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LOX-1, a bridge between GLP-1R and mitochondrial ROS generation in human vascular smooth muscle cells

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ABSTRACT

A growing body of evidence indicates that glucagon-like peptide-1 (GLP-1) agonists or dipeptidyl peptidase-4 (DPP-4) inhibitors play an important role in modulating oxidant stress in vascular beds. However, the underlying mechanism of this process remains unclear. In recent studies, we observed an increase in GLP-1 receptor (GLP-1R) expression in the aorta of LOX-1 knock-out mice. Since LOX-1 is a pivotal regulator of reactive oxygen species (ROS), we conducted studies to identify relationship between LOX-1, ROS and GLP-1 agonism or DPP-4 antagonism. We observed a sustained decrease in GLP-1R expression in human vascular smooth muscle cells (VSMCs) treated with ox-LDL. When VSMCs were treated with different concentration of liraglutide (a GLP-1 agonist) or NVPDPP728 (a DPP-4 inhibitor), expression of ROS decreased compared with ox-LDL alone treatment. To further prove that LOX-1 plays a pivotal role in ROS and GLP-1R expression, we treated VSMCs with LOX-1 antibody or transfected cells with human LOX-1 cDNA. The inhibitory effect of ox-LDL on GLP-1R expression was reversed with anti-LOX-1 antibody treatment, while the inhibitory effect of liraglutide and NVPDPP728 on ROS generation was attenuated when cells were transfected with LOX-1 cDNA. Our results suggest that LOX-1 may play a bridging role in GLP-1 activation and ROS interaction.

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1. Introduction

Atherogenesis is a complex disease process characterized by generation of large amounts of reactive oxygen species (ROS) [1,2]. Lectin-like oxidized low-density lipoprotein scavenger receptor-1 (LOX-1), a C-type lectin superfamily member, which binds, internalizes and degrades ox-LDL, plays a key role in the generation of ROS in several cell types like vascular smooth muscle cells (VSMCs), endothelial cells and macrophages [2–4].

Glucagon-like peptide-1 (GLP-1) is a gut hormone secreted by intestinal L cells after food ingestion [5]. There is much evidence for anti-diabetic function of GLP-1 based on the observations that it activates insulin release. GLP-1 also inhibits glucagon secretion in a glucose-dependent manner, delays gastric emptying, and represses food intake by promoting brain satiety. However, most of the effects of GLP-1 last for a brief period of time due to its catabolization by dipeptidyl peptidase-4 (DPP-4), an enzyme also known as CD26 [6]. This information has led to the introduction of GLP-1 agonists and DPP-4 inhibitors as treatment for diabetes. Besides the glycemic control, GLP-1 has also been reported to exert a variety of effects on the cardiovascular system in animal models and humans with and without diabetes [7–10].

It is thought that GLP-1 agonism decreases ROS production, and it subsequently improves vascular reactivity [11–14]. However, the mechanism linking GLP-1 agonism with inhibition of ROS generation is not clear. In recent studies, we serendipitously observed an increase in GLP-1 receptor (GLP-1R) expression in the aorta of LOX-1 knock-out (KO) mice. Since LOX-1 is a pivotal regulator of ROS generation, we postulated that LOX-1 may be a bridge in the interaction of GLP-1R and ROS.

2. Materials and methods

2.1. Reagents

Primary human aortic smooth muscle cells (VSMCs) and DMEM cell culture medium containing 25 mM glucose were purchased from ATCC (Manassas, VA). The GLP-1 agonist liraglutide was obtained from Sigma Aldrich (St. Louis, MO); GLP-1R antibody was

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purchased from Abcam (Cambridge, MA); anti-human LOX-1 antibody was a gift from T. Sawamura (National Cardiovascular Center Research Institute, Osaka, Japan). High TBARS ox-LDL was obtained from Biomedical Technologies Inc. (Stoughton, MA)., The DPP-4 inhibitor NVPDPP728 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine 2000 Transfection Reagent was bought from Invitrogen (Grand Island, NY).

2.2. Animals

C57BL/6 mice (also called wild-type, WT) were purchased from Jackson Laboratories (Bar Harbor, Maine). Homozygous LOX-1 KO mice were generated using previous protocols [15–17]. Briefly, after using C57BL/6 strain to backcross 8 times, the genetic background was totally replaced. Through brother–sister mating method, LOX-1 KO and C57BL/6 mice were bred and housed in the breeding colony at the animal room of our institution. Male mice, 7–10 weeks of age, were utilized in the present studies. All experimental procedures were approved by the Institutional Animal Care and Usage Committee.

2.3. Mouse vascular smooth muscle tissue isolation

Smooth muscle tissue was removed from the abdominal aorta of both wild type (WT) and LOX-1 KO mice using previously reported method [18]. The tissues were used for subsequent protein isolation and Western blot analysis.

2.4. Cell culture and transfection

VSMCs were maintained in DMEM medium supplemented with 10% FBS (ATCC, Manassas, VA).Cells were incubated in a humidified atmosphere with 5% CO2 at 37 °C. VSMCs between passages 3 and 8 were used for experiments. After seeding in 25 ml flask and grown to semi-confluent density (≈85%), cells were treated with ox-LDL (0, 10, 20, 40 μg/mL), liraglutide (0, 10, 100, 1000 ng/mL) and NVPDPP728 (0, 1, 10, 100 μg/mL) for 24 h. For transfection of LOX-1, cells were seeded in 6-well plates. When confluent, cells were transfected with PCI-neo empty plasmid (vector) or PCI-neo plasmid with human LOX-1 cDNA (hLOX-1) using lipofectamine 2000 (Invitrogen, Grand Island, NY).

2.5. Measurement of intracellular reactive oxygen species

Intracellular mitochondrial ROS generation was evaluated with MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen, Grand Island, NY). Mitochondrial ROS generation was assessed by fluorescence imaging as well as by flowcytometry (Becton Dickinson, Franklin Lakes, NJ) as described earlier [2]. Data were quantitated using FlowJo software.

2.6. Western blot

The relative expression of proteins was assessed as described earlier [19]. The dilutions of GLP-1R and LOX-1 antibodies were 1:1500 and 1:1000, respectively. β-actin was used to normalize the results. Image J software was used to quantify the extent of immunostaining.

2.7. Statistical analysis

All experiments were performed in quadruplicate. Values were analyzed with the use of one-way ANOVA (multiple means) or the Newman-Keuls-Student t test. Results are shown as means ± SD. A P value <0.05 was considered significant.

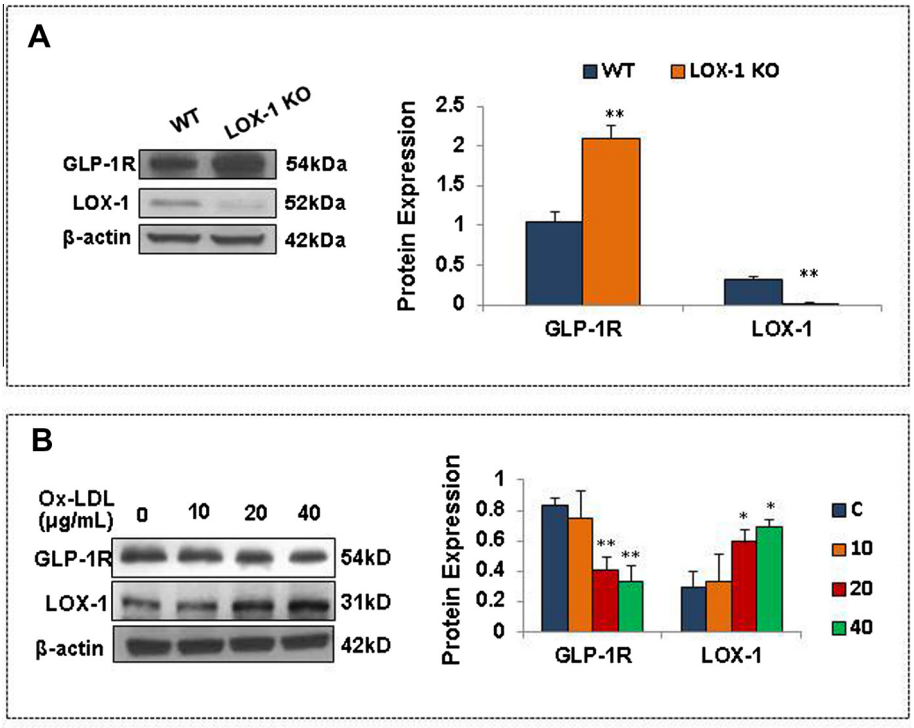

Fig. 1. (A) GLP-1R expression was increased in the aortas of mice with LOX-1 deletion (LOX-KO). (**P < 0.01 vs. WT) (B) GLP-1R protein expression decreased when human vascular smooth muscle cells (VSMCs) were treated with ox-LDL. Left panels show representative Western blots; right panels show summary of data from 4 independent experiments in mean ± SD. (*P < 0.05, **P < 0.01 vs. Control.)

3. Results

3.1. LOX-1 KO mice express more GLP-1R than WT mice and ox-LDL inhibits GLP-1R expression

We observed a marked increase in GLP-1R expression in LOX-1 KO mice compared to WT mice based on Western blot analysis ($P < 0.01$) (Fig. 1A). Note the absence of LOX-1 in the aortas of LOX-1 KO mice, confirming the genotype of the mice. Since LOX-1 is a determinant of ROS generation [1] and GLP-1R activation inhibits ROS generation [13], we examined the effects of different concentrations of ox-LDL as an activator for ROS. As shown in Fig. 1B, expression of GLP-1R decreased following treatment of cells with ox-LDL in a concentration-dependent fashion. As observed earlier [3], ox-LDL treatment simultaneously caused an increase in LOX-1 expression ($P < 0.01$ vs. control). The expression pattern of LOX-1 correlated negatively with the expression pattern of GLP-1R (Fig. 1B).

3.2. Liraglutide and NVPDPP728 inhibit mitochondrial ROS generation

Treatment of cells with ox-LDL resulted in marked mitochondrial ROS generation. Both the GLP_1 agonist liraglutide and the DPP-4 inhibitor NVPDPP728 inhibited mitochondrial ROS generation in the presence of ox-LDL. This effect was observed with fluorescence imaging (Fig. 2A) as well as flowcytometry (Fig. 2B). The effects of liraglutide and of NVPDPP728 were concentration-dependent.

3.3. ROS inhibits GLP-1R through upregulation of LOX-1

To define the role of LOX-1 in the interaction between ROS and GLP-1R expression, we used LOX-1 antibody to block its function. As expected, ROS generation increased in VSMCs after ox-LDL treatment, and this effect was blocked by LOX-1 antibody (Fig. 3A). Of note, there was no change in basal ROS generation in cells treated with LOX-1 antibody. The results of fluorescence imaging were confirmed by Western blot analysis (Fig. 3B). Further, the inhibitory effect of ox-LDL on GLP-1R expression was blocked by LOX-1 antibody ($P < 0.05$ vs. ox-LDL alone) (Fig. 3B). As control, LOX-1 antibody treatment without ox-LDL had no effect on basal GLP-1R expression.

3.4. Liraglutide and NVPDPP728 inhibit ROS through repressing LOX-1

To confirm that LOX-1 is the link between GLP-1 activation and ROS inhibition, we transfected VSMCs with LOX-1 cDNA (transfection efficiency ~80%), and examined if LOX-1 upregulation would block the inhibitory effect of NVPDPP728 and liraglutide on mitochondrial ROS generation. In cells transfected with empty vector, NVPDPP728 and liraglutide both dramatically reduced mitochondrial ROS generation. In keeping with our hypothesis on the role of LOX-1, forced upregulation of LOX-1 (cells transfected with LOX-1 cDNA) completely overcame the effects of NVPDPP728 and liraglutide. This was seen on immunocytochemistry as well as flow cytometry.

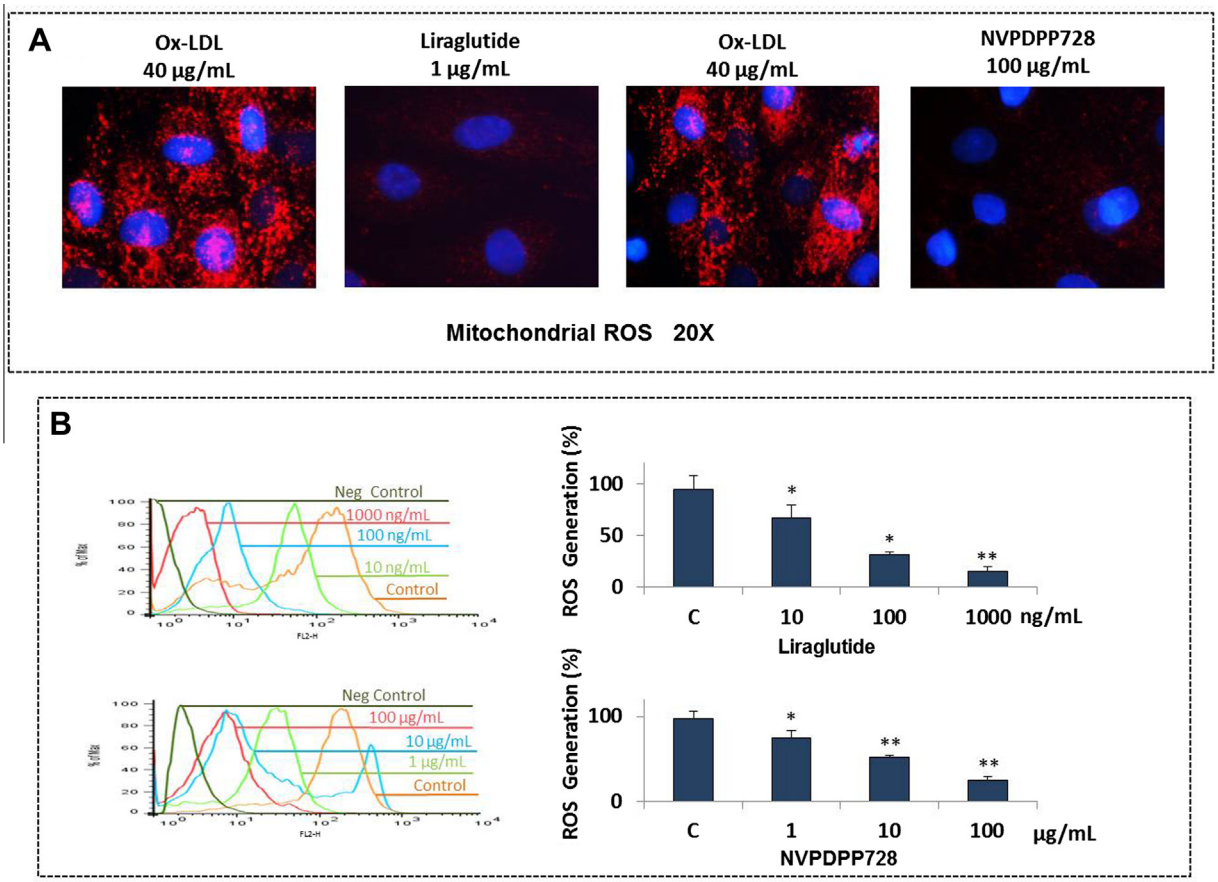

Fig. 2. (A) Representative fluorescence images of reduction in mitochondrial ROS generation (MitoSOX staining) after liraglutide (1 μg/mL) or NVPDPP728 (100 μg/mL) treatment. (B) Mitochondrial ROS generation in response to different concentration of liraglutide or NVPDPP728 treatment as measured by flowcytometry. Left panels show representative flowcytometry measurements; right panels show summary of data from 4 independent experiments in mean ± SD. (*$P < 0.05$, **$P < 0.01$ vs. Control.)

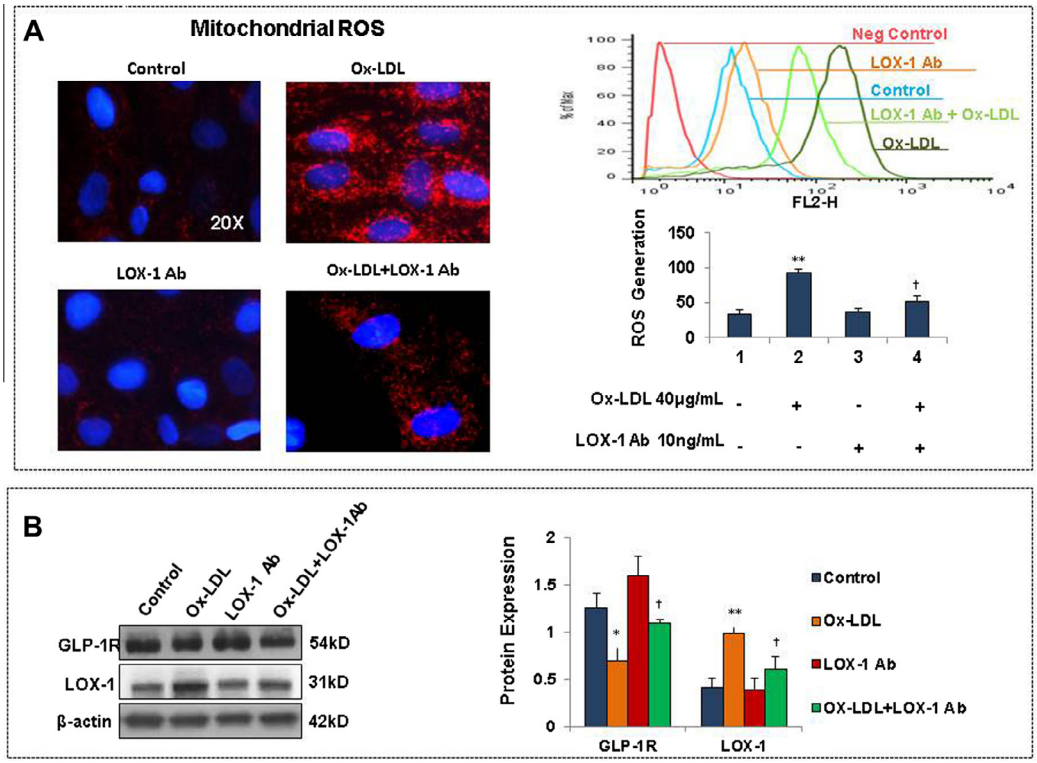

Fig. 3. (A) Representative fluorescence images of enhanced mitochondrial ROS generation (MitoSOX staining and flow cytometry data) following treatment of cells with ox-LDL. Note the reduction in ROS generation upon treatment of cells with LOX-1 antibody. Upper right panels show representative flow cytometry and summary of quantitative data from 4 independent experiments in mean ± SD. (B) LOX-1 blockade with LOX-1 antibody upregulates GLP-1R and reduces LOX-1 expression in ox-LDL treated VSMCs. Left panel shows representative Western blots; right panels show summary of data from 4 independent experiments in mean ± SD. ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $^{\dagger}P < 0.05$ vs. ox-LDL alone treatment.)

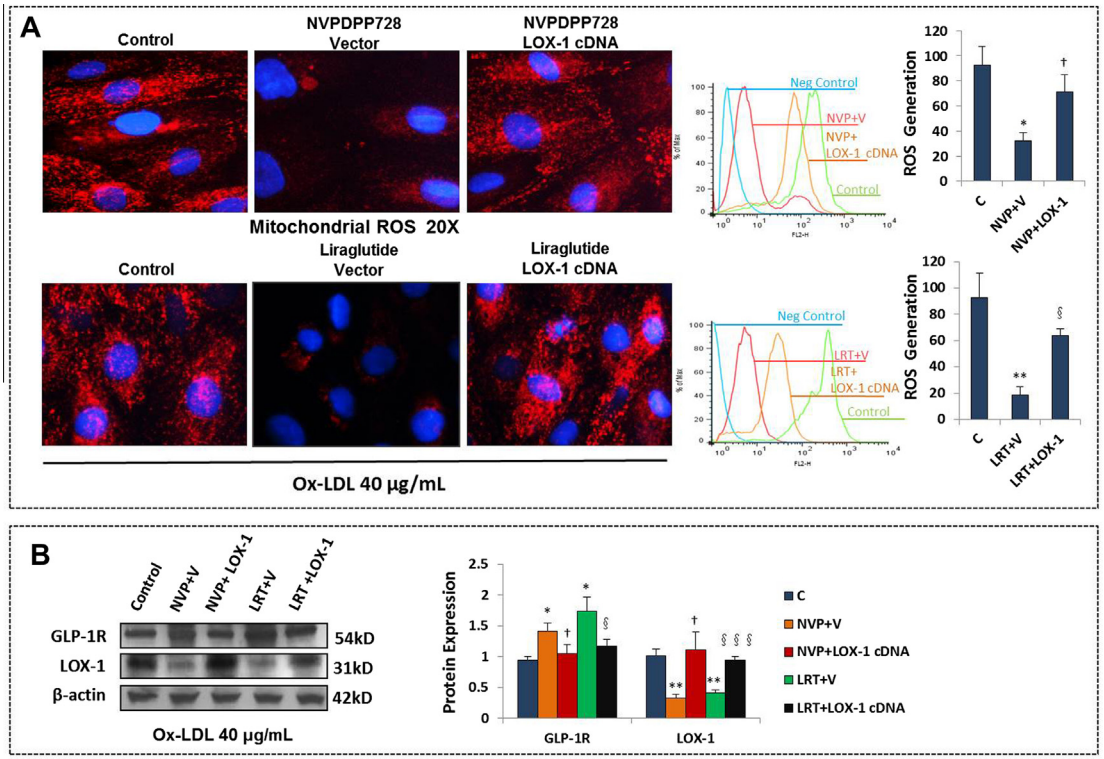

Fig. 4. (A) Suppression of ox-LDL- stimulated mitochondrial ROS generation by NVPDPP728 or liraglutide. The inhibitory effect of NVPDPP728 or liraglutide on mitochondrial ROS generation is countered by forced upregulation of LOX-1 (cells transfected with LOX-1 cDNA). Right panel shows representative flow cytometry experiments and summary of data from 4 independent experiments in mean ± SD. (B) GLP-1R and LOX-1 protein expression in cells treated with NVPDPP728 (NVP) or liraglutide (LRT). Cells were transfected with empty vector (V) or with LOX-1 cDNA. Bar graphs represent data in mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control, $^{\dagger}P < 0.05$ vs. NVP+V, $^{\S}P < 0.05$, $^{\S\S\S}P < 0.001$ vs. LRT+V.

The effect of NVPDPP728 and liraglutide GLP-1R expression were blocked by LOX-1 over-expression ($P < 0.05$ vs. empty vector-transfected group) (Fig. 4B).

4. Discussion

As mentioned in the Introduction, we serendipitously observed that LOX-1 KO mouse aorta expressed more GLP-1R as compared with WT mice aorta. This phenomenon prompted us to study the role of LOX-1 in the relationship between ROS and GLP-1R expression. Indeed, this study shows the existence of LOX-1 as a link between ROS and GLP-1R expression.

First, we observed that exposure of VSMCs to ox-LDL decreased the expression of GLP-1R in a concentration-dependent manner. As expected, ox-LDL treatment was associated with LOX-1 upregulation. Importantly, LOX-1 expression had a negative correlation with GLP-1R expression. Since ox-LDL treatment results in generation of large amounts of ROS [1], these observations imply that ROS inhibit GLP-1R expression in a LOX-1 dependent manner.

Our second major observation was that the GLP-1 agonist liraglutide as well as the DPP-4 inhibitor NVPDPP728 reduced ROS generation in VSMCs. This observation is consistent with previous studies which showed that GLP-1 agonist and DPP-4 inhibitor inhibited ROS in endothelial cells [13], islet cells [14] and vasculature of rats with sepsis [20]. Our study now extends this finding in VSMCs.

Since LOX-1 activation regulates generation of ROS [21], we conducted additional experiments to determine the role of LOX-1 as a link between ROS and GLP-1. We observed that blockade of LOX-1 with LOX-1 antibody resulted in GLP-1R upregulation despite treatment of VSMCs with ox-LDL. Further, when VSMCs were transfected with LOX-1 cDNA resulting in LOX-1 over-expression, the inhibitory effect of the GLP-1 agonist liraglutide and the DPP-4 inhibitor NVPDPP728 on mitochondrial ROS was markedly inhibited. These observations strongly suggest that LOX-1 is an important regulator of GLP-1 and ROS interaction.

Ox-LDL is one of the major contributors in atherogenesis. It stimulates LOX-1 and increases intracellular ROS expression. However, as a scavenger receptor, LOX-1 is not only induced by its ligand ox-LDL, but also it activated by ROS [21]. In this study, we showed that the GLP-1 activator liraglutide as well as DPP-4 inhibitor NVPDPP728 increased GLP-1R expression and simultaneously inhibited LOX-1 expression and mitochondrial ROS generation. On the other hand, ox-LDL which stimulates LOX-1 expression reduced the expression of GLP-1R on cell membrane which would hinder GLP-1 binding to its receptor. Ox-LDL treatment of VSMCs also stimulated mitochondrial ROS generation. The increase in ROS generation may stimulate pro-inflammatory cytokines [22]. Although we did not measure the expression of these cytokines under the influence of liraglutide or NVPDPP728, several reports suggest that this is indeed the case. Shiraki et al. [23] demonstrated that liraglutide could reduce tumor necrosis factor-alpha induced oxidative stress and subsequent inflammation in endothelial cells. Similarly, Erdogdu et al [14] recently showed that exendin-4, another GLP-1 agonist, protected endothelial cells from lipoapoptosis via modulation of ROS/MAPKs pathway. These findings lend support to the results of our study. It is of note that inhibition of LOX-1 abolishes or markedly reduces the generation of pro-inflammatory cytokines [24].

Collectively, these observations imply that GLP-1 agonism and DPP-4 inhibition have an inhibitory effect on mitochondrial ROS generation, while GLP-1R expression is upregulated. The upregulation of GLP-1R and inhibition of ROS can be blocked by ox-LDL as well as by over-expression of LOX-1 in VSMCs. We propose that LOX-1 plays an important, if not the key, role in the interaction between ROS and GLP-1R upregulation by GLP-1 agonists and DPP-4 inhibitors. These effects of GLP-1 agonists and DPP-4 inhibitors may have a bearing on their cardioprotective effects.

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