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Glucagon-like peptide-1 receptor agonist dulaglutide prevents ox-LDL-induced adhesion of monocytes to human endothelial cells: An implication in the treatment of atherosclerosis

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ABSTRACT

Atherosclerosis is a common comorbidity of type II diabetes and a leading cause of death worldwide. The presence of oxidized low-density lipoprotein (ox-LDL) drives atherogenesis by inducing oxidative stress, mitochondrial dysfunction, expression of proinflammatory cytokines and chemokines including interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein 1 (MCP-1), adhesion molecules including vascular cellular adhesion molecule 1 (VCAM-1) and E-selectin, and downregulating expression of the Krüppel-like factor 2 (KLF2) transcription factor. Importantly, ox-LDL induced the attachment of THP-1 monocytes to endothelial cells. In the present study, we demonstrate for the first time that the specific glucagon-like peptide 1 receptor (GLP-1R) agonist dulaglutide may prevent these atherosclerotic effects of ox-LDL by preventing suppression of KLF2 by p53 protein in human aortic endothelial cells. KLF2 has been shown to play a major role in protecting vascular endothelial cells from damage induced by ox-LDL and oscillatory shear, and therefore, therapies capable of mediating KLF2 signaling may be an attractive treatment option for preventing the development and progression of atherosclerosis.

1. Introduction

Among the greatest threats to global health, diabetes mellitus is an increasingly common whole-body disease associated with numerous comorbidities including cardiovascular disease. Compared to the normal population, diabetes patients have more than 4-fold greater risk of developing cardiovascular disease (Martín-Timón et al., 2014). As the leading cause of death among diabetes patients, cardiovascular disease presents an important treatment target, but the mechanisms driving the development and progression of cardiovascular complications are complicated (Abdul-Ghani et al., 2017; Fox et al., 2004). Atherosclerosis is a cardiovascular disease process in which fatty plaque forms on the intima of mid-sized arteries at areas of oscillatory shear. This leads to sustained inflammation, hardening of the arterial wall due to accumulation of macrophages in the intima, narrowing of the artery and eventual arterial occlusion, thereby preventing the vasculature from performing its task of delivering adequate supply of oxygen and nutrients throughout the body and disrupting whole-body homeostasis (Tabas et al., 2015; Chinetti-Gbaguidi et al., 2015; Bäck and Hansson, 2015). Lipoprotein disorders associated with diabetes play an important role in the development of atherosclerosis by promoting accumulation of plaque and oxidized low-density lipoprotein (ox-LDL) in the vasculature (Furukawa et al., 2018). Endothelial dysfunction is closely related to the pathogenesis of atherosclerosis in patients with type II diabetes (Tabit et al., 2010). Importantly, chronic exposure of endothelial cells to ox-LDL and hyperglycemic conditions induces an inflammatory response and expression of vascular adhesion molecules by endothelial cells including vascular adhesion molecule 1 (VCAM-1), which causes neutrophils and monocytes to roll along and adhere to the endothelial wall (Renier et al., 2007).

Recently, the role of the incretin hormone glucagon-like peptide 1 (GLP-1) has been receiving increasing attention as a treatment target for diabetes as well as other diseases. GLP-1 is excreted by intestinal L-cells postprandially and is involved in regulating glucose levels, insulin secretion, gastric emptying and satiety (Renier et al., 2007). The two main methods of modulating GLP-1R are inhibition of the enzyme

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dipeptidyl peptidase 4 (DPP-4), which rapidly degrades native GLP-1, or administration of GLP-1 analogues that are resilient to degradation by DPP-4 (Ahrén and Schmitz, 2004). Exendin-4 is an incretin hormone isolated from the saliva of the Gila monster lizard which has served as the basis for the development of several GLP-1 analogues including exenatide, liraglutide, albiglutide, lixisenatide and most recently, dulaglutide (Jimenez-Solem et al., 2010; Trujillo et al., 2015). Dulaglutide is produced by Eli Lilly and received FDA approval as a once-weekly treatment for type II diabetes in 2014 (Sanford, 2014). Dulaglutide consists of two GLP-1 analogues, one of which is covalently bound to an Fc fragment of human IgG4 antibody (Thompson and Trujillo, 2015). In the present study, we investigate the effects of dulaglutide on the expression of vascular adhesion molecules and subsequent adhesion of THP-1 monocytes to human aortic endothelial cells (HAECs) induced by exposure to ox-LDL.

2. Materials and methods

2.1. Cell culture and treatment

HAECs were purchased from Lonza. HAECs were cultured in EGM-2 endothelial cell growth medium-2 bullet kit supplemented with 10% FBS. Human THP-1 monocytes were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h.

2.2. Assessment of reactive oxygen species (ROS)

Intracellular ROS in HAECs was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h and washed 3 times with PBS. Cells were then loaded with 5 μM DCFH-DA for 15 min in darkness at 37 °C. Fluorescent signals were visualized using a Zeiss fluorescence microscope. Intracellular ROS was calculated using Image J software. Briefly, regions of interest (ROI) were defined in the fluorescent image, and the average number of cells present in the defined ROI was counted. The integrated density value (IDV) in the ROI was calculated and divided by the average number of cells. The results were used to represent the average level of intracellular ROS.

2.3. Reduced glutathione (GSH) assay

Intracellular levels of reduced glutathione (GSH) in HAECs were determined using a fluorometric assay. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. Cells were then collected in ice cold 5% meta-phosphoric acid (MPA). Cells were then sonicated and centrifuged at 14,000 × g for 5 min. Supernatant was incubated with an equal volume of OPAME (Sigma-Aldrich, USA) in methanol and borate buffer and incubated for 15 min at RT. Fluorescent signals were recorded at 350 nm excitation and 420 nm emission.

2.4. Determination of mitochondrial membrane potential (MMP)

Intracellular levels of MMP in HAECs were determined using tetramethylrhodamine methyl ester (TMRM) (Invitrogen, USA) staining. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. Cells were then washed 3 times with PBS and probed with 20 nmol/L TMRM. After incubation for 1 h at 37 °C, cells were washed 3 times and fluorescent signals were visualized using a Zeiss fluorescence microscope.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Culture medium was collected for ELISA assay using human IL-1β, IL-6, MCP-1, HMGB-1, VCAM-1, and E-selectin ELISA kits in accordance with the manufacturer's instructions (R&D Systems, USA).

2.6. Real-time polymerase chain reaction (PCR) analysis

Total RNA from HAECs was isolated using Trizol (Invitrogen, USA). Total intracellular RNA (1 μg) was used to synthesize cDNA with an iScript cDNA synthesis kit (Bio-Rad, USA). Expression of target genes was determined using a real-time PCR analysis kit with SYBR Green quantitative PCR Master Mix (Fermentas, Ontario, Canada) on a StepOne Sequence Detector (Applied Biosystems, USA). Expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH.

2.7. Western blot analysis

HAECs were lysed in cell lysis buffer containing phosphate and protease inhibitors. Protein samples were then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore). Membranes were then blocked with 5% non-fat milk for 1 h at RT. After that, the membranes were sequentially incubated with primary antibodies over night at 4 °C and secondary antibodies at RT. The specific protein bands were detected with ECL-Plus (GE Healthcare, USA).

2.8. Cellular adhesion assay

HAECs were cultured to 80% confluence. Cells were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. A total of 2×10^5 THP-1 monocytes were stained with calcein acetoxymethyl ester (calcein AM, Invitrogen, USA) for 30 min and incubated with HAECs for 2 h. Unattached THP-1 cells were washed away and attached THP-1 cells were visualized using a fluorescence microscope.

2.9. Assessment of cell viability

HAECs were seeded into 6-well plates and stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. After 3 gentle washes, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in phenol-free red medium at the final concentration of 5 mg/ml was added and incubated for 4 h at 37 °C in darkness, the product was dissolved with dimethyl sulfoxide (DMSO). OD value at 570 nm was measured to reflect the viability percentage.

2.10. Measurement of lactate dehydrogenase (LDH) release

HAECs were seeded into 6-well plates and stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. 50 μl supernatant was collected and mixed with 50 μl of the LDH assay reagent in a fresh 96-well plate. After incubation for 30 min in darkness, the reaction was stopped with 50 μl stop buffer. OD value at 490 nm was recorded to assess LDH release.

2.11. Statistical analysis

Experimental data are shown as means ± S.E. Statistical analysis was performed with the software SPSS (version 20). Comparisons between multiple groups were analyzed with analysis of variance (ANOVA). P values less than 0.05 were considered statistically

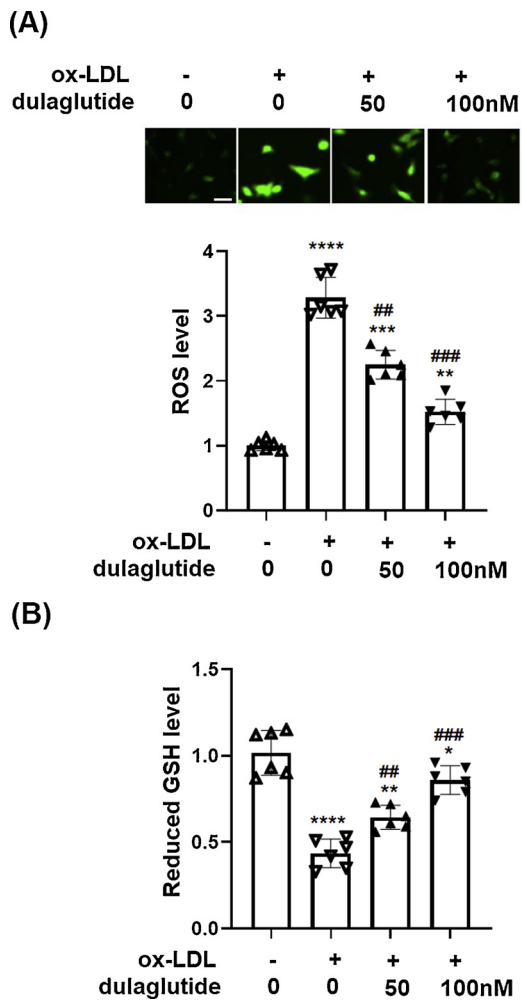

Fig. 1. Dulaglutide ameliorated ox-LDL-induced oxidative stress in human aortic endothelial cells (HAECs). HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. (A). Reactive oxygen species (ROS) was determined by DCFH-DA staining; Scale bar, 100 μm; (B). Reduced GSH was assessed (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ vs. control group; ##, $P < 0.01$, ###, $P < 0.001$ vs. ox-LDL alone group, n = 6).

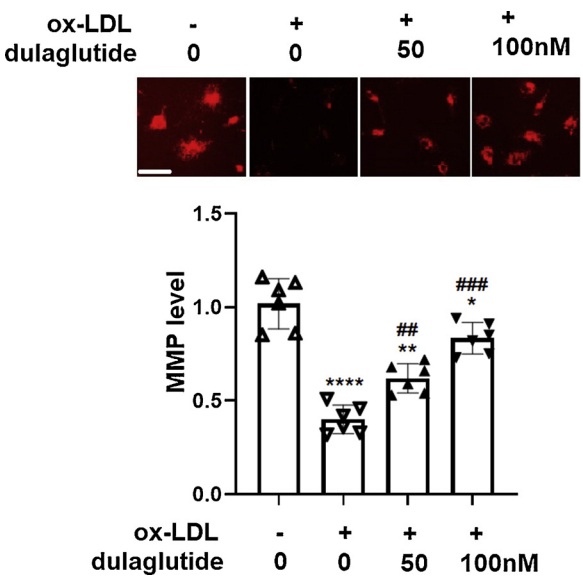

Fig. 2. Dulaglutide suppressed ox-LDL-induced mitochondrial dysfunction in human aortic endothelial cells (HAECs). HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. Mitochondrial membrane potential (MMP) was determined by TMRM staining. Scale bar, 100 μm (****, $P < 0.0001$, ***, $P < 0.001$, *, $P < 0.05$ vs. control group; ##, $P < 0.01$, ###, $P < 0.001$ vs. ox-LDL alone group, n = 6).

significant.

3. Results

3.1. Dulaglutide ameliorates ox-LDL-induced oxidative stress and mitochondrial dysfunction

Oxidative stress is well-recognized as playing a major role in the initiation and progression of atherosclerosis at all stages by triggering inflammatory cellular signaling pathways through the production of reactive oxygen species (ROS) (Bonomini et al., 2008; Singh and Jialal, 2006; Harrison et al., 2003). Dysfunction of the mitochondrial respiratory chain has also been cited as a characteristic of atherosclerosis that contributes to overproduction of ROS (Rosenblat et al., 2002). Here, we assessed the effect of dulaglutide against oxidative stress and mitochondrial dysfunction in HAECs induced by ox-LDL. Briefly, cells were treated with 10 μg/ml ox-LDL in the presence or absence of 50 and 100 nM dulaglutide for 24 h. As shown in Fig. 1A, ox-LDL increased the level of ROS to nearly 3.5-fold baseline, which was suppressed by dulaglutide in a dose-dependent manner with the higher dose of dulaglutide returning the level of ROS to near normal. Glutathione (GSH) is a powerful antioxidant that has been implicated in downregulating oxidation of LDL (Rosenblat et al., 2002). As shown in Fig. 1B, exposure of HAECs to ox-LDL decreased the level of GSH by more than half, which was returned to near baseline by treatment with dulaglutide in a dose-dependent manner.

Next, we assessed the effect of dulaglutide on mitochondrial dysfunction induce by ox-LDL. Mitochondria are a major site of ROS production and also suffer injury and loss of function from prolonged exposure to ROS (Madamanchi and Runge, 2007; Victor et al., 2009). Reduced mitochondrial membrane potential (MMP) is a driving force in numerous diseases including atherosclerosis (Emma and Bennett, 2014). We determined the effects of dulaglutide on MMP using TMRM staining. As shown in Fig. 2, exposure to 100 μg/ml ox-LDL reduced MMP by more than 60%, which was partially rescued by treatment with dulaglutide in a dose-dependent manner. These findings imply that agonism of GLP-1R with dulaglutide may prevent ox-LDL-induced oxidative stress and mitochondrial dysfunction in HAECs by downregulating production of ROS, preserving GSH, and partially preventing loss of MMP.

3.2. Dulaglutide suppresses ox-LDL-induced expression of proinflammatory cytokines and chemokines

Proinflammatory cytokines and chemokines are major players in the inflammatory response. Interleukin (IL)-1β and IL-6 have both been shown to contribute to the development of atherosclerosis (Huber et al., 1999; Kirii et al., 2003). Additionally, IL-6 has been cited as an independent predictor of plaque progression (Eltoft et al., 2018). An important chemokine in atherosclerosis is monocyte chemoattractant protein 1 (MCP-1). MCP-1 has recently been implicated in the development of atherosclerosis by recruiting monocytes to plaques. Furthermore, increased levels of MCP-1 have been shown to exist in atherosclerotic plaque and are considered an independent risk factor for death and atherosclerotic events in patients with chronic kidney disease (Gregg et al., 2018; Komiyama et al., 2016). In the present study, we investigated the effect of GLP-1R agonism on expression of these three cytokines by exposing HAECs to 100 μg/ml ox-LDL in the presence or absence of 50 and 100 nM dulaglutide for 24 h. As shown in Fig. 3,

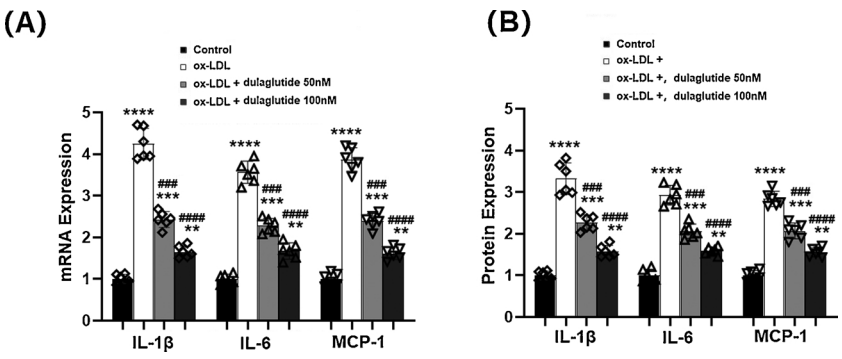

Fig. 3. Dulaglutide suppressed ox-LDL-induced expression and secretion of pro-inflammatory cytokines such as IL-1β, IL-6, and MCP-1. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. (A). mRNA levels of IL-1β, IL-6, and MCP-1 were determined by real time PCR analysis; (B). Secretion of IL-1β, IL-6, and MCP-1 were determined by ELISA (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$ vs. control group; ###, $P < 0.001$, ####, $P < 0.0001$ vs. ox-LDL alone group, n = 6).

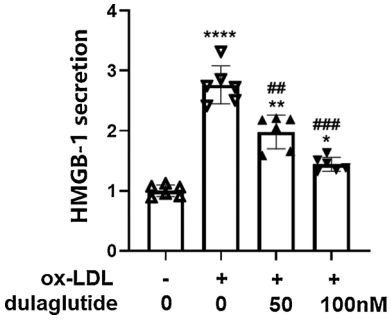

Fig. 4. Dulaglutide suppressed ox-LDL-induced secretion of high-mobility group protein 1 (HMGB-1). HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. Secretion of HMGB-1 was determined by ELISA (****, $P < 0.0001$, **, $P < 0.01$, *, $P < 0.05$ vs. control group; ##, $P < 0.01$, ###, $P < 0.001$ vs. ox-LDL alone group, n = 6).

exposure to ox-LDL nearly quadrupled expression of IL-1β, IL-6 and MCP-1 by HAECs at the mRNA level and increased expression of these three cytokines to approximately 3-, 2.8-, and 2.5-fold baseline at the protein level. Remarkably, treatment with dulaglutide reduced expression of IL-1β, IL-6 and MCP-1 to only roughly 1.5-fold baseline at both the mRNA and protein levels, thus demonstrating a strong inhibitory effect against ox-LDL-induced expression of proinflammatory cytokines.

High-mobility group protein 1 (HMGB-1) is a ubiquitously expressed chromatin protein that is released by damaged or activated cells and acts as a damage-associated molecular pattern (DAMP). HMGB-1 has been shown to be produced by activated vascular smooth muscle cells and is upregulated in cardiovascular and atherosclerotic calcification, atheromatous plaque, and atherosclerotic lesions (Chen et al., 2017; Inoue et al., 2007). To determine the effects of GLP-1R agonism on expression of HMGB-1 induced by ox-LDL, HAECs were exposed to 100 μg/ml ox-LDL in the presence or absence of 50 and 100 nM dulaglutide for 24 h. As demonstrated by the results of ELISA in Fig. 4, ox-LDL nearly tripled expression of HMGB-1, which was returned to near normal levels by dulaglutide in a dose-dependent manner. Thus, GLP-1R may play a role in regulating expression of HMGB-1 by vascular endothelial cells exposed to insult from ox-LDL.

3.3. Dulaglutide increases cell viability

Next, we set out to determine the effects of GLP-1R agonism using dulaglutide on cell viability of HAECs exposed to insult from ox-LDL. Briefly, cells were treated with 100 μg/ml ox-LDL in the presence or absence of 50 and 100 nM dulaglutide for 24 h. As shown in Fig. 5A, ox-LDL reduced cell viability to only roughly 64%, which was restored to roughly 83% and 93% by dulaglutide in a dose-dependent manner. Next, we measured the release of LDH into the medium. As shown in Fig. 5B, ox-LDL increased LDH release by more than 29%, which was drastically reduced by dulaglutide in a dose-dependent manner. These findings implicate a strong protective effect of dulaglutide against ox-LDL-induced cell death.

3.4. Dulaglutide reduces ox-LDL-induced expression of cellular adhesion molecules and adhesion of THP-1 monocytes to endothelial cells

One of the most important events in the development of atherosclerosis is the rolling of monocytes along the intima and subsequent attachment of monocytes to the vascular wall triggered by the expression of cellular adhesion molecules. VCAM-1 is an important adhesion molecule that plays a major role in initiating rolling and attachment of monocytes to endothelial cells (Cybulsky et al., 2001). Ox-LDL has been shown to induce VCAM-1 expression through FAK-dependent activation of IκB kinase β (IKKβ), thereby promoting monocyte adhesion and development of plaque (Yurdagul et al., 2016). E-selectin, another important adhesion molecule, mediates rolling of leukocytes along the endothelium and inflammation in atherosclerosis and cardiovascular disease (Tsoref et al., 2018). Here, we investigated the effect of

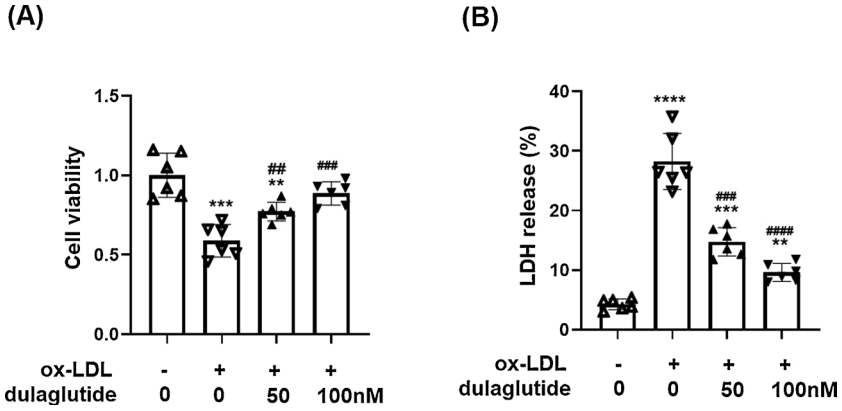

Fig. 5. Dulaglutide suppressed ox-LDL-induced reduction of cell viability and release of lactate dehydrogenase (LDH). HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. (A). Cell viability was determined by the MTT assay; (B). LDH release was determined by a commercial kit (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$ vs. control group; ##, $P < 0.01$, ###, $P < 0.001$, ####, $P < 0.0001$ vs. ox-LDL alone group, n = 6).

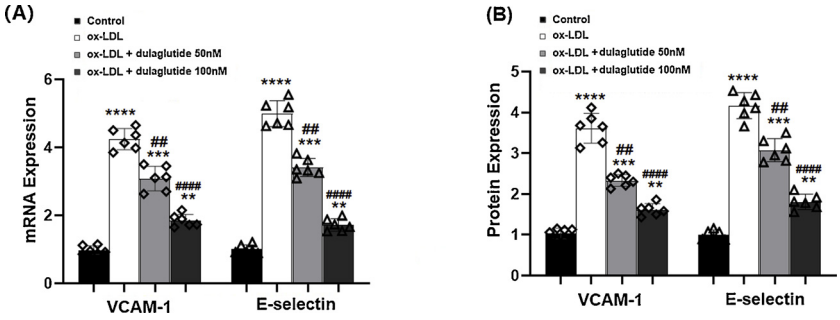

Fig. 6. Dulaglutide suppressed ox-LDL-induced expressions of VCAM-1 and E-selectin. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. (A). Expression of VCAM-1 and E-selectin at the mRNA levels was determined by real time PCR analysis; (B). Expression of VCAM-1 and E-selectin at the protein levels was determined by ELISA analysis (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$ vs. control group; ##, $P < 0.01$, ####, $P < 0.0001$ vs. ox-LDL alone group, n = 6).

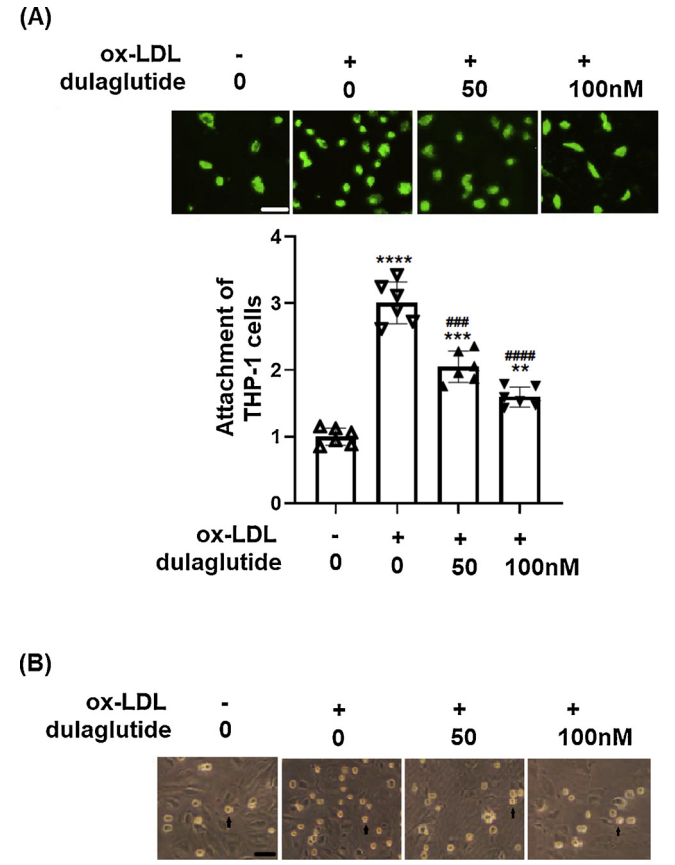

Fig. 7. Dulaglutide suppressed ox-LDL-induced the attachment of THP-1 cells to HAECs. HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. (A). The attachment of THP-1 cells to HAECs in was detected by a fluorescent microscope and quantified; (B). Representative photos for THP-1 cells in a phase contrast field after cell adhesion assay. Adhered cells were indicated by black arrow. Scale bar, 100 μm (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$ vs. control group; ###, $P < 0.001$, ####, $P < 0.0001$ vs. ox-LDL alone group, n = 6).

dulaglutide on expression of VCAM-1 and e-selectin by exposing HAECs to 100 μg/ml ox-LDL in the presence or absence of 50 and 100 nM dulaglutide for 24 h. As shown in Fig. 6A, ox-LDL induced roughly 4.2-ad 5-fold expression of VCAM-1 and e-selectin, respectively, as the mRNA level which was ameliorated to approximately 2-fold baseline by dulaglutide in a dose-dependent manner. These results were confirmed at the protein levels (Fig. 6B). Next, we assessed the effect of dulaglutide on attachment of THP-1 monocytes to HAECs induced by ox-LDL. Briefly, cells were stimulated with 100 μg/ml ox-LDL in the presence or absence of 50 and 100 nM dulaglutide for 24 h. Then, the culture medium was removed, and a total of 2×10^5 THP-1 monocytes in freshly prepared cell culture medium were added and incubated for 2 h. Unattached THP-1 cells were washed away. The results in Fig. 7A demonstrate that there was an approximate 3-fold increase in the number of THP-1 monocytes adhered to HAECs upon exposure to ox-LDL, which was reduced to roughly 1.5-fold baseline by agonism of GLP-1R using dulaglutide. Thus, these findings represent a potential role for dulaglutide in preventing rolling and attachment of monocytes to endothelial cells by downregulating ox-LDL-induced expression of VCAM-1 and e-selectin.

3.5. Dulaglutide rescues ox-LDL-induced reduction of KLF2

Finally, we determined the effect of dulaglutide on an important transcription factor involved in atherogenesis. Krüppel-like factor 2 (KLF2) has been shown to play an important protective role in regulating the inflammatory response in atherosclerosis and other cardiovascular diseases by mediating activation of cells and regulating the transcription of VCAM-1 and e-selectin involved in the development of atherosclerotic plaque (Alberts-Grill et al., 2016). Transcription of KLF2 is downregulated by p53 protein, so overexpression of p53 promotes atherogenesis by hindering the protective effects of KLF2, thereby promoting endothelial dysfunction and inflammation (Takabe et al., 2011). Here, we exposed cells to 100 μg/ml in the presence or absence of 50 and 100 nM dulaglutide for 24 h. As shown in Fig. 8, ox-LDL reduced expression of KLF2 by at least half at both the mRNA and protein levels. However, dulaglutide rescued KLF2 to roughly 80% baseline at both the mRNA and protein levels. Additionally, we show that exposure to ox-LDL roughly tripled phosphorylation of p53, which was reduced to near baseline by GLP-1R agonism using dulaglutide in a dose-dependent manner. Thus, the atheroprotective effects of dulaglutide may be mediated by the KLF2 pathway.

4. Discussion

In the present study, we explored a new role for dulaglutide, a novel GLP-1R agonist, in protecting human aortic endothelial cells against insult from ox-LDL in the context of atherosclerosis. Recently, there has been mounting evidence supporting GLP-1 agonism as a potential treatment target for preventing the development of atherosclerosis and cardiovascular disease related to type II diabetes. For example, the GLP-1 analogue lixisenatide was recently shown to prevent acute cardiovascular events in *Apoe*$^{-/-}$*Irs2*$^{+/-}$ mice by decreasing the size of atheroma plaques, increasing plaque stability, and promoting macrophages to the anti-inflammatory M2 phenotype (Vinué et al., 2017). Another study showed that liraglutide could delay atherogenesis by inducing cell cycle arrest in vascular smooth muscle cells via the AMPK pathway (Jojima et al., 2017). Notably, the findings of a contemporary study comparing dulaglutide and liraglutide suggest that dulaglutide is superior to liraglutide in that it offers improved glucose function, greater convenience to the patient and has similar results to liraglutide in terms of endothelial function and oxidative stress (Álvarez-Villalobos et al., 2016). A recent double-blind trial determined that agonism of GLP-1R using liraglutide reduced the risk of cardiovascular events including death and exerted a beneficial effect on the vascular

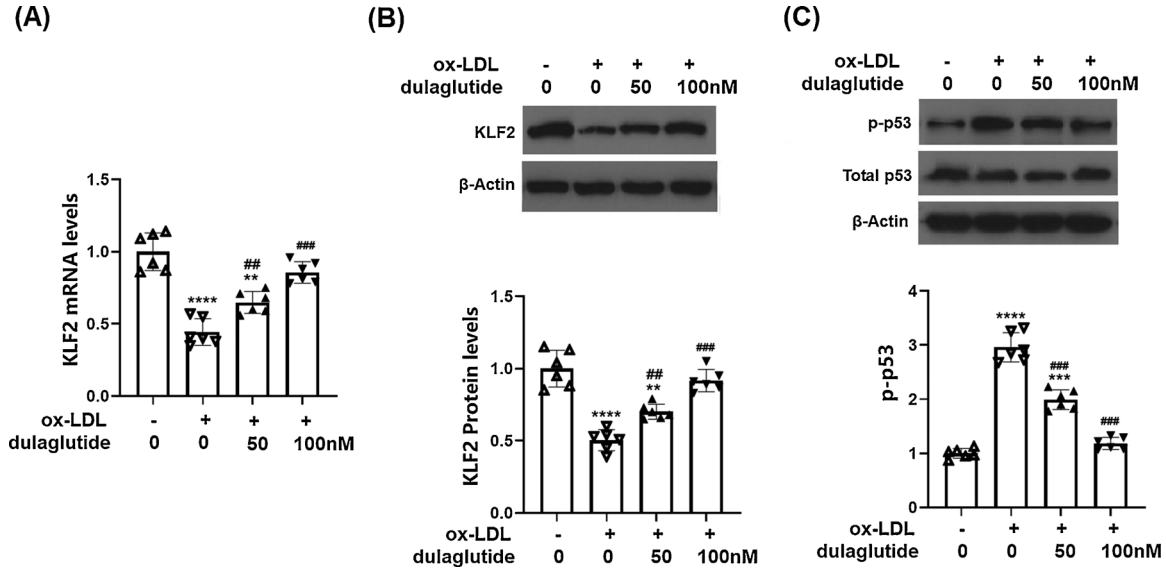

Fig. 8. Dulaglutide suppressed ox-LDL-induced reduction of KLF2 via p53 in HAECs. (A–B). HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 24 h. The expression of KLF2 at the mRNA and protein levels were determined by real time PCR and western blot analysis, respectively; (C). HAECs were stimulated with ox-LDL (100 μg/ml) in the presence or absence of dulaglutide at the concentrations of 50 and 100 nM for 2 h. Phosphorylated p53 were determined (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$ vs. control group; ##, $P < 0.01$, ###, $P < 0.001$ vs. ox-LDL alone group, n = 6).

endothelium in type II diabetes patients. However, the mechanism driving this effect remains elusive (Marso et al., 2016). To our knowledge, the present study is the first to explore the effects of dulaglutide on HAECs. Our findings demonstrate that dulaglutide, a preferred GLP-1R agonist, may exert a protective effect against atherosclerosis and cardiovascular disease by inhibiting oxidative stress and mitochondrial dysfunction, downregulating expression of cytokines, chemokines, and adhesion molecules, preventing attachment of monocytes to endothelial cells, and suppressing downregulation of the KLF2 transcription factor via phosphorylation of p53.

As incretin mimetics represent a relatively new class of drugs, it is important to gain a deeper understanding of the full potential of these safe and effective therapeutic agents. While there has been some controversy over the role of GLP-1 in atherosclerosis (Panjwani et al., 2013), recent research has demonstrated numerous cardiovascular benefits of GLP-1R agonism. However, the mechanisms facilitating these benefits are complicated. To our knowledge, this is the first study to highlight the potential role of the KLF2 transcription factor as a mediator of the anti-atherosclerotic effects of dulaglutide. KLF2 has been demonstrated to exert powerful protective effects against endothelial damage by inhibiting expression of VCAM-1 and e-selectin, thus preventing rolling and adhesion of monocytes to the endothelium, and by inducing expression of nitric oxide synthase by endothelial cells (SenBanerjee et al., 2004). Additionally, sustained oscillatory shear stress has been shown to induce expression of KLF2 in human vascular endothelial cells (Dekker et al., 2002). Notably, we found that dulaglutide significantly rescued KLF2 from downregulation by ox-LDL, most likely by preventing phosphorylation of p53 protein, which is known to suppress expression of KLF2.

In conclusion, our findings demonstrate an important new role of GLP-1R agonism using dulaglutide to prevent adhesion of monocytes to endothelial cells possibly through the KLF2 signaling pathway. Additionally, dulaglutide potently suppressed ox-LDL-induced oxidative stress and mitochondrial dysfunction, significantly inhibited expression of proinflammatory cytokines and chemokines, and exerted a powerful inhibitory effect on expression of VCAM-1 and e-selectin, thereby preventing adhesion of THP-1 monocytes to HAECs. Importantly, we show that the KLF2 pathway may be involved in this effect. Further research using animal models is necessary to better understand the mechanism driving this protective effect of dulasglutide.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abdul-Ghani, M., DeFronzo, R.A., Del Prato, S., Chilton, R., Singh, R., Ryder, R.E., 2017. Cardiovascular disease and type 2 diabetes: has the dawn of a new era arrived? Diabetes Care 40 (7), 813–820.
Ahrén, B., Schmitz, O., 2004. GLP-1 receptor agonists and DPP-4 inhibitors in the treatment of type 2 diabetes. Horm. Metab. Res. 36 (11/12), 867–876.
Alberts-Grill, N., Engelbertsen, D., Bu, D., Foks, A., Grabie, N., Herter, J.M., Kuperwaser, F., Chen, T., Destefano, G., Jarolim, P., Lichtman, A.H., 2016. Dendritic cell KLF2 expression regulates T cell activation and proatherogenic immune responses. J. Immunol., 1600206.
Álvarez-Villalobos, N.A., Treviño-Alvarez, A.M., González-González, J.G., 2016. Liraglutide and cardiovascular outcomes in Type 2 diabetes. N. Engl. J. Med. 375, 1797–1798.
Bäck, M., Hansson, G.K., 2015. Anti-inflammatory therapies for atherosclerosis. Nat. Rev. Cardiol. 12 (4), 199.
Bonomini, F., Tengattini, S., Fabiano, A., Bianchi, R., Rezzani, R., 2008. Atherosclerosis and oxidative stress. Histol. Histopathol. 23 (3), 381–390.
Chen, Q., Wang, Z.Y., Chen, L.Y., Hu, H.Y., 2017. Roles of high mobility group box 1 in cardiovascular calcification. Cell. Physiol. Biochem. 42 (2), 427–440.
Chinetti-Gbaguidi, G., Colin, S., Staels, B., 2015. Macrophage subsets in atherosclerosis. Nat. Rev. Cardiol. 12 (1), 10.
Cybulsky, M.I., Iiyama, K., Li, H., Zhu, S., Chen, M., Iiyama, M., Davis, V., Gutierrez-Ramos, J.C., Connelly, P.W., Milstone, D.S., 2001. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J. Clin. Invest. 107 (10), 1255–1262.
Dekker, R.J., van Soest, S., Fontijn, R.D., Salamanca, S., de Groot, P.G., VanBavel, E., Pannekoek, H., Horrevoets, A.J., 2002. Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2). Blood 100 (5), 1689–1698.
Emma, P.K., Bennett, M.R., 2014. Mitochondrial DNA damage and atherosclerosis. Trends Endocrinol. Metab. 25 (9), 481–487.
Eltoft, A., Arntzen, K.A., Wilsgaard, T., Mathiesen, E.B., Johnsen, S.H., 2018. Interleukin-

6 is an independent predictor of progressive atherosclerosis in the carotid artery: the Tromsø Study. Atherosclerosis 271, 1–8.
Furukawa, S., Suzuki, H., Fujihara, K., Kobayashi, K., Iwasaki, H., Sugano, Y., Yatoh, S., Sekiya, M., Yahagi, N., Shimano, H., 2018. Malondialdehyde-modified LDL-related variables are associated with diabetic kidney disease in type 2 diabetes. Diabetes Res. Clin. Pract. 141, 237–243.
Fox, C.S., Coady, S., Sorlie, P.D., Levy, D., Meigs, J.B., D'Agostino, R.B., Wilson, P.W., Savage, P.J., 2004. Trends in cardiovascular complications of diabetes. JAMA 292 (20), 2495–2499.
Gregg, L.P., Tio, M.C., Li, X., Adams-Huet, B., de Lemos, J.A., Hedayati, S.S., 2018. Association of monocyte chemoattractant protein-1 with death and atherosclerotic events in chronic kidney disease. Am. J. Nephrol. 47 (6), 395–405.
Harrison, D., Griendling, K.K., Landmesser, U., Hornig, B., Drexler, H., 2003. Role of oxidative stress in atherosclerosis. Am. J. Cardiol. 91 (3), 7–11.
Huber, S.A., Sakkinen, P., Conze, D., Hardin, N., Tracy, R., 1999. Interleukin-6 exacerbates early atherosclerosis in mice. Arterioscler. Thromb. Vasc. Biol. 19 (10), 2364–2367.
Inoue, K., Kawahara, K.I., Biswas, K.K., Ando, K., Mitsudo, K., Nobuyoshi, M., Maruyama, I., 2007. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. Cardiovasc. Pathol. 16 (3), 136–143.
Jojima, T., Uchida, K., Akimoto, K., Tomotsune, T., Yanagi, K., Iijima, T., Suzuki, K., Kasai, K., Aso, Y., 2017. Liraglutide, a GLP-1 receptor agonist, inhibits vascular smooth muscle cell proliferation by enhancing AMP-activated protein kinase and cell cycle regulation, and delays atherosclerosis in ApoE deficient mice. Atherosclerosis 261, 44–51.
Jimenez-Solem, E., Rasmussen, M.H., Christensen, M., Knop, F.K., 2010. Dulaglutide, a long-acting GLP-1 analog fused with an Fc antibody fragment for the potential treatment of type 2 diabetes. Curr. Opin. Mol. Ther. 12 (6), 790.
Kirii, H., Niwa, T., Yamada, Y., Wada, H., Saito, K., Iwakura, Y., Asano, M., Moriwaki, H., Seishima, M., 2003. Lack of interleukin-1β decreases the severity of atherosclerosis in ApoE-deficient mice. Arterioscler. Thromb. Vasc. Biol. 23 (4), 656–660.
Komiyama, M., Takanabe, R., Ono, K., Shimada, S., Wada, H., Yamakage, H., Satoh-Asahara, N., Akao, M., Morimoto, T., Shimatsu, A., Takahashi, Y., 2016. GW27-e1046 smoking status-dependent association between monocyte chemoattractant protein-1 and blood pressure. J. Am. Coll. Cardiol. 68 (Suppl. 16), C165.
Madamanchi, N.R., Runge, M.S., 2007. Mitochondrial dysfunction in atherosclerosis. Circ. Res. 100 (4), 460–473.
Martín-Timón, I., Sevillano-Collantes, C., Segura-Galindo, A., Del Cañizo-Gómez, F.J., 2014. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? World J. Diabetes 5 (4), 444–470.
Marso, S.P., Daniels, G.H., Brown-Frandsen, K., Kristensen, P., Mann, J.F., Nauck, M.A., Nissen, S.E., Pocock, S., Poulter, N.R., Ravn, L.S., Steinberg, W.M., 2016. Liraglutide and cardiovascular outcomes in type 2 diabetes. N. Engl. J. Med. 375 (4), 311–322.
Panjwani, N., Mulvihill, E.E., Longuet, C., Yusta, B., Campbell, J.E., Brown, T.J., Streutker, C., Holland, D., Cao, X., Baggio, L.L., Drucker, D.J., 2013. GLP-1 receptor activation indirectly reduces hepatic lipid accumulation but does not attenuate development of atherosclerosis in diabetic male ApoE−/− mice. Endocrinology 154 (1), 127–139.
Renier, G., Maingrette, F., Li, L., 2007. Diabetic vasculopathy and the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). Curr. Diabetes Rev. 3 (2), 103–110.
Rosenblat, M., Coleman, R., Aviram, M., 2002. Increased macrophage glutathione content reduces cell-mediated oxidation of LDL and atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 163 (1), 17–28.
Sanford, M., 2014. Dulaglutide: first global approval. Drugs 74 (17), 2097–2103.
SenBanerjee, S., Lin, Z., Atkins, G.B., Greif, D.M., Rao, R.M., Kumar, A., Feinberg, M.W., Chen, Z., Simon, D.I., Luscinskas, F.W., Michel, T.M., 2004. KLF2 is a novel transcriptional regulator of endothelial proinflammatory activation. J. Exp. Med. 199 (10), 1305–1315.
Singh, U., Jialal, I., 2006. Oxidative stress and atherosclerosis. Pathophysiology 13 (3), 129–142.
Tabas, I., García-Cardeña, G., Owens, G.K., 2015. Recent insights into the cellular biology of atherosclerosis. J. Cell Biol. 209 (1), 13–22.
Tabit, C.E., Chung, W.B., Hamburg, N.M., Vita, J.A., 2010. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. Rev. Endocr. Metab. Disord. 11 (1), 61–74.
Takabe, W., Alberts-Grill, N., Jo, H., 2011. Disturbed flow: p53 SUMOylation in the turnover of endothelial cells. J. Cell Biol. 193 (5), 805–807.
Trujillo, J.M., Nuffer, W., Ellis, S.L., 2015. GLP-1 receptor agonists: a review of head-to-head clinical studies. Ther. Adv. Endocrinol. Metab. 6 (1), 19–28.
Tsoref, O., Tyomkin, D., Amit, U., Landa, N., Cohen-Rosenboim, O., Kain, D., Golan, M., Naftali-Shani, N., David, A., Leor, J., 2018. E-selectin-targeted copolymer reduces atherosclerotic lesions, adverse cardiac remodeling, and dysfunction. J. Control. Release 288, 136–147.
Victor, V.M., Apostolova, N., Herance, R., Hernandez-Mijares, A., Rocha, M., 2009. Oxidative stress and mitochondrial dysfunction in atherosclerosis: mitochondria-targeted antioxidants as potential therapy. Curr. Med. Chem. 16 (35), 4654–4667.
Vinué, Á., Navarro, J., Herrero-Cervera, A., García-Cubas, M., Andrés-Blasco, I., Martínez-Hervás, S., Real, J.T., Ascaso, J.F., González-Navarro, H., 2017. The GLP-1 analogue lixisenatide decreases atherosclerosis in insulin-resistant mice by modulating macrophage phenotype. Diabetologia 60 (9), 1801–1812.
Yurdagul, A., Sulzmaier, F.J., Chen, X.L., Pattillo, C.B., Schlaepfer, D.D., Orr, A.W., 2016. Oxidized LDL induces FAK-dependent RSK signaling to drive NF-κB activation and VCAM-1 expression. J. Cell. Sci. 129 (8), 1580–1591.