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Original Article

Exendin-4 attenuates high glucose-induced endothelial progenitor cells dysfunction through the PI3-K/Akt/eNOS pathway

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Abstract: Background: Recent studies have demonstrated that exendin-4 have beneficial effects on endothelial function and postnatal neovascularization following vascular injury in patients with diabetes mellitus (DM), but the mechanisms underlying these effects remain to be elucidated. Hence, we hypothesized that exendin-4 might restore the impaired functional activities of endothelial progenitor cells (EPC) after ischemia in DM patients to play a beneficial role. Methods: EPCs were respectively incubated with normal glucose (5 mM) or high glucose (25 mM) environment for 3 days, followed by treatment with exendin-4 for 24 hours. Cell proliferation, migration, tube formation and nitric oxide (NO) production were examined in vitro. The protein levels of endothelial NO synthase (eNOS), phospho-eNOS (p-eNOS), Akt, phospho-Akt (p-Akt) and PI3K were analyzed using Western-blot. Results: High glucose reduced EPC functional activities, including cell proliferation, migration and tube formation. Additionally, Akt/eNOS activity and NO production were downregulated in high glucose-stimulated EPCs. Administration of exendin-4 ameliorated high glucose-induced EPC dysfunction. The GLP-1 receptor (GLP-1R) antagonist exendin (9-39), PI3K inhibitor LY-294002 and eNOS inhibitor L-NAME could attenuate the effect of exendin-4. Meanwhile, exendin-4 increased the phosphorylation of Akt/eNOS and secretion of NO in high glucose-treated EPCs, which could be blocked by PI3K inhibitor (LY294002). Conclusions: Our results suggested that exendin-4 could attenuate high glucose -induced EPCs dysfunction. This effect might be mediated through the PI3-K/Akt/eNOS pathway.

Keywords: Exendin-4, high glucose, endothelial progenitor cells, eNOS

Introduction

Diabetes mellitus (DM) is a complex metabolic disorder characterized by impaired glucose metabolism with hyperglycemia [1]. Patients with DM have high risk of developing cardiovascular complications compared with nondiabetic controls [2]. This has been attributed to the occurrence of endothelial dysfunction that leads to the initiation and progression of atherosclerotic vascular disease and impaired neovascularization after ischemia induced by hyperglycemia [2-4].

It is generally known that endothelial progenitor cells (EPCs) play a central role in the process of endothelial repair and postnatal neovascularization at sites of injury or ischemia [5-7]. However, the functional capacities of EPCs are often impaired in DM patients, which are believed to be one of the pathogenesis of vascular complications in diabetic patients [8].

Exendin-4, as a 39 amino acid agonist of GLP-1R, is being used to increase insulin production for the clinical treatment of type 2 diabetes [9]. Recent studies have demonstrated that exendin-4 has been shown to have beneficial effects on endothelial function and postnatal neovascularization following vascular injury, apart from the insulinotropic effects [10]. Nevertheless, the cellular and molecular mechanisms underlying exendin-4 on functional activities of EPCs remain not yet clearly defined. Therefore, we hypothesized that exendin-4 might exert beneficial effect on endothelial function and neovascularization after ischemia by restoring the impaired functional activities of EPC in DM patients. The effects and the molecular mecha-

nisms of exendin-4 on functional capacities of EPC were investigated in this study.

Materials and methods

Late EPCs culture and characterization

Late EPCs were cultured and characterized by following the protocol described by other research labs [11]. Briefly, PBMNCs were cultured on fibronectin-coated 6-well plates in EBM-2 supplemented with endothelial growth medium-SingleQuots (Clonetics, San Diego, CA, USA). After 4 days culture, non-adherent cells were removed by thoroughly washing with culture medium. Medium was changed daily for 7 days, and then every other day until the first passage. After 3 weeks' culture, the late EPC was characterized by immunofluorescence staining for CD133, endothelial nitric oxide synthase (eNOS), and von Willebrand factor (Santa Cruz, USA). Fluorescence images were recorded using an Olympus FV1000 laser scanning confocal microscope (Olympus, Japan).

Cell treatment

EPCs were respectively incubated with normal glucose (5 mM) or high glucose (25 mM) environment for 3 days. EPCs were treated with different concentrations (10, 25, 50 and 100 nM) of exendin-4 (Sigma, St. Louis, MO) for 24 hours. To investigate the involved signaling pathways, cells were administrated with the GLP-1 receptor (GLP-1R) antagonist exendin (9-39) (200 nmol/l; Sigma), phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 or L-NAME (a specific inhibitor of eNOS, 100 M) (Cell Signaling Technology, Beverly, MA) for 1 hour before the treatment of exendin-4.

EPCs proliferation

EPC proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT) assay. Briefly, the late-outgrowth EPCs were supplemented with 20 μl of MTT (0.5 mg/ml, Sigma) and incubated for 4 hours for the proliferation assay. Blue formazan was dissolved with 150 μl/well of dimethyl sulfoxide. Optical density (OD) values were measured at 550 nm (test wavelength) and 650 nm (reference wavelength). Relative cell proliferation rate was calculated as: (OD of the experimental group/OD of the control group) × 100%.

EPCs migration

The migratory function of the EPCs was evaluated by modified Boyden chamber (Transwell, Costar). Briefly, 4×10^4 EPCs were placed in the upper chambers of transwell plates with serum-free endothelial growth medium. In the lower chambers, stromal cell-derived factor 1 (SDF-1) (50 ng/mL) was supplemented to the medium placed at 37°C incubation. After incubation for 24 hours, the membrane of chamber was washed by PBS twice and stained using lectin-FITC (UEA-1 lectin, Sigma). Then the upper membrane side was scraped with a cotton ball and fixed with 2% paraformaldehyde. The migrated cells in lower membrane side were counted by 6 random high-power (× 100) microscopic fields by fluorescence microscopy.

EPCs adhesion

Dishes were coated with fibronectin (10 μg/ml). EPCs (2×10^4) in each well of a 24-well plate were stimulated with or without SDF-1 (100 ng/ml) for 5 hours at 37°C. Non-attached cells were removed with PBS, and adherent EPC were fixed with 4% paraformaldehyde and stained with 0.3% crystal violet. The adherent EPCs were counted by independent investigators blinded to treatment groups randomly.

Tube formation assay

A growth factor-reduced Matrigel (Corning) was warmed up at 4°C overnight. After completely thawed, 60 μl of Matrigel was plated to 96-well plates at the same level to distribute evenly, and incubated for 1 hour at 37°C. EPCs (2×10^4) were resuspended with EBM-2, and loaded on the top of the Matrigel. Each conditional group contained 3 wells. Following incubation at 37°C for 2 hours, each well was imaged directly under a microscope with 10 × phase contrast, and an average of tubules was counted from 3-5 random fields.

Nitric oxide (NO) production

Intracellular NO levels were measured using a NO-sensitive fluorescence probe 3-amino, 4-aminomethyl-2',7'-difluorescein, diacetate (DAF-FM DA; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocols. Cells were incubated in 60-mm plates for 24 h under different treat-

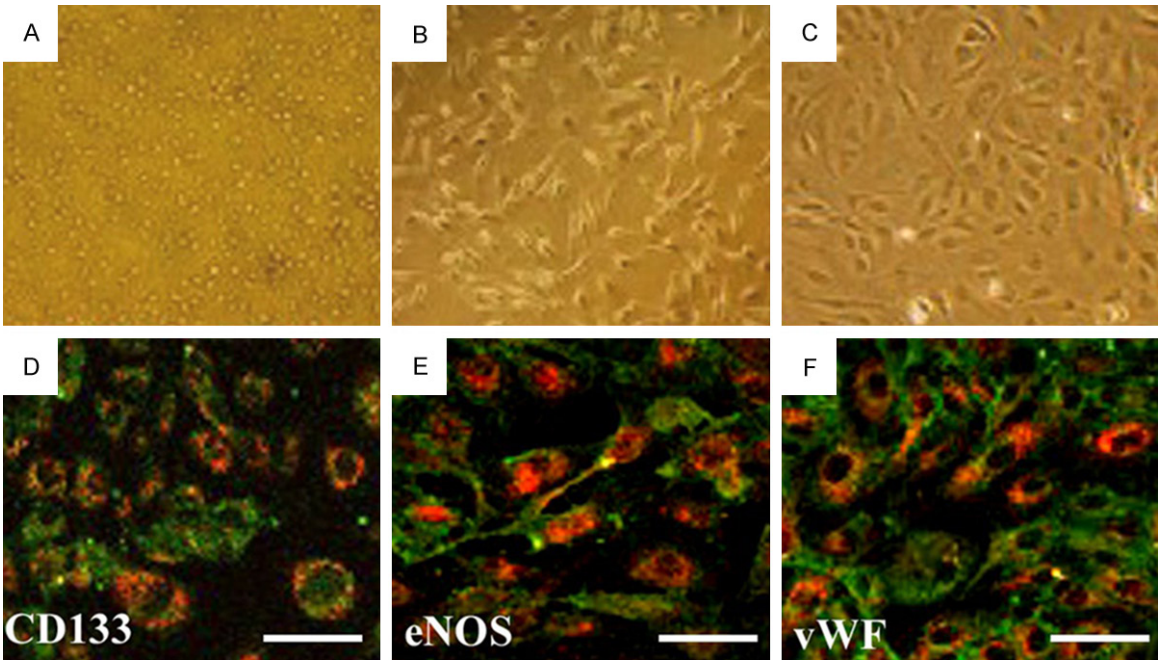

Figure 1. Morphology and characterization of EPCs from peripheral blood. MNCs were isolated and plated on fibronectin-coated culture dishes on day 1 (A). Four days after plating, adherent early EPCs with spindle shape were apparent (B). Twenty-one days after plating, late-outgrowth EPCs with cobblestone-like morphology were apparent (C). Immunofluorescence detection (green) of FITC-labeled CD133 (D), FITC-labeled eNOS (E), FITC-labeled von Willebrand factor (F) are shown for late-outgrowth EPCs. Scale bar: 100 μm.

ment conditions. Subsequently, the cells were washed twice with PBS and incubated with 5 μmol/l of DAF-FMDA in serum-deficient medium for 30 min at 37°C. At the end of the incubation, the cells were washed with PBS and gently trypsinized. Cell fluorescence was measured by a flow cytometer (BD Biosciences, San Jose, CA) at an emission wavelength of 515 nm and an excitation wavelength of 495 nm. Controls were set up as 100% of the intracellular NO level.

Western blot analysis

Total proteins of EPCs were extracted and quantified by protein extraction reagent (Merck) and bicinchoninic acid protein assay kit (Thermo Fisher) separately. Protein extracts were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes (Roche). The following antibodies were used: rabbit anti-Akt antibody (1:250; Abcam), anti-p-Akt antibody (1:500; ImmunoWay), rabbit anti-p-eNOS, anti-eNOS antibody and rabbit anti-GAPDH antibody (1:1000; Cell Signaling Technology). Proteins were visualized with HRP-conjugated anti-rabbit IgG (1:2000; Cell Signaling Technology), followed by use of the ECL chemiluminescence system (Thermo).

Statistical analysis

Results are expressed as mean value ± SD. Comparison of continuous variables in the clinical study was performed by Student's t test. Comparisons between the in vitro experimental groups were performed using ANOVA followed by Fisher's protected least significant difference test. In all of the analyses, $P<0.01$ was considered statistically significant. All statistical analyses used SPSS statistical software (SPSS version 20.0).

Results

EPC characterization

Peripheral blood MNCs initially seeded on fibronectin-coated wells exhibited a round shape (**Figure 1A**). After changing medium on day 4, early EPCs appeared and elongated, as recognized by their characteristic spindle shape (**Figure 1B**). At day 21 after plating, late-outgrowth EPC with cobblestone-like morphology similar to mature endothelial cells were apparent (**Figure 1C**). Late-outgrowth EPC displayed critical markers of mature endothelial cells, including CD133 (**Figure 1D**), eNOS (**Figure 1E**), and von Willebrand factor (**Figure 1F**).

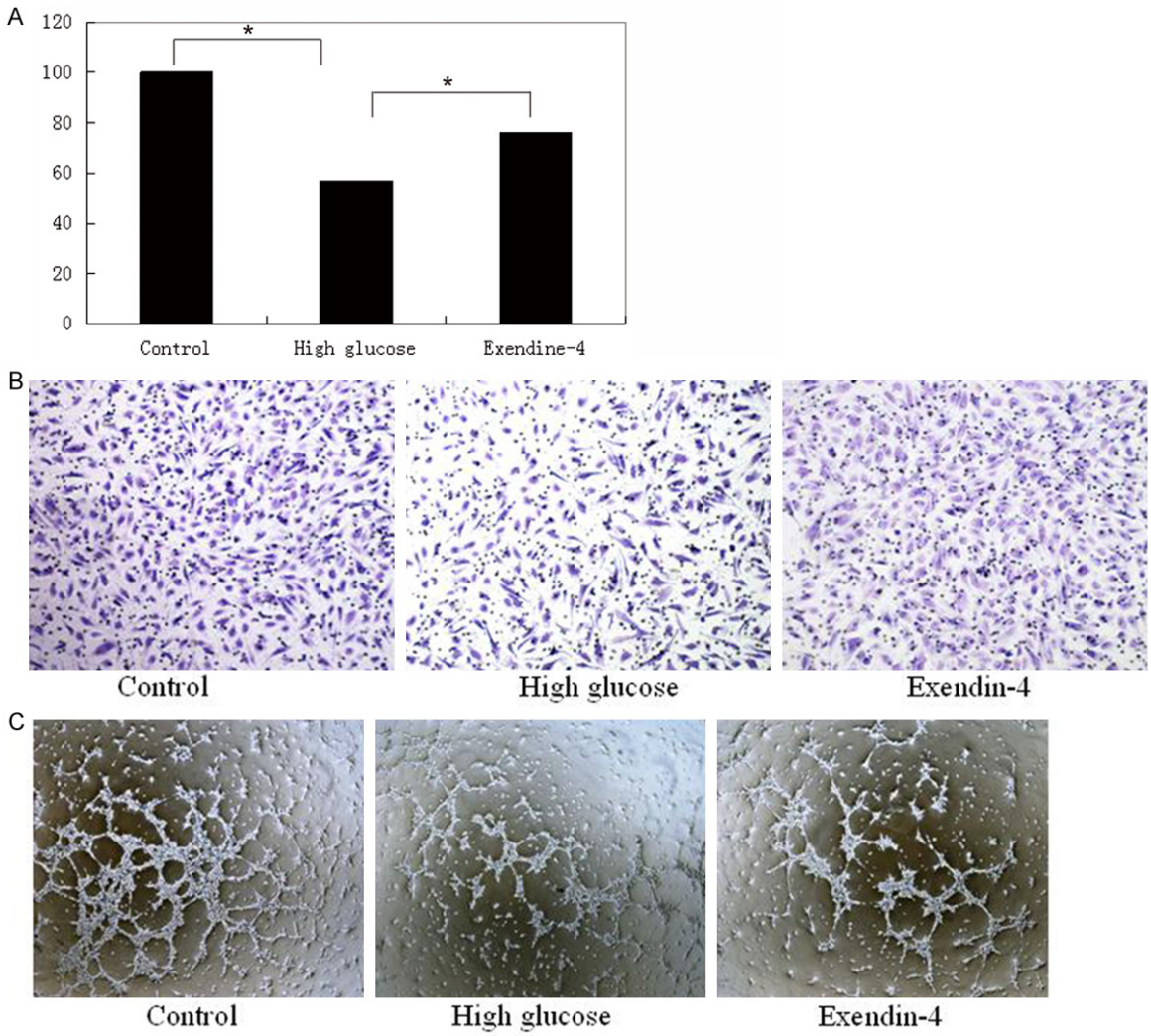

Figure 2. Effect of exendin-4 on EPCs proliferation, migration and tube formation in high glucose conditions. A: The inhibitory effects of high glucose on proliferation were reversed by exendin-4 treatment in high glucose conditions. B: EPC migration was significantly suppressed in high glucose conditions compared with control cells, but exendin-4 alleviated such effect. C: High glucose environment attenuated the tube formation of EPC compared with the control group, exendin-4 improved high glucose-suppressed tube formation. Columns represent mean ± SD. *significant difference vs. high glucose conditions ($P<0.05$).

Effect of exendin-4 on EPC proliferation, migration and tube formation in high glucose conditions

To clarify the effect of exendin-4 on EPC functions in high glucose conditions, MTT and modified Boyden chamber assays were used to evaluate cell proliferation and migration. As shown in **Figure 2**, EPC proliferation and migration were significantly suppressed in high glucose conditions compared with control cells. The inhibitory effects of high glucose on proliferation and migration were reversed by exendin-4 treatment by 27% and 32%, respectively. In addition, we further examined of the change of EPC tube formation under high glucose condition and the supplement of exendin-4. Compared with the control group, high glucose environment attenuated the tube formation of EPC by 37%. But, administration of exendin-4 induced high glucose-suppressed tube formation by 33%.

Exendin-4 promotes eNOS activation and NO production in EPC via PI3-K/Akt/ eNOS pathways

To identify the possible molecular mechanisms of exendin-4 to recover function of EPCs suppressed by high glucose, NO production and

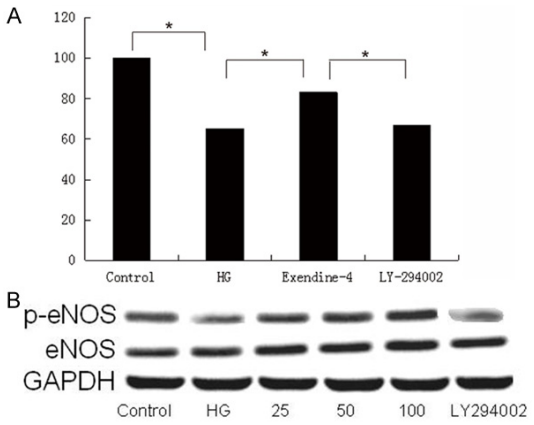

Figure 3. Exendin-4 promotes eNOS activation and NO production in EPC via PI3-K/Akt/eNOS pathways. A: High glucose reduced NO production of EPCs, yet treatment with exendin-4 reversed NO production. B: The phosphorylation of eNOS was decreased in stimulation of high glucose, but treatment with exendin-4 significantly up-regulated the phosphorylation of eNOS. Columns represent mean ± SD. *significant difference vs. high glucose conditions (P<0.05). Columns represent mean ± SD. *significant difference vs. high glucose conditions (P<0.05).

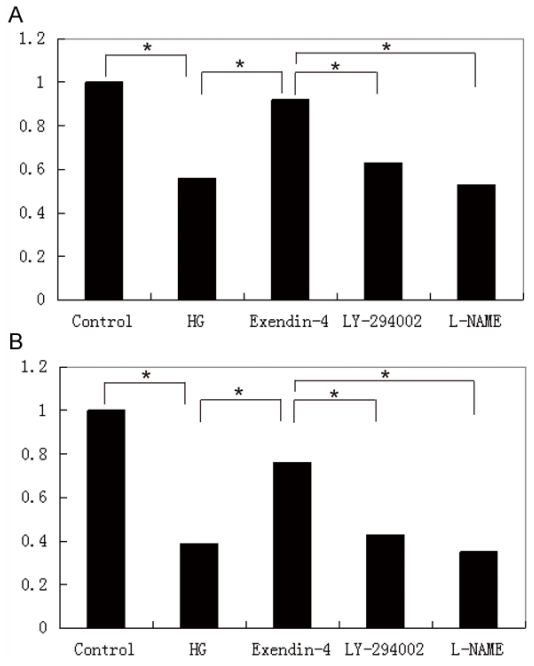

Figure 4. Exendin-4 improves high glucose-suppressed EPCs function through the PI3K/Akt/eNOS signaling pathways. A: Supplement with LY294002 and L-NAME inhibited exendin-4-improved migratory capacity. B: The stimulatory effect of exendin-4 on capillary-like tube formation was significantly suppressed by coincubation with LY294002 and L-NAME. Columns represent mean ± SD. *significant difference vs. high glucose conditions (P<0.05). Columns represent mean ± SD. *significant difference vs. high glucose conditions (P<0.05).

eNOS activation were investigated in cultured EPCs exposed to high glucose. As shown in **Figure 3A**, high glucose reduced NO production of EPC by 35%, yet treatment with exendin-4 reversed NO production by 27%. Moreover, we further examined function of exendin-4 in terms of activation of eNOS. As shown in **Figure 3B**, the phosphorylation of eNOS was decreased in response to high glucose stimulation, but treatment with exendin-4 significantly up-regulated the phosphorylation of eNOS. Similarly, the phosphorylation of Akt was decreased in stimulation of high glucose, and treatment with exendin-4 increased the expression level of phosphorylation of Akt. However, PI3K inhibitor LY-294002 could reduce the effects of exendin-4 on eNOS phosphorylation and NO production. These results suggested that exendin-4 induced eNOS activation and increased NO production in high glucose-Induced EPCs dysfunction via the PI3-K/Akt/eNOS signaling.

Exendin-4 improves high glucose-suppressed EPCs function through the PI3K/Akt/eNOS signaling pathways

To confirm whether PI3K/Akt/eNOS signaling pathways are involved in the effects of exendin-4 on EPCs functions suppressed by high glucose, EPCs were pretreated with LY294002, a specific inhibitor of PI3-K and L-NAME, a specific inhibitor of eNOS (100 μM) before exendin-4 treatment. As shown in **Figure 4**, supplement with LY294002 and L-NAME inhibited exendin-4-improved migratory capacity by 31% and 29%, respectively. The stimulatory effect of exendin-4 on capillary-like tube formation was significantly suppressed by coincubation with LY294002 and L-NAME. These results suggested that the activation of PI3-K/Akt signaling cascades and the consequent phosphorylation of the downstream target eNOS protein were involved in the protective effects of exendin-4 on EPCs dysfunction in high glucose conditions.

Discussion

In the present study, we demonstrated that exendin-4 could restore the impaired functions in high glucose-stimulated EPCs, and the possible mechanisms responsible for these effects involve the activation of the PI3-K/Akt/eNOS pathway.

Studies have confirmed that EPCs play an important role in endothelial repair and neovascularization at sites of injury or ischemia [6, 7, 12]. However, the functional capacities of EPCs were often impaired in patients with DM, which are believed to the reason for the endothelial dysfunction and impaired neovascularization after ischemia in DM patients [8, 13]. Consistent with prior studies, our data showed that both EPCs migration toward SDF-1 and EPCs adhesion were markedly impaired in high glucose-stimulated EPCs [14, 15]. Moreover, EPCs tube formation ability in high glucose condition exhibited a significantly decrease compared with normal glucose environment, which suggested high glucose could reduce EPCs angiogenic capacity.

Exendin-4 is a long-acting glucagon-like peptide-1 receptor (GLP-1R) agonist which exerts beneficial effects on glycemic control by increasing insulin secretion [9, 16-18]. Recently, experimental studies demonstrated that exendin-4 could prevent ischemic injury by inducing angiogenesis, but the cellular and molecular mechanisms underlying this effect remain not yet clearly defined [18, 19]. We hypothesized that exendin-4 might exert beneficial effects on neovascularization by restoring the impaired functional activities of EPCs. In this study, we found that exendin-4 could improve the migration, adhesion and tube formation in high glucose-stimulated EPCs. These results indicated that exendin-4 might exert beneficial effect on endothelial function and neovascularization after ischemia by restoring the impaired functional activities of EPC in DM patients.

It is well-known that the PI3-K/Akt/eNOS signaling pathway is critical to modulate the function of EPCs [20]. In patients with DM, the main cause of EPCs dysfunction is the loss of protection from NO due to reduced synthesis from eNOS [21, 22]. A lot of data suggested that the perturbations in the PI3-K/Akt/eNOS signaling pathways are responsible for the impaired function of EPCs in DM patients [20]. Therefore, we suggested that exendin-4 could restore the impaired functions of EPC in DM through the activation of the PI3-K/Akt/eNOS/NO signaling pathway. In this study, the results showed that Akt/eNOS activity and NO production of EPCs were down-regulated in high glucose conditions; exendin-4 could upregulate Akt, eNOS phosphorylation, as well as the bioavailability of NO in high glucose-stimulated EPCs. Moreover, this augmentation in exendin-4-induced the PI3-K/Akt/eNOS/NO signaling pathways in the DM patients paralleled with the enhanced in vitro migration, adhesion and tube formation activities and could be abrogated by Akt or eNOS inhibition. These findings indicate that exendin-4 could recover the impaired functions of EPC mediated by high glucose via PI3-K/Akt/eNOS pathways.

Conclusions

In conclusion, the present study demonstrated that exendin-4 could restore the dysfunctions of EPCs mediated by high glucose through the PI3-K/Akt/eNOS signaling pathways. Nevertheless, exendin-4 has potential as a new therapeutic agent for endothelial repair and neovascularization in atherosclerosis cardiovascular disease vascular diseases.

Disclosure of conflict of interest

None.

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