Retracted Article: GLP-1 receptor agonist lixisenatide protects against high free fatty acids-induced oxidative stress and inflammatory response

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\textbf{ABSTRACT}

Increased free fatty acids (FFA) are one of the risk factors for type 2 diabetes. FFA also contribute to endothelial dysfunction in both the prediabetes and diabetes conditions. Therefore, FFA are an important link between diabetes and endothelial dysfunction. In therapeutic application, GLP-1 receptor agonists have been implemented to lower blood glucose in diabetes. Here, we investigate the role of the common clinically used GLP-1 receptor agonist lixisenatide in endothelial cells. We demonstrate that lixisenatide could protect endothelial cells from high FFA-induced toxicity and cell death. Lixisenatide also suppresses FFA-caused cellular ROS generation and production of the lipid oxidation byproduct 4-HNE. Lixisenatide inhibits FFA-triggered production of TNF-\(\alpha\), IL-6, VCAM-1 and ICAM-1. The presence of lixisenatide in co-culture experiments suppresses adhesion of monocytes to endothelial cells. Moreover, lixisenatide ameliorates FFA-induced decreased eNOS phosphorylation and NO reduction. We also demonstrate that lixisenatide inhibits FFA-induced IkB\(a\) activation, nuclear p65 translocation and NF-kB activation. This evidence indicates that lixisenatide suppresses activation of the NF-kB pathway in endothelial cells. Collectively, our findings suggest that lixisenatide might have therapeutic potential to modulate diabetes-associated vascular complications.

\textbf{INTRODUCTION}

Obesity is a global epidemic and according to a study that spanned from 2008 to 2013 nearly 30\% of people in the world are obese or overweight \cite{1}. Obesity is the direct result of metabolic abnormalities and is highly associated with metabolic syndrome and insulin resistance, which together make up the prediabetes condition \cite{2}. Most of the obese population has elevated levels of plasma free fatty acid (FFA), which can interfere with insulin action in bone and muscle development, and cause peripheral insulin resistance \cite{3}. Elevated FFA also exerts a toxic effect in \(\beta\)-cells and thus impairs insulin secretion \cite{4}. Therefore, an elevated FFA level becomes a risk factor of diabetes. Endothelial cells line the inner side of vessels. From capillaries to large vessels, endothelial cells are connected to each other and form the largest monolayer organ, which is known as the endothelium. The surface area of the endothelium is estimated to be approximately the size of 6 tennis courts \cite{5}. In prediabetes and diabetes conditions, elevated FFA levels can reduce NO production in endothelium through disturbing the PI3K/eNOS signalling \cite{6}. High FFA exposure also induces excessive ROS generation and inflammatory response in endothelial cells \cite{7}.

Many efforts are made to alleviate the complications caused by type 2 diabetes. The most common medical therapy of this type is targeted to recover abnormal glucose metabolism by controlling two key enzymes, GLP-1 and DPP-4, which activate insulin release. Both GLP-1 analogues and DPP-4 inhibitors are effective in lowering glucose levels in type 2 diabetes patients. Various agents possess cardiovascular protective properties \cite{8}. GLP-1 agonists are more potent than DPP-4 inhibitors. GLP-1 receptors are expressed in the cardiovascular system, including the endothelium, and have been shown to contribute to cardiac protection in a mouse model \cite{9}. The GLP-1/GLP-1R/AMPk\(\alpha\) signalling plays a key role in mediating the inhibitory effects of uncoupling protein 2 (UCP2) on oxidative stress and COX-2 expression \cite{10}. Upregulation of GLP-1 and related agents via administration of the DPP-4 inhibitor sitagliptin improved endothelial function by restoring nitric oxide (NO) bioavailability \cite{11}. Among GLP-1 analogues, lixisenatide has unique pharmacokinetic properties. It has a relatively short half-life and an ability to delay gastric emptying. Lixisenatide has a four-fold activity of...
binding to the receptor as compared to native GLP-1 [12]. Here, we explored the biological effect of the unique GLP-1 receptor agonist, lixisenatide, in vascular endothelial cells.

**Materials and methods**

**Cell culture and treatment**

HUVECs, obtained from Lonza (CC-2635), were maintained in a 2% serum of endothelial growth media (EGM2). Only cells up to passage four were used for this study. The human monocytes cell line THP1 was from ATCC, which were maintained in DMEM containing 10% FBS. For FFA treatment, 1 mM fresh prepared FFA was added to HUVEC cell media for various times depending on the respective experiment. For the lixisenatide treatment experiment, 10 and 20 nM lixisenatide was added to HUVEC cell media for 24 h. THP1 cells were stained with calcein-AM for 20 min. 5 × 10⁶ THP1 were added to 1 × 10⁶ HUVECs for 2 h. The adhesive THP1 cells were visualized with a fluorescent microscope.

**Real-time PCR analysis**

RNA was extracted from HUVECs using a Kit (Cat.74004, Qiagen). 1 µg of extracted RNA was applied to produce cDNA using a kit (#1708840, BioRad). For real-time PCR, an SYBR Green master mix was used to assess mRNA of human ICAM-1, VCAM-1, TNF-α and IL-6 on the ABI 7500 platform.

**Western blot analysis**

HUVECs were lysed by RIPA buffer. The nuclear fragments were extracted from HUVECs using a commercial kit. 20 µg samples were subjected to 8–10% SDS-PAGE. Samples were then transferred to PVDF membranes. Membranes were blocked and sequentially probed with primary and secondary antibodies. Bands were visualized using Pierce ECL Plus (Catalog # 32132). The primary antibodies used were the following: ICAM-1, VCAM-1, eNOS, p-IκBα, IκBα, p65, Laminin and β-actin.

**Measurement of cell viability and LDH release**

The viability of HUVECs was determined using MTT. HUVECs were briefly incubated for a period of 4 h with 1 mg/ml MTT. Product was dissolved with DMSO. OD value at 560 nm was detected to reflect cell viability.

Cytotoxicity was examined by measuring LDH release from the cytoplasm to the medium using a commercial kit. After treatment, 100 µL medium was mixed with an equal amount of reaction buffer and incubated for 20 min. OD value was detected to reflect cell viability.

**ROS and 4-HNE assay**

ROS production in HUVECs was assessed using the dye 2',7'-dichlorofluorescin diacetate dye (DCFH-DA). After stimulation, cells were washed and probed with 10 µM DCFH-DA for 1 h. The fluorescent signals were detected using a fluorescent microscope.

The levels of 4-HNE in HUVECs were assessed by the immunofluorescence to reflect lipid oxidation. After treatment, cells were fixed with 4% paraformaldehyde. After permeabilization with 0.1% triton-x 100, cells were loaded with the anti-HNE antibody and the Alexa 594-conjugated secondary antibody. The fluorescent signals were detected using a fluorescent microscope.

**NO determination**

Intracellular NO was measured with DAF-FM DA. After treatment, HUVECs were loaded with 10 µM DAF-FM DA and incubated for 30 min. The fluorescent signals were detected using a fluorescent microscope.

**Promoter assay**

HUVECs were co-transfected with plasmids containing NF-κB promoter and a firefly luciferase promoter. Twenty-four h later, cells were stimulated with 1 nM FFA with or without 10 and 20 nM lixisenatide for another 24 h. Cells were then lysed to measure luciferase activity of renilla and firefly.

**Statistical analysis**

Results are expressed as means ± standard derivation (SD). All three or more-group comparisons were performed by ANOVA. A p value less than .05 was considered to be statistically significant.

**Results**

**Lixisenatide protects against high FFA-induced cell death of endothelium**

First, we tested the effects of the presence of lixisenatide on FFA-mediated endothelial disturbance. As shown in Figure 1(A), 1 mM high FFA solution reduced HUVEC death by about half. However, roughly 75% and 90% HUVECs were viable when 10 and 20 nM lixisenatide was used. Meanwhile, our cell toxicity experiment also confirmed the protective effect of lixisenatide. As shown in Figure 1(B), about 5% LDH was released into HUVECs at the basal level, and the addition of high concentration FFA solution resulted in about 42% LDH release. However, high FFA treatment resulted in only about 25% and 12% LDH release when 10 and 20 nM lixisenatide was used, respectively.

**Lixisenatide mitigates high FFA-induced oxidative stress**

We examined the change in ROS status in the presence of lixisenatide. Compared to non-treated HUVECs, high FFA caused roughly 3.7-fold cellular ROS production. However, the same FFA treatment led to only roughly 2.5- and 1.5-fold ROS production with 10 and 20 nM lixisenatide, respectively (Figure 2(A)). Compared to non-treated cells, high FFA
treatment caused roughly three-fold 4-HNE release. However, treatment with the same concentration of FFA resulted in only roughly 1.8- and 1.4-fold 4-HNE release with 10 and 20 nM lixisenatide, respectively (Figure 2(B)).

**Lixisenatide mitigates high FFA-induced production of proinflammatory cytokines**

To test the influence of lixisenatide on inflammation, we assessed two major pro-inflammatory cytokines. High FFA treatment increased TNF-α mRNA expression to roughly 5.2-fold basal levels. However, the same FFA treatment only induced roughly 3.5- and 2.3-fold increase in TNF-α with 10 and 20 nM lixisenatide (Figure 3(A)). Similarly, high FFA increased IL-6 expression by roughly five-fold. However, FFA treatment only induced roughly 3- and 2-fold increase in IL-6 expression with 10 and 20 nM lixisenatide (Figure 3(A)). Consistently, high FFA treatment led to roughly 3.5-fold TNF-α release into the media. However, the same FFA treatment caused only roughly 2.2- and 1.5-fold TNF-α release with 10 and 20 nM lixisenatide, respectively (Figure 3(B)). Meanwhile, FFA treatment induced roughly 3-fold IL-6 production. However, the presence of lixisenatide caused only roughly 2- and 1.2-fold IL-6 release, respectively (Figure 3(B)).

**Lixisenatide suppresses high FFA-induced vascular adhesion molecules expression**

Next, we examined the presence of lixisenatide on the effect of FFA on vascular adhesion molecules. High FFA induced roughly 7.5-fold mRNA expression of ICAM-1, but only yielded roughly 5- and 3-fold ICAM-1 expression with 10 and 20 nM lixisenatide, respectively (Figure 4(A)). Similarly, high FFA gave rise to roughly eight-fold VCAM-1 expression, but only yielded roughly 4.5- and 3-fold VCAM-1 expression with 10 and 20 nM lixisenatide, respectively (Figure 4(A)). ICAM-1 is virtually non-detectable by western blot under normal conditions. However, treatment with high FFA triggered the production of a large amount of ICAM-1 protein. Notably, ICAM-1 expression was reduced to 60% and 30% with 10 and 20 nM lixisenatide (Figure 4(B)). Protein expression of VCAM-1 is also very low at basal levels while high FFA treatment triggered the production...
of a large amount of VCAM-1, which was reduced to 50% and 20% with 10 and 20 nM lixisenatide (Figure 4(B)). These experiments confirmed the inhibitory action of lixisenatide on major adhesion molecules.

**Lixisenatide mitigates high FFA-induced adhesion of immune cells to HUVECs**

The inhibitory role of lixisenatide on endothelial adhesion molecules prompted our next experiment, i.e. monocyte and endothelial cell adhesion assay. We relabeled THP-1 cells and added the fluorescence-labelled cells to HUVECs. High FFA treatment caused binding of roughly 3.3-fold more THP-1 to HUVECs. However, normal FFA levels only caused binding of roughly 2- and 1.3-fold more THP-1 to HUVECs with 10 and 20 nM lixisenatide, respectively (Figure 5). In conclusion, lixisenatide suppressed FFA-induced attachment of monocytes to HUVECs.

**Lixisenatide mitigates high FFA-induced reduced eNOS phosphorylation and nitric oxide generation**

To measure the biological effect of FFA on endothelial function, we examined eNOS and NO generation status in our treatment experiment. At baseline, there is a fair amount of eNOS phosphorylation and total eNOS protein while the addition of high FFA solution had no effect on the total eNOS level, but rather reduced the level of phosphorylated eNOS (p-eNOS) by about half. However, the same FFA treatment caused roughly 65% and 80% p-eNOS recovery with 10
and 20 nM lixisenatide, respectively (Figure 6(A)). Compared to basal levels, high FFA treatment resulted in a reduction in cellular NO of about half. However, the presence of 10 and 20 nM lixisenatide recovered cellular NO by roughly 75% and 95% NO (Figure 6(B)).

**Lixisenatide suppresses high FFA-induced activation of NF-κB**

Our next experiment explored the molecular pathways through which the protective effects of lixisenatide are involved in endothelial cells. We tested the effect of lixisenatide on the NF-κB pathway. In cytoplasm, phosphorylation of IκBα is the key process driving activation of NF-κB. In our experiment, referencing non-treated HUVECs, high FFA treatment triggered roughly three-fold p-IκBα. However, high FFA treatment caused only roughly 2- and 1.5-fold p-IκBα with 10 and 20 nM lixisenatide (Figure 7(A)). This experiment suggests that lixisenatide inhibits the activation of IκBα and could influence downstream NF-κB signalling. Next, we tested nuclear NF-κB signalling by examining p65 accumulation and NF-κB promoter activity in nuclear samples. We extracted nuclear samples from HUVECs and compared the respective p65 levels. Compared to non-treated cells, high FFA treatment caused roughly 3.5-fold more accumulation of p65 in nuclear samples, however high FFA treatment induced only roughly 2.5- and 1.5-fold accumulation of p65 with 10 and 20 nM lixisenatide, respectively (Figure 7(B)). In another experiment, we transfected NF-κB luciferase promoter and assessed the effects of the addition of lixisenatide on the action of high FFA treatment on NF-κB promoter. When compared with the promoter control only, high FFA treatment triggered roughly 30-fold NF-κB promoter activity. However, high FFA treatment induced only roughly 18- and 5-fold promoter activity in the addition of 10 and 20 nM lixisenatide, respectively (Figure 7(C)). These results indicate that lixisenatide suppresses activation of NF-κB induced by high FFA levels.

**The effect of lixisenatide against FFA-induced endothelial dysfunction is mediated by the GLP-1 receptor**

To study whether the protective effect of lixisenatide against FFA-induced endothelial dysfunction is mediated by the GLP-1 receptor, the GLP-1R antagonist exendin 9–39 (Ex(9–39)) (100 nM) was used. The inhibitory effects of lixisenatide on FFA-induced LDH release (Supplementary Figure 1A), ICAM-1, and VCAM-1 expression are negated by the presence of Ex(9–39) (Supplementary Figure 1A). Importantly, the presence of Ex(9–39) negated the inhibitory effects of lixisenatide against FFA-induced adhesion of THP-1 cells to HUVECs.

**Discussion**

As an incretin-based therapy, GLP-1 receptor agonists represent an important element of pharmacological management for type 2 diabetes. A comprehensive assessment of cardiovascular outcomes of patients with type 2 diabetes treated with GLP-1 RAs in clinical trials revealed a significantly lower risk of cardiovascular disorders in GLP-1 RA-treated patients compared with placebo-treated patients [8,13]. These clinical outcomes indicate that GLP-1 RAs exert cardiovascular benefits independent of their glucose lowering ability. GLP-1 RAs have been investigated for their efficiency in the management of cardiovascular diseases. Early research shows that administration of GLP-1 into hypertension-afflicted rats inhibits the progression of hypertension and facilitates improvements in endothelial and cardiac function [14]. GLP-1 receptor deficient mice display impaired cardiac function [15]. Short-term GLP-1 infusion has been shown to exert beneficial effects on left ventricular function in acute myocardial infarct patients [16,17]. In healthy non-diabetic subjects, administration of GLP-1 enhanced the increase of forearm blood flow [18]. Treatment with exenatide has been shown to have a consistent effect on blood pressure reduction [19]. This evidence implicates that vascular endothelial cells are indeed involved in GLP-1- and GLP-1 RA-mediated cardiovascular modulation. There have been multiple studies involving GLP-RAs, including exendin-4, exenatide, lixisenatide and liraglutide, in endothelial cells. Both lixisenatide and exenatide are synthetic peptides based on exendin-4. Exendin-4 has been shown to reduce vascular permeability and inhibit vascular inflammatory and oxidative responses [20,21]. Exenatide has direct vascular protective effects on endothelial cells and modulates vascular inflammation [22–24]. Liraglutide is a GLP-1-derived peptide, and it has been shown that liraglutide could also enhance endothelial function and suppress endothelial inflammation and atherosclerosis in animal models [25–28].
Lixisenatide has been evaluated in broad clinical studies. It has been shown that lixisenatide has a favourable level of safety and tolerability as a monotherapy [29]. However, the function of lixisenatide on vascular endothelial cells remains undetermined. Based on the findings of a previous study involving other GLP-1 RAs, it would be highly probable that lixisenatide has vascular effects in endothelial cells. Here, we assessed the role of lixisenatide in HUVECs. We applied high FFA solution to endothelial cells to mimic the common pathological conditions of prediabetes and diabetes. Higher levels of FFA have been known to cause vascular inflammation and ROS production, which are harmful to endothelial cells [7]. These findings show that lixisenatide possesses a significant effect in mitigating high FFA-induced cytotoxicity and cell death. The following are findings to support this proposed protective role of lixisenatide. First, lixisenatide suppresses FFA-induced induction of TNF-α, IL-6, ICAM-1 and VCAM-1. Second, the addition of lixisenatide suppresses adhesion of monocytes to endothelial cells triggered by high levels of FFA. Third, lixisenatide attenuates FFA-induced ROS production and reduces production of the lipid oxidation byproduct 4-HNE. Fourth, the presence of lixisenatide mitigates FFA-induced reduced eNOS activity and NO production. This evidence suggests that lixisenatide has both anti-inflammatory and anti-ROS properties and that it provides protection for endothelial cells against agents induced by injury. Our data demonstrate that lixisenatide could also inhibit FFA-elicited NF-κB activation. This inhibition is reflected by its effect on IκBα activation and p65 deposition in nuclei, as well as NF-κB promoter activity.

Higher levels of FFA are known to cause vascular inflammation and ROS production, which are harmful to endothelial cells.
cells [7]. The interaction between cytokines and the control of adhesion molecules expression are complex [30]. Elevated levels of TNF-α and IL-6 are linked with FFA-related endothelial dysfunction mainly via the NF-κB pathway [31]. Furthermore, FFA plays an important role in inducing inflammatory responses by increasing VCAM-1 and ICAM-1 expression through activation of NF-κB [32]. Interestingly, TNF-α is reported to increase the adhesion of monocytes to endothelial cells by stimulating the expression of VCAM-1, ICAM-1 and E-selectin [33]. However, it is still unknown whether elevated TNF-α and IL-6 increase the expression of adhesion molecules or whether lixisenatide exerted a direct effect.

Taken together, previous reports and our own findings strongly support the notion that GLP-1 RA family members possess endothelial protective effects. Given its potent receptor binding ability, lixisenatide is a potential candidate for application in diabetic patients with elevated FFA. Because of its glucose-lowering and vascular health protective effects, this dual benefit of lixisenatide may be ideal for preventing vascular disasters in diabetic patients.

Disclosure statement
No potential conflict of interest was reported by the authors.

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