

Research Article

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Glucagon-like peptide-1 affects human umbilical vein endothelial cells in high glucose by the PI3K/Akt/eNOS signaling pathway

Glukagon benzeri peptid-1, insan umbilikal ven endotel hücrelerini yüksek glukoz varlığında PI3K/Akt/eNOS sinyal yolu ile etkiler

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Abstract

Objectives: To investigate the effects of glucagon-like peptide-1 (GLP-1) for the apoptosis of human umbilical vein endothelial cells (HUVECs) in high glucose and the related mechanisms.

Material and methods: HUVECs were cultured under different conditions for 48 h. The apoptosis rate of cells was detected by flow cytometry, the expression of p-Akt and p-eNOS was measured by Western blot, and the production of nitric oxide (NO) was detected by NO assay kit.

Results: HUVECs were incubated in high glucose, the apoptosis rate of cells increased, the expression of p-Akt and p-eNOS reduced, and the production of NO decreased. After GLP-1 was added into the high glucose, the apoptosis rate of cells significantly reduced, the expression of p-Akt and p-eNOS and the production of NO obviously increased. After exendin, wortmannine and L-NAME were added into high glucose and GLP-1, respectively, exendin, wortmannine and L-NAME increased the cell apoptosis, down-regulated the expression of p-Akt and p-eNOS and dropped the production of NO, except that L-NAME made no difference on the expression of p-Akt.

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Conclusion: GLP-1 increased the expression of Akt and endothelial nitric oxide synthase (eNOS) in HUVECs via the up-regulation of PI3K/Akt/eNOS pathway and decreased the apoptosis rate of HUVECs in high glucose.

Keywords: Glucagon-like peptide-1; Human umbilical vein endothelial cells; High glucose; Cell apoptosis; PI3K/Akt/eNOS signaling pathway.

Özet

Amaç: Glukagon benzeri peptid-1'in (GLP-1)Y yüksek glukoz varlığında insan göbek bağı ven endotel hücrelerinin (HUVEC'ler) apoptozu üzerine etkilerini ve ilgili mekanizmaları araştırmak.

Gereç ve Yöntem: HUVEC'ler 48 saat boyunca farklı koşullar altında büyütüldü. Hücrelerin apoptoz oranı flow sitometri ile tespit edildi, p-Akt'in ve p-eNOS ekspresyonu, Western blot ile ölçüldü ve NO üretimi, NO assay kiti ile tespit edildi.

Bulgular: HUVEC'ler yüksek glukozda inkübe edildi, hücrelerin apoptoz oranı arttı, p-Akt ve p-eNOS ifadesi azaldı ve NO üretimi azaldı. Yüksek glukozda GLP-1 ilave edildikten sonra, hücrelerin apoptoz oranı, p-Akt ve p-eNOS'un ekspresyonunu önemli ölçüde düşürdü ve NO üretimi belirgin bir şekilde arttı. Exendin takiben, yüksek glukozlu hücrelere wortmannine ve GLP-1 eklenen hücrelere de L-NAME eklendi, ekstendin, wortmannine ve L-NAME, hücre apoptozunu arttıran, p-Akt ve p-eNOS ekspresyonunu aşağı regüle ederek, NO üretimini düşürdü. Ancak L-NAME, p-Akt'in ekspresyonu üzerinde herhangi bir fark yaratmadı.

Sonuç: GLP-1, PI3K/Akt/eNOS yolağının yukarı regülasyonu yoluyla HUVEC'lerde Akt ve eNOS ekspresyonunu

arttırdı ve yüksek glukozda HUVEC'lerin apoptoz oranını düşürdü.

Anahtar Kelimeler: Glikagon benzeri peptid-1; İnsan umbilikal ven endotel hücreleri; Yüksek glukoz; Hücre apoptozu; PI3K/Akt/eNOS sinyal yolağı.

Introduction

Glucagon-like peptide-1 (GLP-1), one of incretin peptides, is secreted by enteroendocrine L cells mainly located in the mucosa of the distal part of the small intestine and colon after ingesting food [1]. GLP-1 can stimulate β -cells in the pancreas to secrete insulin and play the role of lowering blood sugar [2]. Meanwhile, GLP-1 inhibits glucagon secretion, suppresses gastrointestinal peristalsis, increases satiety and reduces appetite and food intake [3]. Thus, GLP-1 is widely used to treat patients with type 2 diabetes.

Vascular complications of diabetes are the leading cause of death and disability in diabetic patients [4]. Chronic hyperglycemia causing endothelial cell apoptosis is the main factor of cardiovascular disease patients with type 2 diabetes mellitus [5]. Therefore, preventing the endothelial cell apoptosis and protecting the endothelial function can control the diabetic vascular complications and improve the prognosis of patients with diabetes.

These studies showed that PI3K/Akt/endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signaling pathways play an important role in preventing endothelial cell apoptosis that hyperglycemia results in Ref. [6]. Phosphatidylinositol 3-kinase (PI3K) activates downstream Akt to prevent cell apoptosis death stimulating factors causing [7]. Akt, also known as protein kinase B, is a serine/threonine kinase family which is an important anti-apoptosis protein in body [8]. Activation of Akt requires the phosphorylation of Thr308 in the activation loop by the phosphoinositide-dependent kinase 1 (PDK1) and Ser473 within the carboxyl-terminal hydrophobic motif by an unknown kinase [9]. The activated Akt in endothelial cells can activate eNOS which plays an important role in regulating a broad spectrum of functions in the cardiovascular system, including vasorelaxation, the inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell (SMC) migration and proliferation, and platelet aggregation [10]. The eNOS synthesizes NO from the substrate L-arginine in endothelial cells after activated. NO served as super oxygen anion scavenger in endothelial cells reacts with super oxygen anion, prevents it from creating hydrogen peroxide (H_2O_2) and preserve endothelial cells from apoptosis [11].

Recent studies have found GLP-1 also has protective effect on endothelial cell [12], while the functional mechanism is still unclear. In order to better understand the mechanism by which GLP-1 may protect endothelial cell, we investigated whether the mechanism is related with up-regulation of PI3K/Akt/eNOS/NO signaling pathways in the present study.

Materials and methods

Reagents

GLP-1, exendin, wortmannin and L-NAME were purchased from Sigma (USA). P-Akt antibody [phospho-Akt (Ser473) antibody: #9271] and p-eNOS antibody [phospho-eNOS (Ser1177) antibody: #9571] were purchased from Cell Signaling Technology, Inc. (USA). Annexin-V/PI double staining kits and NO assay kit were obtained from Beyotime (China). Exendin, wortmannine and L-NAME were purchased from ApexBio (USA).

Phospho-Akt (Ser473) antibody, a mouse polyclonal antibody, can detect endogenous levels of Akt1 only when phosphorylated at Ser473 of Akt from human by Western blot. Phospho-eNOS (Ser1177) antibody, a rabbit polyclonal antibody, can detect endogenous levels of eNOS only when phosphorylated at Ser1177 of Akt from human by Western blot.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs, Catalog Number: C-12200) from normal human placenta umbilical cord tissues were obtained from Promo Cell GmbH (Heidelberg, Germany) as cryopreserved primary cultures. After purchased, HUVECs were recovered and sub-cultured in endothelial cell medium containing 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., USA) and 1% endothelial cell growth supplement in a humidified atmosphere of 95% air and 5% CO_2 at $37^\circ C$. After added different experimental factors, HUVECs in logarithmic growth phase were divided into six groups. It is as follows: physiological concentration of glucose (control: 5 mmol/L of glucose), high glucose (HG: 33 mmol/L of glucose), high glucose and GLP-1 (HG + G: 33 mmol/L of glucose and 10 nmol/L of GLP-1), physiological concentration of glucose and GLP-1 (control + G: 5 mmol/L of glucose and 10 nmol/L of GLP-1), high glucose, GLP-1 and exendin (HG + G + E: 33 mmol/L of

glucose, 10 nmol/L of GLP-1 and 5 nmol/L of exendin), high glucose, GLP-1 and wortmannine (HG+G+W: 33 mmol/L of glucose, 10 nmol/L of GLP-1 and 100 nmol/L of wortmannine), and high glucose, GLP-1 and L-NAME (HG+G+L: 33 mmol/L of glucose, 10 nmol/L of GLP-1 and 100 μ mol/L of L-NAME). Exendin, wortmannine and L-NAME were added in 3 min ago.

The detection of cell apoptosis rate

The flow cytometry was used to detect the cell apoptosis rate. The HUVECs in logarithmic growth phase were inoculated into six-well plates, and a suspension of HUVECs at 4×10^5 cells/mL in a 2-mL volume of culture medium was incubated at 37°C. The experimental groups were based on the above method, and every group was in triplicate. After HUVECs incubated for 48 h, the culture solution was discarded. Next, after washed with D-Hank's and digested with pancreatic enzymes successively, the cells were gathered. After the cell solution was centrifuged (10,000 \times g, 10 min, 4°C), the supernatant was removed. After washed with phosphate buffer saline (PBS) precooling at 4°C, the cell solution was centrifuged (10,000 \times g, 10 min, 4°C) again, and then the supernatant PBS was removed. The washing was repeated twice. According to the operating instructions of Annexin-V/PI double staining kits, the HUVECs were stained and quantitatively analyzed by the flow cytometry (FACS Calibur 95, BD Biosciences). The data were presented as dot plots showing fluorescence intensity of Annexin-V and PI.

The detection of p-Akt and p-eNOS expression

The Western blot was used to detect the expression of p-Akt and p-eNOS. The HUVECs in logarithmic growth phase were inoculated into 25 cm³ of culture bottle, and a suspension of HUVECs at 1×10^6 cells/mL in a 5-mL volume of culture medium was incubated at 37°C. The experimental groups were based on the above method, and every group was in triplicate. After HUVECs incubated for 48 h, the HUVECs was washed twice with PBS precooling at 4°C and collected by cell scraper. Next, according to the operating instructions of protein extraction kit, the total protein of each group was extracted and measured by Bradford method. After 40 μ g of the total protein in each group was denatured at 100°C for 5 min, the protein was separated by 12% SDS-PAGE and

transferred to PVDE membrane, and then sealed by 5% skimmed milk for 2 h. After the membrane was washed, p-Akt antibody (1:1000) and p-eNOS antibody (1:1000) were added and incubated overnight. After that, horseradish peroxidase labeled second antibody was added and incubated at room temperature for 1 h. After washed, the membrane dried, and the target protein was detected by chemiluminescence method. β -Aatin was internal reference and used to calculate the relative content of the target protein. Signal intensity of the bands was analyzed to use Image-Pro Plus Software of version 6.0 (Media Cybernetics, Inc., MD, USA).

The detection of NO concentration

The HUVECs in logarithmic growth phase were inoculated into six-well plates, and a suspension of HUVECs at 4×10^5 cells/mL in a 2-mL volume of culture medium was incubated at 37°C. The experimental groups were based on the above method, and every group was in triplicate. After incubated for 48 h, 0.1 mL of supernatant was taken out. According to the operating instructions of NO assay kit, the absorbance of the supernatant was determined by ELISA at 550 nm to calculate the concentration of NO.

Statistical analysis

The statistical analyses in this work were carried out by SPSS version 17.0. All data were presented as the mean \pm SD. Comparisons between two groups were performed using student's t-test. Values of $p < 0.05$ were considered to indicate a statistically significant difference.

Results

The effect of GLP-1 and high glucose for the apoptosis rate of HUVECs

The percentage of apoptotic cells was examined by Annexin-V/PI double staining. The apoptosis rate of HUVECs in HG was 23.8% and significantly higher than 4.8% of control group ($p < 0.05$). The apoptosis rate of HUVECs in HG+G was 4.5% and significantly reduced in comparison with HG. The apoptosis rate of HUVECs in HG+G+E (23.1%), HG+G+W (24.7%) and HG+G+L (21.8%) was markedly higher than HG+G. The all above results were showed in Figure 1.

The effect of GLP-1 and high glucose for the p-Akt and p-eNOS expression of HUVECs

The expression of p-Akt and p-eNOS in HG reduced ($p < 0.05$) in comparison with control group, but the expressions in HG+G significantly increased ($p < 0.05$), and the expression in HG+G+E with exendin and

HG+G+W with wortmannine also decreased in comparison with HG+G, as showed in Figure 2. The expression of p-Akt in HG+G+L with L-NAME were not significantly different from HG+G ($p > 0.05$), as shown in Figure 2A and B. However, there was a significant difference between HG+G+L and HG+G in the expression of p-eNOS ($p < 0.05$), as shown in Figure 2C and D.

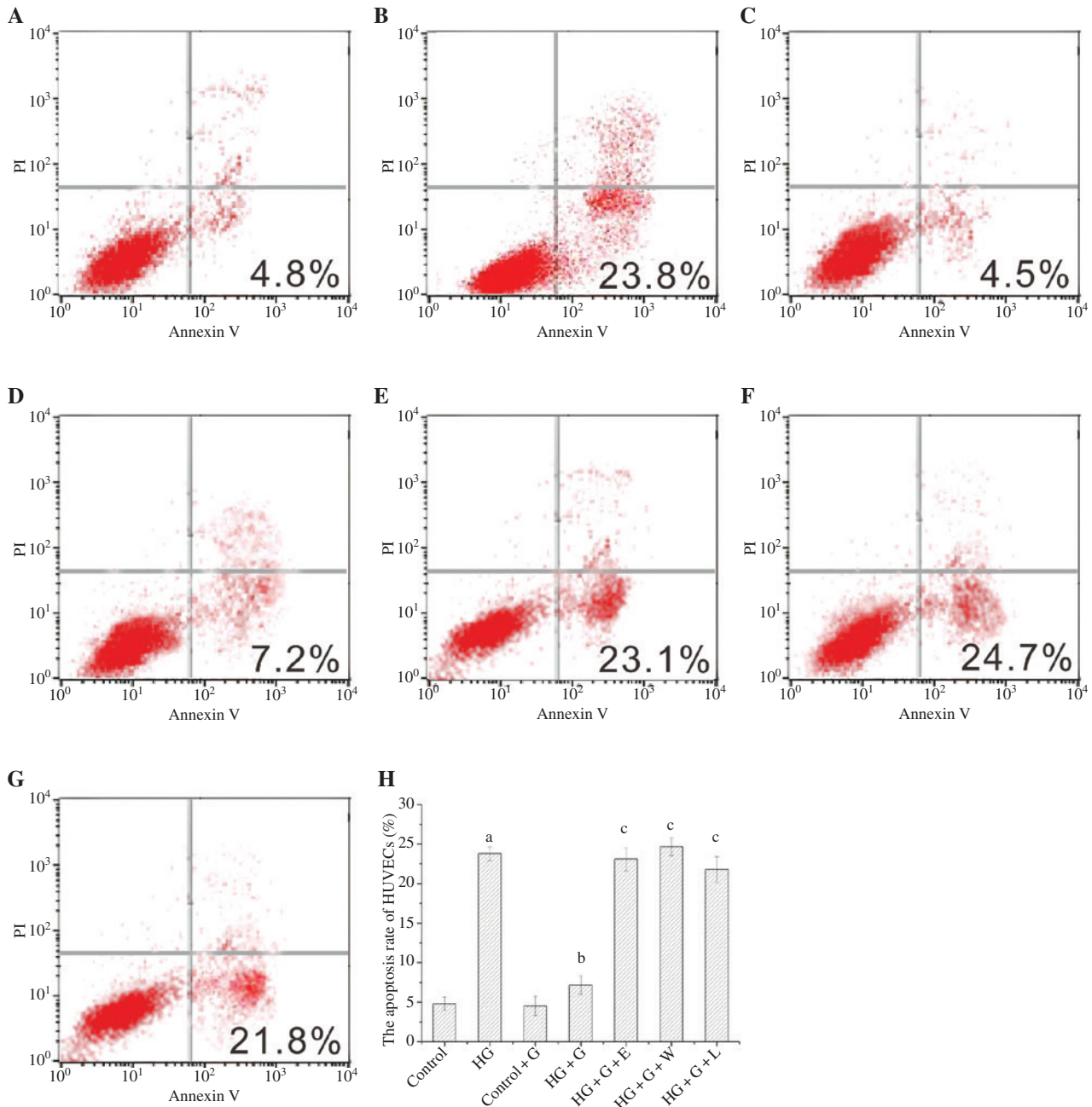


Figure 1: The apoptosis rate of HUVECs in the different conditions was labeled with Annexin V/PI and detected by the flow cytometry.

^a $p < 0.05$ compared to control; ^b $p < 0.05$ compared to HG; ^c $p < 0.05$ compared to HG+G. (A) control, (B) HG, (C) control+G, (D) HG+G; (E) HG+G+E; (F) HG+G+W, (G) HG+G+L and (H) a histogram of the apoptosis rate from A to G.

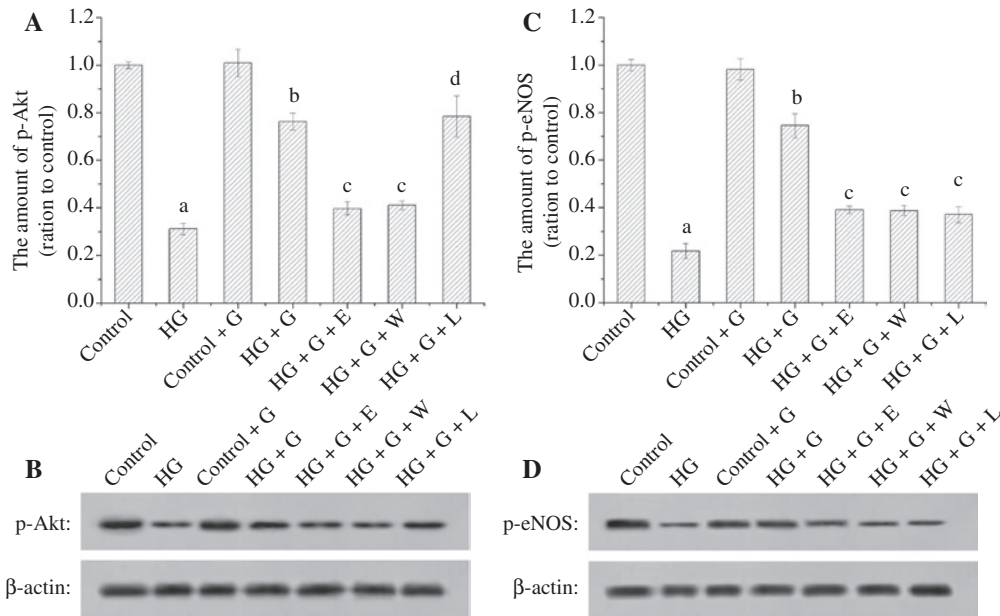


Figure 2: The effect of GLP-1 and high glucose for the p-Akt and p-eNOS expression of HUVECs.

The histogram (A) showed the relative amount of p-Akt in HUVECs. The images (B) showed the analyzed result of p-Akt in HUVECs by Western blot. The histogram (C) showed the relative amount of p-eNOS in HUVECs. The images (D) showed the analysis result of p-eNOS in HUVECs by Western blot. ^a $p < 0.05$ compared to control; ^b $p < 0.05$ compared to HG; ^c $p < 0.05$ compared to HG + G; ^d $p > 0.05$ compared to HG + G.

The effect of GLP-1 and high glucose for the NO production of HUVECs

The NO production of HG was obviously less than the control group ($p < 0.05$), but the HG + G produced more NO than HG ($p < 0.05$). The NO production of HG + G + E with exendin, HG + G + W with wortmannine or HG + G + L with L-NAME obviously reduced ($p < 0.05$). The all above results were showed in Figure 3.

Discussion

GLP-1, a brain-gut insulinotropic peptide, plays an important role in regulating glucose homeostasis, and protecting pancreatic β cell and cardiovascular system [13]. The function of GLP-1 protecting vascular endothelial cells attracts extensive attention, and GLP-1 is applied in clinical treatment of diabetes.

The results of the flow cytometry in Figure 1 showed that GLP-1 could obviously reduce the apoptosis rate of HUVECs, so we further investigated the pathways that GLP-1 protects vascular endothelial cells under hyperglycemia. The results of Figure 2 displayed that the expression of p-Akt and p-eNOS in HG was lower than the control group, meanwhile the production of NO in HG was obviously less than the control group, as shown in Figure 3.

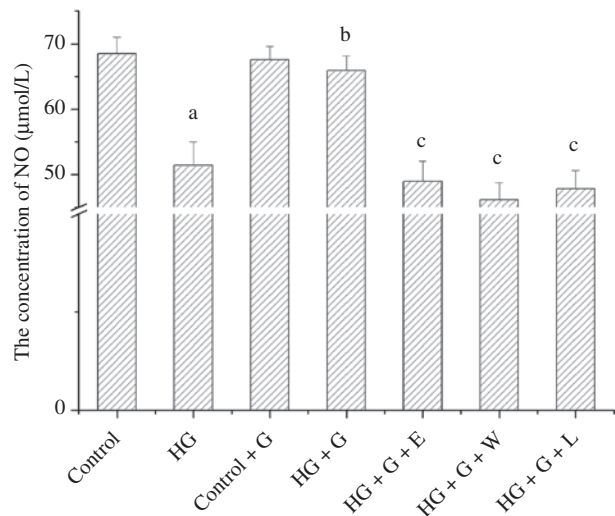


Figure 3: The histogram showed the effect of GLP-1 and high glucose for the NO concentration of HUVECs in the different conditions.

^a $p < 0.05$ compared to control; ^b $p < 0.05$ compared to HG; ^c $p < 0.05$ compared to HG + G.

The p-Akt is one of the downstream effector molecules of PI3K signal pathway and an important anti-apoptosis protein in the body. The excitation of p-Akt plays a significant role in protecting endothelial cells and inhibiting the cells apoptosis [14]. The p-eNOS plays an important role in maintaining the normal function of endothelial cells,

and its dysfunction may be significantly associated with the cardiovascular complications in patients with type 2 diabetes mellitus [15]. After GLP-1 was added into the HG, the expression of p-Akt and p-eNOS in high blood glucose was markedly improved and the production of NO in high blood glucose markedly increased. In order to make certain the relative mechanism, we did the further research. Exendin, a potent GLP-1 receptor antagonist, is capable of competing with GLP-1 for receptor binding [16]. After exendin was added into the HG+G, the apoptosis rate of HUVECs was significantly higher than HG+G in Figure 1, the expression of p-Akt and p-eNOS was significantly lower than HG+G in Figure 2, and the production of NO was observably less than HG+G in Figure 3. Wortmannine, a PI3K inhibitor, specifically inhibits PI3K and represses the PI3K/Akt signal pathway [17]. The experimental results wortmannine produced were similar to the exendin. L-NAME is an eNOS inhibitor. L-NAME caused the increasing of HUVECs apoptosis rate (Figure 1), the down-regulation of p-eNOS expression (Figure 2C and D) and the reduction of NO production (Figure 3), in comparison with HG+G. However, L-NAME had no effect on the expression of p-Akt. This is because p-Akt is the upstream molecular of p-eNOS and L-NAME only inhibit the activity of p-eNOS.

According to the above experimental results, we speculate that the mechanism is as follows: GLP-1 activated GLP-1R of HUVECs and further increased the expression of PI3K; PI3K activated the downstream Akt, and then activated Akt activated and increased eNOS, increased the production of NO and released NO into the HUVECs; the NO reacted with superoxide anion in HUVECs, avoided the production of H_2O_2 and prevented HUVECs from the apoptosis of HUVECs.

In conclusion, GLP-1 can protect the HUVECs through the GLP-1R-dependent and GLP-1 related pathways. In addition, we will focus far more on the protected effects of GLP-1 does factor for high glucose inducing apoptosis of HUVECs.

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Conflict of interest statement: All authors declared that there were no any potential conflicts of interest.

Compliance with ethical standards: This article does not contain any studies with human subjects or animals performed by any of the authors.

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