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Original Article

Glucagon-like peptide 2 promotes angiogenesis in human umbilical vein endothelial cells via akt signaling pathway

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Abstract: Glucagon-like peptide 2 (GLP-2) is a newly discovered gastrointestinal peptide produced from expression of the glucagon gene in the enteroendocrine L-cells of the intestinal mucosa. GLP-2 consists of 33-amino-acid with 33% sequence homology to glucagon. In the present study, we reported a novel role of GLP-2 in promoting angiogenesis of human umbilical vein endothelial cells (HUVECs) *in vitro*. First, we found that GLP-2 enhanced cell proliferation and migration activity of HUVECs in a dose-depend manner. Secondly, GLP-2 also promoted formation capillary tube structures of HUVECs in matrigel tube formation assay. Finally, we revealed that the effects of GLP-2 on HUVECs were mediated by activation of Akt signaling. Collectively, our results indicate that GLP-2 may serve as a novel therapeutic agent to facilitate the treatments of cardiovascular and cerebrovascular diseases by promoting angiogenesis.

Keywords: Angiogenesis, Incretin hormone, glucagon-like peptide 2, endothelial cell, akt signaling

Introduction

Incretin hormones are secreted from the gastrointestinal tract (GIT), and are capable of reducing blood glucose levels, by stimulating insulin release and inhibiting glucagon release [1]. Two main incretin hormones have been identified, including glucagon-like peptide (GLP) and glucose-dependent insulinotropic polypeptide (GIP) [2, 3]. Interestingly, recent studies have also revealed several other effects of incretins, especially GLP, on intestinal growth, angiogenesis and endothelial function [4-6].

GLP is comprised of glucagon-like peptide-1 (GLP-1) and GLP-2 [7, 8]. GLP-1 is a 30-amino acid peptide hormone, which is encoded by glucagon gene [9]. GLP-1 is secreted by the L-cells of the intestine in two main forms: GLP-1-(7-37) and GLP-1-(7-36)NH2 [9]. In addition to its glucose-lowing effect, GLP-1 was reported to promote angiogenesis of human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner in a 3D cell culture system [5]. Furthermore, studies on rodent models have found a cardio-protective role of GLP-1 by shrinking infract size, and protecting from ischemia-reperfusion injury [10]. Although the protective effect of these drugs is still under debate, these findings has revealed a promising treatment to prevent cardiovascular complications in patients with high blood glucose levels, by using GLP-1 and related drugs [11, 12].

GLP-2, another member of glucagon-related family, is also produced from glucagon gene [13]. Human GLP-2 is comprised of 33-amino-acids, and is secreted in a 1:1 ratio to GLP-1 by enteroendocrine L cells [14, 15]. GLP-2 was first identified in the trout, which shares approximately 40% conserved residues compared to human GLP-2 [16]. Because of poor evolutional conservation, GLP-2 was initially not paid much attention [15]. However, recent studies showed GLP-2 is an essential intestinotrophic mediator, and has been used to treat short bowel syndrome in clinical trials [17, 18]. GLP-2 has also been found to protect ischemic bowel injury, and promoted mucosal repair after surgical occlusion of superior mesenteric artery [19]. Since angiogenesis is an important process during wound healing. These findings indicate

GLP-2 may play a role in regulation of angiogenesis. In addition, Bulut *et al.* reported that GLP-2 enhanced release of vascular endothelial growth factor (VEGF) from subepithelial myofibroblasts [20]. Given these findings, we hypothesize that GLP-2 may promote angiogenesis of endothelial cells. To address this hypothesis, we have investigated the effects of GLP-2 treatment on proliferation, migration activity, and tube formation of human umbilical vein endothelial cells (HUVECs). Further, we have revealed that GLP-2 was capable of activating Akt signaling.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from LONZA (Walkersville, MD, USA) and were cultured in endothelial cell medium (Life Technologies, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS, Life Technologies), 100 μg/mL endothelial cell growth supplement, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C under a humidified 95%: 5% (v/v) mixture of air and CO_2. The HUVECs were used for research at 3-7 passages.

Cell proliferation assay

To determine relative cell viability, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed in 96-well plates. HUVECs were seeded at a density of 3×10^3 cells/well into a sterile 96-well plate and grown in 5% CO_2 at 37°C for 24 h. After treatment with GLP-2 (0, 0.5, 1.0 μM) for 1, 2, 3, 4 and 5 days, cell viability was measured by MTT (Sigma, St. Louis, MO, USA) assay. Briefly, 20 μL of 5 mg/mL MTT was added to each well and incubated with cells for 4 h in an incubator. 150 μl dimethyl sulfoxide (DMSO) was then added after removal of the medium. Finally, the optical density was measured at a wavelength of 570 nm. For cell counting, HUVECs (5×10^4 per well) were seeded in 24-well plates and cultured for 24 h. After treatment with GLP-2 at different time points, the cell were harvested and counted by trypan blue exclusion-based methods.

Wounded healing assay

HUVECs (5×10^4 per well) were seeded in 24-well plate in complete medium and culture to reach confluence. A 200 μL pipette tip was used to create a scratch on cell monolayer. After two times of wash with PBS, the cells were then treated with GLP-2 (0, 0.5, 1.0 μM) and cultured for 24 h. Migration was quantified by measuring the distance of cell migration from the two sides of the wound.

Transwell assay

HUVECs invasion were analyzed using 8 μm Matrigel-coated Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, cells were harvested and resuspended in serum-free medium and plated into the top well of a Transwell invasion chamber. The lower chamber was filled with medium supplemented with 10% FBS. After 24 h incubation, the non-invading cells were removed with a cotton swab, and the invading cells on the underside of the membrane were fixed with 4% paraformaldehyde solution, and stained with 0.1% crystal violet. Six random fields from each membrane were counted.

Tube formation assay

Tube formation assay was used for angiogenesis. Briefly, the 96-well plates were coated with 45 μl Matrigel (BD Biosciences) and solidified at 37°C for 30 min. HUVECs (3×10^4 per well) in 100 μl complete medium were added into each well. The cells were treated with GLP-2 (0, 0.5, 1.0 μM) and incubated for 10 h at 37°C in 5% CO_2. We photographed the tubular structure of endothelial cells with a microscope (Olympus; Tokyo, Japan) and calculated the branch length from six random fields.

Western blot

HUVECs cells were treated with GLP-2 (0, 0.5, 1.0 μM) for 30 min and then lysed with an equal volume of RIPA buffer (Beyotime, Shanghai, China), supplemented with 1% proteinase inhibitor cocktail (Santa Cruz Biotechnology, Dallas, TX, USA). Total protein content was determined by BCA method. Equivalent amounts of protein (30 μg) from each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with TBST buffer (20 mM Tris [pH 8.0], 150 mM NaCl and 0.1% Tween-20) containing 5% BSA for 1 h, the membrane was incubated with antibodies against p-Akt and Akt (Santa Cruz Biotechnology) overnight at 4°C, respec-

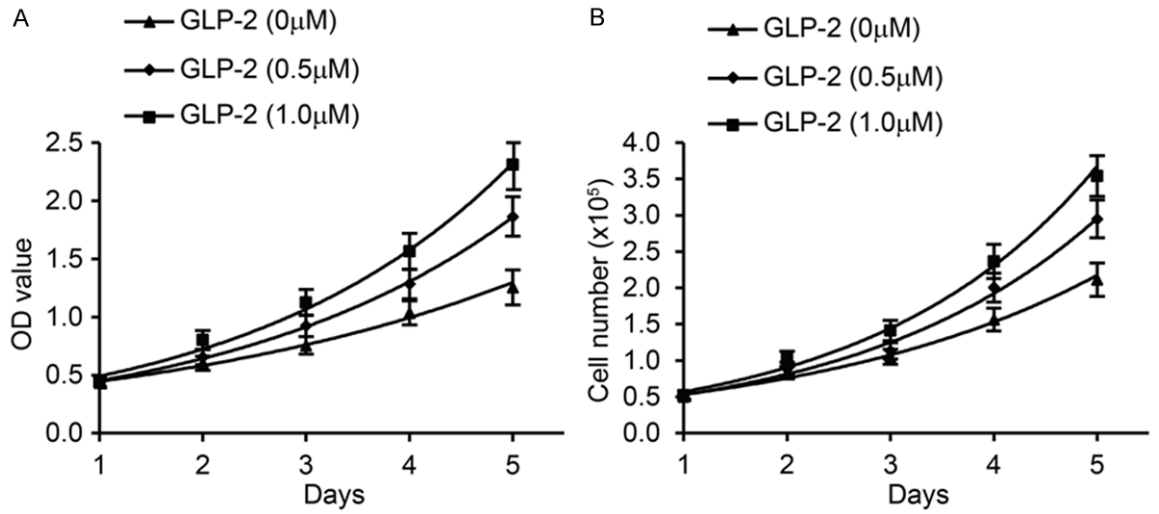

Figure 1. GLP-2 promotes HUVEC proliferation in a dose-dependent manner. HUVECs were incubated with different concentrations of GLP-2 (0, 0.5, 1.0 μM) for five days. MTT A and cell counting; B. Assays were performed.

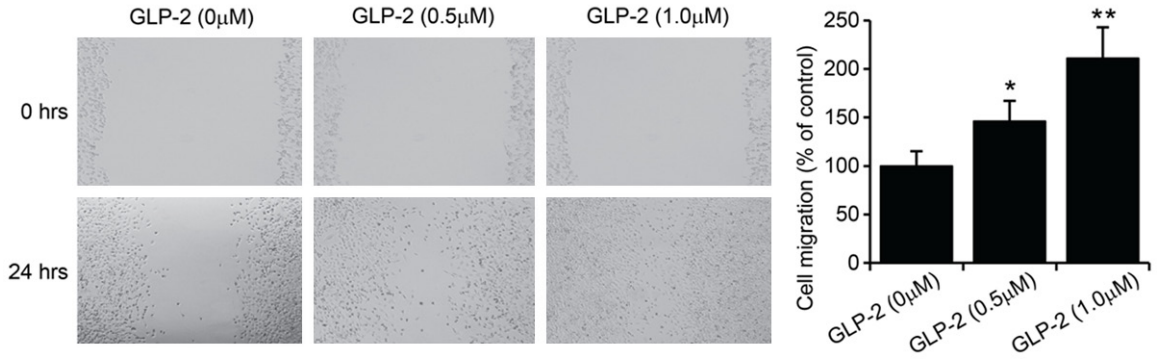

Figure 2. GLP-2 promotes HUVEC migration in a dose-dependent manner. Cell migration was measured by wounded healing assay. HUVECs were treated with different concentrations of GLP-2 for 24 h and the distance of migration was measured. **P*<0.05. ***P*<0.01 versus control group.

tively. Following incubation in horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, enhanced chemiluminescence (ECL) Western blotting substrate (Pierce; Rockford, IL, USA) was used for signal detection.

Statistics

All experiments were performed three times independently. Quantitative data were presented as mean ± standard deviation (SD). Data comparison was based on unpaired Student's t test or analysis of variance (ANOVA), and *P*-values less than 0.05 (**P*<0.05) were considered significant and *P*-values less than 0.01 (***P*<0.01) and 0.001 (****P*<0.001) were considered highly statistically significant.

Results

GLP-2 promotes HUVEC proliferation

To investigate the effect of GLP-2 on angiogenesis, we first employed the cell proliferation MTT assay and cell counting. As shown in **Figure 1A** and **1B**, treatment with GLP-2 (0.5 and 1.0 μM) significantly increased cell OD value and cell numbers. Thus, this finding implicated promotive role of GLP-2 in HUVEC proliferation.

GLP-2 promotes HUVEC migration

To evaluate whether GLP-2 affect the HUVEC ability of migration, we employed wounded healing cell migration assay. As shown in **Figure 2**,

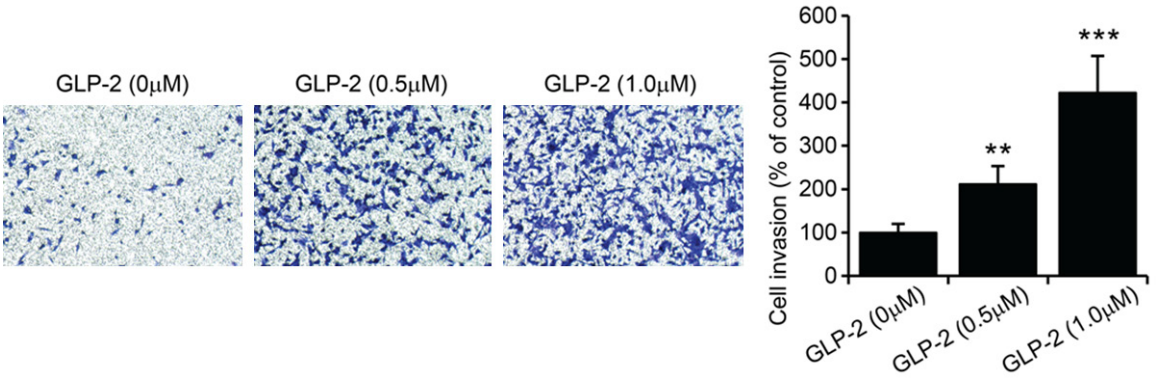

Figure 3. GLP-2 enhances HUVEC invasion in a dose-dependent manner. Cell invasion was measured by transwell assay. HUVECs were treated with different concentrations of GLP-2 for 24 h and the number of invaded cells was quantified. ***P*<0.01. ****P*<0.001 versus control group.

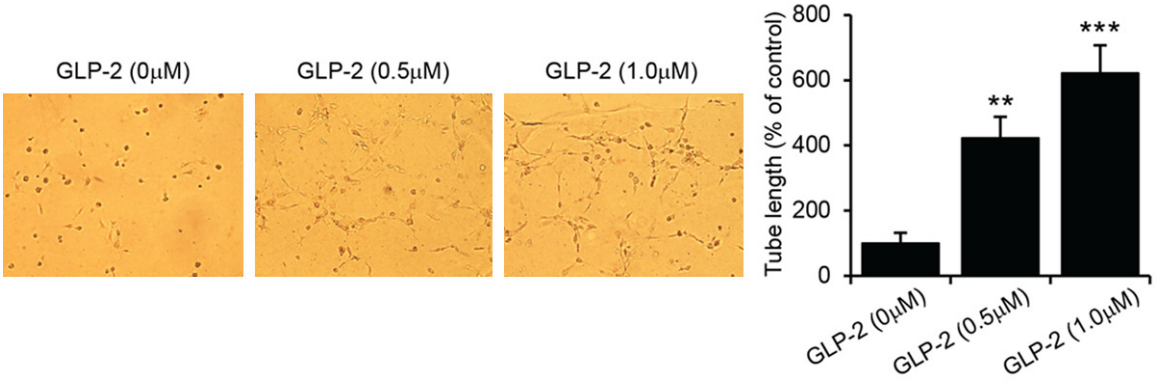

Figure 4. GLP-2 enhances HUVEC tube formation in a dose-dependent manner. HUVECs were seeded on 24-well Matrigel coated plates and treated with different concentrations of GLP-2 for 10 h, capillary tube structures were photographed and the branch length in three random fields per well was measured. Representative capillary tube structures were shown. ***P*<0.01. ****P*<0.001 versus control group.

different concentration of GLP-2 (0.5 and 1.0 μM) significantly promoted the distance of HUVEC migration by 50% and 110%. This result identified that GLP-2 promotes HUVECs migration.

GLP-2 enhances HUVEC invasion

Transwell assays were used to further analyze GLP-2 effect on migration ability of HUVECs. More invasive cells were observed in the 0.5 and 1.0 μM GLP-2-treated groups (**Figure 3**). Therefore, we noted that 0.5 and 1.0 μM of GLP-2 significantly enhanced the invasion of HUVECs by 110% and 320%, respectively, when compared with the control group.

GLP-2 enhances HUVEC tube formation

The tube formation assay is one of the most widely used in vitro assays to model the reorganization stage of angiogenesis. Therefore, we explored the effect of GLP-2 on HUVEC angiogenesis with a tube formation assay. HUVECs were seeded on the growth factor reduced Matrigel, and capillary tube structures were formed (**Figure 4**). We found that GLP-2 significantly increased tubular length of endothelial cells at 0.5 and 1.0 μM by 320% and 520%, respectively.

GLP-2 regulates Akt signaling pathway in HUVECs

Akt signaling pathway has been found mediating HUVEC tube formation [21]. To access whether GLP-2 could regulate Akt signaling pathway in HUVECs, we treated HUVECs with different concentration of GLP-2 (0, 0.5, 1.0 μM) and employed western blot for p-Akt. The data suggested that GLP-2 significantly in-

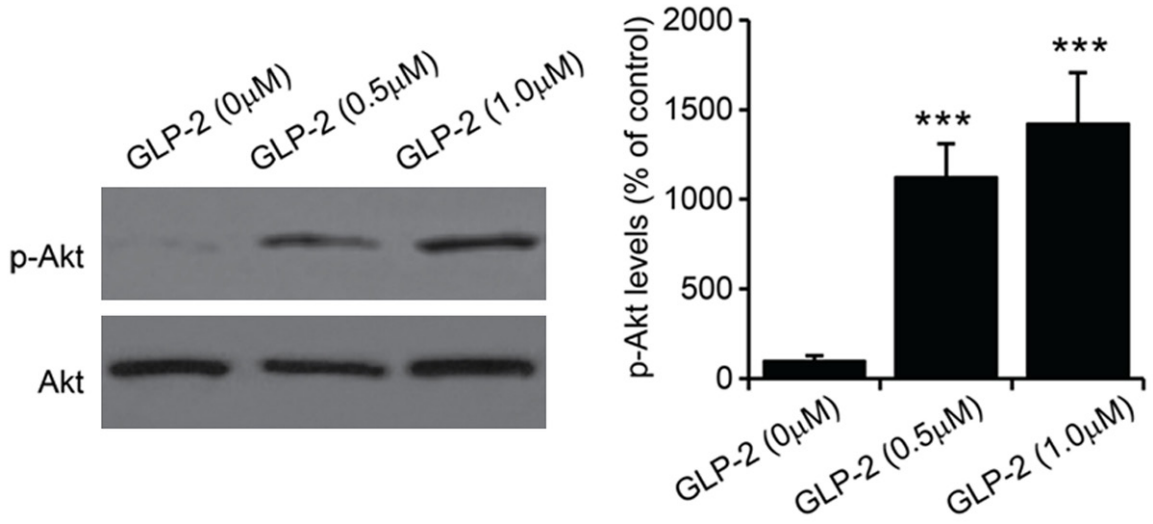

Figure 5. GLP-2 activates Akt signaling pathway in HUVECs. HUVECs were incubated with different concentrations of GLP-2 for 30 min. p-Akt levels were analyzed by western blot and the density of bands were quantified. ***P<0.001 versus control group.

creased p-Akt levels in a dose-dependent manner (**Figure 5**).

Akt signaling is required for the effects of GLP-2 on cell proliferation and migration in HUVECs

GLP2 has been also found to regulate Akt signaling pathway [22]. To verify whether GLP-2 regulate HUVECs through Akt signaling pathway. We first used an Akt signaling inhibitor, MK-2206 to treat HUVECs for 30 min. Then, GLP-2 (1.0 μM) was added into medium and HUVEC MTT, wounded healing, migration and tube formation assays were employed. As shown in **Figure 6**, the promotive role of GLP-2 to HUVECs was blocked after inhibition of Akt signaling, indicating that GLP-2 functions through Akt signaling pathway.

Discussion

Angiogenesis refers to the formation of new blood vessels, which is crucial for wound healing and recovery form cardiovascular and cerebrovascular diseases [23, 24]. GLP-1 was found to promote angiogenesis of HUVECs in a dose-dependent manner in vitro, and showed a protective role of ischemia-reperfusion injury by shrinking infract size in rodent models [5, 10]. In the present study, we revealed that GLP-2, another member of GLP family derived from the proglucagon gene, was also capable of promoting proliferation, migration, invasion of HUVECs. As revealed by tube formation assay, we found GLP-2 treatment dramatically enhanced the formation capillary tube structures *in vitro*. Furthermore, we examined the effects of GLP-2 treatment on activity of Akt signaling in HUVECs, and found an activation of Akt signaling induced by GLP-2 treatment in a dose-dependent manner. Finally, by using chemical inhibitor of Akt signaling, we demonstrated that Akt signaling was required for the effects of GLP-2 on cell proliferation and migration in HUVECs.

Proliferation and migration of endothelial cells are necessary for developing blood vessels [25, 26]. In this study, the proliferation rate of HUVECs was determined by cell counting and MTT assay, which showed a promotive role of GLP-2 in HUVEC proliferation. In sprouting angiogenesis, HUVECs are capable of expressing proteases and degrading the basement membrane [27]. Furthermore, HUVECs can be induced to migrate along chemical gradients that was established by proangiogenic growth factors [28, 29]. We found treatment with GLP-2 strongly induced the migration activity of HUVECs Endothelial cells can differentiate and form tubes to conduct the flow of blood. This process was called tube formation, also known as tubulogenesis. Here, we reported that GLP-2 enhanced tube formation of HUVECs in matrigel tube formation assay. These results indicate that GLP-2 directly enhances angiogenesis by promoting proliferation and migration activity of HUVECs. However, as previously suggested, GLP-2 stimulated VEGF release from sub-

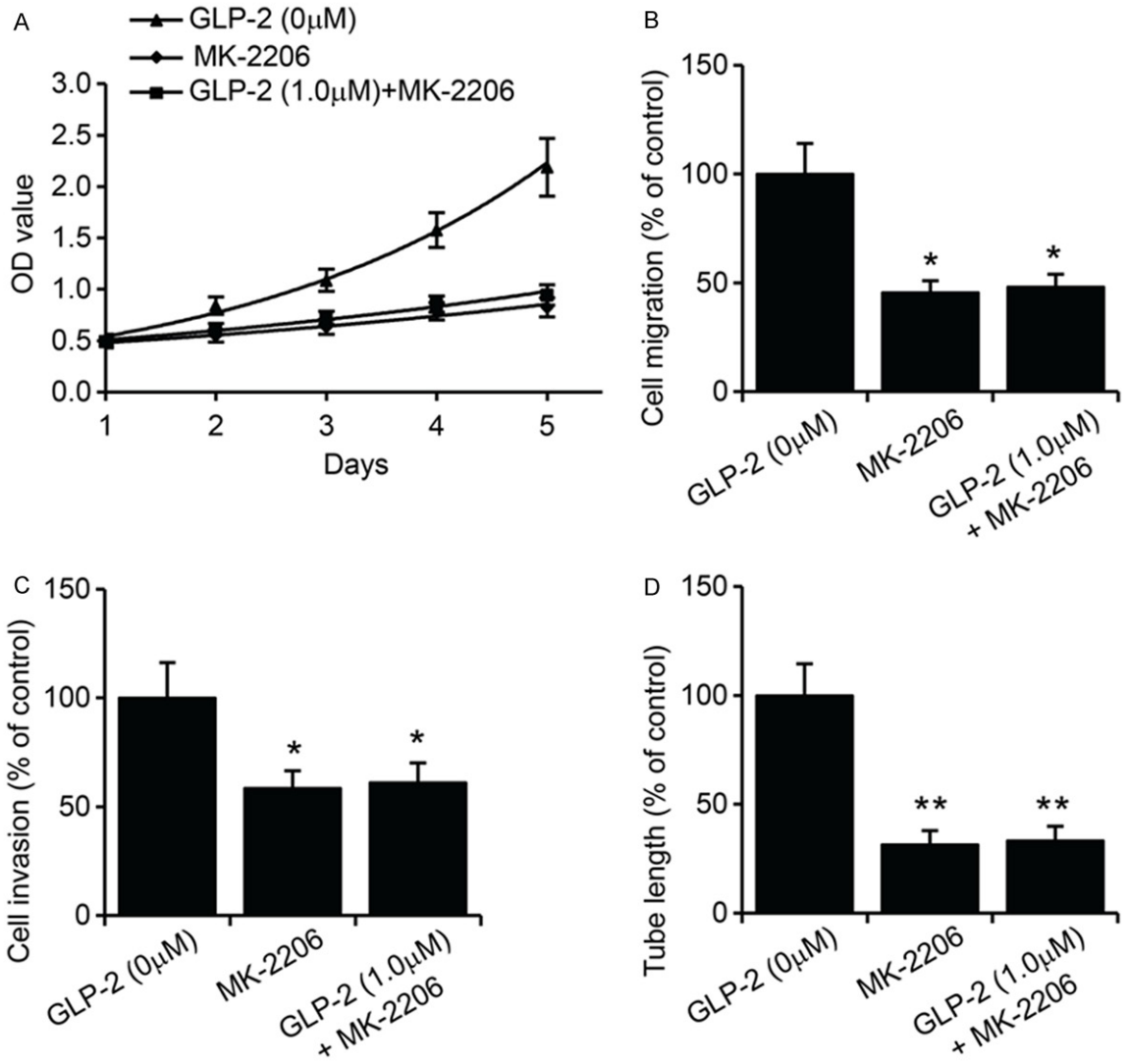

Figure 6. GLP-2 functions through Akt signaling pathway. After HUVECs were treated with MK-2206 with or without 1.0 μM GLP-2, MTT A. Wounded healing; B. Transwell; C. And tube formation assays; D. Were performed. **P*<0.05. ***P*<0.01 versus control group.

epithelial myofibroblasts during intestinal restitution [20]. As VEGF is a highly specific mitogen for endothelial cells, GLP-2 may promote angiogenesis through an indirect mechanism [30].

We further found GLP-2 activated Akt signaling in HUVECs. Conversely, blocking Akt signaling by specific chemical inhibitor completely abrogated the effects of GLP-2 on proliferation and migration of HUVECs. However, more efforts are still needed to investigate the mechanism by which GLP-2 regulates Akt signaling. Although the GLP-2 receptor (GLP-2R) was identified recently, the expression of GLP-2R was mainly restricted in the gastrointestinal tract and the central nervous system [31, 32]. In addition, GLP-2R was also found to be present enteroendocrine cells, and subepithelial myofibroblasts in humans [32]. Interestingly, GLP-2R was reported to be co-localized with serotonin in enteroendocrine cells, and with vasoactive intestinal peptide in enteric neurons [33]. It is possible that GLP-2 may promote angiogenesis by facilitate the release of serotonin and vasoactive intestinal peptide. However, although cardiac expression of GLP-2 in rats has been reported recently, its expression in human endothelial cells remains to be investigated [34]. Given the fact that GLP-2R is similar to

GLP-1R that is widely expressed, it was believed that GLP-2 may act through binding to GLP-1R [35].

In conclusion, we found that GLP-2 enhanced angiogenesis in HUVECs by activation of Akt signaling. Our findings indicate that GLP-2 may represent a promising agent to facilitate the treatments of cardiovascular and cerebrovascular diseases.

Disclosure of conflict of interest

None.

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