Impaired cardiac microvascular function contributes to cardiovascular complications in diabetes. Glucagon-like peptide-1 (GLP-1) exhibits potential cardioprotective properties in addition to its glucose-lowering effect. This study was designed to evaluate the impact of GLP-1 on cardiac microvascular injury in diabetes and the underlying mechanism involved. Experimental diabetes was induced using streptozotocin in rats. Cohorts of diabetic rats received a 12-week treatment of vildagliptin (dipeptidyl peptidase-4 inhibitor) or exenatide (GLP-1 analog). Experimental diabetes attenuated cardiac function, glucose uptake, and microvascular barrier function, which were significantly improved by vildagliptin or exenatide treatment. Cardiac microvascular endothelial cells (CMECs) were isolated and cultured in normal or high glucose medium with or without GLP-1. GLP-1 decreased high-glucose–induced reactive oxygen species production and apoptotic index, as well as the levels of NADPH oxidase such as p47phox and gp91phox. Furthermore, cAMP/PKA (cAMP-dependent protein kinase activity) was increased and Rho-expression was decreased in high-glucose–induced CMECs after GLP-1 treatment. In conclusion, GLP-1 could protect the cardiac microvessels against oxidative stress, apoptosis, and the resultant microvascular barrier dysfunction in diabetes, which may contribute to the improvement of cardic function and cardiac microvascular dysfunction in diabetes. The protective effects of GLP-1 are dependent on downstream inhibition of Rho through a cAMP/PKA-mediated pathway. Diabetes 62:1697–1708, 2013

Diabetes is recognized as a major risk factor for cardiovascular disease, the leading cause for morbidity and mortality in the diabetic population (1). Diabetic cardiovascular disease results from many causes such as microangiopathy, myocardial metabolic abnormalities, and fibrosis (2,3). Under microangiopathy, the vessel wall of microvessels becomes thicker and vulnerable to bleeding, protein leakage, and slow blood flow. Accumulating evidence has demonstrated that microvascular injury plays a very important role in the diabetic cardiovascular dysfunction (4,5). However, there are still few effective strategies to prevent the progress of microvascular dysfunction in diabetes.

Glucagon-like peptide-1 (GLP-1), synthesized and secreted by intestinal L-cells, is a peptide with many protective biological functions. GLP-1 receptor (GLP-1R) is widely expressed in islet cells, kidney, lung, brain, and, interestingly, heart (6,7). It has been shown that administration of GLP-1 at the time of reperfusion was effective in decreasing myocardial infarct size in patients with acute myocardial infarction (8). Furthermore, a study of diabetic patients found that infusion of GLP-1 was associated with improved endothelial function (9). More importantly, recent data have suggested that GLP-1 was capable of exerting a direct cytoprotective effect against oxidative stress in diabetic mice aorta (10). Therefore, GLP-1 is of potential interest as a possible therapeutic regimen for treatment of cardiac microvascular injury in diabetes. However, native GLP-1 has a short half-life of minutes before getting degraded rapidly by dipeptidyl peptidase-4 (DPP-4) (11). To assess the potential role of intact GLP-1, DPP-4 inhibitors such as vildagliptin and GLP-1 analog exenatide are used as a means of preventing its degradation.

It is well-known that diabetes and hyperglycemia could increase intracellular reactive oxygen species (ROS), subsequently inducing apoptotic cell death, inflammation, and injury to endothelial cells (12,13). NADPH oxidase is the most important enzyme responsible for superoxide production in vasculature (14). Recent evidence demonstrates that Rho plays a regulatory role in oxidative stress in many diseases (15,16). Inhibition of Rho may result in the cardioprotective effect on cardiovascular remodeling associated with oxidative stress (17). Therefore, we hypothesize that GLP-1 could protect cardiac microvessels by inhibition of Rho and subsequently induce NADPH oxidase suppression.

To this end, this study was designed to determine the protective effects of GLP-1 on cardiac microvessels in diabetes and to characterize the underlying molecular mechanism. In vivo and in vitro studies were performed to assess cardiac function, glucose metabolism, and microvascular barrier function. The effects of GLP-1 on oxidative stress and apoptosis in microvessels were evaluated in isolated cardiac microvascular endothelial cells (CMECs). Furthermore, the underlying mechanism for GLP-1–induced protective effects was investigated. 

**RESEARCH DESIGN AND METHODS**

**Animal preparation for in vivo experiments.** Male Sprague-Dawley rats (weight, 220–250 g) were made diabetic using intraperitoneal injection of streptozotocin (35 mg/kg) for 3 days (18). Blood glucose levels were tested 1...
abetic rats were randomly assigned to the following groups: vehicle group (n = 15) that received daily treatment of vildagliptin at 1 mg/kg of body weight; exenatide group that received daily treatment of exenatide at 1 mg/kg of body weight; and insulin group that received daily treatment of insulin at 0.5 units, 1 units, 1.5 units, and 2 units. Blood glucose was measured once per day for 2 weeks. After having determined the dosage of insulin, diabetic rats were randomly assigned to the following groups: vehicle group (n = 15) that received daily treatment of vildagliptin at 1 mg/kg of body weight; exenatide group (n = 15) that received daily treatment of exenatide (Tocris Bioscience, Minneapolis, MN) at 1 mg/kg of body weight for 12 weeks; and insulin group (n = 15) that received daily treatment of protamine zinc insulin (Jiangsu Wanbang Biopharma, China) at 1.5 units for 12 weeks. Age-matched normal rats (n = 15) were used as nondiabetic controls. All experiments were conducted under the National Institutes of Health Guidelines on the Use of Laboratory Animal Care and were approved by the Fourth Military Medical University Committee on Animal Care.

Measurement of blood pressure. Blood pressure was monitored with a tail-cuff system (Noninvasive Blood Pressure System; PanLab) as described previously (10). Briefly, rats were placed in a warm chamber (37 °C) for 10 min to rest, and blood pressures and pulse rates were recorded. Five readings were obtained from each rat.

Measurement of insulin levels and insulin resistance. We used commercially available ELISA kits to determine plasma insulin levels (Cusabio Biotech). The euglycemic-hyperinsulinemic clamp technique was performed to assess insulin resistance. Five days before clamp experiments, an intravenous catheter was implanted into the right jugular vein of rats under surgical anesthesia as previously described (20). Rats were overnight-fasted. A priming dose of insulin infusion was administered during the initial 10 min to acutely increase plasma insulin concentration to the desired level, at which it was maintained by continuous insulin infusion at a rate of 2.5 × 10^-3 units/kg body weight per minute. Blood glucose was clamped at 5 mmol/L for the next 180 min by adjusting the rate of 20% glucose infusion according to blood glucose measurements every 5 min. Dihydroethidine staining and cytofluorometric analysis were performed for each group in the day 7 by Image-Pro Plus software version 6.0. The mean fluorescence intensity of each cell was calculated, and the total cell emission signals per field were averaged for data analysis.

Loss of insulin resistance was measured by lucigenin-enhanced chemiluminescence assay under adaptation of the previously described method (25). ROS production was expressed as relative light units per second per million cells. Dihydroethidine staining was used to detect the in situ formation of superoxide according to the oxidative fluorescent microtopography, as described recently (26). Dihydroethidine staining was visualized under a confocal microscope (Olympus). Then, images of cells were analyzed with Image-Pro Plus software version 6.0. The mean fluorescence intensity of each cell was calculated, and the total cell emission signals per field were averaged for data analysis.

Assessment of apoptosis of CMECs. Apoptosis of CMECs was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an in situ apoptosis detection kit (Roche, Penzberg, Germany) according to the manufacturer’s instructions. The index of apoptosis was expressed as the proportion of the TUNEL-positive CMECs to the total CMECs in percentage.

Western blot analysis. Western blot was performed as previously described. Briefly, CMECs from each group were cultured and harvested at the indicated time. Cells were washed and scraped using lysis buffer. Total proteins were loaded onto an SDS-PAGE gel and transferred electrophoretically to nitrocellulose membranes (Millipore, Billerica, MA). After blocking with 5% skim milk, the membranes were incubated with the appropriate primary antibody at 4°C overnight. The membranes were washed and further incubated with previously mixed with benzoyl peroxide as a catalyzer at perfusion pressure no more than 100 mmHg through the aorta and were corrupted in a solution consisting of 5% sodium hydroxide solution at room temperature; the solution was changed every 12 h for 4-5 days. After removal of all connective tissue with 70% ethanol, each section was rinsed twice in 0.1 M phosphate-buffered saline to according with standard instruction were used, including dehydrogenation, desiccation, and gilding. Prepared samples were subsequently observed by a scanning electron microscope (Hitachi S-3400N).

Examination of cardiac microvascular barrier function. Transmission electron microscopy was used to assess CMEC-to-cell junctions as previously described (20). In brief, hearts of anesthetized rats were perfused via the aorta with 50 mL of fixative solution (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 2.5 mM MgO, 5.6 mM KCl, 3.7 mM glucose, and 3.6 mM propranolol), followed by 250 mL of fixation solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2% lanthanum nitrate) using a Langendorff system. After perfusion, the heart tissue was kept in the same fixative solution without lanthanum for 1 h. Then, specimens were rinsed with washing solution (0.15 mol/L NaCl plus 0.2 mol/L sucrose). Subsequently, tissue samples were dehydrated by graded ethanol and embedded in Agar 100. Ultra-thin sections (60-mm-thick) were cut, mounted on copper grids, and observed using a transmission electron microscope (JEOL JEM-2000EX) to check for the location of lanthanum nitrate. Cardiac microvessels were regarded as having increased permeability when the electron-dense marker penetrated into the junctional complex and electron-dense material was identified on the luminal surface.
TABLE 1
Blood glucose levels in different experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Vildagliptin</th>
<th>Exenatide</th>
<th>Insulin (0.5 units)</th>
<th>Insulin (1 units)</th>
<th>Insulin (1.5 units)</th>
<th>Insulin (2 units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>21.4 ± 1.0</td>
<td>22.0 ± 1.1</td>
<td>22.4 ± 1.4</td>
<td>22.4 ± 1.2</td>
<td>21.9 ± 1.2</td>
<td>22.1 ± 1.1</td>
</tr>
<tr>
<td>1 day</td>
<td>19.8 ± 1.1</td>
<td>20.5 ± 1.3</td>
<td>22.0 ± 1.1†</td>
<td>20.1 ± 1.1†</td>
<td>19.2 ± 0.9</td>
<td>18.3 ± 1.2‡</td>
</tr>
<tr>
<td>2 days</td>
<td>20.0 ± 1.5</td>
<td>19.8 ± 1.0</td>
<td>21.6 ± 1.1†</td>
<td>20.0 ± 0.8†</td>
<td>18.7 ± 1.1</td>
<td>17.0 ± 1.0‡</td>
</tr>
<tr>
<td>3 days</td>
<td>18.1 ± 1.1</td>
<td>18.9 ± 0.9</td>
<td>21.7 ± 1.4†</td>
<td>20.7 ± 0.9†</td>
<td>18.7 ± 1.2</td>
<td>16.8 ± 0.9†</td>
</tr>
<tr>
<td>4 days</td>
<td>17.4 ± 1.4</td>
<td>17.9 ± 1.1</td>
<td>21.1 ± 1.2†</td>
<td>20.4 ± 1.3†</td>
<td>18.3 ± 0.9</td>
<td>16.6 ± 1.1†</td>
</tr>
<tr>
<td>5 days</td>
<td>18.1 ± 1.0</td>
<td>18.3 ± 1.3</td>
<td>21.0 ± 1.2†</td>
<td>20.3 ± 1.0†</td>
<td>18.5 ± 0.8</td>
<td>16.8 ± 1.0†</td>
</tr>
<tr>
<td>6 days</td>
<td>17.9 ± 0.9</td>
<td>18.0 ± 0.8</td>
<td>21.2 ± 1.2†</td>
<td>19.1 ± 1.4†</td>
<td>18.4 ± 0.7</td>
<td>16.5 ± 1.1†</td>
</tr>
<tr>
<td>7 days</td>
<td>18.4 ± 0.8</td>
<td>17.8 ± 0.9</td>
<td>21.6 ± 0.9†</td>
<td>19.6 ± 1.3†</td>
<td>18.3 ± 0.8</td>
<td>16.8 ± 0.9†</td>
</tr>
<tr>
<td>10 days</td>
<td>17.5 ± 1.1</td>
<td>18.0 ± 1.0</td>
<td>21.7 ± 0.7†</td>
<td>19.1 ± 1.4†</td>
<td>17.9 ± 1.1</td>
<td>16.7 ± 1.0†</td>
</tr>
<tr>
<td>14 days</td>
<td>17.7 ± 1.0</td>
<td>17.9 ± 1.0</td>
<td>22.0 ± 1.1†</td>
<td>19.8 ± 1.2†</td>
<td>18.3 ± 0.8</td>
<td>16.6 ± 1.2‡</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 15) and expressed as mmol/L. *P < 0.05 vs. vildagliptin group; †P < 0.05 vs. exenatide group.

RESULTS
Basic parameters of rats in different experimental groups. It has been reported that GLP-1 is an intestinally derived hormone acting in vivo to promote the level of circulating insulin, leading to reduced blood glucose levels. We performed experiments to determine the dosage of insulin administration to keep blood glucose in the diabetic group comparable with that in vildagliptin-treated and exenatide-treated groups. According to our results (Table 1), we found that insulin treatment at dosage of 1.5 units per day had the similar effect in blood glucose as those elicited by vildagliptin and exenatide in rats. As shown in Table 2, diabetic rats exhibited hyperglycemia (22.3 ± 1.8 mmol/L vs. 8.4 ± 2.1 mmol/L, P < 0.05) and reduced body weight gain (282.6 ± 7.9 g vs. 440.1 ± 7.3 g, P < 0.05) compared with control group. Administration of vildagliptin and exenatide for 12 weeks significantly decreased plasma glucose level (17.5 ± 1.7 mmol/L vs. 22.3 ± 1.8 mmol/L, P < 0.05; 18.3 ± 2.3 mmol/L vs. 22.3 ± 1.8 mmol/L, P < 0.05) and slightly decreased body weight compared with diabetic group (273.5 ± 6.5 g vs. 282.6 ± 7.9 g, P < 0.05; 265.3 ± 9.5 g vs. 282.6 ± 7.9 g, P < 0.05). Vildagliptin or exenatide treatment increased the level of plasma insulin in diabetes (1.22 ± 0.35 ng/mL vs. 0.54 ± 0.13 ng/mL, P < 0.05; 1.17 ± 0.21 ng/mL vs. 0.54 ± 0.13 ng/mL, P < 0.05). Administration of 1.5 units insulin per day had the similar effects in blood glucose and body weight as those elicited by vildagliptin and exenatide in rats. However, blood pressure and insulin resistance exhibited little difference between diabetic and the treatment groups.

TABLE 2
Basic parameters of rats in different experimental groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Con</th>
<th>Diabetes + vehicle</th>
<th>Diabetes + insulin</th>
<th>Diabetes + exenatide</th>
<th>Diabetes + vildagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>237.3 ± 9.2</td>
<td>243.6 ± 8.9</td>
<td>245.5 ± 9.3</td>
<td>248.4 ± 10.3</td>
<td>243.5 ± 6.2</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>8.2 ± 1.9</td>
<td>23.9 ± 2.1*</td>
<td>21.9 ± 2.3*</td>
<td>21.2 ± 2.4*</td>
<td>22.1 ± 1.8*</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>100.06 ± 5.2</td>
<td>103.81 ± 4.8</td>
<td>106.71 ± 5.1</td>
<td>102.34 ± 4.2</td>
<td>104.61 ± 4.7</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.52 ± 0.70</td>
<td>0.67 ± 0.22*</td>
<td>0.71 ± 0.23*</td>
<td>0.64 ± 0.20*</td>
<td>0.69 ± 0.18*</td>
</tr>
<tr>
<td>Insulin resistance (ng/kg/min)</td>
<td>12.1 ± 1.8</td>
<td>13.2 ± 1.9</td>
<td>12.6 ± 1.8</td>
<td>11.9 ± 1.5</td>
<td>12.7 ± 1.6</td>
</tr>
<tr>
<td>12 weeks after treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>440.1 ± 7.3</td>
<td>282.6 ± 7.9*†</td>
<td>259.9 ± 7.8*†</td>
<td>265.3 ± 9.5*†</td>
<td>273.5 ± 6.5*†</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>8.4 ± 2.1</td>
<td>22.3 ± 1.8*</td>
<td>17.8 ± 2.5*†</td>
<td>18.3 ± 2.3*†</td>
<td>17.5 ± 1.7*†</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>105.51 ± 4.9</td>
<td>106.92 ± 5.1</td>
<td>107.53 ± 4.8</td>
<td>106.23 ± 4.5</td>
<td>108.02 ± 5.6</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.48 ± 0.65</td>
<td>0.54 ± 0.13*</td>
<td>1.23 ± 0.38*†</td>
<td>1.17 ± 0.21†</td>
<td>1.22 ± 0.35††</td>
</tr>
<tr>
<td>Insulin resistance (ng/kg/min)</td>
<td>11.5 ± 1.4</td>
<td>11.8 ± 1.7</td>
<td>11.2 ± 2.3</td>
<td>10.9 ± 1.7</td>
<td>12.0 ± 1.6</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 15). Con, control group; Diabetes, diabetes group. *P < 0.05 vs. control group; †P < 0.05 vs. baseline group.
FIG. 1. Effects of vildagliptin or exenatide on cardiac function and cardiac glucose metabolism in diabetes. A: Representative mitral flow patterns from pulsed Doppler. B: Quantification of E/A ratio. C: Measurement of left ventricular end-diastolic diameter after 12 weeks of drug treatment. D: Measurement of fractional shortening (FS) after 12 weeks of drug treatment. E: Representative PET/CT scan images from coronal, sagittal, and transverse view from each group. Higher glucose uptake level is shown as an increase in the intensity of red color as indicated on the color scale bar shown. F: Quantification of accumulated 18F-FDG in the heart. Data are expressed as mean ± SD (n = 6). *P < 0.05 vs. control group; #P < 0.05 vs. Diabetes + vehicle group; &P < 0.05 vs. Diabetes + insulin group. DM, diabetes; E, early mitral diastolic wave; A, late mitral diastolic wave; LVEDD, left ventricular end-diastolic diameter.
Vildagliptin or exenatide treatment improved cardiac diastolic function in diabetes. After 12 weeks of treatment with vildagliptin or exenatide, rats were anesthetized and cardiac function was determined using echocardiography. Compared with the control group, diabetic rats exhibited significantly dampened diastolic function, as manifested by increased E/A ratio (Fig. 1A, B) and left ventricular end-diastolic diameter (Fig. 1C), the effects of which were attenuated in vildagliptin or exenatide treatment (Fig. 1D, E).

FIG. 2. Effects of vildagliptin or exenatide on cardiac microvascular integrity and permeability. A: The surface of cardiac microvessels in control animals was smooth and well-integrated. B: Cardiac microvessels in diabetic animals were visualized to be highly irregular and rough. C: Cardiac microvessels treated with insulin. D and E: Cardiac microvessels with vildagliptin or exenatide treatment exhibited integrity and vessel integration. F: Lanthanum nitrate was regulated to the blood vessel lumen in control animals. G: Lanthanum nitrate was found to diffuse across vessel lumen to the basal lamina in diabetic animals. H: Cardiac microvessels were treated with insulin. I and J: Diffusion of lanthanum nitrate across CMECs was attenuated in vildagliptin or exenatide treatment (scale bar, 2 μm). The extracellular tracer lanthanum nitrate is highlighted with arrows. DM, diabetes.

FIG. 3. Characterization of CMECs and detection of GLP-1R. A: CMEC monolayer presents cobblestone appearance by phase-contrast microscopy (scale bar, 40 μm). B: Uptake of Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) by immunofluorescence (red, Dil-Ac-LDL; blue, DAPI). C: Expression of GLP-1R by immunofluorescence (green, GLP-1R; scale bar, 10 μm). D: Merge between B and C. E: Western blot analysis for GLP-1R. β-Cells were used as positive control.
FIG. 4. Effects of GLP-1 on oxidative stress in high-glucose–induced CMECs. A: GLP-1 inhibits high-glucose–induced superoxide generation in CMECs dose dependently. B: GLP-1 inhibits high-glucose–induced NADPH oxidase activity in CMECs dose dependently. C: Representative images.
mitigated by vildagliptin or exenatide treatment. Compared with insulin-treated group, vildagliptin or exenatide further improved cardiac diastolic function in diabetic rats. However, there was little difference in fractional shortening among the four groups studied (Fig. 1D). In summary, vildagliptin or exenatide was capable of improving cardiac diastolic function in diabetes.

Vildagliptin or exenatide treatment improved cardiac glucose metabolism in diabetes. To examine the role of cardiac glucose metabolism after vildagliptin or exenatide treatment in diabetes, $^{18}$F-FDG was used to analyze the level of myocardial glucose uptake. $^{18}$F-FDG method has been widely used in both experimental and clinical settings to quantify cardiac glucose metabolism. Our data revealed that diabetes led to a defective $^{18}$F-FDG uptake in the heart, the effect of which was significantly improved by vildagliptin or exenatide treatment. Insulin treatment exhibited less $^{18}$F-FDG uptake in the heart compared with administration of vildagliptin or exenatide (Fig. 1E, F).

Vildagliptin or exenatide treatment maintained cardiac microvessel integrity in diabetes. Scanning electron microscopy was used to evaluate cardiac microvascular integrity (28). In control animals, the surface of cardiac microvessels was smooth and well-integrated (Fig. 2A). However, numerous irregular exvacuations and invaginations were identified in cardiac microvessels from diabetic animals. Cell junction integrity was greatly compromised and cells were not well-integrated with the vessel (Fig. 2B). Vildagliptin or exenatide treatment effectively attenuated the changes, preserving cardiac microvascular integrity and vessel integration, in a manner more pronounced than the insulin treatment (Fig. 2C–E).

Vildagliptin or exenatide treatment protected cardiac microvascular barrier function in diabetes. Lanthanum nitrate has been widely used to assess the barrier function of endothelial cells (29). Under healthy conditions, lanthanum nitrate is unable to cross endothelial cell-to-cell junctions, but it may traverse cell boundaries via paracellular diffusion when such barriers are in pathologic conditions. Lanthanum nitrate was regulated to the blood vessel lumen in control animals (Fig. 2F); however, significant deficit in barrier integrity of cardiac microvessels was found in diabetic rats (Fig. 2G). Diffusion of lanthanum nitrate across endothelial cells was attenuated by administration of vildagliptin (Fig. 2I) or exenatide (Fig. 2J) in a manner more pronounced than that of the insulin (Fig. 2H). These findings indicated that vildagliptin or exenatide treatment was capable of protecting diabetes-induced cardiac endothelium injury independent of its glucose-lowering property.

Identification of CMECs and detection of GLP-1R. The cells displayed "flagstone" morphology (Fig. 3A) and tested positive on Dil-acetylated low-density lipoprotein intake assay (Fig. 3B), indicating that the cultured cells were CMECs. Both immunofluorescence staining (Fig. 3C, D) and Western blot (Fig. 3E) showed that GLP-1R was expressed on CMECs.

GLP-1 attenuated high-glucose–induced oxidative stress in CMECs. ROS are known to play a major role in diabetic cardiovascular disease. To determine whether GLP-1 attenuated high-glucose–induced oxidative stress in CMECs, ROS production and NADPH activity was measured. As shown in Fig. 4A and B, high glucose increased ROS production and NADPH activity after 24-h incubation in CMECs, whereas GLP-1 could dose-dependently suppress ROS production and NADPH activity in high-glucose–induced CMECs. We noticed that GLP-1 at $10^{-8}$ mol/L significantly suppressed ROS production and NADPH activity. Dihydroethidine staining also showed that ROS production was elevated in the high-glucose group, whereas it decreased in the GLP-1–treated ($10^{-8}$ mol/L) group (Fig. 4C, D). To further determine whether GLP-1 protected against high-glucose–induced oxidative stress, the protein expression of p47phox, gp91phox, p22phox, and p40phox subunits of NADPH oxidase was measured. The expression of p47phox, gp91phox, p22phox, and p40phox were significantly increased in high-glucose–induced CMECs compared with control group. After treatment with GLP-1 ($10^{-8}$ mol/L), the expression of p47phox, gp91phox, p22phox, and p40phox was significantly decreased compared with high-glucose–induced CMECs (Fig. 4E–H).

GLP-1 decreased high-glucose–induced apoptosis in CMECs. To investigate the role of GLP-1 on high-glucose–induced apoptosis in CMECs, TUNEL assay was performed. High glucose induced a significant increase in TUNEL-positive cells. Compared with cells cultured in high glucose with vehicle, the GLP-1–treated group showed a significant decrease in apoptotic cells (46.1 ± 8.5% vs. 29.8 ± 5.3%; P < 0.05; Fig. 5A, B). We also detected the expression of caspase-3 and found that GLP-1 statistically decreased the caspase-3 expression in high-glucose–induced CMECs (P < 0.06; Fig. 5C). Taken together, these results demonstrated that GLP-1 exerted an antiapoptotic effect on high-glucose–induced CMECs.

GLP-1 suppressed high-glucose–induced activation of Rho in CMECs through cAMP/PKA pathway. Rho is a key regulator for oxidative stress (30). ROCK is a serine/threonine kinase, which is a known downstream effector of Rho. We examined the effect of GLP-1 on Rho expression in CMECs. Incubation of CMECs with high glucose significantly upregulated Rho expression and elicited a significant increase in ROCK expression. Treatment of CMECs with GLP-1 significantly alleviated high-glucose–induced increase in Rho and ROCK expression without affecting these parameters under normal glucose conditions. Incubation of cells with the PKA selective inhibitor H89 abrogated GRP-1–induced effect on Rho suppression (Fig. 6A, B).

GLP-1 decreased high-glucose–induced ROS production in CMECs via a cAMP/PKA/Rho-dependent mechanism. To further determine the mechanism of action behind the beneficial effect of GLP-1 on CMECs, fasudil (Selleck, Houston, TX) was used. Chronic exposure of CMECs to high glucose promoted intracellular ROS levels, the effect of which was obliterated by GLP-1. Incubation of CMECs with fasudil inhibited ROS production. However, combination of GLP-1 and fasudil failed to further attenuate ROS production. The inhibitory effect of GLP-1 on high-glucose–induced ROS accumulation was significantly attenuated by treatment with H89 (Fig. 7A–C). In line with...
the changes in intracellular ROS accumulation, the inhibitory effect of GLP-1 on high-glucose–induced activation of p47phox and gp91phox was reduced by H89. The combined treatment with GLP-1 and fasudil failed to produce any additive effects on high-glucose–induced p47phox and gp91phox expression and oxidative stress (Fig. 7D–F). Overexpression of Rho in CMECs potentiated p47phox and gp91phox activity and ROS production. Taken together, these results suggested that GLP-1 may decrease high-glucose–induced ROS accumulation in CMECs through a cAMP/PKA/Rho-dependent mechanism.

DISCUSSION
Pharmacological modulation of GLP-1 has emerged as a novel therapeutic option for diabetes (31–33). The therapeutic use of GLP-1 is severely compromised by its rapid degradation by DPP-4 (34). Exenatide is a peptide incretin mimic that shares many biological functions with GLP-1. Clinical efficacy of DPP-4 inhibitor such as vildagliptin has been well-documented in many studies (35).

It has been reported that GLP-1 is an intestinally derived hormone acting in vivo to promote the level of circulating insulin, leading to reduced blood glucose levels (36). In addition to its glucose-lowering property, GLP-1 also exhibits potential cardioprotective properties (37). For example, GLP-1 is capable of protecting the heart against ischemia/reperfusion injury (38). In addition, GLP-1 was shown to retard atherosclerotic lesion formation through enhancing GLP-1 activity in macrophages and endothelium (39). In this study, GLP-1 was found to improve cardiac function and glucose metabolism in hearts from diabetic rats, consistent with a recent clinical report in which GLP-1–based therapy reduces overall cardiovascular events in diabetic patients (40).

Tight control of cardiac microvascular function is essential to the maintenance of circulatory homeostasis and physiological function of the heart. Impaired cardiac microvascular function is thought to contribute greatly to diabetic cardiovascular disease (41,42). There is convincing evidence suggesting that endothelial apoptosis occurs at an earlier time point compared with pathological changes in cardiomyocytes in diabetes (43). The increased rate of endothelial apoptosis in diabetes may contribute to microvascular barrier dysfunction and rarefaction, which lead to myocardial metabolism abnormalities and impaired intracellular calcium homeostasis (44). In addition, microvascular injury aggravates the myocardium stiffness because of reduced blood supply, which directly prompts diastolic dysfunction (45). Our results from the present study exhibited that vildagliptin or exeninade significantly...
attenuated microvascular barrier defect and diastolic dysfunction in the diabetic heart.

A number of scenarios may be considered that underscore the beneficial effects of these agents in diabetes. Physiological levels of intracellular ROS production are deemed necessary for normal cellular development and function, whereas excessive ROS generation may facilitate pathophysiological progression in many diseases, including diabetes. This is in line with the speculation for a pivotal role of oxidative stress in the onset and development of diabetes complications, both microvascular and macrovascular (46). High glucose facilitated ROS production in CMECs, mainly through NADPH oxidase activation, involving formation and translocation of cytosolic NADPH oxidase subunits (p47phox, p67phox, and p40phox) and membrane-bound subunits (p22phox and gp91phox). Intriguingly, GLP-1 significantly suppressed high-glucose–induced ROS accumulation in CMECs, as well as levels of p47phox and gp91phox. Similarly, GLP-1 was effective in rescuing against high-glucose–induced apoptosis, possibly through inhibition of oxidative stress in CMECs.

Our data further revealed that GLP-1 prevented high-glucose–induced CMECs oxidative stress, possibly through inhibition of activation of the Rho/ROCK pathway. Rho/ROCK has been considered a key mediator for oxidative stress–mediated cell injurious process. Burger et al. (47) reported an important role of Rho/ROCK in angiotensin II–induced upregulation of endothelial NADPH oxidase and subsequently increased endothelial superoxide production. Moreover, accumulating evidence has depicted that the adverse endothelial outcome in diabetes is corroborated with increased oxidative stress, whereas Rho/ROCK may govern the downstream effects of hyperglycemia–induced oxidative stress. We have shown that exposure of CMECs to high glucose promoted Rho/ROCK activity. Rho/ROCK activity was suppressed by GLP-1 in high-glucose–induced CMECs. These findings demonstrated that the protective effects of GLP-1 may be mediated through inhibition of Rho/ROCK activation, which in turn alleviated high-glucose–induced oxidative stress in CMECs.

It is noteworthy that the upstream molecules and mechanisms responsible for GLP-1–associated cardiac microvascular protection remain elusive in diabetes. It was recently revealed that activation of the cAMP/PKA pathway may be involved in GLP-1–offered protective effects. Xiao et al. (48) demonstrated that GLP-1 promoted voltage-gated l-type Ca\(^{2+}\) current in the heart possibly through a cAMP/PKA-dependent pathway and may contribute, in part, to prolonged action potential duration. DPP-4 inhibition was found to protect against vascular senescence, which was attenuated by GLP-1 in a receptor-dependent manner involving downstream PKA signaling (49). Interestingly, cAMP/PKA was an essential negative regulator of Rho (50). In this study, our results revealed that cAMP/PKA may contribute to GLP-1–mediated suppression of high-glucose–evoked Rho/ROCK activity in CMECs. This conclusion was supported by the fact that GLP-1–induced suppression of high-glucose–induced Rho/ROCK activation was abrogated when the cAMP/PKA pathway was inhibited. Also, data from our study showed that Rho inhibitor fasudil suppressed ROS production and NADPH oxidase upregulation. However, joint therapy with GLP-1 and fasudil failed to further suppress ROS production and NADPH oxidase upregulation. Taken together, these results suggested that GLP-1 may inhibit high-glucose–induced oxidative stress in CMECs through a cAMP/PKA/Rho-dependent mechanism.

This study provides both in vivo and in vitro evidence supporting the protective effects of GLP-1 against diabetes-induced microvascular defect. These findings indicate that pharmacological intervention targeting on GLP-1 may represent a promising therapeutic strategy to
FIG. 7. Effects of GLP-1, fasudil, and H89 on high-glucose (HG)-induced oxidative stress in CMECs. A: Representative diagram showing dihydroethidine (DHE) staining of CMECs (red, DHE; blue, DAPI; scale bar, 25 μm). B: The average fluorescence intensity from five fields was summarized. C: Superoxide generation of CMECs in different groups. D: Western blot assay for p47phox protein and gp91phox protein expression. E and F: Quantitative analysis of p47phox and gp91phox expression. *P < 0.01 vs. control group; #P < 0.01 vs. HG group; &P < 0.05 vs. HG plus GLP-1 group. Con, control; H89, PKA inhibitor.
maintain microvascular function and cardiac function in patients with diabetes and cardiovascular disease. Nonetheless, it is noteworthy that our findings were mainly based on rodent models and in vitro experiments. Therefore, caution must be taken in evaluating the effects of GLP-1 on microvascular and cardiac function in patients with diabetes.

In conclusion, GLP-1 could protect the cardiac microvessels against oxidative stress, apoptosis, and the resultant microvascular barrier dysfunction in diabetes rats, en route to improved cardiac diastolic function and cardiac glucose metabolism. The protective effects of GLP-1 are dependent on downstream inhibition of Rho through a cAMP/PKA-dependent manner, resulting in a subsequent decrease in the expression of NADPH oxidase. These findings should provide important implications for diabetes with cardiovascular disease, for which GLP-1 may hold promise for prevention and treatment.

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D.W., J.R., and F.C. designed the experiments. D.W., P.L., and Y.W. performed the experiments. D.W. wrote the manuscript. P.L. researched data. Y.W. and W.L. contributed to the discussion. C.W. and D.S. contributed to the CMEC isolation. R.Z. and T.S. assisted in the preparation of the experiments. X.M. and C.Z. contributed to PET/CT assessment. H.W. and W.L. reviewed the manuscript. J.R. and F.C. analyzed the results and edited the manuscript. F.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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