Introduction

Endothelial nitric oxide synthase (eNOS), which synthesizes nitric oxide (NO) from the substrate L-arginine in endothelial cells (ECs), plays an important role in regulating a broad spectrum of functions in the cardiovascular system, including vasorelaxation, the inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell (SMC) migration and proliferation, and platelet aggregation[1]. NO is the most important endothelium-derived vasodilator and has a potent anti-atherosclerotic effect because of its anti-oxidative, anti-inflammatory and anti-coagulatory properties[2, 3]. Pathological changes such as insulin resistance and the metabolic alterations in type 2 diabetes mellitus (T2DM) can lead to eNOS dysfunction and relatively low NO production, which are now considered the major mechanisms of macrovascular complications in T2DM.

Glucagon-like peptide-1 (GLP-1) is a gut hormone that is released into the bloodstream after feeding. Potentiating the insulinotropic action of glucose, GLP-1 is effective in controlling glucose by stimulating insulin secretion. The impaired secretion of GLP-1 in patients with T2DM has been considered to be one of the mechanisms underlying their abnormal glucose metabolism[4–7]. Thus, its analogues have been used clinically to treat T2DM[8]. Recently, many researchers have turned their attention to the cardiovascular effects of GLP-1 and have found that GLP-1 can induce endothelium-dependent vasorelaxation[9–11] and improve the response of vessels to other vasodilators[12–14]. Furthermore, GLP-1 can elevate the NO levels in coronary effluent from mouse hearts, and its vasorelaxing effect can be blocked by an eNOS inhibitor[11], suggesting that GLP-1 upregulates eNOS. Taken together, we reason that GLP-1 may be an endogenous vascular-protective hormone and an extremely effective drug target for pharmacological intervention in T2DM patients.

GLP-1 exerts its actions through GLP-1 receptor (GLP-1R), which is also expressed in the endothelium[11, 13, 15] . Native GLP-1, existing mainly as an intact, biologically active form
GLP-1 [7–36 amide]), is rapidly degraded after secretion by dipeptidyl peptidase-4 (DPP-4) to its inactive form, N-terminally truncated GLP-1(9–36). GLP-1(9–36) has a weak affinity to GLP-1R and was previously considered to have no biological function. However, some research has suggested that GLP-1(9–36), which offers significant cardioprotection against ischemia-reperfusion injury and induces vasodilation[10, 11, 16], may affect GLP-1 function, at least in the cardiovascular system.

In the present study, to improve our understanding of the mechanism by which GLP-1 may exert cardiovascular-protective effects, we investigated the effect of GLP-1 on the activation and expression of eNOS in human umbilical vein endothelial cells (HUVECs). In addition, we investigated whether the GLP-1R-dependent or GLP-1(9–36)-related pathways are involved in these effects.

**Materials and methods**

**Cell culture**

Primary human umbilical vein endothelial cells (HUVECs) derived from normal human placenta umbilical cord tissues were purchased from Pricells Company (Wuhan, China). They were verified with vWF, FactorVIII and CD31 (P-CAM), and frozen in liquid nitrogen at the end of primary culture. After the purchase, they were recovered and sub-cultured in endothelial cell medium containing 5% fetal bovine serum (FBS) and 1% endothelial cell growth supplement (ScienCell, San Diego, CA, USA) in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. The cells from the 3rd–6th passages were used in all of the experiments. When grown to 70%–90% confluency, the cells were recovered in the presence or absence of GLP-1 (ProSpec, Rehovot, Israel), exenatide (a GLP-1R agonist) (Lilly, Indianapolis, IN, USA), GLP-1(9–36) (Chinese Peptide, Hangzhou, China), exendin(9–39) (a GLP-1R antagonist) (Sigma, St Louis, MO, USA) or sitagliptin (an inhibitor of DPP-4) (HKBY, Beijing, China) for various times and then collected for further analysis.

**Measurement of eNOS activity**

The activities of eNOS were assayed with an NOS assay kit (Beiyotime, Haime, China) according to the manufacturer’s instructions. The cells were grown to 90% confluency in 96-well microplates and incubated with or without 5-5000 pmol/L GLP-1, GLP-1(9–36) or exenatide in a 200 μL system containing sufficient substrates of NOS (εG, NADPH and L-arginine) and 3-Amino, 4-aminomethyl-2',7'-difluorescein, diacetate (DAF-FM DA), a cell-permeable fluorescent substance. After incubation at 37 °C for 30 min, the fluorescence intensity (FI) was detected using a microlate reader (Infinite®200 Pro, Tecan, Switzerland) with an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Each plate also included at least 2 negative control wells to which no cells were added. The relative activities (RA) of eNOS were calculated as follows:

\[ RA = \frac{FI_{\text{sample}} - FI_{\text{negative}}}{FI_{\text{control}} - FI_{\text{negative}}} \]

**Western blot analysis**

Western blot analysis was used to quantify the total and phosphorylated (ser-1177) levels of eNOS proteins and to detect the expression of GLP-1R and DPP-4 in HUVECs. Cells were washed 3 times with ice-cold PBS and then lysed with ice-cold RIPA lysis buffer (Beyotime) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride (Beyotime) and phosphatase inhibitors (YuanPingHao Bio, Beijing, China). The cell lysates were centrifuged (12000g, 12 min, 4 °C), and the supernatants were collected. Protein concentrations were determined using a BCA kit (Beyotime). Equal amounts of samples (50 μg) were denatured and subjected to 10% SDS-PAGE. The separated proteins were then electro-transferred onto a PVDF membrane blocked with 5% (w/v) fat-free milk powder in Tris-buffered saline with Tween (TBST) for 2 h. The membranes were incubated overnight at 4 °C with primary antibodies as follows: anti-eNOS (1:400), anti-phospho-eNOS (ser-1177) (1:200), anti-GLP-1R (1:400), anti-DPP-4 (1:400) and anti-β-actin (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) in TBST for 2 h at room temperature. The immunostained proteins were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). The intensities of the bands were quantified using a Gel Doc™ XR imaging system (Bio-Rad Laboratories, Hercules, CA, USA) and Quantity One software.

**Real-time RT-PCR**

HUVECs were lysed on ice with TRIzol (1 mL/well) (GCBBio, Guangzhou, China), and total RNA was extracted with chloroform and isopropanol according to the manufacturer’s protocol. The extracted RNA was quantified using NaNoDrop (NaNoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The levels of eNOS transcripts were quantified with real-time RT-PCR using SYBR® Premix Ex Taq™ (Takara Bio) according to the manufacturer’s instructions. The primer pairs used in the real-time RT-PCR for human eNOS were 5’-TCCACGATGTTGACTTGGCTA-3’ (sense) and 5’-GCTGTCTGATGGACCTTGA-3’ (antisense), and the primer pairs used for the human housekeeping gene β-actin were 5’-TGGCACCCCCACAAATGGA-3’ (sense) and 5’-CATAGTCATGCCGCTAGAAGC-3’ (antisense). The PCR reactions were carried out in a Rotor–Gene 6000 system (Corbett Life Science, Australia) under the following conditions: 95 °C for 30 s, 45 cycles at 95 °C for 5 s and 55 °C for 30 s. The expression level of eNOS relative to β-actin (the internal reference gene) was calculated and adjusted against the expression levels of untreated samples, which were deemed to be 1.0. The calculation formula is as follows: eNOS relative gene expression=(eNOS gene concentration in treatment group/β-actin gene concentration of the same sample)/(eNOS gene concentration in control group/β-actin gene concentration of the same sample).
Statistical analysis
A statistical analysis was performed with SPSS version 13.0 (SPSS Inc, Chicago, IL, USA). Data are presented as the mean±SD. Multiple comparisons were evaluated by the one-way ANOVA followed by the S-N-K test. \( P<0.05 \) was considered statistically significant.

Results
GLP-1 promotes eNOS activities in HUVECs
The incubation of HUVECs with GLP-1 for 30 min increased eNOS activity (Figure 1A). GLP-1 increased eNOS activity at 5 pmol/L, but the difference was not significant. Higher concentrations of GLP-1 (50–5000 pmol/L) significantly enhanced eNOS activity. As indicated in Figure 1B, GLP-1-induced NO production was blocked by N\(^{G}\)-nitro-L-arginine methyl ester (L-NAME) (1 mmol/L), suggesting that eNOS activation mediated the effect of GLP-1 on NO release in HUVECs.

GLP-1 stimulates the phosphorylation of eNOS in HUVECs
The activity of eNOS is regulated by phosphorylation at multiple sites. The most thoroughly studied site is the activation site ser-1177\([17]\]. Several protein kinases, including Akt/PKB, PKA, and AMPK, activate eNOS by phosphorylating ser-1177 in response to various stimuli, such as insulin and fluid shear stress\([18, 19]\). We thus investigated whether GLP-1 increases ser-1177 phosphorylation. The incubation of HUVECs with GLP-1 (5000 pmol/L) for 5, 10, and 30 min increased the phosphorylation of eNOS (ser-1177), with the most significant effect being obtained at 5 min (Figure 2A). For the dose course study, 500–5000 but not 50 pmol/L GLP-1 significantly enhanced the phosphorylation of eNOS at ser-1177 (Figure 2B).

The effects of GLP-1 on eNOS mRNA and protein levels in HUVECs
Cells were incubated with 5000 pmol/L GLP-1 for 6, 12, 24, and 48 h. Real-time RT-PCR revealed no significant difference in eNOS mRNA expression between these groups (Figure 2C). Western blot analysis indicated that GLP-1 could upregulate eNOS protein expression after a 48-h incubation (Figure 2D).

The GLP-1R and GLP-1(9–36)-related pathways are involved in the effect of GLP-1 on eNOS
Exenatide is a synthesized GLP-1 receptor agonist, and it may mimic GLP-1R-mediated effects, such as GLP-1-stimulated insulin release. To determine whether a GLP-1R-dependent or GLP-1(9–36)-related pathway (ie, GLP-1R independent) is responsible for the effects of GLP-1 on eNOS, we investigated the influence of exenatide and GLP-1(9–36) on eNOS in HUVECs. We also confirmed the expression of GLP-1R and DPP-4 proteins in HUVECs\([15, 20]\) by Western blot analysis (Figure 3A). Like GLP-1, exenatide or GLP-1(9–36) treatment (5000 pmol/L) effectively promoted eNOS activity (Figure 3B), the phosphorylation of eNOS at ser-1177 (Figure 3C) and eNOS protein levels (Figure 3D) in HUVECs. Exenatide and GLP-1 had no effect on eNOS mRNA expression, but GLP-1(9–36) increased the mRNA levels approximately twofold after an incubation period of 48 h (Figure 3E). To further confirm the involvement of the GLP-1R-dependent and GLP-1(9–36)-related pathways in the effects of GLP-1 on eNOS, we investigated the influence of GLP-1R blockage and DPP-4 inhibition on the effects of GLP-1. The synthesized GLP-1R antagonist exendin(9–39) and the DPP-4 inhibitor sitagliptin, which can abolish GLP-1(9–36) formation, were employed in this study. The incubation of 5000 pmol/L exendin(9–39) or sitagliptin (Figure 3F–3H) partially blocked the GLP-1-induced enhancement of eNOS activity, phosphorylation and total eNOS protein levels. Unexpectedly, cotreatment with exendin(9–39) and sitagliptin, compared with each single treatment, further attenuated but could not completely abolish the aforementioned upregulatory effects of GLP-1 on eNOS (Figure 3F–3H).

Discussion
Because GLP-1 induces endothelium-dependent vasodilation in human and rat arteries, we speculate that it may activate eNOS and increase NO production. eNOS plays an important role in maintaining normal endothelial function, and its dysfunction may be significantly associated with the cardiovascular complications in T2DM. Here, we investigated the effects of GLP-1 on the activation and expression of eNOS in HUVECs and whether GLP-1R and GLP-1(9–36) mediate these effects. Administration of 500–5000 pmol/L of GLP-1 promoted eNOS activity and phosphorylation at ser-1177 after 5
min in HUVECs. After a 48-h incubation, GLP-1 elevated total eNOS protein but had no effect on eNOS mRNA levels. Both exenatide and GLP-1(9–36) increased eNOS activity, phosphorylation and total protein expression. In addition, GLP-1(9–36) elevated eNOS mRNA levels. Exendin(9–39) and sitagliptin, alone or in combination, partially blocked the GLP-1-induced enhancement of eNOS activity, phosphorylation and total protein levels. Taken together, our results suggest that GLP-1 upregulates eNOS activity and protein expression through the GLP-1R-dependent and GLP-1(9–36)-related pathways in HUVECs.

The concentration of 5–5000 pmol/L covers the physiological and pharmacological concentrations of GLP-1, thus this study seeks to observe the effects of GLP-1 within this range of concentration. Figure 1 shows that 50–5000 pmol/L of GLP-1 can increase the concentration of NO. In addition, we observed the effect of GLP-1 on eNOS phosphorylation, mRNA and protein expression (Figures 2 and 3). The observation of these three changes needs to explore the right time point. Taking into account that the relative high concentration of treatment factors may more easily produce the effects, we thus selected the 5000 pmol/L concentration for the experiment. After clarifying that this concentration could enhance eNOS phosphorylation and protein levels and determining the appropriate time point, we further observed the effect of other concentrations of GLP-1. Figure 2B shows that within 5–5000 pmol/L range, only 500–5000 pmol/L of GLP-1 can cause elevated levels of phosphorylation. However, a lower concentration (5–500 pmol/L) of GLP-1 does not cause changes in protein levels (data not shown), suggesting that only high concentrations of GLP-1 can result in eNOS expression upregulation. As indicated in Figure 1, 5000 pmol/L of GLP-1 did not cause eNOS mRNA change in the time period we observed, thus we did not further observe the effect of other concentrations.
Previous research has suggested that a 30-s infusion of GLP-1 can induce pulmonary vasodilation in isolated rat lungs that can be blocked by L-NAME, an eNOS inhibitor\(^9\). Thus, GLP-1 may increase NO release by directly increasing eNOS activity rather than protein levels in a short time. In this study, to understand the effect of GLP-1 on eNOS activity, we observed intracellular NO levels in HUVECs incubated with GLP-1 for 30 min and found that GLP-1 (50–5000 pmol/L) could increase eNOS activity. In normal individuals, the concentration of GLP-1 during the fasting state is 5–10 pmol/L and can increase to 20–40 pmol/L after meals whereas pharmacological concentrations of GLP-1 can be more than 100 pmol/L\(^{21,22}\). Thus, GLP-1 may increase eNOS activity at both physiological and pharmacological concentrations. Among

Figure 3. GLP-1R-dependent pathway and GLP-1(9–36)-related pathway are involved in effects of GLP-1 on eNOS levels in HUVECs. (A) GLP-1R and DPP-4 proteins were detected in HUVECs by Western blot analysis. Cells were incubated in the presence or absence (control) of GLP-1, GLP-1(9–36) or exenatide (GLP-1R agonist) (all at 5000 pmol/L) for the following times (B, C, D, E). Cells were incubated with GLP-1 in the presence of exendin (9–39) (G+E) or sitagliptin (G+S) or both (G+E+S) for the following times (F, G, H). (B, F) After 30-min incubation, eNOS activity was determined by NO content in cells. (C, G) After 5-min incubation, phosphorylation of eNOS at ser-1177 was examined by western blot analysis. (D, H) After 48-h incubation, total eNOS protein level was measured by western blot analysis. The upper parts of C, D, G, and H show representative experiments. Data are mean±SD after normalization to β-actin level. (E) After 48-h incubation, eNOS mRNA level was quantified by real-time RT-PCR. Data are mean±SD after normalization to β-actin level. \( {^b} P < 0.05, {^c} P < 0.01 \) compared to control.
the rapid regulatory mechanisms of eNOS activity, eNOS ser-1177 phosphorylation is the most important pathway and is most frequently investigated. In the present study, GLP-1-induced eNOS ser-1177 phosphorylation was time dependent, i.e., phosphorylated eNOS levels increased rapidly in a few minutes and then decreased within a short amount of time. Thus, GLP-1-induced eNOS activation may be restricted by negative feedback mechanisms, thereby limiting NO synthesis within an appropriate range to avoid adverse effects of excessive NO in endothelium.

GLP-1-induced NO synthesis and release via the activation of eNOS can explain the direct vasodilation by GLP-1. Moreover, GLP-1 can facilitate the effects of other vasodilator stimuli, such as fluid shear stress and acetylcholine[8−14]. However, in Daul salt-sensitive rats, after continuous infusion of GLP-1 for 14 d, the vasorelaxing reaction of the isolated thoracic aorta ring in response to acetylcholine was twice that in non-GLP-1-infused rats[15], indicating that GLP-1 has a long-term effect on eNOS, especially at the mRNA and protein levels. Here, our results reveal that a 48-h incubation of GLP-1 increases eNOS protein but not mRNA levels in HUVECs. Thus, GLP-1 regulates eNOS expression at the translational level and has no effects on gene transcription or mRNA stability.

GLP-1R mediates the main effect of GLP-1 on its target tissues, such as pancreatic islets, and plays an important role in maintaining glucose homeostasis. Indeed, GLP-1R is also expressed in endothelial cells[11, 13, 15], thus, we hypothesize that the GLP-1R-dependent pathway may be involved in the endothelial effects of GLP-1. On the other hand, evidence indicates that an unknown signal pathway, which is independent of GLP-1R but is related to GLP-1(9–36), may exist and mediate certain biological effects of GLP-1. The half-life of GLP-1 in vivo is very short (only several minutes), and the cycling GLP-1 is usually present in the form of GLP-1(9–36)[25]. GLP-1(9–36) has a weak affinity with GLP-1R but cannot activate GLP-1R. Thus, it was considered either to have no biological activity or to antagonize GLP-1R to some extent[24]. Recent studies have found that GLP-1 can protect myocardium and relax blood vessels in mutagenic mice without GLP-1R. GLP-1(9–36) has a similar effect in normal and GLP-1R –/– mice. DPP-4 inhibitors, which block the conversion of GLP-1 to GLP-1(9–36), can weaken the above effects of GLP-1[21]. Studies have also found that GLP-1(9–36) has a more intense relaxing effect on isolated mouse femoral artery rings compared with GLP-1 and that single GLP-1R agonists have no relaxing effect[20, 21]. Together, these studies indicate that a GLP-1(9–36)-related, non-GLP-1R-dependent signaling pathway may mediate the biological effect of GLP-1. However, whether a GLP-1R-dependent or GLP-1(9–36)-related pathway mediates the effects of GLP-1 on eNOS was unknown. We thus treated HUVECs with GLP-1, exenatide (a GLP-1R agonist) and GLP-1(9–36) at the same concentrations and found that all of them could increase the activity and phosphorylation of eNOS as well as the total eNOS protein levels. In addition, like GLP-1, exenatide had no effect on eNOS mRNA levels, but GLP-1(9–36) could increase eNOS mRNA expression after a 48-h incubation. Thus, both the GLP-1R-dependent and GLP-1(9–36)-related pathways may be involved in the effect of GLP-1 on eNOS in HUVECs. Moreover, both the GLP-1R antagonist and the DPP-4 inhibitor that abolishes GLP-1(9–36) formation partially suppressed the GLP-1-induced eNOS activation, ser-1177 phosphorylation and upregulation of eNOS protein levels, further confirming the involvement of these two signaling pathways.

GLP-1 can upregulate eNOS in HUVECs, implying that, as a physiological hormone, it is involved in maintaining normal endothelial function. Thus, decreased GLP-1 levels may be a mechanism underlying the endothelial dysfunction in T2DM patients. Previous studies have demonstrated that GLP-1 and GLP-1(9–36) can lead to the vasodilation of femoral and mesenteric arteries in rats, but the GLP-1R agonist exendin-4 and exenatide has no such effects[12, 13]. This observation can be explained by the lack of GLP-1R expression in mesenteric arteries in rats, but the GLP-1R agonist exendin-4 and exenatide has no such effects[12, 13]. This observation can be explained by the lack of GLP-1R expression in mesenteric arteries in rats, but the GLP-1R agonist exendin-4 and exenatide has no such effects[12, 13]. This observation can be explained by the lack of GLP-1R expression in mesenteric artery intima[13]. Thus, the increased GLP-1(9–36) may be necessary to improve the pathophysiologic changes caused by the decreased secretion of GLP-1 in T2DM patients, especially in tissues without GLP-1R expression. To date, there are few studies on the GLP-1(9–36)-related signaling pathway. Further research can provide a better understanding of the physiological functions and pharmacological actions of GLP-1(9–36).

In summary, GLP-1 can increase eNOS activity and protein levels in HUVECs via both the GLP-1R-dependent and GLP-1(9–36)-related pathways. New antidiabetic drugs, including GLP-1 analogues, GLP-1R agonists and DPP-4 inhibitors, improve glucose control in T2DM patients and have direct cardiovascular-protective effects. In addition, close attention should also be paid to the difference between the cardiovascular effects of GLP-1R agonists, GLP-1 analogues and DPP-4 inhibitors.

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Author contribution
Li DING and Jin ZHANG designed and performed the research and analyzed the data; and Li DING wrote the paper.

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