

# Exenatide exerts direct protective effects on endothelial cells through the AMPK/Akt/eNOS pathway in a GLP-1 receptor-dependent manner

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**Wei R, Ma S, Wang C, Ke J, Yang J, Li W, Liu Y, Hou W, Feng X, Wang G, Hong T.** Exenatide exerts direct protective effects on endothelial cells through the AMPK/Akt/eNOS pathway in a GLP-1 receptor-dependent manner. *Am J Physiol Endocrinol Metab* 310: E947–E957, 2016. First published April 12, 2016; doi:10.1152/ajpendo.00400.2015.—Glucagon-like peptide-1 (GLP-1) may have direct favorable effects on cardiovascular system. The aim of this study was to investigate the effects of the GLP-1 analog exenatide on improving coronary endothelial function in patients with type 2 diabetes and to investigate the underlying mechanisms. The newly diagnosed type 2 diabetic subjects were enrolled and given either lifestyle intervention or lifestyle intervention plus exenatide treatment. After 12-wk treatment, coronary flow velocity reserve (CFVR), an important indicator of coronary endothelial function, was improved significantly, and serum levels of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were remarkably decreased in the exenatide treatment group compared with the baseline and the control group. Notably, CFVR was correlated inversely with hemoglobin A<sub>1c</sub> (Hb A<sub>1c</sub>) and positively with high-density lipoprotein cholesterol (HDL-C). In human umbilical vein endothelial cells, exendin-4 (a form of exenatide) significantly increased NO production, endothelial NO synthase (eNOS) phosphorylation, and GTP cyclohydrolase 1 (GTPCH1) level in a dose-dependent manner. The GLP-1 receptor (GLP-1R) antagonist exendin (9–39) or GLP-1R siRNA, adenyl cyclase inhibitor SQ-22536, AMPK inhibitor compound C, and PI3K inhibitor LY-294002 abolished the effects of exendin-4. Furthermore, exendin-4 reversed homocysteine-induced endothelial dysfunction by decreasing sICAM-1 and reactive oxygen species (ROS) levels and upregulating NO production and eNOS phosphorylation. Likewise, exendin (9–39) diminished the protective effects of exendin-4 on the homocysteine-induced endothelial dysfunction. In conclusion, exenatide significantly improves coronary endothelial function in patients with newly diagnosed type 2 diabetes. The effect may be mediated through activation of AMPK/PI3K-Akt/eNOS pathway via a GLP-1R/cAMP-dependent mechanism.

AMP-activated protein kinase; endothelial nitric oxide synthase; coronary flow velocity reserve; exenatide; glucagon-like peptide-1; human umbilical vein endothelial cells; type 2 diabetes mellitus

PATIENTS WITH TYPE 2 DIABETES are at a high risk of developing coronary atherosclerosis, a leading cause of death in these patients. Endothelial dysfunction contributes to the development and progression of coronary atherosclerotic heart disease in type 2 diabetes. It has been demonstrated that nitric oxide

(NO) plays a central role in regulating endothelial function homeostasis. Loss of NO bioavailability is a key feature of endothelial dysfunction preceding atherosclerosis (20). NO production by endothelial nitric oxide synthase (eNOS) requires the enzymatic cofactor tetrahydrobiopterin (BH4). Deficiency of vascular BH4 leads to impaired endothelial function, whereas restoration of vascular BH4 bioavailability results in an attenuated endothelial dysfunction (19). GTP cyclohydrolase 1 (GTPCH1) is the rate-limiting enzyme in the BH4 biosynthetic pathway regulating intracellular BH4 levels. Overexpression of GTPCH1 is sufficient to augment BH4 levels in cultured endothelial cells, whereas exposure of endothelial cells to GTPCH1 inhibitors or siRNA markedly reduces the levels of BH4 and NO (37). Thus, the expression of GTPCH1 may play an important role in regulating NO-mediated endothelial function.

Improved understanding of the critical role of endothelium in the pathophysiology of vascular disease has led to the development of clinical tests for its functional evaluation. Coronary flow velocity reserve (CFVR), derived from the ratio of maximal hyperemia to basal coronary blood flow, is an important noninvasive indicator of coronary endothelial function and microcirculation (28). Reduced CFVR is an early prognostic indicator of coronary atherosclerotic heart disease (28). Our previous studies also demonstrated a positive association between CFVR and endothelial function in patients either with type 2 diabetes-associated coronary artery disease or with hyperhomocysteinemia (18, 27, 39).

Glucagon-like peptide-1 (GLP-1)-based therapy (including GLP-1 analogs and dipeptidyl peptidase-4 inhibitors) has been approved recently as a new therapeutic option for patients with type 2 diabetes, owing to its ability to stimulate glucose-dependent insulin secretion by pancreatic  $\beta$ -cells. In addition to its established glucose-lowering action, GLP-1 exerts a number of extrapancreatic effects, including improvement of cardiovascular function. Emerging evidence indicates that GLP-1 and its analogs have direct effects on vascular endothelium (6, 15, 22, 24, 29, 31). Exenatide (a GLP-1 analog) treatment has been demonstrated to induce a significant improvement of brachial artery endothelial function as evaluated by flow-mediated dilation (FMD) in patients with type 2 diabetes (22). Exendin-4 (a form of exenatide) reduced monocyte adhesion to the endothelium, suppressed atherosclerosis in apolipoprotein E-deficient mice (6, 15), and promoted the proliferation of human coronary artery endothelial cells (6, 15). Nevertheless, the precise protective mechanisms of GLP-1 or exenatide on endothelium have yet to be clearly established.

Therefore, in the present study, we investigated the therapeutic effect of exenatide in improving coronary endothelial

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function and reducing inflammatory potential in patients with newly diagnosed type 2 diabetes. Furthermore, we used the cultured primary human umbilical vein endothelial cells (HUVECs) to investigate the protective effects of exenatide on the endothelial function and elucidated the underlying signaling mechanisms.

## MATERIALS AND METHODS

### *In Vivo Studies*

**Subjects.** All participants were recruited from the Outpatient Clinic at the Department of Endocrinology and Metabolism of Peking University Third Hospital (Beijing, China). Subjects aged between 30 and 60 yr with newly diagnosed diabetes (without prior medical treatment) according to World Health Organization diabetes criteria in 1999 (2) and glycosylated hemoglobin A<sub>1c</sub> (Hb A<sub>1c</sub>)  $\leq 10\%$  (86 mmol/mol) were included. The exclusion criteria were diabetic retinopathy, nephropathy and related diseases, cardiovascular disease, heart failure, severe arrhythmia, bronchial asthma, chronic obstructive pulmonary disease, malignant disease, systemic inflammatory disease, infectious disease, inflammatory bowel disease, and taking lipid-lowering or anti-hypertension drugs. The following individuals were also excluded: women who were breast-feeding or pregnant and individuals who were allergic to adenosine or receiving folic acid, vitamin, or nitrates that affected the accuracy of the CFVR determination used in the study. This study (ChiCTR-IPR-15006558) was approved by the Ethics Committee of Peking University Third Hospital. The purpose of the study and potential complications were explained to the participants, and written, informed consent was obtained.

**Study design.** Eligible participants were divided into receiving either lifestyle intervention, consisting of exercise and dietary components directed by health educators as recommended (4), or lifestyle intervention plus exenatide treatment according to the patient's intention. All subjects underwent 12 wk of treatment. Exenatide was administered initially by subcutaneous injection at 5  $\mu\text{g}$  twice daily. Compliance with the prescribed therapy and possible treatment-related side effects were monitored at 1-mo follow up. After 4 wk, exenatide dosage was increased to 10  $\mu\text{g}$  twice/day and continued until the end of the study. Fasting blood samples were collected at baseline and at the 12th wk after treatment for clinical biochemistry analyses. The primary endpoint of this study was the improvement of coronary artery endothelial function, and secondary end points were the levels of inflammatory cytokines.

**Laboratory measurements.** After centrifugation at 3,500 rpm, 4°C for 10 min, the plasma or serum was collected and stored at  $-80^{\circ}\text{C}$  until further use. The levels of fasting blood glucose, serum total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and C-reactive protein were measured by standard procedures on an Olympus AU5400 automatic biochemical analyzer (Olympus, Tokyo, Japan). Hb A<sub>1c</sub> measurements were performed using high-performance liquid chromatography (Tosoh, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) was used to measure soluble intercellular adhesion molecule-1 (sICAM-1; Invitrogen, Carlsbad, CA) and soluble vascular cell adhesion molecule-1 (sVCAM-1; R & D Systems, Minneapolis, MN).

**Assessment of coronary endothelial function.** Coronary artery endothelial function was assessed by measuring CFVR using transthoracic Doppler echocardiography. The peak coronary flow velocities in the distal left anterior descending coronary artery were recorded at rest and during hyperemia after intravenous infusion of adenosine ( $0.14 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) in all subjects. CFVR was calculated using the following formula:  $\text{CFVR} = \frac{\text{peak coronary flow velocity during hyperemia}}{\text{peak coronary flow velocity at rest}}$ . Two

cardiologists did this assessment, and they were blinded from the treatment module.

### *In Vitro Studies*

**Cell culture and treatment.** Human umbilical cord samples were collected from healthy donors. Written, informed consent was obtained from pregnant women before labor. The umbilical cord samples were collected and washed three times with phosphate-buffered saline (PBS). Endothelial cells were isolated by collagenase digestion and cultured in Medium 199 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS), as described previously (26). The cells of five to six passages were used in all experiments, which were examined to ensure specific characteristics of endothelial cells by cytochemical staining. HUVECs were treated with different concentrations (0–50 nmol/l) of exendin-4 (Sigma, St. Louis, MO), GLP-1 (7–36) amide (100 nmol/l; R & D Systems, Minneapolis, MN), or GLP-1 (9–36) amide (100 nmol/l; R & D Systems) for 24 h or with 20 nmol/l exendin-4 for different times. In some experiments, 200  $\mu\text{mol/l}$  homocysteine (Sigma) was used for a 24-h incubation to induce endothelial dysfunction. Pretreatment of HUVECs with exendin-4 (20 nmol/l) was performed 1 h prior to the homocysteine treatment. To clarify the involved signaling pathways, cells were incubated with the GLP-1 receptor (GLP-1R) antagonist exendin (9–39) (200 nmol/l; Sigma), adenylyl cyclase activator forskolin (10  $\mu\text{mol/l}$ ; Sigma) or inhibitor SQ-22536 (100  $\mu\text{mol/l}$ ; Sigma), AMP-activated protein kinase (AMPK) inhibitor compound C (10  $\mu\text{mol/l}$ ; Sigma), or phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 (10  $\mu\text{mol/l}$ ; Cell Signaling Technology, Beverly, MA) for 30 min before other treatments. Levels of sICAM-1 in cell supernatant were detected by ELISA, as mentioned above.

In the knockdown experiment, HUV-EC-C (Cell Resource Centre of Shanghai Life Science Research Institute), a cell line of HUVECs, was cultured in DMEM (Invitrogen) supplemented with 10% FBS. To silence *GLP-1R* gene expression, cells were transfected with siRNAs (synthesized by RiboBio, Guangzhou, China) using lipofectamine RNAi MAX Reagent (Invitrogen). After transfection for 48 h, cells were treated with exendin-4 (20 nmol/l) for 24 h.

**Measurement of intracellular NO.** Intracellular NO levels were measured using a NO-sensitive fluorescence probe 3-amino, 4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM DA; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocols. Cells were incubated in 60-mm plates for 24 h under different treatment conditions. The cells were subsequently washed twice with PBS and incubated with 5  $\mu\text{mol/l}$  of DAF-FM DA in serum-deficient medium for 30 min at  $37^{\circ}\text{C}$ . At the end of the incubation, the cells were washed with PBS and gently trypsinized. Cell fluorescence was measured by a flow cytometer (BD Biosciences, San Jose, CA) at an emission wavelength of 515 nm and an excitation wavelength of 495 nm. Controls were set up as 100% of the intracellular NO level.

**Measurement of intracellular reactive oxygen species.** A reactive oxygen species (ROS) detection kit (Sigma) was used to determine intracellular oxidant production in the cells based on oxidation of ROS probe dye 2',7'-dichlorofluorescein diacetate (DCF-DA; 20  $\mu\text{mol/l}$ ) by intracellular ROS, resulting in formation of the fluorescent compound 2',7'-dichlorofluorescein (DCF). DCF fluorescence was monitored with a confocal microscope (LSM 510 META; Carl Zeiss, Jena, Germany). Controls were set up as 100% of the intracellular ROS level.

**cAMP determination.** HUVECs were seeded at a density of  $10^6$ /dish and incubated overnight for attachment. The cells were incubated with exendin-4 at various concentrations (0–50 nmol/l) for 15 min or with 20 nmol/l exendin-4 for various durations (0–30 min). To determine the mechanism of cAMP production, HUVECs were stimulated for 15 min with exendin-4 (20 nmol/l) or forskolin (10  $\mu\text{mol/l}$ ) with or without 30-min pretreatment of exendin (9–39) (500 nmol/l)

Table 1. Clinical and metabolic profiles of type 2 diabetic patients at baseline and after 12 wk of treatment with or without exenatide

| Metabolic Parameters               | Control Group (n = 13) |                          | Exenatide Group (n = 18) |                              |
|------------------------------------|------------------------|--------------------------|--------------------------|------------------------------|
|                                    | Baseline               | After 12 wk              | Baseline                 | After 12 wk                  |
| Age, yr                            |                        | 46.4 ± 5.6               |                          | 42.9 ± 8.8                   |
| Sex (male/female)                  |                        | 10/3                     |                          | 12/6                         |
| Hypertension (yes/no)              |                        | 3/10                     |                          | 3/15                         |
| Hyperlipidemia (yes/no)            |                        | 7/6                      |                          | 12/6                         |
| Smoking (yes/no)                   |                        | 4/9                      |                          | 4/14                         |
| Drinking (yes/no)                  |                        | 3/10                     |                          | 3/15                         |
| Weight, kg                         | 73.7 ± 12.1            | 70.9 ± 13.7 <sup>b</sup> | 84.3 ± 14.9 <sup>a</sup> | 79.6 ± 14.3 <sup>a</sup>     |
| Body mass index, kg/m <sup>2</sup> | 25.6 ± 3.1             | 24.6 ± 3.4               | 32.3 ± 12.2 <sup>a</sup> | 28.2 ± 3.9 <sup>a</sup>      |
| Waist circumference, cm            | 90.8 ± 8.9             | 84.5 ± 7.8 <sup>b</sup>  | 96.5 ± 7.0 <sup>a</sup>  | 90.4 ± 11.3 <sup>a</sup>     |
| Systolic blood pressure, mmHg      | 117.9 ± 13.9           | 113.9 ± 8.9              | 119.1 ± 10.3             | 117.1 ± 10.5                 |
| Diastolic blood pressure, mmHg     | 65.5 ± 9.7             | 69.8 ± 9.9               | 70.2 ± 6.3               | 72.1 ± 8.5                   |
| Total cholesterol, mmol/l          | 5.41 ± 0.86            | 4.64 ± 1.52              | 4.93 ± 0.85              | 4.30 ± 0.89 <sup>b</sup>     |
| Triglycerides, mmol/l              | 2.37 ± 1.47            | 1.79 ± 0.63              | 2.37 ± 1.29              | 1.49 ± 0.67 <sup>b</sup>     |
| HDL-C, mmol/l                      | 1.22 ± 0.25            | 1.27 ± 0.26              | 0.96 ± 0.20 <sup>a</sup> | 1.02 ± 0.16 <sup>a</sup>     |
| LDL-C, mmol/l                      | 3.36 ± 0.76            | 3.20 ± 0.81              | 3.14 ± 0.70              | 2.76 ± 0.74 <sup>b</sup>     |
| C reactive protein, mg/l           | 1.19 (0.13–20.29)      | 1.14 (0.46–2.24)         | 1.96 (0.17–8.81)         | 2.45 (1.3–17.5) <sup>a</sup> |
| Homocysteine, μmol/l               | 12.4 (10.8–13.7)       | 11.9 (9.8–14.9)          | 12.7 (9.4–13.2)          | 12.6 (9.6–13.9)              |
| Fasting blood glucose, mmol/l      | 7.90 ± 1.23            | 5.95 ± 1.41 <sup>b</sup> | 8.63 ± 2.09              | 6.54 ± 1.31 <sup>b</sup>     |
| Hb A <sub>1c</sub> , %             | 7.52 ± 1.33            | 6.40 ± 0.62 <sup>b</sup> | 7.91 ± 1.09              | 6.13 ± 0.56 <sup>b</sup>     |

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Hb A<sub>1c</sub>, hemoglobin A<sub>1c</sub>. Categorical data were analyzed by  $\chi^2$  test. Plasma levels of C-reactive protein and homocysteine did not follow a normal distribution, were expressed as medians and interquartile ranges, and were assessed by nonparametric tests (Mann-Whitney *U*-test). Other continuous variables are expressed as means ± SD. Intragroup differences were evaluated using the paired-samples *t*-test. The independent-samples *t*-test was used to compare the mean values of parameters between 2 groups. <sup>a</sup>*P* < 0.05 compared with control group; <sup>b</sup>*P* < 0.05 compared with baseline.

or SQ-22536 (100 μmol/l). Cells were washed twice with ice-cold PBS, lysed in lysis buffer (300 μl/3 × 10<sup>6</sup> cells), and subjected to three freeze-thaw cycles. Intracellular cAMP levels were determined using a cAMP assessment kit (R & D Systems) according to the manufacturer's instructions.

**Western blot.** HUVECs were lysed using cell lysis buffer (Applygen Technologies, Beijing, China) containing protease inhibitor cocktail (Roche, Basel, Switzerland). The protein content was assayed by a BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA). Proteins of the cell lysate were separated by SDS-PAGE and transferred from the gel to a nitrocellulose membrane. After incubation in blocking solution (5% BSA in TBS-T; Sigma), membranes were incubated with primary antibodies (all at 1:1,000 dilutions) overnight at 4°C, followed by incubation with IRDye 800CW-conjugated goat anti-rabbit or anti-mouse IgG (1:10,000; LI-COR Biosciences, Lincoln, NE) according to primary antibodies. Protein bands were visualized by an Odyssey Infrared Imaging System (LI-COR Biosciences), and the intensity of each band was measured using the accompanying software. The following primary antibodies were used: anti-eNOS monoclonal antibody, anti-phospho-Ser<sup>1177</sup> eNOS antibody (BD Biosciences), anti-AMPK antibody, anti-phospho-Thr<sup>172</sup> AMPK antibody, anti-Akt antibody, anti-phospho-Ser<sup>473</sup> Akt antibody (all from Cell Signaling Technology), anti-GTPCH1 antibody (Abnova, Taibei, China; or Abcam, Cambridge, UK), and anti-GAPDH antibody (ComWin Biotech, Beijing, China).

#### Statistical Analysis

Categorical data (sex difference, hypertension, hyperlipidaemia, smoking, and drinking) were analyzed by  $\chi^2$  test. Plasma levels of C-reactive protein and homocysteine did not follow a normal distribution and were expressed as medians and interquartile ranges and were assessed by nonparametric tests (Mann-Whitney *U*-test). Other continuous variables were normally distributed, and their values were expressed as means ± SD. Intragroup differences were evaluated using the paired-samples *t*-test. The independent-samples *t*-test was used to compare the mean values of parameters between two groups. Association of CFVR with the anthropometric and biochemical pa-

rameters was examined by multivariate linear regression analysis. For the in vitro studies, all experiments were performed four times with each group in triplicate. Data were presented as means ± SE. Data of multiple groups were compared by one-way ANOVA followed by the post hoc Tukey-Kramer test. *P* < 0.05 (2-tailed) was considered statistically significant. All analyses were performed using SPSS 16.0J for Windows (SPSS Japan, Tokyo, Japan).

## RESULTS

### Clinical Characteristics and Metabolic Parameters at Baseline and at the 12th Week After Treatment in Patients with Type 2 Diabetes

During the period of July 2011 to June 2012, a total of 36 potentially eligible participants were registered and assigned, with 17 subjects in the control group and 19 in exenatide group. In the control group, 3 subjects (17.65%) had poor compliance, one (5.88%) chose to withdraw, and 13 (76.47%) completed the treatment and measurement. In the exenatide group, one subject (5.26%) withdrew consent, and 18 (94.74%) completed the treatment and measurement. The clinical characteristics and metabolic parameters at baseline and posttreatment are summarized in Table 1. The body weight, body mass index, and waist circumferences were significantly higher, and HDL-C was significantly lower in the exenatide group than in the control group at baseline. The subjects were likely younger, and there seemed to be a higher proportion of hyperlipidemia and a lower proportion of smoking in the exenatide group, although there was no statistical significance. After 12 wk of exenatide treatment, glycemic levels (as determined by Hb A<sub>1c</sub> and fasting blood glucose) were significantly improved (*P* < 0.05) compared with the baseline levels in both groups. Compared with the baseline levels, serum total cholesterol, triglycerides, and LDL-C levels at the end of the 12-wk exenatide



treatment were significantly decreased ( $P < 0.05$ ); a reduction in waist circumference (from  $96.5 \pm 7.0$  to  $90.4 \pm 11.3$  cm,  $P = 0.08$ ) and an elevation in HDL-C (from  $0.96 \pm 0.20$  to  $1.02 \pm 0.16$  mmol/L,  $P = 0.06$ ) demonstrated marginal significance; and body weight, body mass index, blood pressure, and plasma homocysteine level were not strikingly altered in the exenatide group.

#### Effects of Exenatide Treatment on Coronary Endothelial Function in Patients With Type 2 Diabetes

After 12 wk of exenatide treatment, CFVR was significantly improved in the exenatide group compared with the baseline (from  $2.89 \pm 0.60$  to  $3.36 \pm 0.58$ ,  $P < 0.05$ ) and the control group ( $3.36 \pm 0.58$  vs.  $3.03 \pm 0.45$ ,  $P < 0.05$ ). An increased CFVR level, although not statistically significant, was also apparent in the control group (Fig. 1A). Conversely, the serum level of vascular adhesion molecule sICAM-1 was significantly reduced after 12 wk of exenatide treatment compared with that of the baseline (from  $287.0 \pm 44.6$  to  $229.7 \pm 45.5$  ng/mL,  $P = 0.0002$ ). Concurrently, a significantly reduced sVCAM-1 level was also observed (from  $491.4 \pm 163.4$  to  $438.6 \pm 149.0$  ng/mL,  $P = 0.0047$ ) at the end of the 12-wk exenatide treatment. However, the levels of sICAM-1 and sVCAM-1 did not change much before or after treatment in the control group (Fig. 1, B and C).

#### Association of CFVR with the Anthropometric and Biochemical Parameters

We next investigated the relationship between CFVR and various other parameters. Although CFVR did not show any correlation with all the parameters in whole subjects (data not shown), it was negatively correlated with Hb A<sub>1c</sub> and positively with HDL-C in the exenatide group (Fig. 2 and Table 2). We also performed multiple stepwise regression analysis to determine the variables that were independently associated with CFVR. The results showed that Hb A<sub>1c</sub> and HDL-C were the factors independently related to CFVR (Table 2). The multiple regression equation was:  $Y_{CFVR} = 2.986 + 1.341X_{HDL-C} - 0.169X_{HbA_{1c}}$ . However, these two factors accounted for only the partial effects on CFVR ( $r^2 = 0.288$ ,  $P = 0.037$ ), indicating that other factors may also function on CFVR.

#### Exenatide Increases NO Production and Upregulates GTPCH1 Level and eNOS Phosphorylation in HUVECs

Incubation of HUVECs with 1–50 nmol/L exenatide (a form of exenatide) for 24 h induced a dose-dependent increase in NO production of  $\leq 20$  nmol/L exenatide (Fig. 3A). Similarly, incubation with exenatide (1–20 nmol/L) for 15 min induced the phosphorylation of eNOS in a dose-dependent manner. The activation of eNOS could be observed as early as 10 min after the administration of exenatide (at 20 nmol/L) and reached its maximum at 15 min. Additionally, the total eNOS level was stable at the time points of interest (Fig. 3, B and C). Exenatide administered at doses ranging from 1 to 20 nmol/L for 24 h significantly increased the level of GTPCH1 (Fig. 3D). The time course of exenatide-induced upregulation of GTPCH1 was also examined over a range of 6 to 48 h. Exenatide enhanced GTPCH1 levels starting from 6 h and reaching a peak at 24 h (Fig. 3E).

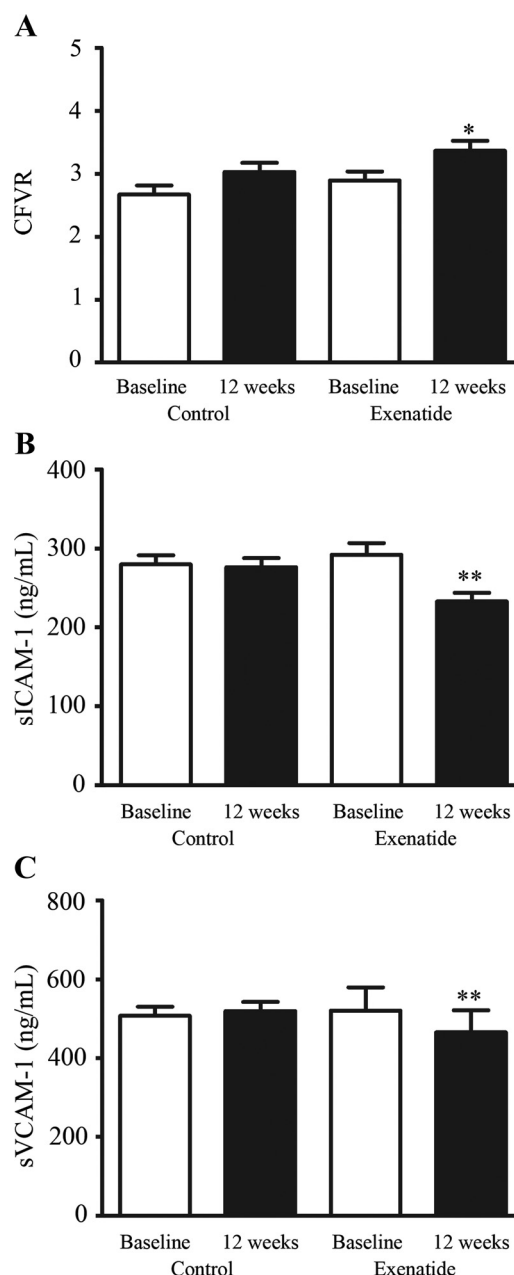


Fig. 1. Effects of 12-wk treatment on coronary flow velocity reserve (CFVR) and circulating levels of soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in patients with type 2 diabetes. A: CFVR, detected by transthoracic Doppler echocardiography. B: sICAM-1. C: sVCAM-1. Values are expressed as means  $\pm$  SD. Intragroup differences were evaluated using the paired-samples *t*-test. The independent-samples *t*-test was used to compare the mean values of parameters between two groups. \* $P < 0.05$  and \*\* $P < 0.01$  compared with baseline.

#### Exenatide Increases NO production, GTPCH1 Level, and eNOS Activation Through GLP-1R/cAMP Signaling Pathways

In our study, GLP-1R mRNA and protein were detected in HUVECs (data not shown). It has been shown that GLP-1R and its downstream signaling are known as the mediators in most of the GLP-1 actions. Therefore, we determined whether GLP-1R was involved in the exenatide-mediated endothelial

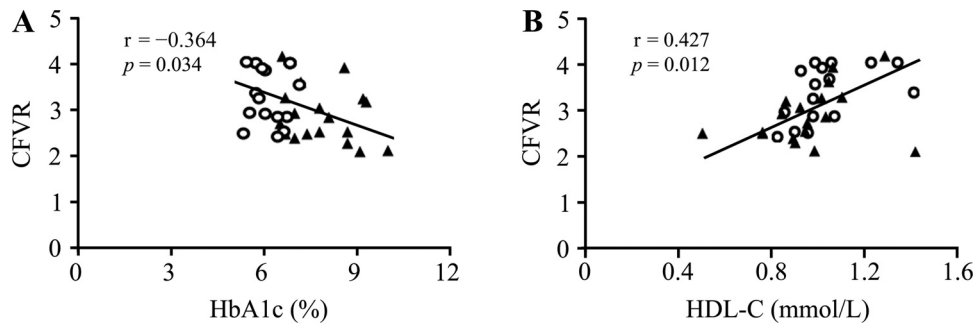


Fig. 2. Association of CFVR with hemoglobin A<sub>1c</sub> (Hb A<sub>1c</sub>) and high-density lipoprotein cholesterol (HDL-C) in the exenatide treatment group. A: CFVR was negatively correlated with Hb A<sub>1c</sub>. B: CFVR was positively correlated with HDL-C. ▲, Baseline; ○, posttreatment.

protective effects. As mentioned above, exendin-4 increased NO production, upregulated GTPCH1 level, and promoted eNOS activation in HUVECs (Fig. 3). GLP-1R antagonist exendin (9–39) (200 nmol/l) or GLP-1R siRNA blocked the effects of exendin-4 (Fig. 4, A–F), suggesting that the above effects of exendin-4 were GLP-1R dependent. To further determine the function of GLP-1R in the protective effects of exendin-4 in HUVECs, we detected the effects of GLP-1 (7–36) amide, an active form of GLP-1 with high affinity for GLP-1R, and GLP-1 (9–36) amide, an NH<sub>2</sub>-terminal truncated metabolite of the active GLP-1 formed by dipeptidyl peptidase-4 cleavage. Results showed that GLP-1 (7–36) amide displayed similar influences as exendin-4, demonstrated by upregulation of NO production, increment of GTPCH1 level, and activation of eNOS, whereas GLP-1 (9–36) amide had no such effects (Fig. 4, A–C). All of these results suggested that GLP-1R had an important role in the endothelial protective effects of exendin-4.

To confirm that GLP-1R in the endothelial cells was functional, we assessed cAMP production after exendin-4 stimulation. Results showed that incubation of HUVECs with 1–50 nmol/l exendin-4 for 15 min induced a dose-dependent increase of intracellular cAMP concentrations of  $\leq 20$  nmol/l exendin-4 (Fig. 4G). Besides, 20 nmol/l exendin-4 could upregulate cAMP levels and reached its maximum as early as 5 min after administration (Fig. 4H). Not surprisingly, the

GLP-1R antagonist exendin (9–39) (200 nmol/l) or adenylyl cyclase inhibitor SQ-22536 (100  $\mu$ mol/l) attenuated the effect of exendin-4 (20 nmol/l) on the upregulation of cAMP levels, and the adenylyl cyclase activator forskolin (10  $\mu$ mol/l) increased cAMP levels significantly (Fig. 4I). Notably, the adenylyl cyclase inhibitor SQ-22536 (100  $\mu$ mol/l) blocked the effect of exendin-4 on the upregulation of NO production, increment of GTPCH1 level, and activation of eNOS (Fig. 4, J–L), whereas the adenylyl cyclase activator forskolin (10  $\mu$ mol/l) showed similar effects with exendin-4, suggesting that the above effects of exendin-4 were cAMP signaling dependent.

#### Exendin-4 Induces GTPCH1 Upregulation and eNOS Activation in HUVECs Via AMPK and PI3K/Akt Pathways

As shown in Fig. 5, A and B, exendin-4 induced AMPK and Akt phosphorylation in a time-dependent manner. However, exendin-4 did not result in significant changes in total Akt or AMPK level at the time points of interest. Since exendin-4 induced phosphorylation of AMPK and Akt, we investigated the roles of these kinases in the exendin-4-induced activation of eNOS using the specific AMPK inhibitor compound C or the PI3K inhibitor LY-294002. As shown in Fig. 5C, exendin-4 stimulated phosphorylation of eNOS, which was blocked by compound C (10  $\mu$ mol/l) or LY-294002 (10  $\mu$ mol/l), indicating a requirement of these kinases in the exendin-4-induced eNOS activation. Consistent with the above results, the level of GTPCH1 was dramatically decreased after exposure to compound C or LY-294002 compared with administration of exendin-4 alone. (Fig. 5D).

#### Exendin-4 Protects Against Homocysteine-Induced Endothelial Dysfunction in HUVECs

In our previous study, CFVR was significantly lower in patients with hyperhomocysteinemia, and plasma homocysteine levels were negatively correlated with CFVR, indicating that homocysteine might impair coronary artery endothelial function (18). Therefore, in this study, we used homocysteine to induce endothelial dysfunction in HUVECs and investigated whether the protective effect of exendin-4 on endothelial function was also found in the in vitro injury model. As expected, homocysteine induced endothelial dysfunction, including increased intracellular ROS generation, decreased NO production, and modestly reduced phosphorylation of eNOS (Fig. 6, A–D). Moreover, homocysteine also increased the sICAM-1 levels of the cell supernatant (Fig. 6E). Exendin-4 could reverse the homocysteine-induced endothelial dysfunction

Table 2. Linear and multiple regression analysis of variables associated with CFVR in subjects treated with exenatide

| Variable                           | Simple   |          | Multiple |          |
|------------------------------------|----------|----------|----------|----------|
|                                    | <i>r</i> | <i>P</i> | <i>b</i> | <i>P</i> |
| Body mass index, kg/m <sup>2</sup> | −0.082   | 0.646    | —        | —        |
| Waist circumference, cm            | −0.107   | 0.553    | —        | —        |
| Systolic blood pressure, mmHg      | 0.025    | 0.888    | —        | —        |
| Diastolic blood pressure, mmHg     | 0.061    | 0.730    | —        | —        |
| Total cholesterol, mmol/l          | −0.200   | 0.258    | —        | —        |
| Triglycerides, mmol/l              | −0.165   | 0.358    | —        | —        |
| HDL-C, mmol/l                      | 0.427    | 0.012    | 1.341    | 0.019    |
| LDL-C, mmol/l                      | −0.092   | 0.605    | —        | —        |
| Fasting blood glucose, mmol/l      | −0.103   | 0.564    | —        | —        |
| Hb A <sub>1c</sub> , %             | −0.364   | 0.034    | −0.169   | 0.042    |
| sICAM-1, ng/ml                     | −0.098   | 0.650    | —        | —        |
| sVCAM-1, ng/ml                     | −0.041   | 0.851    | —        | —        |

CFVR, coronary flow velocity reserve; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1. In multiple linear stepwise regression analysis, variables included for analysis were waist circumference, systolic blood pressure, total cholesterol, triglycerides, HDL-C, LDL-C, Hb A<sub>1c</sub>, sICAM-1, and sVCAM-1.

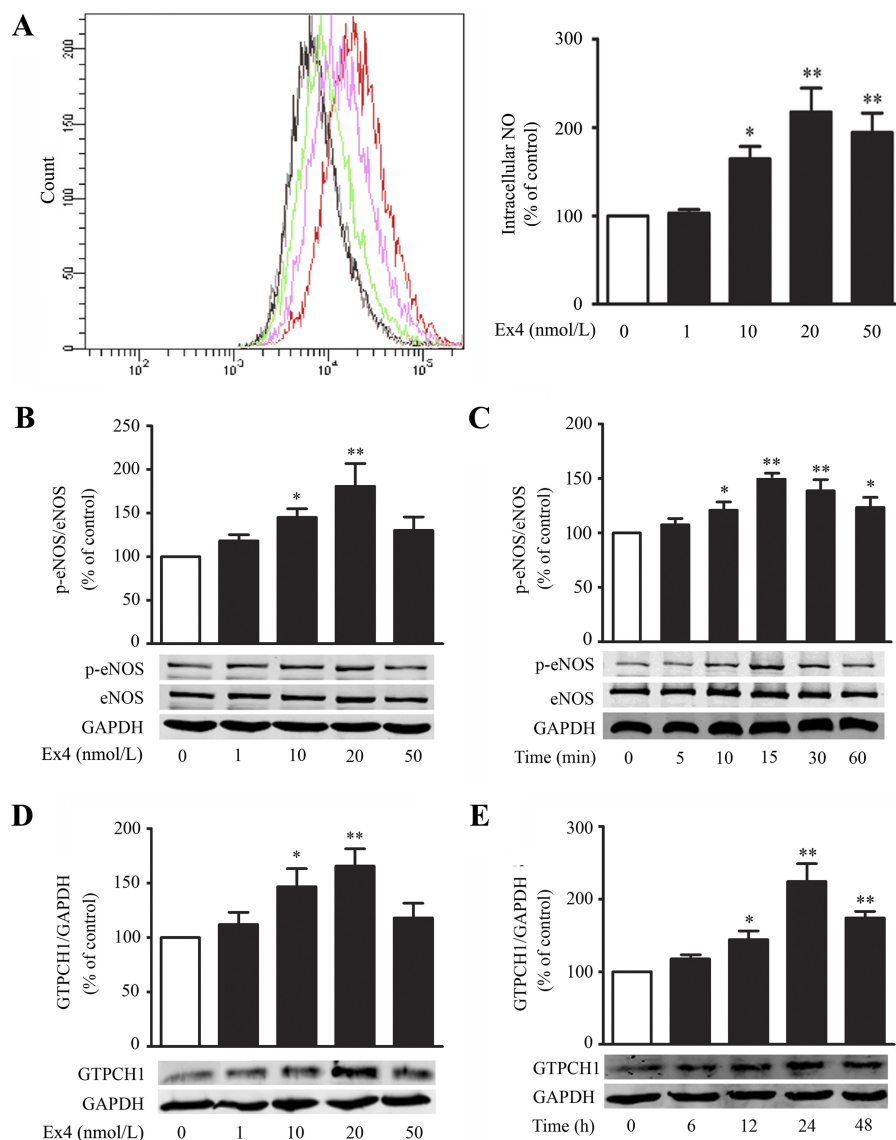


Fig. 3. Exendin-4 (Ex-4; a form of exenatide) increases nitric oxide (NO) production, endothelial nitric oxide synthase (eNOS) phosphorylation, and GTP cyclohydrolase 1 (GTPCH1) level in human umbilical vein endothelial cells (HUVECs). **A:** Ex-4 dose-dependently increased NO production in HUVECs, as detected by flow cytometry analysis. Black line, control; gray line, 1 nmol/L; green line, 10 nmol/L; red line, 20 nmol/L; purple line, 50 nmol/L. **B:** dose-dependent stimulation of Ex-4 on eNOS phosphorylation (p-eNOS) after 15-min incubation. **C:** time course of Ex-4 (20 nmol/L) on eNOS phosphorylation. **D:** dose-dependent stimulation of Ex-4 on GTPCH1 level after 24-h incubation. **E:** time course of Ex-4-induced GTPCH1 upregulation. Values are expressed as means  $\pm$  SE. All experiments were performed 4 times with each group in triplicate. Data were compared by 1-way ANOVA, followed by the post hoc Tukey-Kramer test. \* $P < 0.05$  and \*\* $P < 0.01$  compared with the control group.

(Fig. 6, A–E). Furthermore, the GLP-R antagonist exendin (9–39) was able to diminish the protective effects of exendin-4 on the endothelial dysfunction (Fig. 6, A–E), suggesting that the protective effects of exendin-4 in the homocysteine-induced endothelial injury model were also GLP-1R dependent.

## DISCUSSION

Our clinical study demonstrated that exenatide improved coronary artery endothelial function reflected by increased CFVR and reduced inflammatory markers of sICAM-1 and sVCAM-1 in newly diagnosed patients with type 2 diabetes. In our in vitro study, exendin-4 (a form of exenatide) increased NO production, eNOS phosphorylation, and GTPCH1 level in the cultured HUVECs. Addition of the GLP-1R antagonist exendin (9–39) or GLP-1R siRNA, adenylyl cyclase inhibitor SQ-22536, AMPK inhibitor compound C, and PI3K inhibitor LY-294002 to the culture cells completely diminished the effects of exendin-4 on NO production, eNOS phosphorylation, and GTPCH1 level, respectively. These results indicate that exenatide treatment improves coronary endothelial func-

tion in patients with type 2 diabetes. This effect may be mediated primarily through activation of the AMPK and PI3K/Akt pathways and subsequent upregulation of GTPCH1 level and improvement of eNOS coupling in a GLP-1R/cAMP-dependent manner.

FMD is designated as an indicator for the assessment of endothelial function in different clinical study populations (31). Although FMD is recognized as an independent predictor for future cardiovascular events, it has potential technical variations. CFVR assessed by transthoracic Doppler echocardiography is an effective method to evaluate coronary endothelial function (21). Our previous studies demonstrated a positive association between CFVR and endothelial function in patients either with type 2 diabetes-associated coronary artery disease or with hyperhomocysteinemia (18, 27, 39). CFVR could be upregulated by some hypoglycemic and lipid-lowering drugs such as the peroxisome proliferator-activated receptor (PPAR)- $\gamma$  agonist rosiglitazone and the PPAR $\alpha$  agonist fenofibrate (35, 40). In this study, we found that after 12 wk of exenatide treatment, endothelial function of the coronary ar-

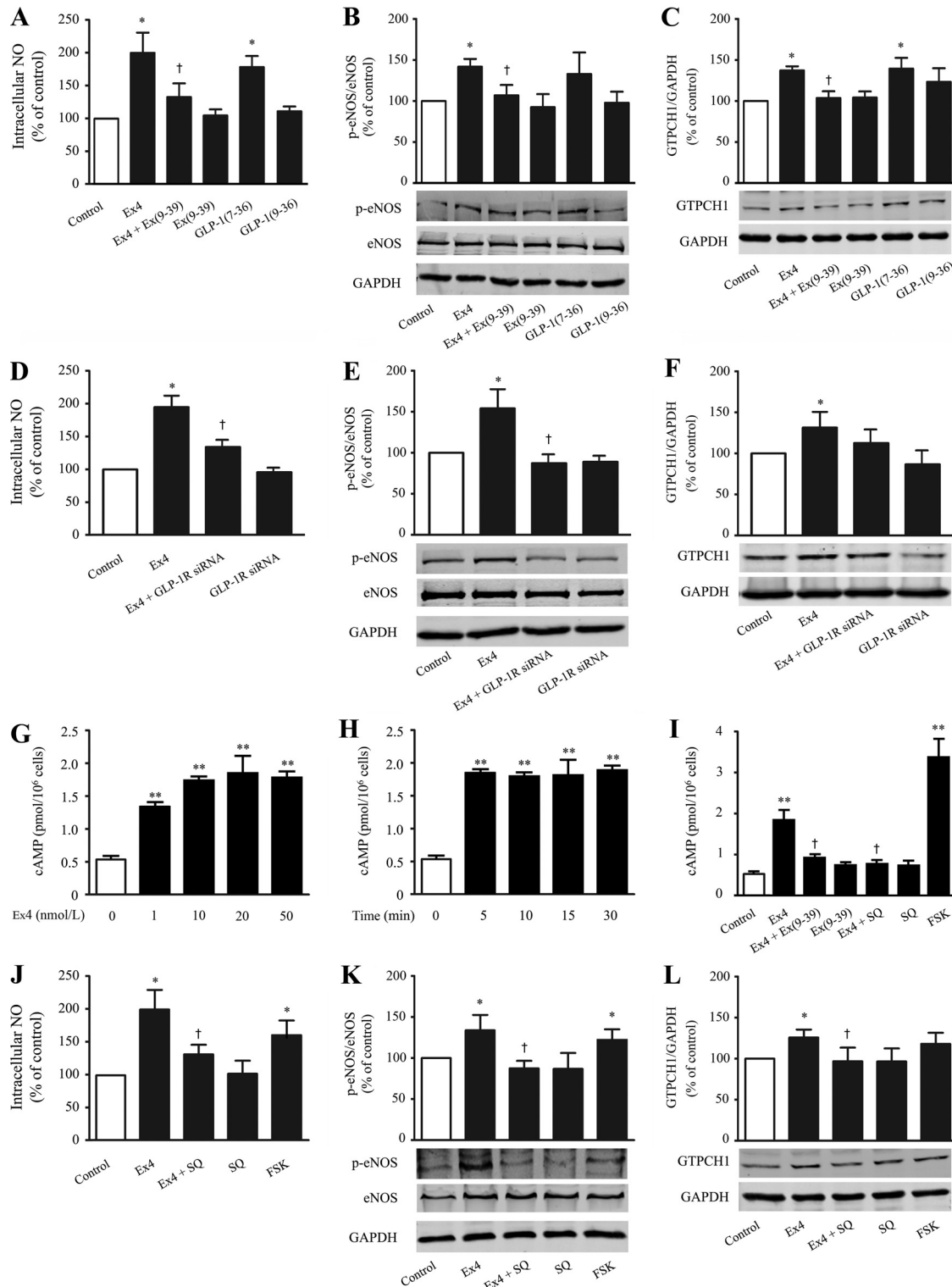


Fig. 4. Ex-4 increases NO production, p-eNOS, and GTPCH1 level via activation of GLP-1 receptor (GLP-1R)/cAMP signaling in HUVECs. A–C: effects of Ex-4 (20 nmol/l), GLP-1 (7–36) amide (100 nmol/l), or GLP-1 (9–36) amide (100 nmol/l) on intracellular NO levels (A), p-eNOS (B), and GTPCH1 level (C) with or without cotreatment of exendin (9–39) [Ex(9–39); 200 nmol/l], after 24-h incubation. D–F: effects of Ex-4 (20 nmol/l) with or without GLP-1R siRNA on intracellular NO levels (D), p-eNOS (E), and GTPCH1 level (F) after 24-h incubation. G: dose-dependent stimulation of Ex-4 on intracellular cAMP levels after 15-min incubation. H: time course of Ex-4 (20 nmol/l) on intracellular cAMP levels. I: effects of Ex-4 (20 nmol/l) or forskolin (FSK; 10  $\mu$ mol/l) on intracellular cAMP levels in the presence or absence of Ex(9–39) (200 nmol/l) or SQ-22536 (SQ; 100  $\mu$ mol/l) after 15-min incubation. J–L: effects of Ex-4 (20 nmol/l) or FSK (10  $\mu$ mol/l) on intracellular NO levels (J), eNOS phosphorylation (K), and GTPCH1 level (L) in the presence or absence of SQ-22536 (100  $\mu$ mol/l) after 24-h incubation. Values are expressed as means  $\pm$  SE. All experiments were performed 4 times with each group in triplicate. Data were compared by 1-way ANOVA followed by the post hoc Tukey-Kramer test. \* $P$  < 0.05 and \*\* $P$  < 0.01 compared with the control group; † $P$  < 0.05 compared with the Ex-4 treatment group.



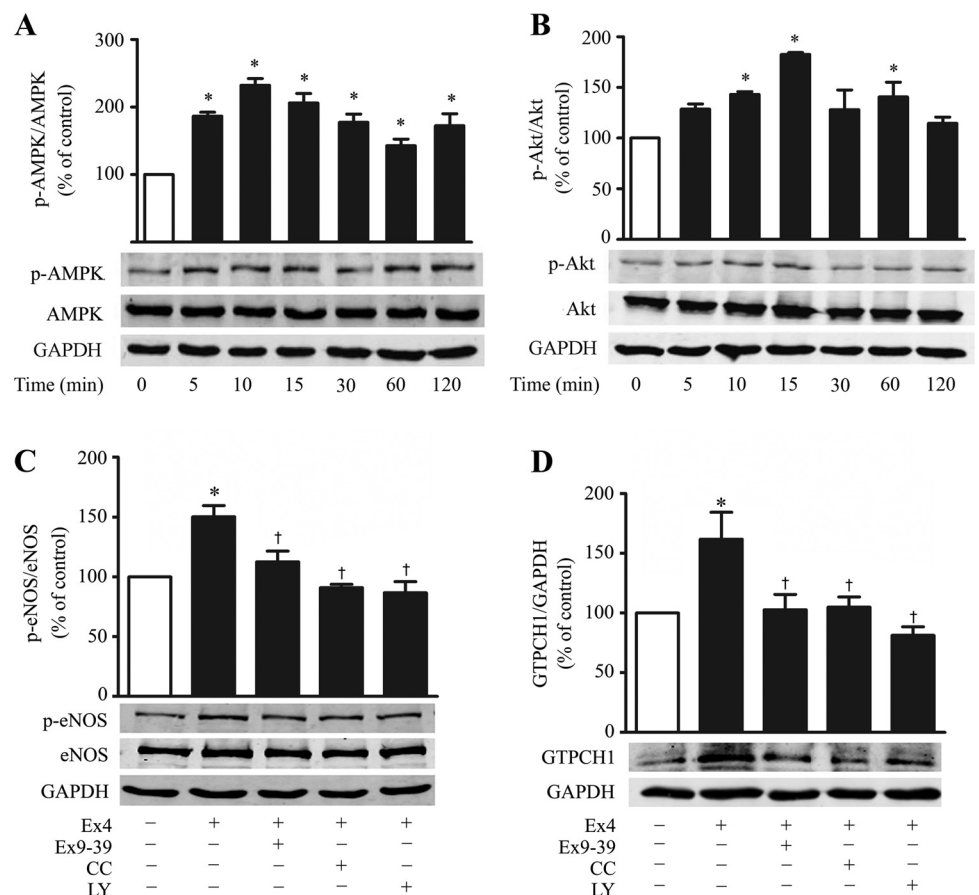


Fig. 5. Ex-4 activates AMP-activated protein kinase (AMPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways and subsequently upregulates GTPCH1 level and p-eNOS HUVECs. *A* and *B*: time course of Ex-4 (20 nmol/l) on AMPK (*A*) and Akt (*B*) phosphorylation. *C* and *D*: effects of Ex-4 (20 nmol/l) on p-eNOS (*C*) and GTPCH1 level (*D*), with cotreatment of Ex(9–39) (200 nmol/l) or compound C (CC; an AMPK inhibitor) (10  $\mu$ mol/l) or LY-294002 (LY; a PI3K inhibitor) (10  $\mu$ mol/l). Values are expressed as means  $\pm$  SE. All experiments were performed 4 times with each group in triplicate. Data were compared by 1-way ANOVA followed by the post hoc Tukey-Kramer test. \* $P$  < 0.05 compared with the control group; † $P$  < 0.05 compared with the exenatide treatment group.

teries was significantly improved compared with the baseline levels. To our knowledge, this is the first study to investigate the effects of GLP-1-based therapy on coronary endothelial function determined by CFVR. Since obesity, high blood glucose, and dyslipidemia are often associated with type 2 diabetes, improved glycemic control and lipid profiles often result in prevention of cardiovascular diseases, which is usually achieved by an increased endothelial function. In the present study, we found that after 12 wk of exenatide treatment, total cholesterol, triglycerides, LDL-C, fasting blood glucose, and Hb A<sub>1c</sub> levels were significantly reduced in the exenatide group. In addition, after exenatide treatment, a decline in body weight, body mass index, or waist circumference, although not statistically significant, was consistent with previous exenatide trials demonstrating significant weight loss (9, 32). Furthermore, we also found that CFVR was negatively correlated with Hb A<sub>1c</sub> and positively with HDL-C, indicating that improved glycemic control and lipid profiles might participate in the improvement of CFVR due to exenatide treatment. Nevertheless, although improved glycemic control and lipid profiles also occurred in the control group, CFVR was unchanged after 12 wk of treatment, suggesting that other factors may contribute to the improvement of CFVR in the exenatide group.

GLP-1 has been shown to have direct beneficial effect on vascular endothelium in several human studies. GLP-1 infusion enhanced the endothelial vasodilation in healthy subjects (8) and significantly increased brachial artery flow and its diameter without affecting serum glucose in type 2 diabetic

patients with stable coronary disease (30). Unlike metformin, exenatide treatment for 16 wk induced a significant improvement in brachial artery endothelial function in the type 2 diabetic patients without an adequate glycemic control (22). These studies differ from our present study in that they measured brachial artery endothelial function instead of coronary artery endothelial function.

Expression of adhesion molecules in endothelial cells plays a critical role in the pathogenesis of atherosclerosis. Endothelial function may be assessed by measuring plasma levels of endothelial products such as soluble adhesion molecules. It has been shown that vascular adhesion molecules, including sICAM-1 and sVCAM-1, are elevated in patients with diabetes (33). GLP-1 and its analogs downregulate adhesion molecules and nuclear factor- $\kappa$ B or activator protein-1 signaling, which are the major redox-sensitive transcriptional pathways responsible for proinflammatory cytokine expression in human vascular endothelial cells and in animal models and diabetic patients (12, 16, 24, 25). In this study, we showed that treatment with exenatide for 12 wk suppressed systemic inflammation in patients with type 2 diabetes by reducing circulating levels of sICAM-1 and sVCAM-1. These findings suggest that exenatide treatment in patients with type 2 diabetes may confer antiatherogenic effects.

There are some limitations in the clinical study. First, our study is an open-labeled paralleled study rather than a double-blinded randomised controlled trial. The baseline demographic and clinical characteristics between two groups did not match well. Second, sample size is relatively small so that our study



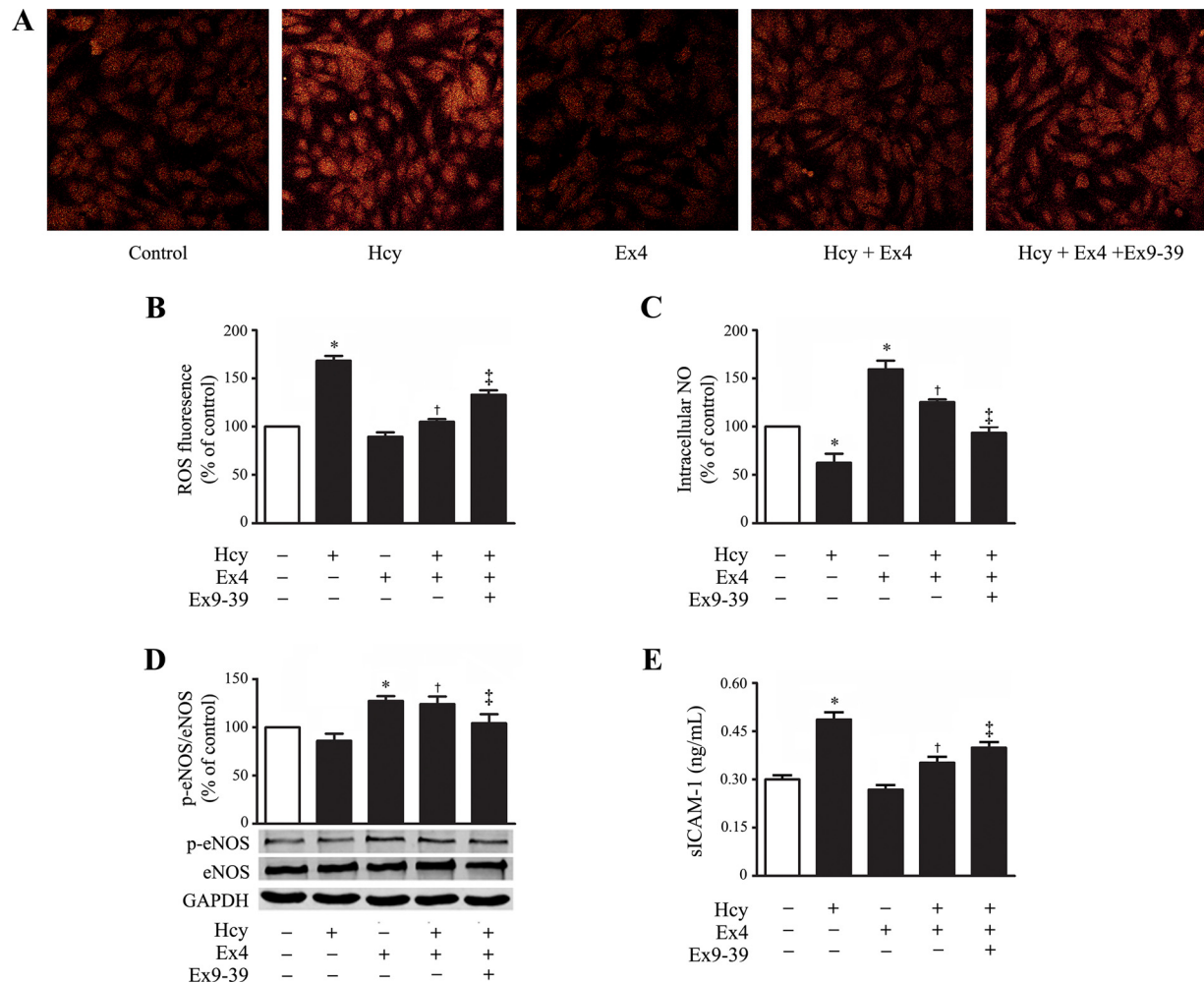


Fig. 6. The protective effects of Ex-4 on homocysteine (Hcy)-induced endothelial dysfunction in HUVECs. **A**: representative photographs of reactive oxygen species (ROS) analysis. **B**: quantification of ROS fluorescence. **C**: NO production detected by flow cytometry analysis. **D**: p-eNOS. **E**: sICAM-1 levels in cell supernatant detected by ELISA. Values are expressed as means  $\pm$  SE. All experiments were performed 4 times with each group in triplicate. Data were compared by 1-way ANOVA followed by the post hoc Tukey-Kramer test. \* $P < 0.05$  compared with the control group; † $P < 0.05$  compared with the Hcy treatment group; ‡ $P < 0.05$  compared with the Hcy + Ex-4 treatment group.

may be not powerful enough to account for potentially confounding factors in our analysis. Nevertheless, even based on the data in any large-scale randomized controlled trial, we are still unable to make the conclusion that exenatide has direct effects on endothelial cells, owing to the complicated regulation in vivo. Therefore, we performed the in vitro experiment to investigate the direct protective effect of exenatide in the cultured primary HUVECs and elucidated the underlying molecular and signaling mechanisms.

Endothelium-derived NO is a potent vasodilator and possesses various vasoprotective effects. Ample evidence indicates that NO-dependent vasodilation is impaired in the coronary arteries and contributes to vascular resistance in patients with diabetes (17, 20). Several studies have shown that GLP-1 and its analogs have beneficial effects on vascular endothelium via increased eNOS phosphorylation and NO production (15, 41). In our in vitro experiment, we showed that exenatide significantly enhanced the phosphorylation of eNOS and increased the NO production in cultured primary HUVECs, which are consistent with previous reports (15, 41).

The expression of GTPCH1, which is the rate-limiting enzyme in BH4 synthesis, may play an important role in regulating NO-mediated endothelial function (10, 37). A reduced expression or activity of GTPCH1 has been shown in the coronary endothelial cells of diabetic rats and the aortas of insulin-resistant rats, resulting in diminished BH4 levels (11). Gene transfer of GTPCH1 increased BH4 levels and eNOS activity in the human aortic endothelial cells incubated with high glucose and in diabetic mice (3). It has been reported that atorvastatin improves vascular BH4 bioavailability by upregulating GTPCH1 gene expression and activity, resulting in an improved endothelial function in patients with coronary artery disease (5). Another study suggested that metformin improved endothelial function by suppressing 26S proteasome-mediated GTPCH1 degradation to increase the level of BH4 (36). Furthermore, we reported previously that PPAR $\alpha$  agonist fenofibrate upregulated BH4 level by increasing the expression of GTPCH1 in HUVECs (26). Our results suggested that exenatide increased the level of GTPCH1 in a dose- and time-dependent manner and thus might lead to improvement of the

downstream cascades, such as the upregulation of BH4 level, the coupling of eNOS, and increment of NO production. These findings provide new insights into the mechanisms underlying the coronary artery benefit of exenatide in patients with type 2 diabetes. Nevertheless, in our study, exendin-4 promoted eNOS phosphorylation as soon as in 10–15 min and lasted at least 24 h, whereas the upregulation of GTPCH1 level required 12–24 h. This suggests that activation of eNOS is not dependent only on GTPCH1-derived BH4. Exendin-4 may also have direct effects on eNOS activation.

Although GLP-1 has direct beneficial effects on myocardial and endothelial cells mediated via GLP-1R-independent pathways (1, 7), most actions of GLP-1 are mediated via the GLP-1R/cAMP pathway (14–16, 23, 31). Our data showed that exendin-4 increased intracellular cAMP level in a dose- and time-dependent manner, indicating that GLP-1R in HUVECs was functional upon exendin-4 stimulation. Similarly to exendin-4, GLP-1 (7–36) amide, an active form of GLP-1, upregulated intracellular NO levels, eNOS phosphorylation, and GTPCH level. However, GLP-1 (9–39) amide, a metabolite of the active GLP-1, had no obvious protective effects on HUVECs. Moreover, the GLP-1R antagonist exendin (9–39) or GLP-1 siRNA and the adenylyl cyclase inhibitor SQ-22536 reversed the above protective effects of exendin-4. These results suggest that the effect of exendin-4 is GLP-1R/cAMP dependent.

Several lines of evidence support the involvement of AMPK and Akt in mediating the beneficial effects of GLP-1 and its analogs on vascular function and eNOS phosphorylation (15, 24, 38). It has been demonstrated that incubation of bovine aortic endothelial cells with GLP-1 increases Akt phosphorylation and subsequently stimulates eNOS phosphorylation (13). Another study showed that exendin-4 stimulated proliferation of human coronary artery endothelial cells via activation of the PI3K/Akt pathway (15). In addition, it has been reported that liraglutide, another GLP-1 analog, exerts anti-inflammatory effect on endothelial cells and improves cardiac function via AMPK pathway (24). Our results demonstrated that exendin-4 could activate AMPK and Akt phosphorylation. The AMPK inhibitor compound C and PI3K inhibitor LY-294002 blocked the effects of exendin-4 on eNOS activation and GTPCH1 upregulation. These results suggest that both AMPK and PI3K/Akt pathways might be involved in the exendin-4-mediated endothelial function improvement.

In our previous study, decreased plasma levels of NO and BH4 and impaired coronary artery endothelial function were found in patients with hyperhomocysteinemia (18). Moreover, homocysteine has been shown to induce eNOS uncoupling through reduction of BH4 in cultured HUVECs (34). Therefore, in this study, homocysteine was used to induce endothelial dysfunction in HUVECs. We demonstrated that exendin-4 decreased supernatant sICAM-1 concentration and intracellular ROS level and increased intracellular NO production and eNOS phosphorylation in the homocysteine-induced endothelial injury model in a GLP-1R-dependent mechanism, similar to that found in the uninjured endothelial cells.

In conclusion, our study shows that the GLP-1 analog exenatide, currently in clinical use against type 2 diabetes, significantly improves coronary artery endothelial function (as indicated by CFVR) in patients with newly diagnosed type 2 diabetes. The improvement effect may be mediated through

activation of the AMPK/PI3K-Akt/eNOS pathway via a GLP-1R/cAMP-dependent mechanism. The study may prove beneficial in GLP-1-based treatment of type 2 diabetic patients, in whom endothelial dysfunction and coronary artery disease adversely affect their survival, and provide insight into a new molecular mechanism underlying the therapeutic effect of GLP-1 agents in the cardiovascular disease.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

R.W., S.M., C.W., J.K., W.L., and X.F. performed experiments; R.W., C.W., J.K., J.Y., Y.L., and W.H. analyzed data; R.W., S.M., J.Y., Y.L., W.H., X.F., G.W., and T.H. interpreted results of experiments; R.W. prepared figures; R.W. and S.M. drafted manuscript; R.W., S.M., C.W., J.K., J.Y., W.L., Y.L., W.H., X.F., G.W., and T.H. approved final version of manuscript; C.W., J.Y., and T.H. edited and revised manuscript; G.W. and T.H. contributed to conception and design of research.

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