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## Glucagon-like peptide-1 protects against cardiac microvascular endothelial cells injured by high glucose

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### ABSTRACT

**Objective:** To investigate the protective effect of glucagon-like peptide-1 (GLP-1) against cardiac microvascular endothelial cell (CMECs) injured by high glucose. **Methods:** CMECs were isolated and cultured. Superoxide assay kit and dihydroethidine (DHE) staining were used to assess oxidative stress. TUNEL staining and caspase 3 expression were used to assess the apoptosis of CMECs. H89 was used to inhibit cAMP/PKA pathway; fasudil was used to inhibit Rho/ROCK pathway. The protein expressions of Rho, ROCK were examined by Western blot analysis. **Results:** High glucose increased the production of ROS, the activity of NADPH, the apoptosis rate and the expression level of Rho/ROCK in CMECs, while GLP-1 decreased high glucose-induced ROS production, the NADPH activity and the apoptosis rate and the expression level of Rho/ROCK in CMECs, the difference were statistically significant ( $P < 0.05$ ). **Conclusions:** GLP-1 could protect the cardiac microvessels against oxidative stress and apoptosis. The protective effects of GLP-1 are dependent on downstream inhibition of Rho through a cAMP/PKA-dependent manner, resulting in a subsequent decrease in the expression of NADPH oxidase.

## 1. Introduction

Diabetic mellitus is a kind of metabolism disorder disease which threatened human's health severely. It is listed as one of the three most dangerous diseases worldwide by the world health organization[1,2]. The chronic raising of blood glucose level caused by diabetic mellitus can lead to a series of complications[3]. Among them, the cardiovascular complication is the leading cause of death. There's evidence that microvascular injury plays a very important role in the diabetic cardiovascular dysfunction[4].

The microvascular located at the terminal of the circulation, and it determined the myocardial perfusion level and the coronary reserve to some extent. Microvascular injury may also responsible for complications such as microvascular angina, adiabatic cardiomyopathy, no-reflow phenomena after the percutaneous coronary interventional and myocardial ischemia-reperfusion[5]. Clinical studies indicate that the microvascular injury happened much earlier than the damage of large vessels and myocardial cells. Some scholars deemed that finding the effective targeting point to alleviate the microvascular injury is of great importance to the prognosis of diabetic mellitus patients[6]. Recent studies have showed that the glucagon like peptide 1 (GLP-1) can protect pancreas by lowering the blood glucose level, and it also of benefit to cardiovascular functions such as alleviating the injury caused by myocardial ischemia-reperfusion, promoting the recovery of the ventricular function[7,8]. The native GLP-1 can't be

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used in the treatment of diabetic mellitus due to its short half-life; however, the much long lasting GLP-1 analogue has been widely used as hypoglycemic agent in clinic<sup>[9,10]</sup>. Nowadays, the function of GLP-1 has become more and more clear, but the specific molecular mechanism of how it affected the myocardial microvascular is still unclear. The unclearness also restricted the application of GLP-1 on the cardiovascular complications of diabetes.

In this research, we studied the protective effort of GLP-1 on the myocardial microvascular of rats from the cellular level. The molecular mechanism was also studied which may provided theoretical support for the treatment of cardiovascular complication in diabetic mellitus.

## 2. Materials and methods

### 2.1. Material

Reagents: GLP-1 (Tocris Bioscience), DMEM/low glucose (Hyclone), fetal calf serum (Zhejiang Tianhang Biological Technology), Dil-Ac-LDL (Molecular probe), Superoxide detection kit (Beyotime), NADPH activity detection kit (YouLi), Colorimetric TUNEL Apoptosis Assay (Roche), Superoxide anion detection kit (Beyotime), BCA Protein assay kit (Thermo), HRP-labeled sheep Anti-rabbit IgG (Beyotime), ECL chemiluminescence detection kit (Biolegend), etc were utilized in this study.

Instrument: CO<sub>2</sub> incubator (Thermo), western blot system (Bio-Rad), confocal microscope (Leica), etc were utilized in this study.

### 2.2. Isolation and culture of cardiac microvascular endothelial cells

The cardiac microvascular endothelial cells were isolated from Sprague-Dawley rat heart by the enzyme dissociation method. Briefly, two weeks old SD rats were anesthetized by pentobarbital injection. The heart was removed from the rat under sterile conditions and then the left ventricular was isolated. After washed with PBS, the epicardial was removed and the remaining myocardium was then minced. The minced tissue was immersed in 0.2% collagenase in 37 °C for 10 minutes, and then 0.02% trypsin was added for another 10 minutes to digest the tissue. DMEM/low glucose with 10% FBS was added to stop digestion, and the solution was filtered through an 100 μ m nylon mesh to remove the undigested tissue. The solution was centrifuged, resuspended in DMEM/low glucose supplemented with 15% FBS and then seeded on dishes. After the cell was cultured in humidified environment at 37 °C and 5% CO<sub>2</sub> for 72 h, the medium was exchanged. Thereafter, the medium was changed every 48 h and the cell was passaged when they reached the exponential phase. Cardiac microvascular endothelial cells (CMECs) were seeded in coverslips,

acetylated low density lipoprotein (15 μ g/mg) was added when the cells reached about 80% confluence. After cultured together for 8 h, the coverslip was washed with PBS, fixed with 4% paraformaldehyde solution. The cells was incubated with DAPI (1:500) for 5 min, after washed with PBS, the coverslip was dried in the air and then followed by mounting with neutral glycerine and microscopic examination with a confocal microscopy.

### 2.3. Cell grouping for in vitro experiment

CMECs were seeded in 6 well plates (1.5×10<sup>5</sup> cells/well), and divided them into three groups, namely the control group, high glucose group, GLP-1 group and GLP-1 plus high glucose group. The final concentration of the glucose in the control group and the high glucose group were 5.5 mmol/L and 25 mmol/L, respectively. The final concentration of GLP-1 was 10 nmol/L.

### 2.4. Index detection

#### 2.4.1. Detection of reactive oxygen species

CMECs were seeded in 96 well plates (5×10<sup>5</sup> cells/well), as it reached about 80% confluence, and the medium was discarded. After washed with PBS, the cell was incubated with superoxide detection working solution for 3 min, and then the optical density (450) was tested by a microplate reader.

#### 2.4.2. Detection of superoxide anion

CMECs were seeded in the coverslips when it reached 80% confluence, after it attached, deal it as we described in the grouping part. Dihydroethidium (DHE 8 μ mol/L, in 0.1% DMSO) was added in the medium, and then incubated for 1 h. After washed with PBS, DAPI (1:500) was added and followed by incubation at room temperature for 5 min. The coverslip was washed with PBS again and dried in the air and then followed by mounting with neutral glycerine and microscopic examination with a confocal microscopy. If the red fluorescence was enhanced, it suggested the level of superoxide anion was elevated.

CMECs were seeded in 6 well plates (1.5×10<sup>5</sup> cells/well), after it attached, deal it as we described in the grouping part. TRIzol was added to lyse the cell, and placed at -70 °C for further use. NADPH peroxidase activity was detected using a chemiluminescence apparatus according to the instruction of the kit. The activity of the NADPH peroxidase was computed by the formulation: RLU the real activity of the sample =  $RLU_{\text{total activity of the sample}} - RLU_{\text{specific activity of the sample}} - RLU_{\text{control group}}$ .

#### 2.4.3. Detection of apoptosis

CMECs were seeded in the coverslips when it reached 80% confluence, after it attached, deal it as we described in the grouping part. The coverslips was fetched out, washed with PBS, and then fixed in 4% paraformaldehyde. After washed with PBS, 3% hydrogen peroxide was added and incubated

for 20 min to remove the endogenous peroxidase. Triton 100 was added and then put on the ice. Before and after the Triton 100 was added, the coverslips should be washed with PBS carefully. The apoptosis rate of the CMECs was detected by using the TUNEL apoptosis detection kit, and the working liquid was prepared according to the instruction. Briefly, the working liquid was dropwise added onto the coverslips, after incubated in 37 °C for 1 h. Then it was washed with PBS, and the cells were incubated with DAPI (1:500) for 5 min at room temperature. It was washed with PBS again, and then followed by mounting and microscopic examination.

#### 2.4.4. Molecular mechanism of GLP-1 on CMECs

CMECs were seeded in 6 well plates ( $1.5 \times 10^5$  cells/well), after it attached. They were divided into 6 groups, namely control group, high glucose group, GLP-1 plus high glucose group, GLP-1 plus H89 plus high glucose group, fasudil plus high glucose group and GLP-1 plus fasudil plus high glucose group. Western blot was used to detect the expression level of Rho and ROCK. Briefly, the cell was washed with PBS and then, M2 was added and the plate was left on ice for 30 min to lyse the cell. Cells were collected into a centrifuge tube with a cell scraper, and then lysed with ultrasonic irradiation for 3 s, 2 times. Cell debris was removed by centrifugation of the lysates at 12 000 *g* for 2 min at 4 °C, and protein concentration was determined by using the BCA Protein assay kit. The working liquid was prepared according to the instruction, and the standard curve was made, the plate was incubated in 37 °C for half an hour, and then the optical density (570) was tested by a microplate reader, and finally the protein concentration was computed according to the standard curve. All the specimen were adjusted to the same concentration, after the protein supernate was mixed with the buffer, the liquid was boiled for 10 min, followed by cooling, centrifugation, 12% SDS-PAGE and the protein was transferred to nitrocellulose membrane. The membrane was then washed and incubated with goat anti-rabbit IgG for 60 min at room temperature. After washing, the chemiluminescence reagent was added and incubated together for 3 min. The protein was tested with an ECL western blot analysis detection system, and then the gray value was computed.

#### 2.5. Statistical analysis

SPSS 17.0 software was used for statistical analysis in this study. All data are expressed as mean $\pm$ SD, and *t*-test were applied to perform statistical analysis.  $P < 0.05$  were considered significantly different.

### 3. Results

#### 3.1. Culture and evaluation of CMECs

The CMECs was spindle or polygonal shaped and displayed typical cobblestone-like morphology (Figure 1). DiL-Ac-

LDL phagocytic test showed that the cell can phagocytize DiL-Ac-LDL, which suggests that the cell we harvested in our experiment is CMECs.

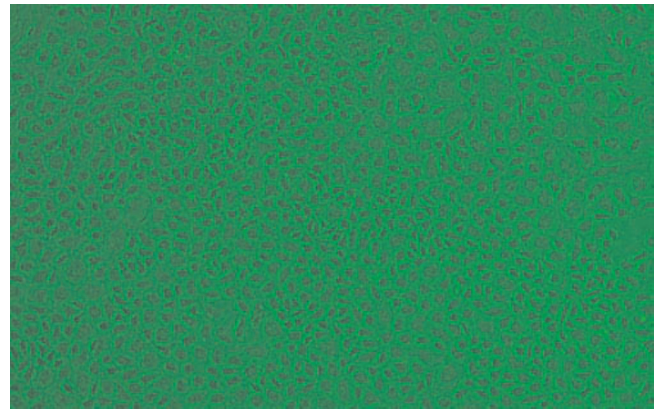


Figure 1. Cobblestone-like CMECs isolated from rats.

#### 3.2. Inhibition effect of GLP-1 on generation of ROS in CMECs

Figure 2 showed that CMECs in high glucose group produced more ROS than control group. Compared with the control group, the fluorescence intensity of CMECs in the high glucose group was greatly improved, while after added with GLP-1, the fluorescence intensity was declined clearly, and the difference were all statistically significant ( $P < 0.05$ ).

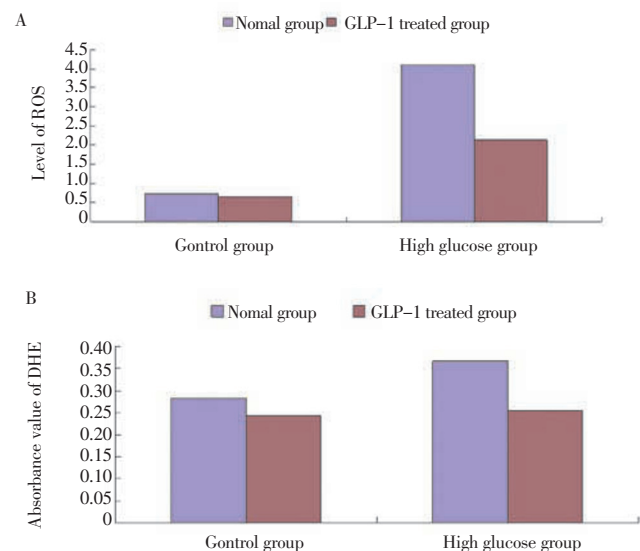
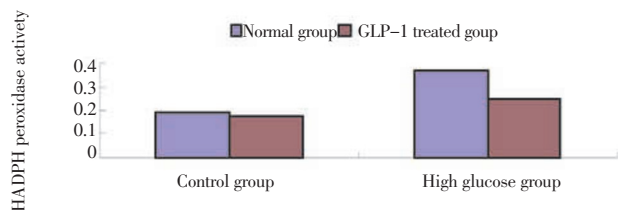


Figure 2. Influence of GLP-1 on the generation of ROS in control group and high glucose group.

A: There was no statistical difference between the levels of ROS in the control group before and after treated with GLP-1 ( $P > 0.05$ ). The level of ROS before treated with GLP-1 was significantly higher than before in the high glucose group ( $P < 0.05$ ); B: After treated with GLP-1, the absorbance value of DHE was declined in both group, and there was no significant difference in the control group ( $P > 0.05$ ); the difference in the high glucose group was statistically significant,  $P < 0.05$ .

### 3.3. Inhibition effect of GLP-1 on activity of NADPH peroxidase in CMECs

Figure 3 showed the activity of NADPH peroxidase in CMECs was much higher in the high glucose group than the control group, the difference was statistically significant ( $P<0.05$ ). After treated with GLP-1, the activity of NADPH peroxidase was declined significantly ( $P<0.05$ ).



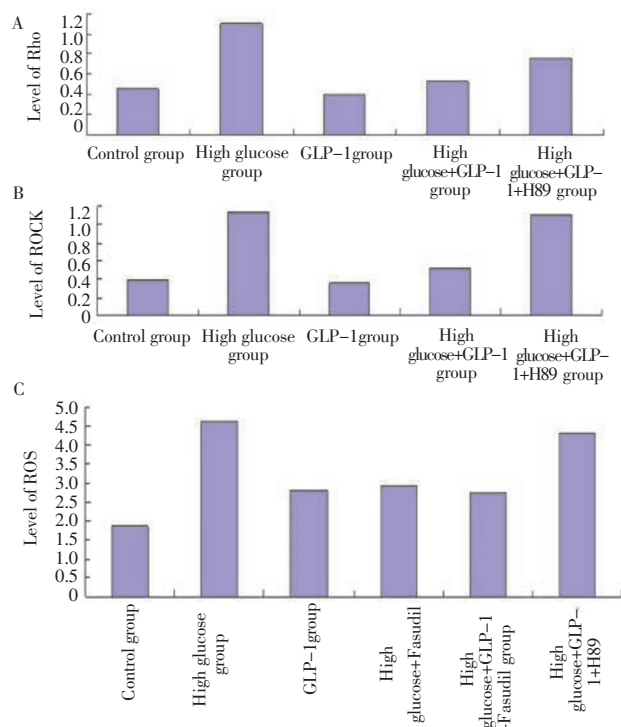
**Figure 3.** Influence of GLP-1 on the activity of NADPH peroxidase. The activity of NADPH peroxidase was higher in the high glucose group than in the control group. After treated with GLP-1, the activity of NADPH peroxidase was declined significantly ( $P<0.05$ ).

### 3.4. Inhibition effect of GLP-1 on apoptosis of CMECs

TUNEL staining result showed that the apoptosis rate of CMECs in the high glucose group was much higher than in the control group ( $11.20\pm 2.64$  vs.  $40.06\pm 2.81$   $P<0.05$ ). After treated with GLP-1, the apoptosis rate of CMECs in the high glucose group was declined significantly, compared with group with normal glucose level [ $(40.06\pm 2.81)\%$  vs.  $(27.11\pm 2.62)\%$ ],  $P<0.05$ ).

### 3.5. Suppressive effect of GLP-1 on activation of Rho/ROCK through cAMP/PKA pathway in CMECs

Compared with the control group, the expression level of Rho/ROCK was improved significantly in the high glucose group. After treated the cell with GLP-1, the expression level of Rho/ROCK was declined greatly, and the difference were all statistically significant ( $P<0.05$ ). When we combined GLP-1 and PKA inhibitor H89, there was some improvement in the expression level of Rho/ROCK ( $P<0.05$ ). As showed in the former experiment, GLP-1 can decrease the expression level of ROS induced by high glucose in CMECs; treated the cell with Rho inhibitor fasudil can suppress the expression of ROS induced by high glucose, while when used GLP-1 and fasudil together, there were no significant difference in the expression level of ROS compared with GLP-1 used alone (Figure 4).



**Figure 4.** Influence of GLP-1 on activity of Rho/ROCK in CMECs through cAMP/PKA pathway.

A, B: High glucose induced the expression of Rho and ROCK; when treated with GLP-1, the level of Rho and ROCK was declined significantly ( $P<0.05$ ); when GLP-1 and H89 were added together, the level of Rho and ROCK was improved significantly compared with the GLP-1 treated group ( $P<0.05$ ). C: CMECs in the high glucose group treated with GLP-1, fasudil, and GLP-1 plus fasudil can significantly lower the level of ROS, compared with the untreated group ( $P<0.05$ ).

## 4. Discussion

In this study, we isolated and cultured CMECs from rats *in vitro*, and observed the protective effort of GLP-1 on CMECs under high glucose, and tried to elucidate the molecular mechanism of protective effort. We found that GLP-1 can lower the expression level of ROS, NADPH peroxidase, Rho and ROCK in CMECs exposed to high glucose. The results further confirmed that GLP-1 can inhibit the oxidative stress effort and apoptosis of CMECs, and it is achieved by cAMP/PKA/Rho signaling pathway

GLP-1 is a kind of transmembrane G-protein-coupled receptor made up of 463 amino acids. It is produced by L cells present in the mucosa of the distal small intestine and the colon which is widely spread in the organs such as pancreas, gastrointestinal, kidney and brain<sup>[11,12]</sup>. GLP-1 is considered as an ideal drug for the treatment of diabetes as it can regulate glucose levels by stimulating glucose dependent insulin secretion and biosynthesis, and by promoting the proliferation of beta cells in the pancreas, suppressing glucagon secretion, delaying gastric



emptying<sup>[13]</sup>. Betsy *et al*<sup>[14]</sup> found by animal experiment that GLP-1 can improve the function of myocardial microcirculation after CPR with no increase of ventricular fibrillation. Lesven *et al*<sup>[15]</sup> also found that GLP-1 can ameliorate clinical symptoms effectively when it used in the acute phase of amyocardia. Nowadays, GLP-1 analogues has been widely used clinically for its advantages in all the aspects, and has achieved satisfactory curative effect<sup>[16,17]</sup>. But how GLP-1 exerts a protective function in the cell level is still unclear. CMEC is the most common cell line used in the laboratory to study the cardiovascular disease. The shape, structure and function of CMECs is quiet different from endothelial cells in the large vessels for the force (sheer stress, blood perfusion pressure and the extrusion in myocardial contraction) applied on it<sup>[18]</sup>.

Studies showed that the organism will generate a great deal of free radicals under the stimulation of high glucose, and then start the oxidative stress reaction<sup>[19,20]</sup>. Some animal experiment suggested tissues in mouse with spontaneous diabetes are under different state of oxidative stress. The level of lipid peroxide in the liver, heart and kidney of rats with STZ induced diabetes also rose obviously<sup>[21,22]</sup>. Teodoro *et al*<sup>[23]</sup> found in their cell experiment that, exposed the HepG2 to high glucose less than an hour, the damage of mitochondria can be observed, and the level of NADH and the marker of oxidative stress ROS also improved greatly. In our experiment, we discovered that high glucose can induce the over expression of ROS in CMECs, while when GLP-1 was added, ROS declined obviously. The results suggested that GLP-1 can suppress the oxidative stress induced by high glucose to some degree. NADPH peroxidase is the main resource of ROS produced by endothelial cells; therefore, we detected the level of NADPH peroxidase to further verify our results and also found GLP-1 can obviously suppress the activation of NADPH peroxidase induced by high glucose.

Oxidative stress is the initial factor of vascular complications of diabetis. Cells exposed to high glucose will generate a great deal of ROS, activate a series of biological efforts such as cell proliferation, apoptosis, migration and inflammatory response. Those biological efforts will damage the vascular endothelial cell, increase the vascular permeability, lead to the pathological angiogenesis and the disorder of angiokinesis, which may results in vascular complications of diabetis finally<sup>[24,25]</sup>. In our study, we also discovered high glucose can improve the activation of caspase 3 in CMECs, and improve the apoptosis rate. All of this lead to the conclusion that the anti-oxidative stress effort of GLP-1 can inhibits the apoptosis of CMECs.

Several stimuli can activate the Rho/ROCK pathway, and it can interact with some other signaling pathways, and then regulate the transcription of some specific genes<sup>[26]</sup>. Lin *et al*<sup>[27]</sup> discovered that the cardiovascular injuries were much lighter in the diabetic rats with ROCK 1 deficiency than the

wild type. Which means lower the activation of ROCK and arginine can improve the function of cardiovascular system in diabetic. In our experiment, high glucose can induce the over expression of Rho/ROCK in CMECs, while when the GLP-1 was added, the level of Rho/ROCK declined obviously. The phenomenon suggested that GLP-1 exert the anti-oxidative stress effort through inhibiting the expression of Rho/ROCK. In addition, we found, when the cell treated with PKA inhibitor H89, the level of Rho/ROCK declined obviously. This indicate that GLP-1 suppress the expression of Rho/ROCK through cAMP/PKA pathway, and further inhibit the oxidative stress effort. GLP-1 can combined with GLP-1R in the membrane of pancreatic  $\beta$ -cell, thus improve the intracellular cAMP level, and the voltage dependent calcium channel opened, induce the influx of extracellular  $Ca^{2+}$ , and stimulate the insulin secretion. GLP-1 can inhibit the inflammatory effort through cAMP/PKA pathway, and thus attenuates endothelial dysfunction induced by lipopolysaccharide<sup>[28,29]</sup>. We can infer cAMP/PKA is a major pathway for the protective effort of GLP-1. At last we detected the influence of H89 on the generation of ROS, we discovered, H89 can reduce the anti-oxidative stress effort of GLP-1. The results suggested that the protective effort of GLP-1 on CMECs is achieved by cAMP/PKA/Rho signaling pathway.

There were flaws in our research. In our study, we only conducted cell experiment, and the animal experiment is still needed in the future.

In conclusion, GLP-1 can inhibit the oxidative stress effort and apoptosis of CMECs, and it is achieved by cAMP/PKA/Rho signaling pathway.

### Conflict of interest statement

We declare that we have no conflict of interest.

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