GLP-1 Agonists Liraglutide Improved Vascular Endothelial Function in Type 2 Diabetes Rats

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Abstract
Objective: Liraglutide (LIRA), a Glucagon-like peptide-1 (GLP-1) receptor agonist, showed potential vascular protective effects with the mechanism remained incompletely understood. Therefore, this study aimed to investigate whether LIRA exerts its effect on vascular endothelial function in rats with type 2 diabetes mellitus (T2DM) via caveolin-1/ endothelial oxide synthase (eNOS) expression.

Methods: T2DM rats were used as study subjects and randomly divided into four groups: 1) Veh group, 2) Veh+LIRA group, 3) T2DM group, and 4) T2DM+LIRA group. All rats received either saline or LIRA 0.2 mg/kg (by i.p. injection) per day for 4 weeks. After the model was successfully established, vascular endothelial function was determined the effect of vasodilator to mesenteric artery rings. Immunofluorescence and western blot were performed to understand the molecular mechanism. Cultured HUVECs with small interfering RNA (siRNA) under high glucose (HG), NO concentration, and western blot were performed to understand the molecular mechanism between LIRA and vascular endothelial function.

Results: Based on our results, the LIRA reduced hyperglycemia and ameliorated vascular endothelial dysfunction in type 2 diabetic mice. LIRA activated eNOS phosphorylation, suppressing oxidative stress and enhancing endothelium-dependent vasorelaxation of mesenteric arteries. Besides, from its anti-oxidative capacity, LIRA activated eNOS to dilate the mesenteric arteries via the downregulation of Cav-1.

Conclusion: LIRA ameliorates vascular endothelial dysfunction in rats with type 2 diabetes mellitus via anti-oxidative and activated eNOS by downregulated Cav-1.

Keywords
Oxidative Stress, Liraglutide, Glucagon-like Peptide-1 Receptor, Type 2 Diabetes, Endothelial Oxide Synthase

Introduction
Type 2 diabetes mellitus (T2DM), is a type of disease in which clinical manifestations are mainly heterogeneous of insulin resistance with relative insulin deficiency. T2DM carries a high risk of cardiovascular diseases (CVDs) (myocardial infarction, stroke, and
peripheral vascular disease) and microvascular complications. Among this vasculopathy, endothelial dysfunction is thought to be the early event initiated by metabolic stresses, including hyperglycemia, insulin resistance [1]. Therefore, identification of novel anti-diabetic therapies strategies and maintenance of the endothelial function to be controlled during diabetes is a top clinical priority.

Caveolin protein can stabilize the specialized type of lipid raft-Caveolae. Caveolae have also been proposed as the vesicle carriers responsible for transcellular transport in endothelial cells. Among the caveolae family, Cav-1, the principal component of caveolae, is abundantly expressed in many different kinds of cell types, including endothelial cells, vascular smooth muscle (VSM), adipocytes, and fibroblasts. Cav-1 can play an important role in different pathways via directly interact with many proteins within the lipid rafts via its scaffolding domain. Recently, we have shown that increased Cav-1 expression of mesenteric arteries in type 1 diabetes [2]. Vascular endothelium has also been found to express abundant GLP-1 receptors. GLP-1 infusion can increase acetylcholine-induced vasodilatation, improves flow-mediated dilatation, muscle microvascular recruitment and glucose use independent of insulin secretion via endothelial eNOS activation. Liraglutide, an analog of human glucagon-like peptide 1 (GLP-1) [3], has been approved for the treatment of type 2 diabetes. LIRA can lower the rate of the first occurrence of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke among patients with T2DM [4]. As the American Diabetes Association (ADA) reported, insulin plus liraglutide was highly recommended to treat the T2DM patients because it can significantly improve the patients’ condition, especially with glucose-lowering actions and less weight gain and hypoglycemia compared with intensified insulin regimens [5].

Oxidative stress is an important player in the pathogenesis of diabetes. Many pathological changes increasing the production of reactive oxygen species (ROS) in endothelial cells, thereby [6,7]. Accumulating amounts of ROS further lead to exacerbate the oxidative burden and impairment of mitochondrial function for the cells [6]. The oxidative stress becomes more severe and endothelial function is largely inhibited due to the insufficiency of nitric oxide (NO) bioavailability by forming peroxynitrite, inducing endothelial eNOS uncoupling [8].

Therefore, we hypothesized that liraglutide might exert anti-oxidative activities and in mesenteric endothelial cells, and these effects could be prevented reduce NO bioavailability via Cav-1 and eNOS pathways on T2DM.

Materials and Methods

Induction of Type 2 Diabetic Models:

Male SD rats 4 weeks, 120-160 g, were provided by the Center for Laboratory Animals of Wenzhou Medical University (License No. SCXK2015-0001). All rats allowed free access to food and water, placed under a standard 12-hour dark/light cycle, and were housed in a certified animal care facility. All institutional and NIH guidelines for the use of animals were followed. These study protocols were approved by the Animal Research Committee of Wenzhou Medical University.

After 3 days of adaptive feeding, the rats were randomized into 2 groups: Veh group and T2DM group. Rats in the T2DM group were fed with a high-fat sugar diet (HFSD, 45% kcal as fat primarily derived from lard), while rats in Veh group were fed with a standard diet (4.25% kcal as fat). After 8 weeks, blood samples were collected from the tail vein to test fasting blood glucose and insulin. Insulin sensitivity index (ISI) =1/(blood glucose*insulin). The ISI results showed a significant difference in two groups (p<0.05), and then rats in T2DM group were intraperitoneally injected with a low dose of streptozotocin (STZ) (Sigma Co., St. Louis, MO, USA) 35 mg/kg in order to induce hyperglycemia. The rats in the Veh group were intraperitoneally injected with the same dose of citrate buffer. After 3 days of injection, rats in T2DM groups with fasting blood glucose ≥ 16.7 mmol/L were considered as T2DM rats. Rats in the Veh group and T2DM group were received either saline or LIRA 0.2 mg/kg per day for 4 weeks [9].

Vascular Reactivity in Mesenteric Arterioles:

Three arterioles rings (1 mm) from the third branch of the mesenteric arterioles per rats and each ring was
suspended between two stainless steel wires and performed in a 5 ml vessel myograph containing physiological salt solution (PSS) of following composition (in mM): NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11, and CaCl₂ 1.8, as previously described [10]. Vessels chamber was continuously gassed with a mixture (95% O₂ and 5% CO₂) and maintaining at 37°C for isometric tension recordings. An optimal preload 2.1 mN was determined to the mesenteric artery rings, we found that KCl gave the maximal constrictor response. After a 60-min equilibration, phenylephrine (PE) 1 µM was added to the bath to test vessel viability (13.2-20.0 mN of developed force). Once a stable contraction was obtained, cumulative concentration-response relationships were evaluated of acetylcholine (ACH) \((10^{-10}-10^{-6} \text{ mol/L})\) and insulin \((10^{-10}-10^{-6} \text{ mol/L})\) using mesenteric artery rings from each group.

**Immunofluorescence:**

The frozen mesenteric arteries sections were incubated with a 5% goat-serum blocker to inhibit non-specific binding, then double-immunostained of Cav-1 and eNOS with anti-Cav-1 (1:500) and anti-eNOS (1:200) (Cell Signaling Technology) for 24h in a humidified chamber at 4°C. Followed incubated with a mixture of FITC-conjugated goat anti-mouse secondary antibody (1:50) for 1 h at room temperature and washed three times with PBS. The results were performed by the Olympus BX60f immunofluorescent microscope (Olympus Optical Co. Ltd., Japan) at a magnification of \(20\times\).

**Western Blot:**

The mesenteric arteries of rats or cells were lysed with lysis buffer, which contained 10 mM Tris, 0.25 M sucrose, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 40 µM leupeptin. The supernatants were collected after centrifuged at 4°C for 15 min. The samples were heated for 5 min at 99°C and the proteins were separated by electrophoresis on SDS-PAGE after measuring concentration. Blocked with 5% nonfat milk in Tris-buffer saline containing 0.1% Tween-20 for 1 h, then the membranes were incubated with primary antibodies overnight. These antibodies included anti-Akt (1:2000, Cell Signaling Technology), anti-phospho(p)-Akt (1:1000, Cell Signaling Technology), Cav-3 antibodies (1:2000, BD Technology) eNOS antibodies (1:2000, Cell Signaling Technology), p-eNOS antibodies (1:1000, Cell Signaling Technology). The blots were visualized with ECL-plus reagent (Clarity Western ECL Substrate, Bio-Rad) and exposed using the ChemiDoc XRS System with Image Lab software (Bio-Rad).

**Measurement of NO Production:**

Nitrite, a stable metabolite of NO with the Griess reaction for NO (Nanjing Jiancheng Biological Co., China). The nitrite detection kit was used according to instructions, 100 µl of medium or standard NaNO₂ was mixed with 100 µl of Griess reagent in 96-well plate, optical density was read in a microplate reader at 540 nm after 15 min. Plasma NO levels and NO production by Human Umbilical Vein Endothelial Cell (HUVECs) were determined a standard curve generated for each experiment for quantification.

**In Situ DHE (Dihydroethidium) Staining of the Left Ventricle:**

Mesenteric arteries ROS levels were examined by DHE staining, as previously described [11]. The frozen mesenteric arteries sections were incubated with DHE (10 µM) for 20 min in the dark. A fluorescence microscope (Leica, Microsystems, Germany) used to visualize the fluorescence of DHE-stained signals after washing with PBS.

**Endothelial Cell Culture:**

HUVECs were cultured as described [12]. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented 10% fetal bovine serum (FBS) equilibrated with a humidified cell incubator with 5% CO₂ at 37°C for 24h. In order to mimic the high glucose level in diabetes, normal concentration glucose, 5.5 mM glucose (in mM) (D-glucose 5.5, mannitol 14.5, KCl 4.0, NaCl 81, CaCl₂ 1.6, pH 7.4), high concentration glucose (HG), 33.3 mM glucose (in mM) (D-glucose 33.3, KCl 4.0, NaCl 81, CaCl₂ 1.6, pH 7.4). The cells were bathed in Hepes-buffered solution for 24 h. NormalHepes-buffered solution (in mM) (KCl 5, NaCl 135, MgCl₂ 1, CaCl₂ 1, Hapes 10, pH 7.4). To examine the effect of liraglutide or Cav-1 inhibition on
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HG cells, HUVECs were treated with PBS, HG, HG+LIRA (1 μg/mL) after transfected with Cav-1 siRNA or scramble siRNA. Cells were harvested for western blot.

Transfection of siRNA:
Before the transfection, HUVEC were plated in 6-well plates to form a monolayer. Then these cells were transfected with a scrambled siRNA or Cav-1 siRNA at a final concentration of 80 nM (Santa Cruz Biotechnology, Santa Cruz, CA) for 6h by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols [13]. The treated cells were used after 48h of incubation.

Data Analysis:
All of the results were presented as mean ± SEM. The data were statistically analyzed using one-way or two-way analysis of variance (ANOVA) or paired or unpaired Student’s t-test. When the ANOVA results revealed a significant difference, pairwise comparisons between means were tested by the least significant difference method (LSD). Significance was set at P < 0.05. All statistical tests were performed with GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, CA).

Results
Establishment and Characterization of the Rat Type 2 Diabetic Models:
After 8 weeks of HFSD, blood glucose levels were not increased significantly, while insulin levels and insulin resistance were successfully elicited of the T2DM group (P < 0.01, Fig-1a, Fig-1b, and Fig-1c). At 3 days after STZ injection, rats in the T2DM group had a higher blood glucose level (>16.7 mmol/L) compared with the Veh group. The high blood level can be maintained for at least four weeks (P < 0.01, Fig-1d). These data indicated that the type 2 diabetic model was successfully established. The blood glucose level in the T2DM group was decreased after receiving 4 weeks of LIRA treatment.

Fig-1:
Insulin levels (a), insulin sensitivity (b) and blood glucose levels (c) after 8 weeks on high fat sugar diet. The effect of LIRA treatment on blood glucose levels for 4 weeks after STZ (p (d). The data are shown as the mean ± SEM; N = 8 rats per group. **p<0.01 vs. Veh, ##p<0.01 vs. Veh+LIRA.
LIRA Alleviates Endothelial Dysfunction in Mesenteric Arteries of Type 2 Diabetic Rats:

The whole ex vivo data showed that T2DM endothelium-dependent relaxation to ACh was highly repressed \(10^{-10}-10^{-6}\)mol/L \((P < 0.01, \text{Fig-2})\). Moreover, T2DM + LIRA exhibited a recovered level in vasorelaxation compared to T2DM. T2DM showed an attenuated of endothelium-dependent relaxation to insulin \(10^{-10}-10^{-6}\)mol/L and T2DM + LIRA will help to bounce the relaxation. The NO donor SNP was examined to test the response of VSM to NO. After treated the T2DM with LIRA, the endothelium-independent relaxation level was recovered \((P < 0.05)\). The whole data was obtained after 4 weeks of LIRA treatment. All these results indicated that the LIRA helps to recover from the impairment of endothelium-dependent and independent stimulation of T2DM rats.

LIRA Improved the Endothelium Function Via Akt-Enos Pathway:

To evaluate whether this pathway was the mechanism in which LIRA can improve the endothelium function, we measured Akt/eNOS expression and phosphorylation in the mesenteric arteries. As the results showed in \textbf{Fig-3}, the p-Akt expression level was lower in the T2DM group than the Veh group, and it was recovered after treated with LIRA (\textbf{Fig-3d}). The eNOS expression level and p-eNOS/eNOS were much lower in the T2DM group compared with the T2DM+LIRA group (\textbf{Fig-3b} and \textbf{Fig-3c}). Herein, we can draw a conclusion that the Akt-eNOS pathway was impaired in the T2DM and they were partly recovered after treated with LIRA. The Cav-1 expression level was higher in the T2DM compared with the Veh group, while its expression decreased after LIRA treatment. The eNOS phosphorylation and Cav-1 expression were negatively related as our results showed. Based on the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure.png}
\caption{ACh-induced (a), insulin-induced (b), and SNP-induced (c) vasodilatation in small mesenteric arteries in rats. The data are shown as the mean ± SEM; \(N = 8\) rats per group. \(*p<0.01\) vs. Veh, \#\#\#p<0.01 vs. Veh+LIRA.}
\end{figure}
previous publication, NO has played an important role in the regulation of the vascular. So, we found that NO decreased in the T2DM and it recovered when treated with LIRA (Fig-4c). We also found hyperglycemia-induced ROS increase, LIRA treatment decreased ROS generated (Fig-4a and Fig-4b).

Co-Location of Cav-1 and Enos in Mesenteric Artery:

Fig-5 clearly showed that both Cav-1 and eNOS were co-located in mesenteric artery endothelial cells. The results were perfectly matched with our western data. Namely, in the T2DM group, Cav-1 in mesenteric artery endothelial cells was increased compared with that of the Veh group, while its expressions of eNOS were lower than that of Veh and T2DM+LIRA group.

Interaction of Cav-1 and Enos:

These data led us to hypothesize a potential interaction between Cav-1 and eNOS in mesenteric arteries in T2DM with LIRA treatment. We further tested whether the downregulation of Cav-1 expression affects eNOS phosphorylation in HUVEC with high glucose using specific siRNA. Cav-1 expression was decreased with siRNA transfection of HUVEC. In high glucose, the presence of Cav-1 siRNA, the eNOS phosphorylation in HUVEC was significantly increased compared with that in cells treated with control siRNA. The expression of Cav-1 was significantly decreased while p-eNOS and NO were increased with LIRA incubation in high glucose cells (Fig-6).

Discussion

The effects of liraglutide on impaired endothelial dysfunction of blood vessels in diabetes remain unclear. In the present study, we demonstrate that liraglutide improves the dilation of mesenteric arteries in T2DM rats, probably via anti-oxidative, activated eNOS in vitro, and that Cav-1 is essential for liraglutide-stimulated endothelial NO
production in vivo.

GLP-1 agonists are not only potent glucose-lowering agents but also be effective to other traditional (body weight, BP, and LDL cholesterol) and non-traditional risk factors (low-grade inflammation and endothelial dysfunction). Liraglutide is a long-acting analogue of a GLP-1 RA, used for lowering blood glucose in patients with diabetes and also believed to possess cardiovascular protective effects in patients with

Fig-4:
The effect of LIRA treatment on DHF fluorescent intensity of mesenteric arteries in rats (a). Relative density values of DHF fluorescent (b). NO production of plasma in rats (c). The data are shown as the mean ± SEM; n =3 repeats per group. **p<0.01 vs. Veh, ###p<0.01 vs. Veh+LIRA.

Fig-5:
The localization of cav-1 or eNOS was determined in the mesenteric artery by immunofluorescent double staining with antibodies specific to caveolin 1 (red) and eNOS (green) as indicated. Co-localization was determined by overlaying the red and green staining. In the Veh group (upper panels), fluorescent intensity of cav-1 was weak, fluorescence of eNOS was bright.
diabetes [4]. Hyperglycemia is a common pathological change occurring in T2DM [14] and it causes endothelial dysfunction, which is the initial process in the development of diabetic vascular complications. In the present study, we found that liraglutide improves hyperglycemia and endothelium-dependent relaxation of mesenteric arteries in T2DM. We observed that liraglutide prevented high blood glucose-induced impairment of endothelial function in the in vitro model, suggesting that liraglutide directly acts on endothelial cells to exert its protective effect. Previous studies have shown that high blood glucose induced VSMC alterations are prevented by treatment with liraglutide [15]. We also observed that liraglutide improved the endothelium-independent relaxation of mesenteric arteries.

Impairment of the Akt/eNOS signaling pathway plays an essential role in endothelial dysfunction of blood vessels in diabetes [2]. Abnormalities in the p-eNOS expression are an important common pathway that links diverse cardiovascular risks (such as DM, obesity, and metabolic syndrome) with endothelial dysfunction [15]. In the present study, Akt/eNOS phosphorylations and NO productions were all decreased in the T2DM while these were all increased after treatment with liraglutide, which suggested that the alleviation of high blood glucose-induced endothelial dysfunction in diabetes with liraglutide was possibly attributable to improve the Akt/eNOS/NO pathway. Many studies have shown that hyperglycemia-induced oxidative stress in diabetes is a major etiologic factor of several diabetic complications including microvasculopathy [14,16,17]. The oxidative stress usually results from either excessive ROS production. We found that oxidative stress was exacerbated in the endothelium of mesenteric arteries in T2DM. Conversely, the worsened oxidative burden
was mostly abrogated by treatment with liraglutide.

As shown in Western blot analysis and immunofluorescent double staining of mesenteric arteries, both Cav-1 and eNOS were co-located in mesenteric artery endothelial cells. Cav-1 was increased in the type 2 diabetes group. However, liraglutide reversed this increase. Cav-1 anchors eNOS to plasma membrane caveolae in the endothelium, thus limits translocation of eNOS in the cytoplasm, consequently, eNOS phosphorylates, and full activation. The segregation of eNOS from caveolae and associated unclamping of eNOS from caveolin were the processes of eNOS activating [18]. Loss of Cav-1 caused vascular, cardiac abnormalities can be rescued by inhibitors of Nos. So, increased Cav-1 clamps more eNOS, and the segregation of eNOS from caveolae and NO production was decreased, which contributes to endothelial dysfunction [19]. As our data shows, the amount of Cav-1 were increased while phosphorylations of eNOS and plasma NO were decreased in type 2 diabetic rats, however, liraglutide reversed them. It indicated that liraglutide improved the endothelium function may decrease the interaction of Cav-1 and eNOS.

Conclusions

Previous studies have shown an indirect regulation of liraglutide on vasculopathy [20,21]. In the present study, we found that liraglutide activated Akt/eNOS, to inhibit oxidative stress and to enhance eNOS phosphorylation in endothelial cells of mesenteric arteries. Furthermore, we found that liraglutide decreased the interaction of Cav-1 and eNOS and also to increase eNOS phosphorylation and NO production. Taken together, our data provide a novel mechanistic understanding of the biological functions of liraglutide on vascular diseases and suggest that liraglutide may exert a protective effect on alleviating vascular damage in type 2 diabetes.

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Conflicts of Interest

All authors have read and approved the final version of the manuscript. The authors have no conflicts of interest to declare.

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