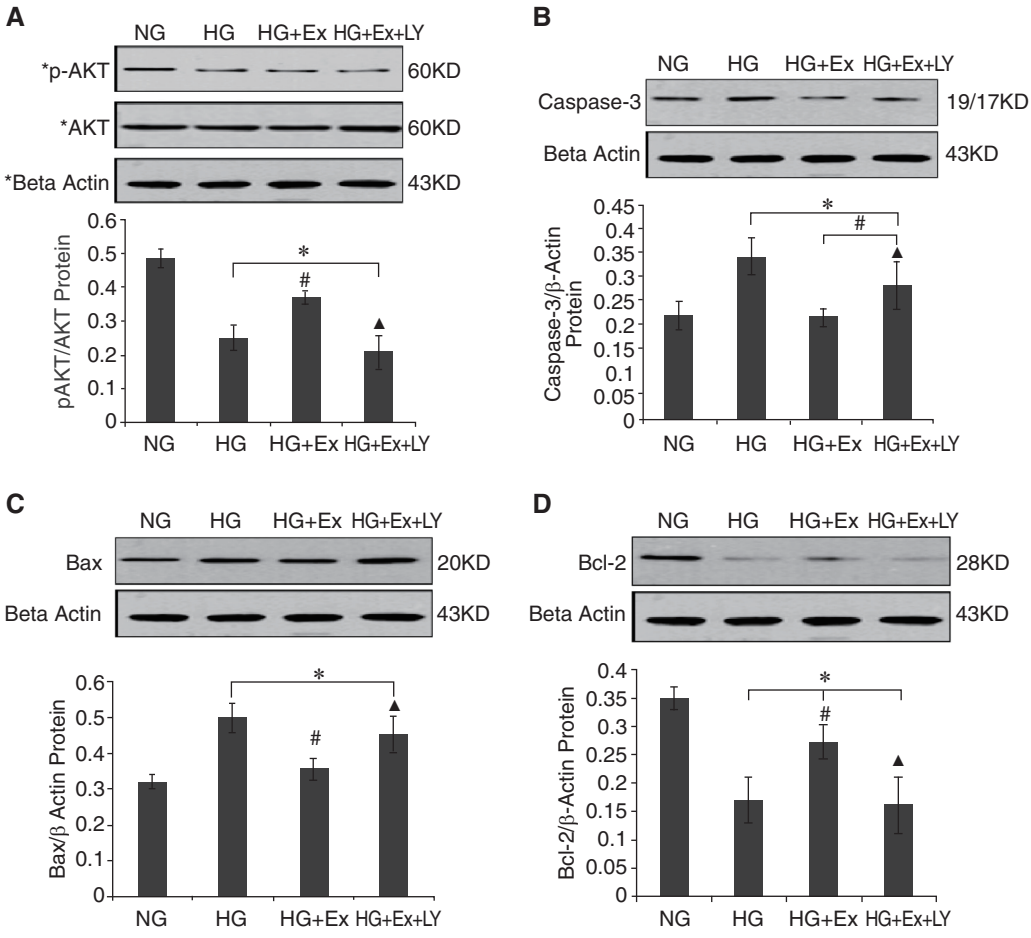

Fig. 5. Western blot analysis of expression of cell apoptosis-associated proteins. Expression of p-AKT/AKT (A), cleaved caspase-3 (B), Bax (C), and Bcl-2 (D) in different groups. $^{*}P < 0.05$, compared with the NG group; $^{\#}P < 0.05$, compared with the HG group; $^{\blacktriangle}P < 0.05$, compared with the HG+Ex group.

in the HG+Ex Group, but the effect was attenuated by LY294002 co-incubation. Compared with the HG+Ex Group, p-AKT/AKT level displayed a significant reduction again in the HG+Ex+LY Group (Fig. 5A).

Discussion

Exendin-4, which has a 53% homology with GLP-1, has become one of the potent anti-diabetic drugs in recent years. Increasing current interests have focused on its effects on cytoprotection, including the protection of cardiomyocyte (1, 3). As shown by Wen *et al.* (25), exendin-4 can inhibit cardiomyocyte injury in high glucose incubation, which is achieved through significant inhibition in the expression of receptor for advanced glycation end products (RAGE). In the present study, it was observed that exendin-4 alleviated high glucose-induced cardiomyocyte apoptosis and improved cells viability. The mechanisms may involve the suppression of oxidative stress *via* the HO-1/Nrf-2 system, and *via* the intervention of apoptosis-associated signal pathways, such as the PI3K/AKT pathway. Firstly, it was shown in this study that exendin-4 ameliorated cardiomyocyte viability and suppressed the high glucose-induced cell apoptosis. The reduced cell viability by high glucose incubation was significantly restored in the presence of exendin-4 (Fig. 1B), and there were no obvious differences in cardiomyocyte viability among the NG group and the Ex-4 (20 nM) and Ex-4 (30 nM) incubation groups. In HG+Ex group, cell apoptosis rate and expressions of pro-apoptosis proteins, Bax and cleaved caspase-3, declined, and the anti-apoptotic protein, Bcl-2, increased in expression level.

Hyperglycemia can activate oxidative stress and causes overproduction of intracellular ROS, which plays a key role in the development and progression of diabetic cardiomyopathy (7). The current study revealed that treatment with exendin-4 decreased high glucose-induced excessive ROS production, which meant the inhibition of oxidative stress. The result demonstrated the antioxidant properties of exendin-4 in high glucose environment.

The HO-1/Nrf-2 system has anti-oxidation and anti-

apoptosis effects (6, 13). It has also been reported that exendin-4 may interfere with the expressions of HO-1/Nrf-2 and exerts its biological effects in different cell lines (14, 24). In the present study, western blot analysis showed that HO-1 and Nrf-2 protein expression levels of cardiomyocytes treated with exendin-4 were decreased when incubated in high glucose medium, while the protein levels were elevated significantly in HG+Ex group. HO-1 is the rate-limiting enzyme in heme catabolism. Activation of HO-1 can mediate complex biological functions, exert cytoprotective, anti-inflammatory, anti-oxidative and anti-apoptotic effects (6, 13). Previous studies have revealed the relationship between modulation of HO-1 and the production of ROS in cardiomyocyte and other cell lines (8, 18). Therefore, upregulation of HO-1 *via* exendin-4 might be partly responsible for the anti-oxidative effect, and consequently attenuation of the production of intracellular ROS in the HG+Ex group. Nrf-2 belongs to the Cap'n' Collar (CNC) family of basic leucine zipper (bZip) transcription factors. Once activated, Nrf-2 could be translocated to the nucleus, where it binds to the anti-oxidant response element to up-regulate the expression of numerous cytoprotective phase II detoxifying enzymes and antioxidant genes, such as HO-1 (21). Hence, Nrf-2 is a master transcription factor contributing to HO-1 expression. In the study, exendin-4 exposure significantly elevated the expression of Nrf-2 when compared to high-glucose incubation alone. These results support that exendin-4 may have an effect on the HO-1/Nrf-2 system, and HO-1/Nrf-2 system may play a role in the inhibition of oxidative stress by exendin-4.

The PI3K/AKT signal pathway is central to many cellular survival mechanisms, and some of the biological effects of the HO-1/Nrf-2 system are related to the PI3K/AKT signal pathway (27, 28). Previous studies have indicated that PI3K is the most important regulatory factor for AKT in its upstream pathway, and some phytochemicals could protect against oxidative stress-induced cells damage *via* the PI3K/AKT signal pathway (19, 20). In the present study, we showed that exposure to exendin-4 significantly promoted phosphorylation levels of AKT, which demonstrated AKT activation and consequent inhibition in the expression of downstream apoptosis-associated genes. Interestingly, LY294002 incubation counteracted the effects of exendin-4 on the AKT pathway and anti-apoptosis in the HG+Ex+LY group. LY294002, a specific inhibitor of PI3K pathway, attenuated the protective effects of exendin-4 in the study, which suggested that exendin-4 might play some biological roles *via* PI3K and its downstream pathways. Taken together, the data suggest that the PI3K/AKT pathway, at least in part, contributed to exendin-4 inhibition of cardiomyocyte apoptosis induced by hyperglycemia.

Based on the findings of this study, it may be speculated that exendin-4 may be a promising therapeutic agent not only for the glucose control, but also for the intervention of diabetic cardiomyopathy.

Acknowledgments

The study was conducted with the support of Capital Medical University, College of Basic Medicine, and the Basic and Clinical Medicine Funding (No. 13-JL58) from Capital Medical University (2013).

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