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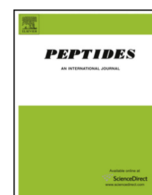

# Liraglutide restores angiogenesis in palmitate-impaired human endothelial cells through PI3K/Akt-Foxo1-GTPCH1 pathway

Jing Ke [1], Rui Wei [1], Fei Yu, Jingjing Zhang, Tianpei Hong *

*Department of Endocrinology and Metabolism, Peking University Third Hospital, Beijing, China*

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) and its analogues have a beneficial role in cardiovascular system. Here, we aimed to investigate whether liraglutide, a GLP-1 analogue, modulated angiogenesis impaired by palmitic acid (PA) in cultured human umbilical vein endothelial cells (HUVECs). Cells were incubated with liraglutide (3–100 nmol/L) in the presence of PA (0.5 mmol/L), and endothelial tube formation was observed and quantified. The protein levels of signaling molecules were analyzed and the specific inhibitors were used to identify the signaling pathways through which liraglutide affected angiogenesis. Results showed that liraglutide ameliorated endothelial tube formation impaired by PA in HUVECs in a dose-dependent manner. Meanwhile, liraglutide increased the phosphorylation of Akt and forkhead box O1 (Foxo1), and upregulated the levels of guanosine 5′-triphosphate cyclohydrolase 1 (GTPCH1) and endothelial nitric oxide synthase (eNOS) in PA-impaired HUVECs. Notably, addition of the PI3K inhibitor LY294002, Foxo1 nuclear export inhibitor trifluoperazine dihydrochloride (TFP), GTPCH1 inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) or NOS inhibitor N-nitro-L-arginine-methyl ester (L-NAME) eliminated the angiogenic effect of liraglutide. Moreover, either LY294002 or TFP abolished the liraglutide-induced upregulation of GTPCH1 and eNOS protein levels. In conclusion, liraglutide restores angiogenesis in PA-impaired HUVECs. The effect is mediated via upregulation of GTPCH1 and eNOS levels in a PI3K/Akt-Foxo1-dependent mechanism.

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## 1. Introduction

Patients with type 2 diabetes are at increased risk of cardiovascular diseases and associated clinical complications. Their compensatory ability in ischemic tissue is also markedly impaired, which is partly related to the remodeling of the pre-existing vasculature and impaired vessel formation [1,2]. Clinical studies have convincingly shown that the presence and extent of existing collateral vessels is of functional significance [3,4]. Moreover, the formation of coronary collateral vessels is significantly reduced in patients with diabetes, which may contribute to the increased risk of mortality in diabetic patients with coronary artery disease [5]. Therefore, the formation of collateral vessels is an important compensatory mechanism to chronic ischemia. Elevated circulating level of free fatty acids, which is a characteristic factor of type 2 diabetes, has been reported as one of negative regulators of endothelial function and angiogenesis [6,7]. Moreover, it has been shown that palmitic acid (PA), one of the fatty acids most commonly found in the western diet, can impair endothelial tube formation *in vitro* [6].

Vasculogenesis, the *de novo* formation of vessels from mesoderm-derived endothelial precursor cells, is responsible for the formation of the first and primitive blood vessels in the embryo [8]. Angiogenesis is defined as the process of sprouting new blood vessels from pre-existing vasculature [9]. Among angiogenesis, endothelial cells first form the tunica intima, a single-cell-layered endothelium that is exposed to the vessel lumen. Next, smooth muscle cells and pericytes are attached to the abluminal surface and help to stabilize the vessel wall and regulate the vascular tone [10]. Finally, connective tissue, collagen and elastic fibers form the vascular adventitia [11]. Notably, the main driver of angiogenesis is the arrangement of endothelial cells. Although the mechanisms underlying angiogenesis are not well known, phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and endothelial function related molecules, including endothelial nitric

*Abbreviations:* DAHP, 2,4-diamino-6-hydroxypyrimidine; DPP-4, dipeptidyl peptidase 4; eNOS, endothelial nitric oxide synthase; Foxo1, forkhead box O1; GLP-1, glucagon-like peptide-1; GTPCH1, guanosine 5′-triphosphate cyclohydrolase 1; HUVECs, human umbilical vein endothelial cells; L-NAME, N-nitro-L-arginine-methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; PA, palmitic acid; TFP, trifluoperazine dihydrochloride.

* Corresponding author.
*E-mail address:* tpho66@bjmu.edu.cn (T. Hong).

[1] The authors contributed equally to this work.

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oxide synthase (eNOS) and nitric oxide (NO), have been identified as important mediators [12,13]. Forkhead box O1 (Foxo1), regulated by PI3K/Akt, has also been suggested as an important regulator in vessel formation in the adult [14].

Glucagon-like peptide-1 (GLP-1), an incretin hormone that is released from intestinal L cells in response to nutrient ingestion, can stimulate glucose-dependent insulin secretion from pancreatic β cells [15]. Besides its glucose-lowering effect, GLP-1 exerts favorable actions on cardiovascular system [16]. GLP-1-based agents, including GLP-1 analogues and dipeptidyl peptidase 4 (DPP-4) inhibitors, have been recently approved as new therapeutic options for patients with type 2 diabetes. Liraglutide is a DPP-4 resistant human GLP-1 analogue that exhibits a prolonged pharmacokinetic profile. Recently, LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of cardiovascular outcome Results) study has shown that liraglutide treatment reduces the rates of cardiovascular events and death in patients with type 2 diabetes [17]. Moreover, our previous study has demonstrated that exenatide, another GLP-1 analogue, significantly improves coronary endothelial function in patients with newly diagnosed type 2 diabetes, and exerts direct protective effects on human umbilical vein endothelial cells (HUVECs) via PI3K/Akt-eNOS pathway [18]. Likewise, other studies have also shown that liraglutide can protect endothelial cells [19]. However, whether GLP-1 and its analogues have a direct effect on angiogenesis is not fully understood. In the present study, we investigated the effect of the GLP-1 analogue liraglutide on endothelial tube formation impaired by PA in HUVECs, and explored the underlying molecular mechanisms involving PI3K/Akt-Foxo1 signaling pathway, and endothelial function related molecules including eNOS and guanosine 5′-triphosphate cyclohydrolase 1 (GTPCH1), a rate-limiting enzyme in eNOS coupling.

## 2. Materials and methods

### 2.1. Reagents

Liraglutide was provided by Novo Nordisk (Bagsvaerd, Denmark). PA, Foxo1 nuclear export inhibitor trifluoperazine dihydrochloride (TFP), GTPCH1 inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) and nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine-methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO). PI3K inhibitor LY294002, rabbit anti-phospho-Ser256 Foxo1 (p-Foxo1), anti-Foxo1, anti-phospho-Ser473 Akt (p-Akt) and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit anti-GTPCH1 antibody was from Abcam (Cambridge, UK). Mouse anti-eNOS antibody was from BD Biosciences (San Joe, CA). Mouse anti-GAPDH antibody was from Zhongshan Biotechnology (Beijing, China). Matrigel basement membrane matrix was from Corning (Bedford, MA).

### 2.2. PA preparation

PA of 256 mg was dissolved in 5 mL absolute ethanol and then titrated with 0.1 mol/L sodium hydroxide 5 mL at 70 °C. Finally, 10 mL PA mixture was added with 190 mL 10% bovine serum albumin (BSA) at 55 °C to make a complex at the concentrations of 5 mmol/L. The stock solution was filter-sterilized and stored at −20 °C. Control solution containing 5% ethanol and 9.5% BSA was similarly prepared.

### 2.3. Cell culture and treatment

HUVECs were obtained from healthy donors with written informed consent. The protocol of our study was approved by the Ethical Committee of Peking University Third Hospital. The umbilical cord samples were collected and washed three times with phosphate-buffered saline (PBS). The endothelial cells were isolated by collagenase digestion and cultured in Medium 199 (Hyclone, Logan, UT) supplemented with 10% FBS (fetal bovine serum) at 37 °C in a 5% $CO_2$ humidified incubator as described previously [20]. The medium was renewed every 2 d until confluence (3–4 d). Cells were harvested by 0.05% trypsin containing 0.03% EDTA for 1–2 min at room temperature and reseeded into 96-well plate for tube formation experiment or into 60-mm culture dishes for either protein extraction or immunofluorescence staining, respectively. The cells of five to six passages were used in all experiments. In this study, exposure of HUVECs to 0.5 mmol/L PA was used as an injured model. To detect the effects of liraglutide, cells were treated with different concentrations of liraglutide (3–100 nmol/L) and/or 0.5 mmol/L PA. To further clarify the involved signaling pathways, cells were pre-incubated for 30 min with the PI3K inhibitor LY294002 (20 μmol/L), Foxo1 nuclear export inhibitor TFP (5 μmol/L), GTPCH1 inhibitor DAHP (0.5 mmol/L) or NOS inhibitor L-NAME (300 μmol/L), followed by co-treatment with 100 nmol/L liraglutide and/or 0.5 mmol/L PA for additional 23.5 h. Solvent DMSO (dimethylsulfoxide) or PBS was used in the control group.

### 2.4. Tube formation assays

The matrigel tube formation assay is an accepted method of *in vitro* model for the study of critical endothelial features required for angiogenesis. HUVECs ($1 \times 10^4$) were cultured in a 96-well plate coated with 60 μL matrigel basement membrane matrix. At the end of treatment with the above-mentioned conditions, the images were obtained with a computer-assisted microscope. The cumulative tube length and tube branch point numbers of endothelial tubes were quantified by using the program image-pro plus. Controls were set up as 100%.

### 2.5. Western blot analysis

Cells were harvested from 60-mm culture dishes and lysed by RIPA containing proteinase inhibitor and phosphatase inhibitor (Applygen Technologies Inc. Beijing, China). Proteins were then isolated by using centrifugation (12,000 rpm, 20 min). Protein concentration was examined by BCA (bicinchoninic acid) protein assay method. Denatured proteins were separated by 10% (*wt*/*vol*) SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed with primary antibodies (all at 1:1000 dilution) overnight at 4 °C, and subsequently incubated with IRDye 800CW-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (both at 1:10,000 dilution; LI-COR Biosciences, Lincoln, NE) for 1 h. Protein bands were visualized with Odyssey 290 infrared imaging system (LI-COR Biosciences). Phosphorylated Akt (p-Akt) and Foxo1 (p-Foxo1), total Akt (t-Akt) and Foxo1 (t-Foxo1), GTPCH1 and eNOS were examined. GAPDH was used as a loading control. Each experiment repeated at least 3 times.

### 2.6. Detection of supernatant NO

NO was determined by nitrate reductase method, using a kit from Beyotime Biotechnology (Shanghai, China). At the end of treatment, the cell culture supernatants of each group were collected. To set up the assay, tubes with 60 μL double distilled water, standard solution and the treatment samples were prepared, and then NADPH (2 mmol/L), flavin adenine dinucleotide and nitrate reductase were added into each tube. After incubation for 30 min at 37 °C, lactate dehydrogenase (LDH) and LDH buffer were added and incubated for another 30 min at 37 °C. Finally, two Griess reagents were added for incubation for 10 min at room temperature. Absorbance at 540 nm in each tube was taken using a microplate reader. The

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