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ORIGINAL RESEARCH

Liraglutide Accelerates Ischemia-Induced Angiogenesis in a Murine Diabetic Model

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BACKGROUND: Severe hindlimb ischemia is a chronic disease with poor prognosis that can lead to amputation or even death. This study aimed to assess the therapeutic effect of liraglutide on hind-limb ischemia in type 2 diabetic mice and to elucidate the underlying mechanism.

METHODS AND RESULTS: Blood flow reperfusion and capillary densities after treatment with liraglutide or vehicle were evaluated in a mouse model of lower-limb ischemia in a normal background or a background of streptozotocin-induced diabetes. The proliferation, migration, and tube formation of human umbilical vein endothelial cells were analyzed in vitro upon treatment with liraglutide under normal-glucose and high-glucose conditions. Levels of phospho-Akt, phospho-endothelial nitric oxide synthase, and phospho-extracellular signal-related kinases 1 and 2 under different conditions in human umbilical vein endothelial cells and in ischemic muscle were determined by western blotting. Liraglutide significantly improved perfusion recovery and capillary density in both nondiabetic and diabetic mice. Liraglutide also promoted, in a concentration-dependent manner, the proliferation, migration, and tube formation of normal glucose– and high glucose–treated human umbilical vein endothelial cells, as well as the phosphorylation of Akt, endothelial nitric oxide synthase, and extracellular signal-related kinases 1 and 2 both in vitro and in vivo. The liraglutide antagonist exendin (9–39) reversed the promoting effects of liraglutide on human umbilical vein endothelial cell functions. Furthermore, exendin (9–39), LY294002, and PD98059 blocked the liraglutide-induced activation of Akt/endothelial nitric oxide synthase and extracellular signal-related kinases 1 and 2 signaling pathways.

CONCLUSIONS: These studies identified a novel role of liraglutide in modulating ischemia-induced angiogenesis, possibly through effects on endothelial cell function and activation of Akt/endothelial nitric oxide synthase and extracellular signal-related kinases 1 and 2 signaling, and suggested the glucagon-like peptide-1 receptor may be an important therapeutic target in diabetic hind-limb ischemia.

Key Words: angiogenesis ■ diabetes ■ glucagon-like peptide-1 ■ hindlimb ischemia ■ liraglutide

Type 2 diabetes is a chronic, multiple-system metabolic disorder caused by impaired insulin secretion or insulin sensitivity.[1] Since the body has been in a hyperglycemic state for a long time, chronic diabetes may also lead to several clinical complications caused by the metabolic syndrome.[2] Clinical trials have shown that diabetes-induced angiopathy promotes the development of various vascular complications, including coronary and peripheral artery disease.[3] The most common symptoms of peripheral artery disease include intermittent claudication, cramping, or aching, which may develop into acute lower-limb ischemia or even lead to amputation.[4] Hind-limb ischemia is caused mainly by chronic tissue ischemia, which is particularly impaired in patients with type 2 diabetes. Although the formation of collateral vessels is an important compensatory mechanism of chronic tissue ischemia, current medication cannot improve impaired collateral circulation. In contrast, treatment of occluded blood vessels with repeated surgical interventions leads to low patency in the long

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CLINICAL PERSPECTIVE

What Is New?

- Liraglutide promotes blood flow perfusion and capillary densities in a mouse model of lower-limb ischemia.
- The treatment with liraglutide increases proliferation, migration, and tube formation of human umbilical vein endothelial cells in vitro under normal-glucose and high-glucose conditions.
- The activation of the Akt/endothelial nitric oxide synthase and extracellular signal-related kinases 1 and 2 signaling pathways may be the underlying mechanism.

What Are the Clinical Implications?

- The current study identifies a novel role of liraglutide in modulating ischemia-induced angiogenesis, possibly through effects on endothelial cell function and activation of Akt/endothelial nitric oxide synthase and extracellular signal-related kinases 1 and 2 signaling and suggests that glucagon-like peptide-1 receptor may be an important therapeutic target in diabetic hindlimb ischemia.

Nonstandard Abbreviations and Acronyms

eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-related kinases 1 and 2
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
HG	high glucose
HUVECs	human umbilical vein endothelial cells
MAPK	mitogen-activated protein kinase
NG	normal glucose
p-eNOS	phospho–endothelial nitric oxide synthase
p-ERK1/2	phospho–extracellular signal-related kinases 1 and 2
PI3K	phosphoinositide 3-kinase

term and is associated with a high risk of renal failure.[5] Therefore, stimulating angiogenesis is a promising approach for relieving ischemic limb symptoms and preventing amputation.

Angiogenesis is an important physiological process that involves the generation of new blood vessels by endothelial cells of primitive vascular beds during wound healing and restoration of blood flow after injury. Functional responses of endothelial cells, such as proliferation, migration and tube formation, are critical during angiogenesis.[6] However, endothelial cells show poor proliferation and migration and contribute little to blood vessel formation in the diabetic state.[7] Hyperglycemia can further damage their physiological properties and induce endothelial dysfunction, ultimately impairing angiogenesis.[8–10]

Glucagon-like peptide-1 (GLP-1) is a hormone that is released from intestinal L cells[11] and stimulates insulin secretion in a glucose-dependent manner and reduces blood glucose levels by inhibiting postprandial glucagon secretion and gastric emptying via the gut-pancreas-liver axis.[12,13] Liraglutide is a synthetic acylated GLP-1 analogue with 97% sequence homology to endogenous GLP-1 that serves as an agonist of the GLP-1 receptor (GLP-1R), a Gs protein–coupled receptor found in multiple cell types, including vascular endothelial cells.[14] Interestingly, liraglutide has a half-life >12 hours because of its strong resistance to degradation by dipeptidyl peptidase-4. This enzyme plays a crucial role in glucose and insulin metabolism and is therefore considered a promising target in the treatment of type 2 diabetes.[2,14] Earlier studies have also shown that liraglutide can improve angiogenesis in impaired endothelial cells by activating the phosphoinositide 3-kinase/Akt–forkhead box protein O 1–GTP cyclohydrolase 1 axis or inhibition of PTEN-induced kinase 1/Parkin-dependent mitophagy[15,16] and restoring coronary no-reflow induced by endothelial cell dysfunction via mitogen-activated protein kinase (MAPK)/extracellular signal-related kinases 1 and 2 (ERK1/2) signaling.[17] However, the effects of liraglutide on lower-limb ischemia have not yet been assessed. In the present study, we evaluated, in nondiabetic and diabetic mice, the potential therapeutic effects of liraglutide on lower-limb ischemia and explored the underlying mechanisms in human umbilical vein endothelial cells (HUVECs), the most widespread cell type for in vitro studies of the vasculature and angiogenesis.

METHODS

The authors declare that all supporting data are available within the article.

Animals

C57BL/6J 6- to 8-week old mice were purchased from the Chongqing Medical University Animal Center (Chongqing, China). Mice were maintained in an animal facility equipped with 12:12-hour light–dark cycles and allowed free access to normal chow and water. All protocols for animal use were reviewed and approved by the Animal Care Committee of Southwest Medical University in accordance with Institutional Animal Care and Use Committee guidelines.

Reagents

Liraglutide was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Anti-CD31 antibody was from Abcam (Cambrige, UK). The antibodies against Akt, phospho-Ser473 Akt, ERK1/2, phospho-ERK1/2 (p-ERK1/2), endothelial nitric oxide synthase (eNOS), and phospho-Ser 1177 (p-eNOS) were from Cell Signaling Technology (Beverly, MA) and Abmart (Shanghai, China). The GLP-1R inhibitor exendin (9-39) was purchased from Sigma (St. Louis, MO). The phosphoinositide 3-kinase (PI3K)/Akt inhibitor LY294002 and MAPK/ERK inhibitor PD985002 were obtained from MedChemExpress (Monmouth Junction, NJ). Anti-GAPDH antibody, HRP-labeled goat anti-rabbit IgG (H + L), and HRP-labeled goat anti-mouse IgG (H + L) were obtained from Beyotime Biotechnology (Shanghai, China). RIPA lysis buffer was from Sigma (St. Louis, MO). BCA protein assay kit was from Beyotime Biotechnology (Shanghai, China). Matrigel basement membrane matrix and transwell inserts were from Corning (Bedford, MA). Streptozotocin, L-glucose, DMSO, and mannitol were from Sigma (St. Louis, MO).

Mouse Model of Type 2 Diabetes

For the establishment of the type 2 diabetic mouse model, all animals were acclimatized for 1 week, then injected intraperitoneally with streptozotocin for 5 consecutive days (40 mg/kg), and then given a high-fat diet for 4 weeks (D12451; Research Diet, New Brunswick, NJ; Figure 2A). Blood samples were then collected via tail prick, and glucose levels were measured using an automatic glucometer (Accu-Check; Roche, Mannheim, Germany). Mice with blood glucose ≥11 mmol/L were considered diabetic. Control mice were fed a standard laboratory normal chow diet.

Mouse Model of Hind-Limb Ischemia

To establish the hind-limb ischemic model, diabetic (n=22) and nondiabetic (n=22) mice were weighed, then anesthetized by intraperitoneal injection of 1% pentobarbital sodium (44 mg/kg). After disinfection of the surgical area with 75% alcohol, a 5-mm longitudinal incision was made in the groin of the left hind limb to expose the femoral artery and separate the surrounding femoral veins, nerves, and adipose tissue. A 5-0 silk suture was used to ligate the femoral vessels distally and proximally, and the ligated vessels were resected between the ligatures without damaging the nervus femoralis. The skin wound was closed with a discontinuous suture.

Nondiabetic and diabetic mice were then randomly divided in a 1:1 ratio into groups treated with liraglutide at 200 μg/kg per day (nondiabetic liraglutide, diabetic liraglutide) or with vehicle (nondiabetic control, diabetic control). Blood flow in the bilateral lower limb was assessed before surgery; immediately after surgery; and at 3, 7, 11, and 14 days after surgery using laser Doppler perfusion imaging (Moor Instruments, Devon, UK). Comparisons between groups were performed at each time point. Before each scan, all mice were anesthetized; the excess hair on limbs, lower abdomen, and back was removed with hair removal cream; and the mice were then placed on a heating blanket at 40 °C. After 14 days of treatment, all mice were euthanized, and bilateral gastrocnemius muscles were collected, fixed with paraformaldehyde, and stored for further analysis. The mean blood flow ratio of the ischemic and nonischemic sides was used to evaluate the reperfusion rate.

Metabolic Parameters

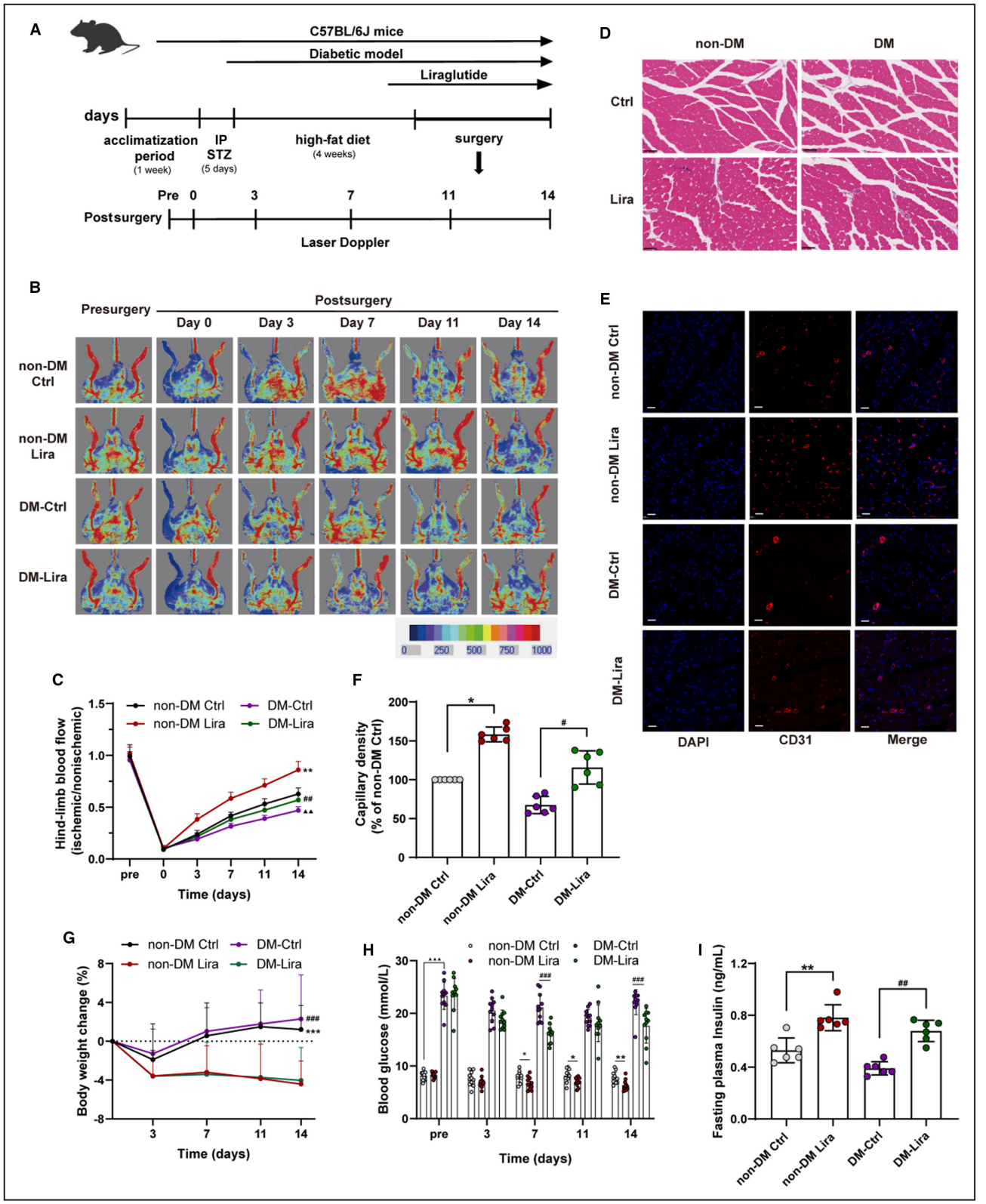
During the whole experiment, the body weight and blood glucose of mice were measured 5 times, before and 3, 7, 11, and 14 days after liraglutide administration. Glucose measurement was performed by a blood glucose meter (Accu-Check, Roche) using blood collected from the tail vein of mice. At the end of the experiment, blood collected from the inferior vena cava after overnight fasting was centrifuged at 2000*g* for 15 minutes at 4 °C. Plasma obtained was used for insulin level measurement by ELISA (Ultrasensitive Mouse Insulin Kit; Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

Histological Analysis

The collected gastrocnemius muscles were fixed with 4% paraformaldehyde at room temperature for at least 24 hours, embedded in paraffin, and then cut into sections 3 μm thick. The microvessel morphology was observed by hematoxylin–eosin staining using a Nikon Eclipse C1 microscope (Nikon, Tokyo, Japan). The microvessel density was assessed by immunofluorescence staining. Briefly, the paraffin sections were dewaxed, rehydrated, and microwaved in the presence of Tris-EDTA buffer (pH 8.0) to retrieve antigens. Slides were blocked with BSA for 30 minutes, then incubated overnight with rabbit polyclonal antibody against CD31 (1:200), followed by incubation with the Cy3-conjugated goat anti-rabbit secondary antibody and DAPI. Fluorescence micrographs were obtained with a DS-U3 system (Nikon), and the area of neovascularization was measured using ImageJ software (US National Institutes of Health, Bethesda, MD).

Cell Culture and Treatment

HUVECs were obtained from Scien Cell Research Laboratories (San Diego, CA) and cultured in endothelial



cell medium (Scien Cell Research Laboratories) supplemented with 5% FBS and 1% endothelial growth factor at 37 °C in an atmosphere containing 5% CO_2.

To examine the effects of liraglutide on HUVECs, the cells were treated with the GLP-1 analogue (0–200 nmol/L) under normal-glucose (NG) or high-glucose (HG) conditions. The HG model was established to mimic the hyperglycemic state of patients with diabetes by treating HUVECs with 35 mmol/L glucose for 72 hours before the administration of liraglutide.

Figure 1. Liraglutide improves blood perfusion recovery and angiogenesis after hind-limb ischemia in both nondiabetic and type 2 diabetic mice.
A, A schematic diagram of establishment of the type 2 diabetic mice model and the experimental design. **B**, Representative laser Doppler images of the hind limbs of nondiabetic mice and diabetic mice treated with liraglutide (nondiabetic liraglutide, diabetic liraglutide) or vehicle (nondiabetic control, diabetic control) before and after surgery. **C**, Quantification of blood flow recovery at the indicated time points, as determined from the ischemic/nonischemic limb perfusion ratio (n=11 per group); 2-way ANOVA was used to compare treatments at each time point. **D**, Hematoxylin-eosin staining of ischemic gastrocnemius muscle sections. Representative pictures are shown (magnification ×200; scale bar, 100 μm). **E**, CD31 immunofluorescence staining on sections of ischemic gastrocnemius muscles from mice in each group (magnification ×400; scale bar, 20 μm). **F**, Quantification of CD31-positive area (n=6 per group). Data are shown as mean±SD, expressed as a percentage of the vehicle value (first bar). **G**, Body weight change as percentage of original body weight at the indicated time points during a 14-day treatment with once daily liraglutide or saline control injection (n=11 per group); 2-way ANOVA was used to compare treatments at each time point. **H**, Random (nonfasting) blood glucose levels at the indicated time points in each group (n=11 per group). **I**, Fasting plasma insulin levels at the end point of experiment (day 14; n=6 per group, each dot represents one mouse). Data are shown as mean±SD. $^{*}P<0.05$; $^{**}P<0.01$; $^{▲▲}P<0.01$ vs nondiabetic Ctrl; $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ vs DM Ctrl. Ctrl indicates control; DM, diabetes mellitus; Lira, liraglutide; and STZ, streptozotocin.

Mannitol (35 mmol/L) was also used as a control to exclude the effect of osmotic pressure.

To clarify the role of GLP-1R, Akt, and ERK1/2 in the angiogenic function of liraglutide, the cells were first treated for 1 h with the GLP-1R inhibitor exendin 9–39 (500 nmol/L), the PI3K/Akt inhibitor LY294002 (20 μmol/L), or the MAPK/ERK inhibitor PD98059 (50 μmol/L), and then treated with liraglutide (100 nmol/L) under NG or HG conditions. DMSO-treated cells were used as vehicle control. All experiments were performed on cells between passages 5 and 10.

Cell Proliferation Assay

The effect of liraglutide on HUVEC proliferation was assessed using the Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Shanghai, China). Cells were first seeded in 96-well plates (1×10^4 cells/well) and cultured overnight under NG or HG conditions. Afterward, the cells were treated with liraglutide in the presence or absence of exendin (9–39) for 24 hours. Then, 100 μL of fresh medium and 10 μL of the Cell Counting Kit-8 solution were added to each well, followed by incubation at 37 °C for 2 hours. The optical density was measured at 450 nm using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA).

Cell Migration Assay

The migration ability of HUVECs was assessed by wound-healing and transwell assays. For the wound-healing assay, HUVECs were seeded in 12-well plates and cultured until 90% confluence. The wound areas were then scratched with the tip of a 200-μL sterile pipette and washed with PBS to remove exfoliated cells. Afterward, the cells were treated with liraglutide in the presence or absence of exendin (9–39), LY294002, or PD985002 and incubated at 37 °C in an atmosphere containing 5% CO_2. Images were acquired at 0 and 12 hours after scratching with an EVOS inverted microscope (AMG, Mill Creek, WA) and then analyzed using ImageJ software.

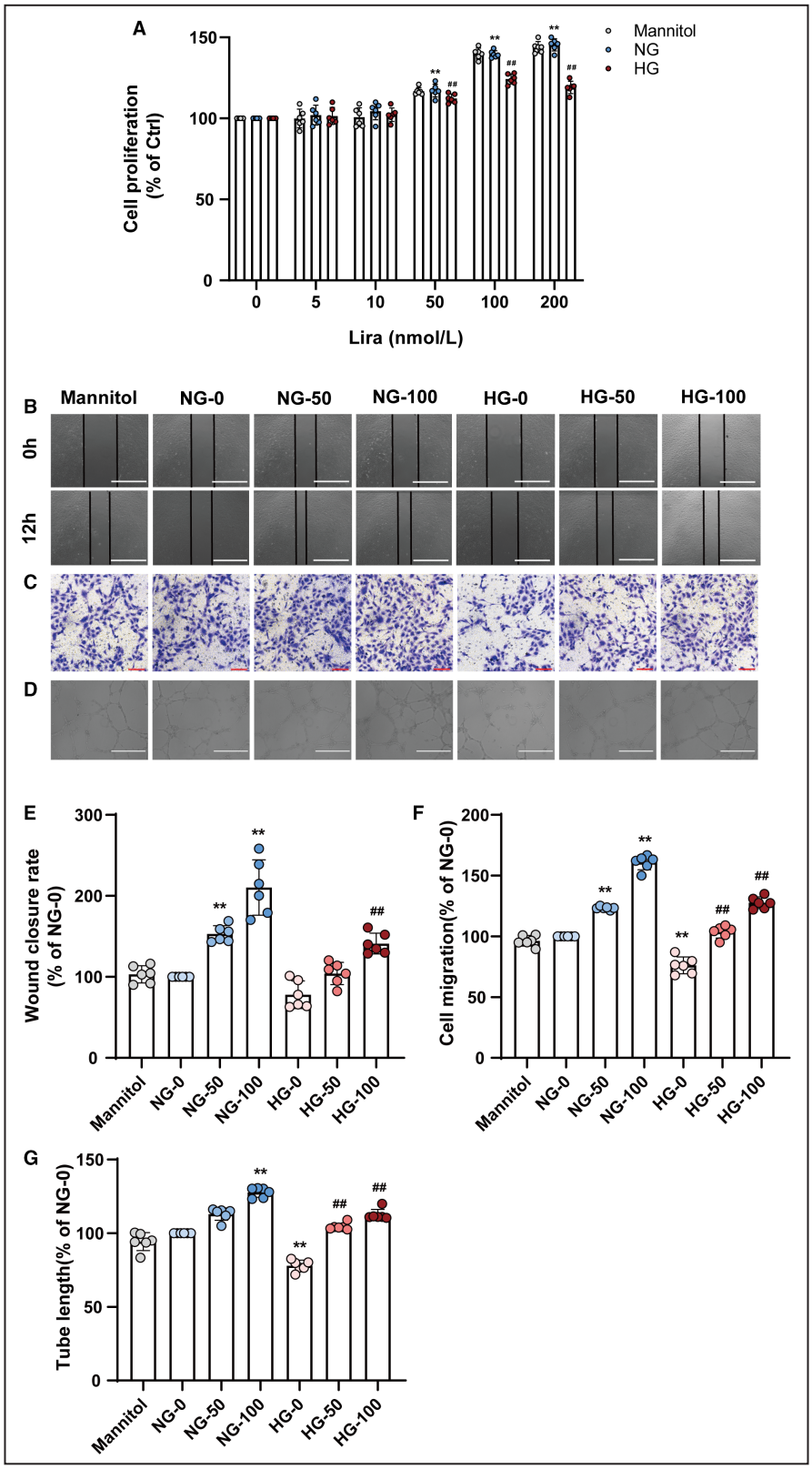
For the transwell assay, we used transwell inserts with an 8-μm pore size. HUVECs (3×10^4 cells/well) were loaded into the upper chamber, and 600 μL of medium containing 5% FBS was added to the lower chamber. After incubation for 24 hours, the nonmigratory cells in the upper chamber were removed, and the migratory cells were fixed with 4% paraformaldehyde for 30 minutes and stained with 0.1% crystal violet solution for 15 minutes. The number of stained migrated cells was measured with an EVOS inverted microscope.

Tube Formation Assay

For the tube formation assay, 48-well cell culture plates were coated with Matrigel (200 μL/well) and incubated at 37 °C until polymerization. After being treated with liraglutide in the presence or absence of exendin (9–39), LY294002, or PD985002. HUVECs were resuspended in endothelial cell medium and then seeded on Matrigel at a density of 2×10^4 cells/well. The formation of capillary-like tube structures was observed with an EVOS inverted microscope, and total tube length was quantified using ImageJ software.

Western Blotting

HUVECs grew to 80% confluence and were then treated with liraglutide with or without exendin (9–39), LY294002, or PD985002 for 6 hours. The culture plates were washed 3 times with ice-cold PBS buffer, and total protein was obtained with ice-cold protein lysis buffers (Cell Lysis for Western and IP, Beyotime Biotechnology) containing 1% (v/v) protease and phosphatase inhibitor cocktail. Levels of relevant proteins were assessed using western blotting. In brief, proteins were separated by 10% SDS-PAGE and then electrophoretically transferred onto Immobilon polyvinylidene fluoride membrane (Merck Millipore, Bayswater, Australia). After being blocked with 5% (m/v) skim milk at room temperature for 1 hour, membranes were incubated at 4 °C overnight with the following



primary antibodies: phospho-Akt Ser473 (1:1000), Akt (1:1000), phospho-ERK1/2 (1:1000), ERK1/2 (1:1000), phospho-eNOS (1:1000), eNOS (1:1000), or GAPDH (1:1000). Then the corresponding HRP-coupled secondary antibodies were incubated at room temperature for 1 hour: HRP-labeled goat anti-rabbit IgG (H+L; 1:1000) and HRP-labeled goat anti-mouse IgG (H+L; 1:1000). Signals were enhanced using the

Figure 2. Liraglutide promotes angiogenesis in vitro in a concentration-dependent manner.
A, Proliferation of human umbilical vein endothelial cells (HUVECs) after treatment with different liraglutide concentrations under normal-glucose (NG) or high-glucose (HG) conditions for 24 h. Cells were exposed to NG or HG conditions for 72 h before the administration of liraglutide. Control: NG-0, HG-0, mannitol-0. (n=6 per group). **B** through **D**, Representative images of wound closure (magnification ×40; scale bar 1000 μm) (**B**), migrating cells (magnification ×200; scale bar, 50 μm) (**C**), and tube formation in Matrigel (n=6 per group; magnification ×100; scale bar, 400 μm) (**D**). **E**, Relative wound-healing rate after treatment with different liraglutide concentrations under NG or HG conditions for 12 h. **F**, Cell migration of HUVECs after treatment with different liraglutide concentrations under NG or HG conditions for 12 h (n=6 per group). **G**, Cumulative tube length after treatment with different liraglutide concentrations under NG or HG conditions for 24 h (n=6 per group). Data are presented as the mean±SD of triplicate experiments. $^{**}P<0.01$ vs NG-0; $^{\#\#}P<0.01$ vs HG-0.

chemiluminescence detection kit (Omni-ECL™ Femto Light Chemiluminescence Kit, EpiZyme Scientific, Shanghai, China) and then visualized with the ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). In some experiments, gastrocnemius muscles were homogenized in RIPA buffer. Total protein concentration of the homogenates was measured with the BCA reagent and used to perform the western blotting analysis. The protein expression level was quantified using Image J.

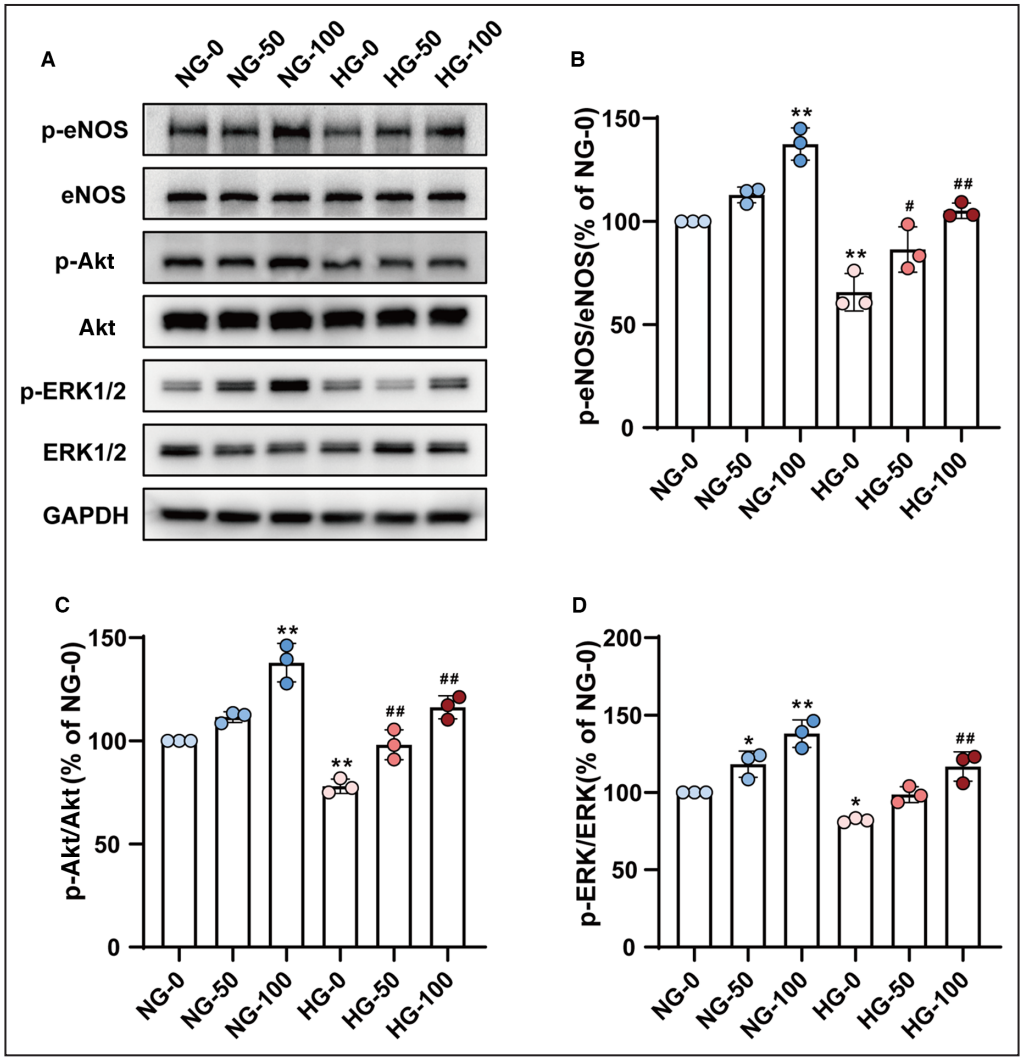
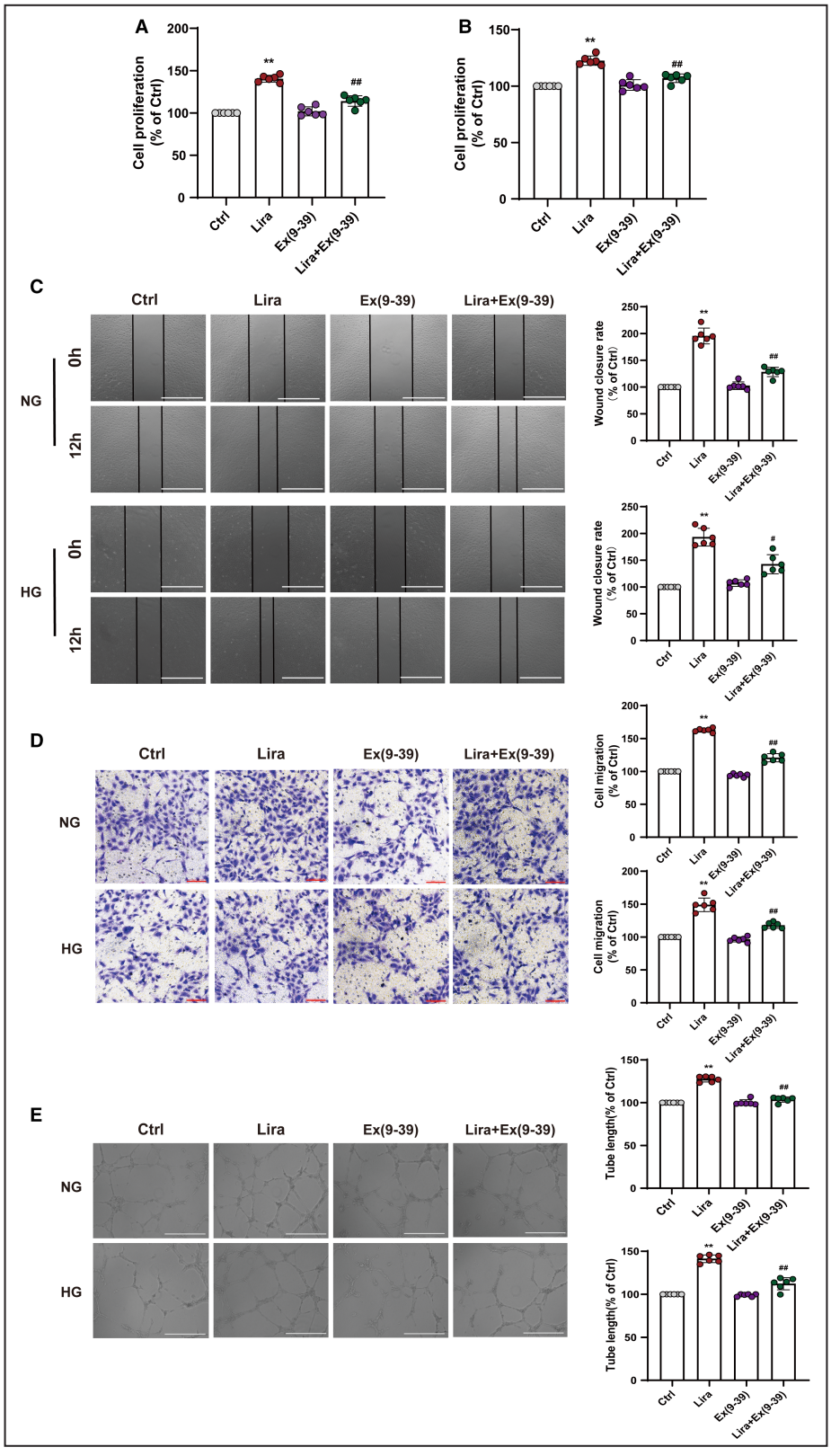


Figure 3. Liraglutide upregulates phospho-Akt (p-AKT), phospho-endothelial nitric oxide synthase (p-eNOS), and phospho-extracellular signal-related kinases 1 and 2 (p-ERK1/2) in human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner.
A, Representative western blots against p-eNOS, p-Akt, and p-ERK1/2. **B** through **D**, Levels of p-AKT, p-ERK1/2, and p-eNOS in human umbilical vein endothelial cells after treatment with different liraglutide concentrations for 2 h under normal-glucose (NG) or high-glucose (HG) conditions. Cells were exposed to NG or HG conditions for 72 h before the administration of liraglutide. Data are presented as the mean±SD of triplicate experiments. $^{*}P<0.05$; $^{**}P<0.01$ vs NG-0; $^{\#\#}P<0.01$ vs HG-0.



Statistical Analysis

Data are expressed as mean±SD. The Shapiro–Wilk normality test was performed to evaluate data distribution. Normally distributed data with 1 variable were analyzed by the unpaired Student *t* test to evaluate the statistical significance between the 2 groups; 1-way ANOVA followed by the Tukey post hoc test was used for ≥3 groups. Two-way ANOVA was used to

Figure 4. Liraglutide exerts its angiogenic properties in vitro via the glucagon-like peptide-1 receptor (GLP-1R).
A and **B**, Proliferation of human umbilical vein endothelial cells (HUVECs) after treatment with liraglutide in the presence or absence of the specific GLP-1R inhibitor exendin (9–39) for 1 h under normal-glucose (NG) (**A**) and high-glucose (HG) (**B**) conditions. Cells were exposed to NG or HG conditions for 72 h before the administration of exendin (9–39) (n=6 per group). (**C** through **E**) Representative images of (**C**) wound closure (magnification ×40; scale bar, 1000 μm) (n=6 per group) (**C**), migrating cells (magnification ×200; scale bar, 50 μm) (n=6 per group) (**D**), and tube formation in Matrigel (magnification ×100, scale bar 400 μm; n=6 per group) (**E**), and the corresponding quantification results in HUVECs after treatment with liraglutide in the presence or absence of exendin (9–39) under NG or HG conditions. Data are presented as the mean±SD of triplicate experiments. **P*<0.05; ***P*<0.01 vs control; #*P*<0.05; ##*P*<0.01 vs liraglutide. Exendin (9–39), 500 nmol/L; liraglutide, 100 nmol/L. Ctrl indicates control; DM, diabetes; Ex, exendin; and Lira, liraglutide.

assess the hind-limb blood flow in Figure 1C and 1G. Prism software version 8.0 (GraphPad Software Inc, San Diego, CA) was used. *P*<0.05 were considered to be significantly different.

RESULTS

Liraglutide Accelerates Reperfusion After Hind-Limb Ischemia in Both Nondiabetic and Type 2 Diabetic Mice

To examine the angiogenic activity of liraglutide in vivo, we established a type 2 diabetes model by a combination of intraperitoneal injection of low-dose streptozotocin and the feeding of a high-fat diet in mice (Figure 1A). Then the mice were subjected to unilateral hind-limb surgery, thereby mimicking peripheral vascular disease in patients with diabetes. Both wild-type and type 2 diabetic mice with hind-limb ischemia were treated with liraglutide or vehicle for 14 days. Laser Doppler perfusion images showed that the surgery reduced blood flow in the ischemic hindlimbs in each group. In addition, liraglutide remarkably accelerated perfusion recovery, such that a significant difference was observed between the liraglutide-treated group compared with the vehicle control group at 14 days after surgery in both nondiabetic (0.628±0.054 versus 0.861±0.076) and type 2 diabetic mice (0.469±0.032 versus 0.569±0.054; Figure 1B and 1C). Hematoxylin–eosin staining showed that the structural integrity of ischemic muscles was better in the liraglutide group than in control groups (Figure 1D). Consistent with these results, immunofluorescence for the angiogenic marker CD31[18] in ischemic gastrocnemius muscles 14 days after the induction of ischemia was more intense in liraglutide-treated mice compared with the controls (Figure 1E and 1F), indicating increased formation of capillary. These results indicate the ability of liraglutide to promote angiogenesis and enhance perfusion recovery after ischemic injury in vivo.

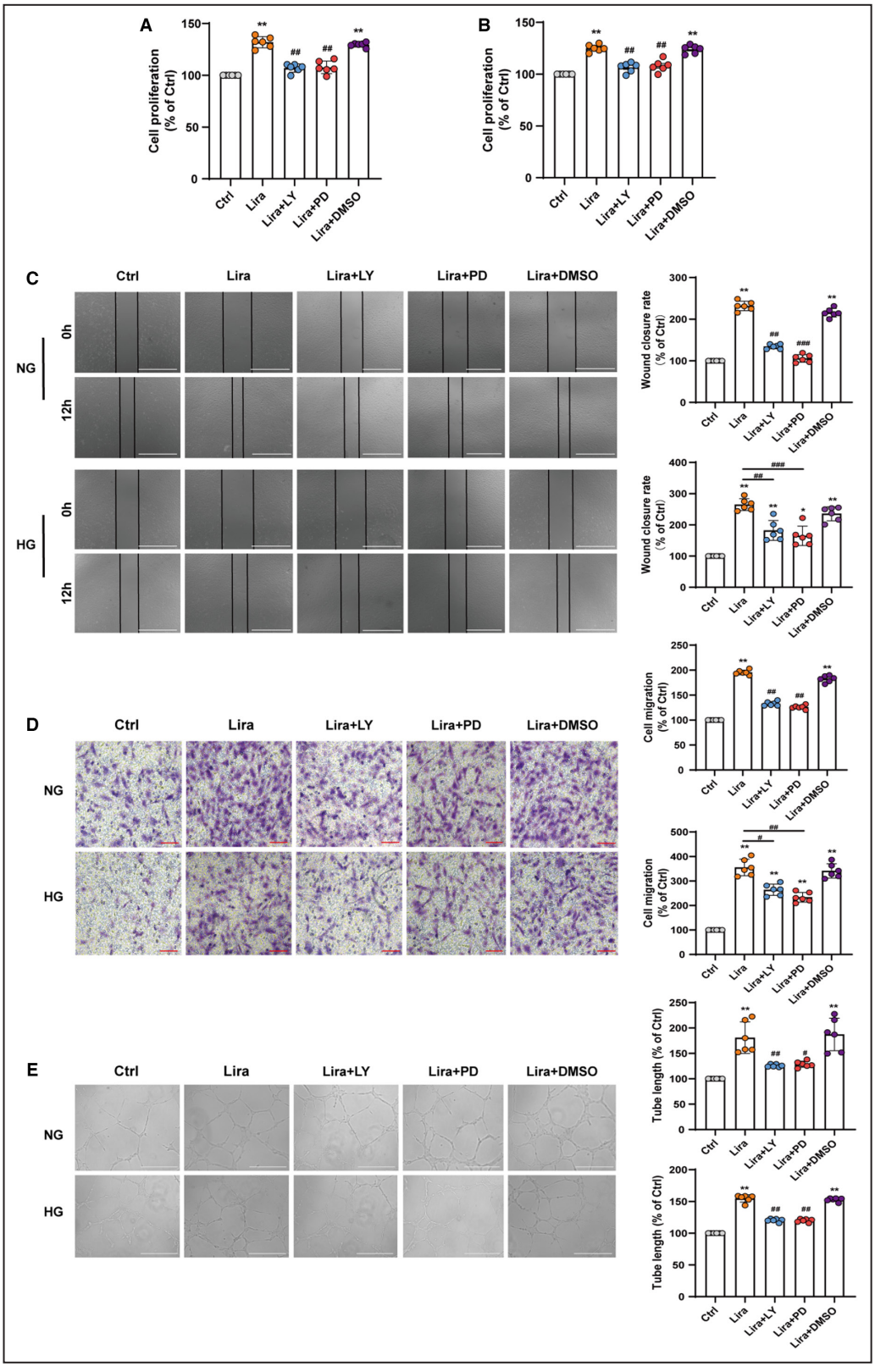
We next evaluated the changes in body weight and blood glucose of the experimental mice. The results showed that the body weight of the liraglutide-treated groups was significantly reduced compared with that of the vehicle groups (Figure 1G). The diabetic mice had blood glucose levels as high as nearly 25 mmol/L before liraglutide treatment. Daily liraglutide injection prevented the rise in blood glucose levels to a certain extent. The difference in levels between vehicle and liraglutide groups was statistically significant at day 14 (6.345±0.953 versus 7.782±1.058 in the nondiabetic groups; 17.645±3.129 versus 22.118±2.247 in the diabetic groups; Figure 1H). In accord with the improved hyperglycemia, liraglutide-treated mice showed higher fasting plasma insulin levels than vehicle-treated mice at the end of the experiment (0.679±0.075 versus 0.391±0.046; Figure 1I).

Liraglutide Promotes HUVEC Proliferation

The severity of limb ischemia has been attributed to the impaired collateralization of vascular ischemic beds, while hyperglycemia has been found to impair the viability and angiogenic capability of vascular endothelial cells.[19] To verify the protective role of liraglutide on angiogenesis, we used the Cell Counting Kit-8 assay to examine the proliferation of HUVECs treated with different liraglutide concentrations under NG or HG conditions. The results indicated that liraglutide significantly promoted the proliferation of HUVECs in a dose-dependent manner (Figure 2A), with a concentration of 100 nmol/L showing the strongest effect. Therefore, subsequent experiments were performed using 0, 50, and 100 nmol/L of liraglutide.

Liraglutide Enhances the Migratory Potential of HUVECs

The role of liraglutide on the migration capacity of HUVECs was measured in vitro using the wound healing and transwell migration assays. The wound healing assay showed that treatment with 50 or 100 nmol/L of liraglutide under NG conditions resulted in a significantly higher wound closure rate than the control group, whereas only 100 nmol/L of liraglutide significantly increased the wound-healing rate under HG conditions (Figure 2B and 2E). The transwell assay showed that HG pretreatment significantly reduced the number of migrating cells. Interestingly, liraglutide reversed this effect and significantly promoted the migration of NG- and HG-treated HUVECs compared with control (Figure 2C and 2F). In both assays, mannitol alone had no effect on the wound-healing or cell migration rates.



Liraglutide Promotes the Tube Formation Ability of HUVECs

The Matrigel tube formation assay was used to examine the cell reorganization ability of HUVECs in vitro. Our results showed that HG treatment significantly reduced the cumulative tubular length of HUVECs. However, liraglutide reversed these effects and promoted the tube formation of NG- and HG-treated

Figure 5. Inhibition of Akt and extracellular signal-related kinases 1 and 2 (ERK1/2) signaling attenuates the angiogenic properties of liraglutide in vitro.
A and **B**, Proliferation of human umbilical vein endothelial cells (HUVECs) after treatment with liraglutide in the presence or absence of the phosphoinositide 3-kinase/Akt inhibitor LY294002 or mitogen-activated protein kinase/ERK inhibitor PD985002 for 1 h under normal-glucose (NG) (**A**) and high-glucose (HG) (**B**) conditions. Cells were exposed to NG or HG conditions for 72 h prior to the administration of LY294002 or PD985002 (n=6 per group). **C** through **E**, Representative images of wound closure (magnification ×40; scale bar, 1000 μm; n=6 per group) (**C**), migrating cells (magnification ×200; scale bar, 50 μm; n=6 per group) (**D**), and tube formation in Matrigel (magnification ×100, scale bar 400 μm; n=6 per group) (**E**), and the corresponding quantification results in HUVECs after treatment with liraglutide in the presence or absence of LY294002 or PD985002 under NG or HG conditions. Data are presented as the mean±SD of triplicate experiments. **P*<0.05; ***P*<0.01 vs control; #*P*<0.05; ##*P*<0.01; ### *P*<0.001 vs liraglutide. LY294002, 20 μmol/L; PD98059, 50 μmol/L; liraglutide, 100 nmol/L. Ctrl indicates control; and Lira, liraglutide.

HUVECs in a dose-dependent manner (Figure 2D and 2G).

Liraglutide Promotes Angiogenesis via Akt/eNOS and ERK1/2 Signaling Pathways

Previous studies have shown that activated AMP-activated protein kinase/PI3K-Akt/eNOS and MAPK/ERK1/2 pathways play a crucial role in angiogenesis.[20] In view of this, we explored whether the effects of liraglutide on the proliferation, migration, and tube formation ability of HUVECs are related to the activation of the signaling molecules eNOS, Akt, and ERK1/2. Exposure of cells to HG before liraglutide administration significantly downregulated p-Akt, p-ERK1/2, and p-eNOS. Liraglutide significantly increased the levels of p-AKT, p-ERK1/2, and p-eNOS under both NG and HG conditions, especially at 100 nmol/L (Figure 3A through 3D), indicating that this GLP-1 analogue promotes angiogenesis by activating the Akt/eNOS and ERK1/2 axes.

GLP-1R Mediates the Angiogenic Effect of Liraglutide on HUVECs

To determine whether GLP-1R is required for the effect of liraglutide on the proliferation, migration, and tube formation capacity of HUVECs. HUVECs were treated with 100 nmol/L of liraglutide and the GLP-1R inhibitor exendin (9–39). Exendin (9–39) alone had no significant effect on the functions of HUVECs, but it partially reversed the angiogenic effects of liraglutide under both NG and HG conditions (Figure 4A through 4E). These results suggest that liraglutide promotes angiogenesis via a pathway involving GLP-1R.

Inhibition of PI3K/Akt and ERK1/2 Activity Alleviated the Angiogenic Effect of Liraglutide

To verify whether liraglutide could also promote angiogenesis when the PI3K/Akt and ERK1/2 signaling pathways were suppressed. HUVECs were treated with the PI3K/Akt inhibitor LY294002 (20 μmol/L), or the MAPK/ERK inhibitor PD98059 (50 μmol/L) for 1 hour, and then treated with liraglutide (100 nmol/L) under NG or HG conditions. The results showed that the angiogenic effect of liraglutide on HUVECs was partially attenuated by LY294002 and PD98059 (Figure 5A through 5E). These results suggest that liraglutide promotes angiogenesis via a pathway involving PI3K/Akt and ERK1/2.

Liraglutide Promotes Angiogenesis by Activating Akt/eNOS and ERK1/2 Signaling

To further explore the role of GLP-1R and the PI3K/Akt and ERK1/2 signaling pathways in the angiogenic properties of liraglutide, we treated HUVECs with Exendin (9–39), LY294002, and PD98059 to suppress the respective activation of p-eNOS, p-AKT and p-ERK1/2. Our experiments showed that exendin (9–39) can partially reverse the effect of liraglutide on the upregulation of p-eNOS, p-Akt, and p-ERK1/2 under NG and HG conditions. In addition, LY294002 significantly downregulated p-Akt and p-eNOS, whereas PD98059 almost completely blocked the effect of liraglutide on p-ERK1/2, without significantly affecting p-eNOS levels (Figure 6A through 6H). These results confirm that liraglutide improves angiogenesis by activating GLP-1R as well as the Akt/eNOS and MAPK/ERK1/2 signaling pathways.

Liraglutide Activates Akt/eNOS and ERK1/2 Signaling in Ischemic Muscle

Our experiments above demonstrated that liraglutide exerts the angiogenic effects on endothelial cells via GLP-1R as well as the Akt/eNOS and ERK1/2 signaling pathways in vitro. To further explore the in vivo mechanisms in liraglutide-mediated improvement of ischemia-induced revascularization, western blot analysis was employed to assess the expression and phosphorylation of Akt/eNOS and ERK1/2 in ischemic gastrocnemius muscle at day 14 after surgery. Although there was no significant difference among the 4 experimental groups in relation to the total protein expression in ischemic muscles, there

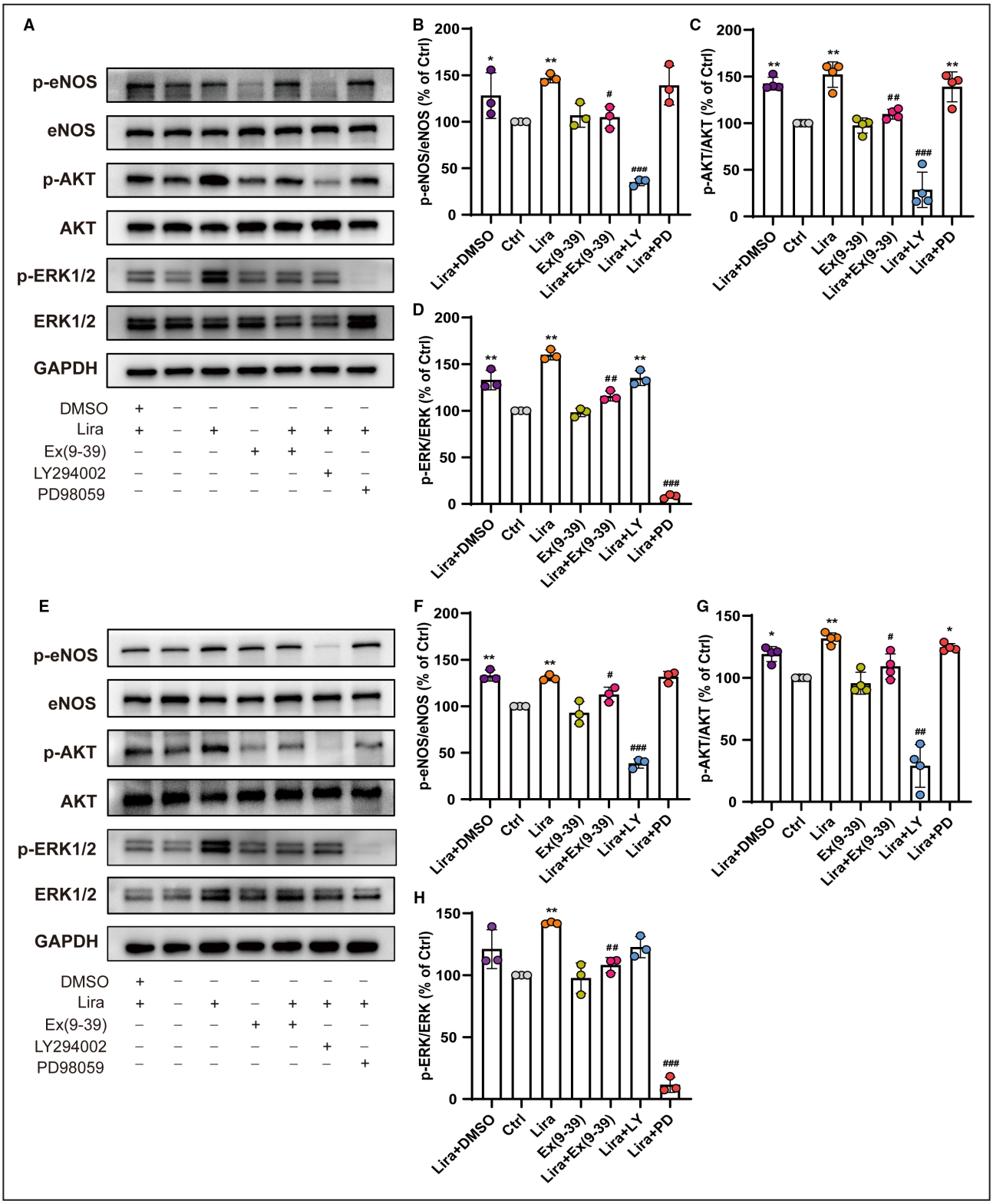


Figure 6. Liraglutide promotes angiogenesis by activating Akt/endothelial nitric oxide synthase (eNOS) and extracellular signal-related kinases 1 and 2 (ERK1/2) signaling in human umbilical vein endothelial cells (HUVECs).
A and **E**, Representative western blots against phospho-endothelial nitric oxide synthase (p-eNOS), phospho-Akt, and phospho-extracellular signal-related kinases 1 and 2 (p-ERK1/2) under (**A**) normal-glucose (NG) and (**E**) high-glucose (HG) conditions. **B** through **D** and **F** through **H**, Levels of p-eNOS, p-Akt, and p-ERK1/2 in HUVECs after treatment with various formulations for 1 h under NG (**B** through **D**) and HG (**F** through H) conditions. Cells were exposed to NG or HG conditions for 72 h before the administration of inhibitors. DMSO was used as vehicle control. Data are presented as the mean±SD of triplicate experiments. $^{**}P<0.01$ vs control; $^{\#\#}P<0.01$ vs liraglutide. Exendin (9–39), 500 nmol/L; LY294002, 20 μmol/L; PD98059, 50 μmol/L; liraglutide, 100 nmol/L. Ctrl indicates control; Ex, exendin; and Lira, liraglutide.

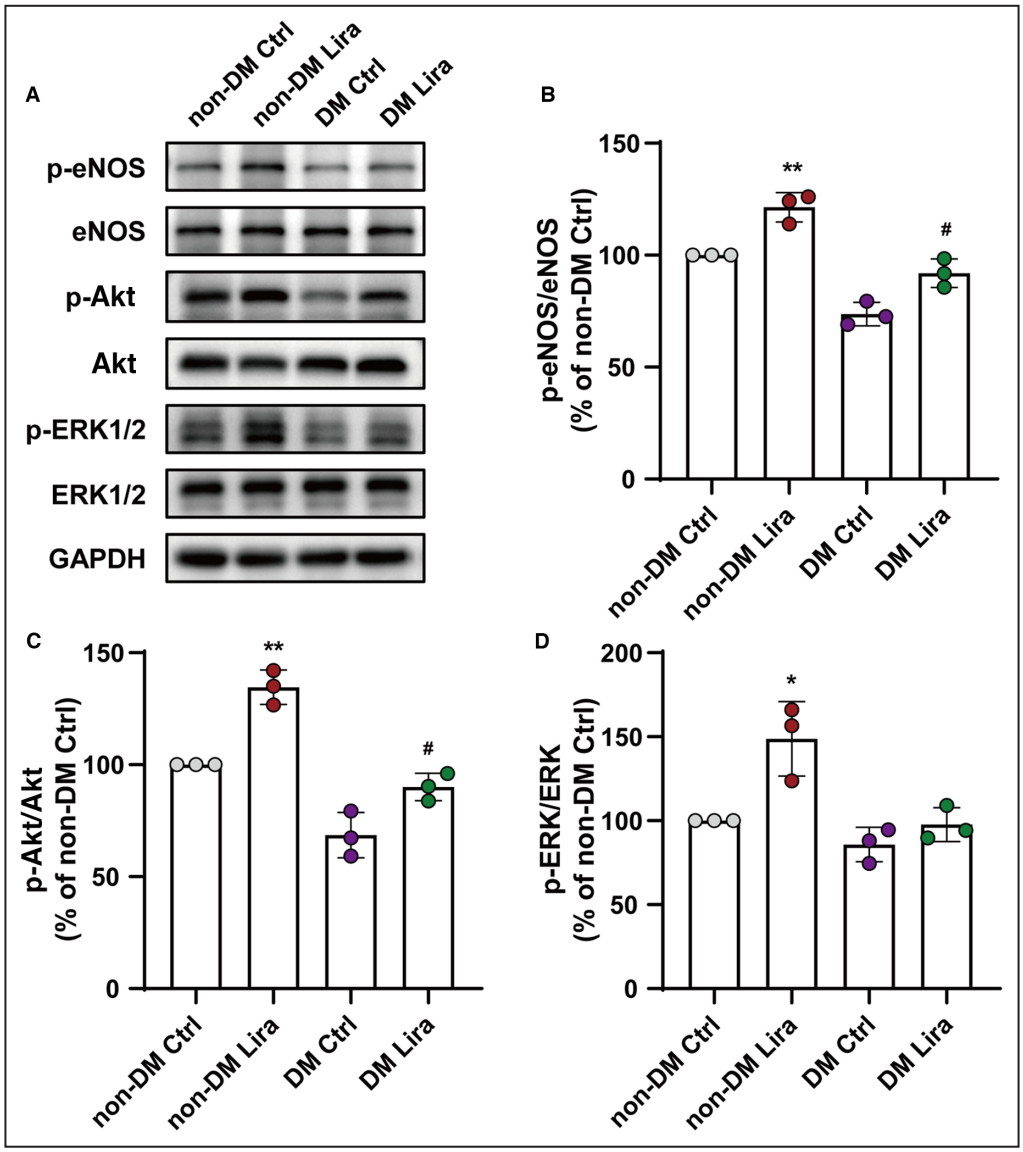


Figure 7. Liraglutide promotes phosphorylation of Akt/endothelial nitric oxide synthase (eNOS) and extracellular signal-related kinases 1 and 2 (ERK1/2) in ischemic muscle in both nondiabetic and type 2 diabetic mice.

Western blotting with the indicated antibodies was performed on the ischemic gastrocnemius muscle of nondiabetic and type 2 diabetic mice treated with or without liraglutide at 14 days after surgery. Representative immunoblots (**A**) and quantitative analysis (**B** through **D**) of relative changes in phospho-Akt (p-Akt)/phospho-eNOS (p-eNOS) and phospho-ERK1/2 (p-ERK1/2). Data are presented as the mean±SD of triplicate experiments. **P*<0.05, ***P*<0.01 vs nondiabetic control; #*P*<0.05 vs diabetic control. Ctrl indicates control; and Lira, liraglutide.

was significantly greater phosphorylation of Akt and eNOS in the ischemic muscle in the liraglutide-treated mice in both the nondiabetic and diabetic groups, consistent with the in vitro results, whereas the level of p-ERK1/2 was upregulated only in the nondiabetic mice when treated with liraglutide. The upregulation of p-ERK 1/2 was only slightly enhanced by liraglutide may be attributed to the inhibitory effects of high glucose (Figure 7A through D).

DISCUSSION

Peripheral artery disease is an obstructive atherosclerotic disease that often manifests as lower-limb ischemia, leading to amputation, multiple organ dysfunction, and even mortality.[2,21,22] Type 2 diabetes causes ischemia in the lower extremities, attenuating vasoconstriction and promoting vasodilation, thus reducing vascular remodeling.[23] In recent years,

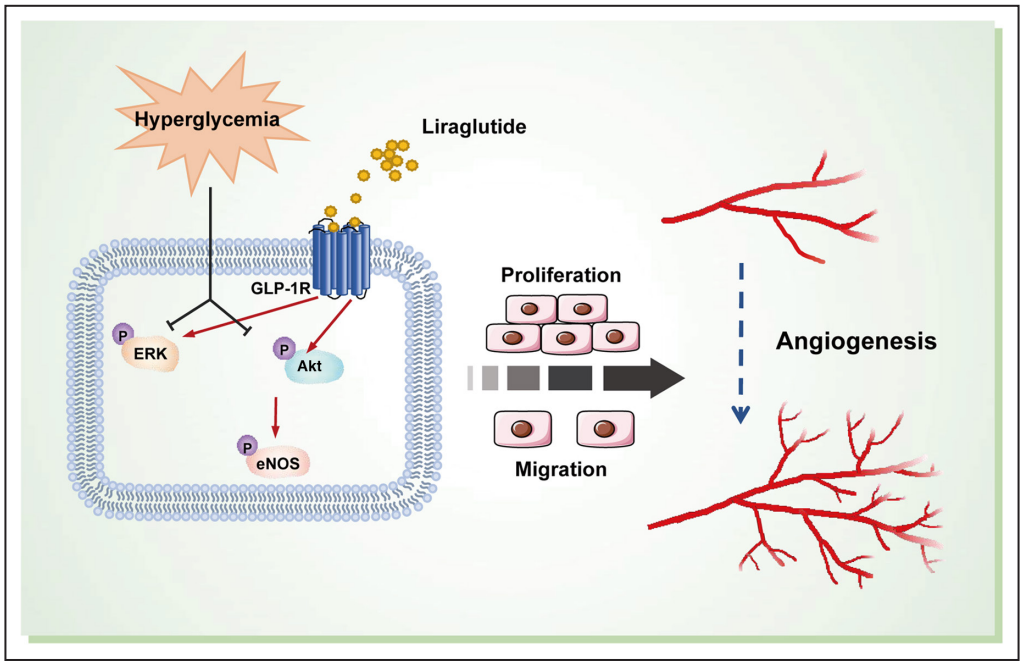


Figure 8. Potential mechanisms through which liraglutide may promote angiogenesis in hindlimb ischemia.
GLP-1R indicates glucagon-like peptide-1 receptor; p-Akt, phospho-Akt; p-eNOS, phospho-endothelial nitric oxide synthase; and p-ERK, phospho–extracellular signal-related kinase.

therapeutic angiogenesis has emerged as a promising new strategy for treating lower-limb ischemia.[24] In the present study, we examined the role of the GLP-1 analogue liraglutide in hindlimb ischemia in type 2 diabetic mice and investigated the underlying mechanism. Our results showed that liraglutide treatment could significantly enhance blood flow in lower extremities and promote angiogenesis after hind-limb ischemia potentially attributable to activating the Akt/eNOS and MAPK/ERK1/2 signaling pathways both in vitro and in vivo (Figure 8). In addition, we also monitored the body weight, blood glucose during the experiment, and the fasting plasma insulin at the end of the experiment. Consistent with the results reported in the literature,[25,26] liraglutide suppressed the body weight gain, lowered plasma glucose levels, and enhanced insulin secretion. It is noteworthy that these metabolic improvements may be beneficial for the proangiogenic effect of liraglutide. Several literatures revealed that GLP-1 preserves the improvement action of microvacular or endothelial function in the insulin-resistant states.[27-29] The enhanced angiogenesis observed by liraglutide might likely be a direct effect on endothelial cells rather than a consequence of the metabolic improvement at this stage.

GLP-1 is known for its glycemic control by promoting insulin secretion via stimulating the adenylyl cyclase pathway in β cells, and it has been found to protect against various cardiovascular diseases, including hypertension, atherosclerosis, and myocardial hypertrophy.[30] Another study showed that GLP-1 could improve angiogenesis in a 3-dimensional in vitro model.[31] However, endogenous GLP-1 is rapidly degraded by dipeptidyl peptidase-4.[32]

Liraglutide has emerged as a long-lasting GLP-1 analogue and agonist of GLP-1R that serves as an effective antiglycemic agent for the treatment of type 2 diabetes.[33] Notably, liraglutide has been recruited in an ongoing clinical trial for patients with type 2 diabetes and peripheral artery disease.[34] Recent studies have shown that liraglutide can alleviate ischemia/reperfusion-induced microvascular endothelial cell injury and inhibit cell apoptosis,[35] and that its cardioprotective properties are mediated by GLP-1R.[36] Consistent with these results, we found that liraglutide promotes the proliferation, migration, and tube formation of HUVECs in a dose-dependent manner, even under HG conditions. It should be noted that administration of liraglutide leads to the correction of microcirculation in rats with the absolute insulin deficiency.[37] The beneficial effects on cell function are regulated by the GLP-1R axis.

We also showed that liraglutide exerts angiogenic effects in HUVECs by upregulating the levels of p-eNOS, p-Akt, and p-ERK1/2. eNOS is an enzyme highly expressed in endothelial cells that catalyzes

the production of nitric oxide via phosphoinositol-3 kinase signaling,[38] thereby mediating vasodilation and vascular function.[9,39] However, long-term hyperglycemia leads to eNOS uncoupling, while insulin resistance leads to overproduction of free fatty acids, which inhibits phosphoinositol-3 kinase, thus blocking the activity of eNOS.[10,19] As a result, the production and bioavailability of nitric oxide is reduced, inducing endothelial cell dysfunction and vascular disease.[40] Here, we confirmed that HG conditions impair the proliferation, migration, and tube formation of HUVECs and significantly downregulate p-eNOS. Furthermore, a previous study in patients with type 2 diabetes showed that liraglutide did not significantly impact endothelial function during long-term treatment.[41]

Akt plays a key role in the long-term regulation of vascular growth, as it promotes the synthesis of nitric oxide via eNOS phosphorylation.[42] A previous study has shown that the activation of Akt/eNOS signaling improves angiogenesis by stimulating the proliferation, migration, and tube formation of endothelial cells.[43] In addition, the GLP-1R analogue exenatide was found to improve coronary flow velocity reserve via AMP-activated protein kinase/PI3K-Akt/eNOS signaling.[44] Both exendin (9–39) and the dipeptidyl peptidase-4 inhibitor partially suppressed the effect of GLP-1 on eNOS levels, suggesting that GLP-1 upregulates eNOS via a GLP-1R–dependent pathway.[45]

ERK proteins, such as ERK1 and ERK2, are involved in Ras–Raf–MEK–ERK signaling, which regulates various processes, including cell proliferation, migration, and differentiation during wound healing and regeneration.[46] Liraglutide can also attenuate hydrogen peroxide-mediated endothelial cell damage through ERK1/2 signaling.[47]

Overall, our results broaden our understanding of the roles of liraglutide in regulating angiogenic signaling. They also support proceeding to additional preclinical studies involving liraglutide as therapeutic strategies to improve hind-limb ischemia reperfusion in vivo.

ARTICLE INFORMATION

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Disclosures

None.

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