Glucagon-Like Peptide-1 Receptor Agonist Reduces di(2-ethylhexyl) Phthalate-Induced Atherosclerotic Processes in Vascular Smooth Muscle Cells

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Summary

Glucagon-like peptide-1 receptor (GLP1R) agonist is an incretin hormone and regulates glucose metabolism. However, phthalates, known as endocrine disruptors, can interfere with hormone homeostasis. In the present study, we aimed to estimate the impact of GLP1R agonist on di(2-ethylhexyl) phthalate (DEHP)-induced atherosclerosis. For this purpose, the effects of GLP1R agonist on various atherogenesis-related cellular processes and pathways were assessed in vascular smooth muscle cells (VSMCs). DEHP-induced cell proliferation and migration were significantly decreased by GLP1R agonist in VSMCs. Protein levels of matrix metalloproteinase (MMP)-2 and MMP-9 were significantly decreased in cells exposed to GLP1R agonist, compared with DEHP-treated cells. Expression levels of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 were also reduced in GLP1R agonist-treated cells. Similarly, DEHP-associated phosphorylation of protein kinase B and extracellular signal-regulated kinase 1/2 was decreased in GLP1R agonist-treated cells, compared with DEHP-treated cells. Our findings suggest that treatment with GLP1R agonist counteracts the activation of pathways related to atherosclerosis.

Key words

Glucagon-like peptide-1 receptor (GLP1R) agonist • di (2-ethy-lhexyl) phthalate (DEHP) • Vascular smooth muscle cells (VSMCs) • Atherosclerosis

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Introduction

Cardiovascular disease (CVD) is a major cause of death and disability in diabetes (Laakso *et al*. 1999, Angeli FS and Shannon RP 2014). Atherosclerotic CVD includes myocardial infarction, peripheral arterial disease, and stroke and is a common cause of death in 80 % of the patients with type 2 diabetes (Martín-Timón *et al*. 2014). Atherosclerosis accompanied by vascular smooth muscle cell (VSMC) dysfunction is correlated to CVD risks (Abdul-Ghani *et al*. 2017, Doran *et al.* 2008). VSMCs play a pivotal role in vascular morphogenesis and regulation of vascular homeostasis (Ross 1995). Abnormal VSMC phenotype is a characteristic of vascular disorders, including atherosclerosis. Aberrant VSMCs in atherosclerosis promote the induction of highly proliferative VSMC phenotypes and plaque formation in the vessel environment (Ross 1995, Martin *et al*. 2016).

Di(2-ethylhexyl) phthalate (DEHP) is one of the most common phthalates acting as environmental endocrine disruptors and is widely used as a plasticizer to make plastic flexible (Hauser R 2005). Many studies show that DEHP affects the human reproductive system, the development of certain types of cancer, hepatotoxicity, and atherosclerosis-related CVD (Latini *et al*. 2006, Zhu *et al*. 2010, Ghosh *et al*. 2010). In addition, DEHP was shown to be associated with atherosclerosis-related CVD complications by promoting oxidative stress and increasing the expression of pro-inflammatory mediators (Zhao *et al*. 2016).

Glucagon-like peptide-1 receptor (GLP1R)

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agonists have emerged as effective treatments for diabetes and improve insulin sensitivity in liver by reducing macrophage infiltration and inhibiting inflammation (Chai *et al*. 2012, Gao *et al*. 2007, Lee *et al*. 2012). GLP1R agonist may also impact biological functions in CVD. Recently, the clinical trial Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) showed a striking reduction in the relative risk of cardiovascular (CV) death and all-cause mortality in patients with type 2 diabetes at high CV risk on liraglutide therapy compared with patients administered with placebo (Marso *et al*. 2016). In a randomized clinical trial, treatment with 3.0 mg of liraglutide decreased the energy intake and weight loss in patients with type 2 diabetes (Pi-Sunyer *et al*. 2015, Lim *et al.* 2018).

In this study, for the first time, the ability of GLP1R agonist to alleviate vascular complications was determined by investigating its effect on atherosclerosis-associated processes in DEHP-treated VSMCs.

Methods

Cell culture

Rat aortic smooth muscle cells (RAoSMCs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagles's medium (DMEM) with 10 % fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. The medium was replaced every two days. Prior to the experiments, cells were plated in 96-well plates at a density of ~1.5×10^4 cells per well (cell proliferation assay) or in six-well plates at 5×10^6 cells per well (all other assays). For the experiments, the cells were incubated with the agents for 24 h at 37 °C. In each experiment, treatments were performed in triplicate.

Cell viability assay

Cells were cultured to 80-90 % confluence and then starved in serum-free DMEM for 24 h. The medium was then replaced with fresh medium containing different concentrations of GLP1R agonist (Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of DEHP (Sigma-Aldrich). Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay (CellTiter 96®AQueous Cell Proliferation Assay kit; Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Briefly, cells were cultured for 24 h, and 20 µl of MTS solution was added to 100 µl of culture medium. Cells were subsequently incubated at 37 °C for 4 h, and the absorbance was measured at 490 nm using a microplate reader.

Wound healing migration assay

Cells were grown to 90 % confluence and then subjected to scratching using a sterile pipette tip. Medium was replaced with fresh medium containing different concentrations of GLP-1R agonist in the presence or absence of DEHP. The scratch wound was allowed to heal for 24 h in the presence or absence of the indicated chemicals. Phase-contrast microscopy images (Optika, Ponteranica, Italy) of each sample were captured at 0 and 24 h, and VSMC ability to migrate was evaluated by measuring the area of the scratch wound at both time points using ImageJ software (version 1.29x, National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Cells (1×10^6/ml) were resuspended in a lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing a protease inhibitor cocktail (cOmplete™ Mini Protease Inhibitor Tablet; Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was measured in the supernatant using a Pierce BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc., Waltham, MA, USA). In total, 40 µg of protein was loaded per lane, separated by 10 % SDS-PAGE, and transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.). Membranes were blocked for 2 h at room temperature with 5 % skimmed milk in Tris-buffered saline-Tween-20 (TBST; 20 mM Tris, 500 nM NaCl, 0.1 % Tween-20), and then incubated with primary antibodies against matrix metalloproteinase (MMP)-2 (1:1000; Cell Signaling Technology, Boston, MA, USA), MMP-9 (1:1000; Cell Signaling Technology), intercellular adhesion molecule (ICAM)-1 (1:1000; Cell Signaling Technology), vascular cell adhesion molecule (VCAM)-1 (1:1000; Cell Signaling Technology), phospho-Akt (1:1000; Cell Signaling Technology), phospho-ERK1/2 (1:1000; Cell Signaling Technology), phospho-p38 (1:1000; Cell Signaling Technology), phospho-JNK (1:1000; Cell Signaling Technology), and β-actin (1:5000; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C.

Following three washes with TBST, membranes were incubated with secondary horseradish peroxidase-conjugated anti-IgG antibodies (1:5000; Thermo Fisher Scientific, Inc.) for 2 h at room temperature and visualized using a Pierce enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.). Densitometric quantification of the protein bands was performed using ImageJ software (version 1.29x, National Institutes of Health).

Statistical analysis

Quantitative data are presented as mean ± SEM. Differences between mean values were compared statistically using the two-tailed Student's t-test or one-way analysis of variance followed by Tukey's post hoc comparison. Statistical analysis was performed using SPSS windows software 22.0 (IBM Corp., Armonk, NY, USA). A P value of <0.05 was considered statistically significant. All experiments were performed at least three times.

Results

GLP1R agonist reduces DEHP-induced cell proliferation in VSMCs

To determine whether GLP1R agonist affects VSMC proliferation, we performed an MTS cell proliferation assay. DEHP-induced proliferative effects were significantly decreased by GLP1R agonist at a concentration of 100 nM after 24 h and at 50 nM and 100 nM after 48 h (Fig. 1). Subsequent experiments were performed using a cellular concentration of 100 nM of GLP1R agonist. In the next experiments, we focused our attention on further defining the therapeutic potential of GLP1R agonist.

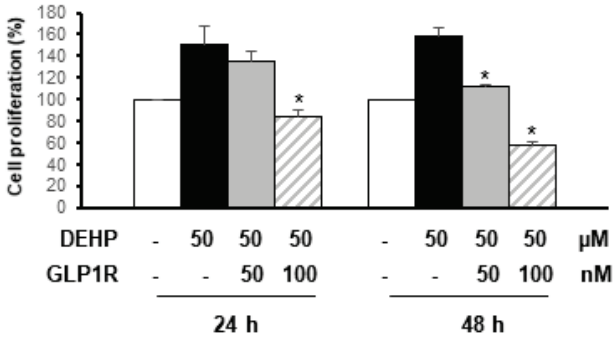

Fig. 1. GLP1R agonist reduces cell proliferation. An MTS proliferation assay was performed to determine VSMC viability. VSMCs were exposed to the indicated concentrations of GLP1R agonist for 24 h, followed by treatment with DEHP (50 μM) (means ± SEM; *P<0.05).

GLP1R agonist reduces DEHP-induced cell migration in VSMCs

A wound-healing migration assay was performed to determine the effects of GLP1R agonist on DEHP-stimulated VSMC migration. DEHP-induced VSMC motility was significantly reduced by GLP1R agonist after 24 h, as compared with DEHP-treated cells in the absence of GLP1R agonist (Fig. 2).

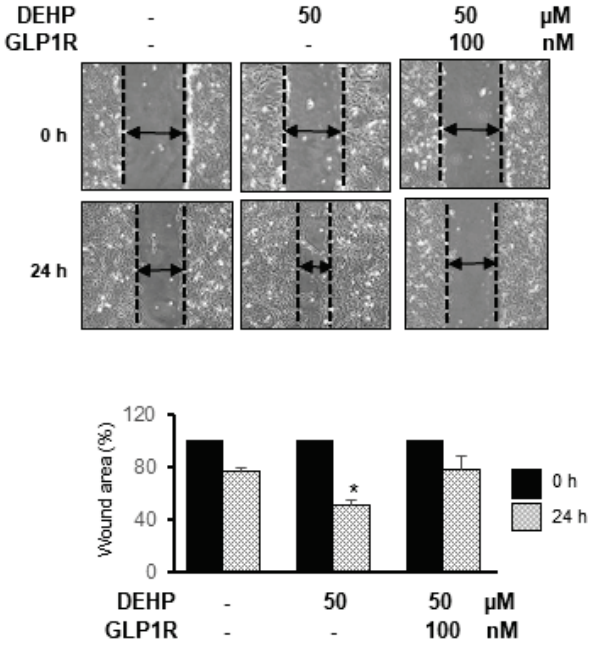

Fig. 2. GLP1R agonist reduces cell migration. Quantification of the wound area. The effect of a 24-h treatment with GLP1R agonist on the migration of VSMCs was determined using a scratch wound healing migration assay. Each experiment was performed in triplicate (means ± SEM; *P < 0.05).

GLP1R agonist reduces DEHP-induced expression of inflammation and adhesion molecules in VSMCs

Expression of MMPs is increased in VSMCs during the development of atherosclerosis. The initiation of atherosclerosis involves the up-regulation of molecules such as ICAM-1 and VCAM-1 (Senior *et al*. 1991, Newby and Zaltsman 2000). MMP-2 and MMP-9 protein expression levels were measured by western blot. MMP-9 expression was markedly decreased following exposure to 100 nM of GLP1R agonist for 24 h and 48 h compared with that in DEHP-treated cells in the absence of GLP1R (P<0.05). However, MMP-2 expression was markedly decreased following exposure to 100 nM of GLP1R agonist for 24 h but not 48 h compared with that in DEHP-treated cells in the absence of GLP1R agonist (Fig. 3).

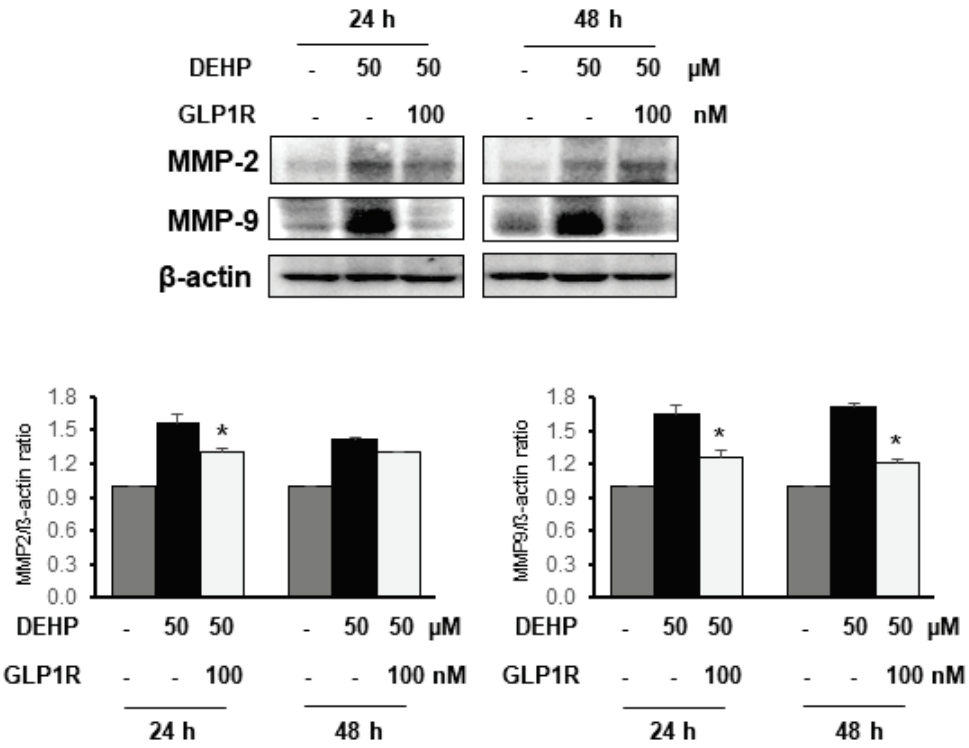

Fig. 3. Effect of GLP1R agonist on the expression of inflammation factors in VSMCs**.** DEHP-induced MMP-2 and MMP-9 expression was markedly reduced in the presence of GLP1R agonist in VSMCs when compared with DEHP-stimulated cells in the absence of GLP1R agonist (means ± SEM; **P*<0.05).

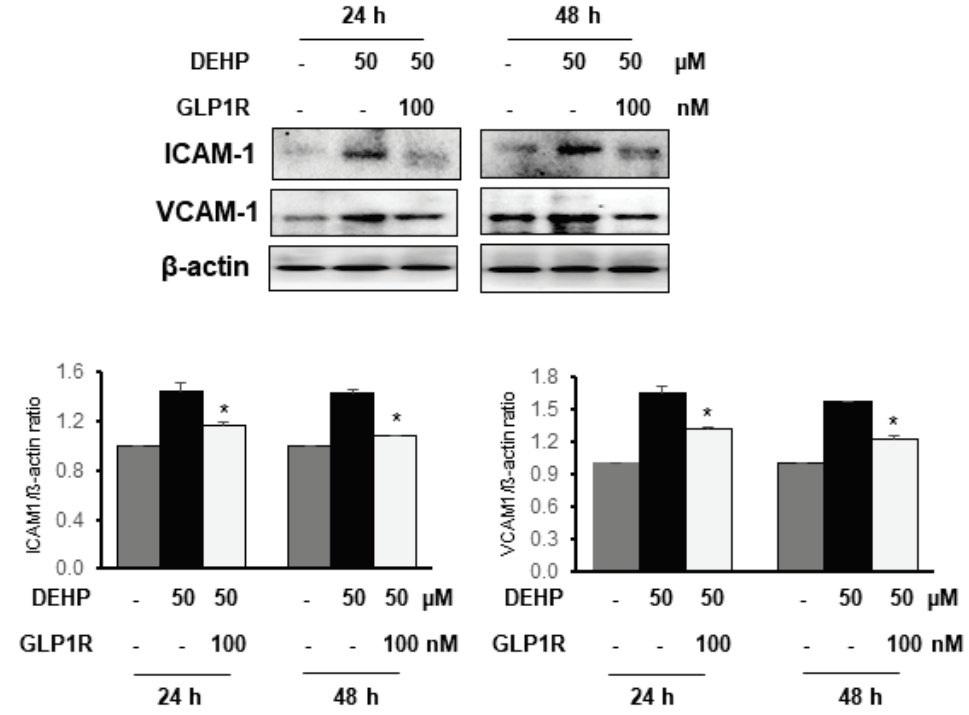

Fig. 4. Effect of GLP1R agonist on the expression of adhesion molecules in VSMCs**.** DEHP-induced ICAM-1 and VCAM-1 expression was markedly reduced in the presence of GLP1R agonist in VSMCs when compared with DEHP-stimulated cells in the absence of GLP1R agonist (means ± SEM; **P*<0.05).

ICAM-1 and VCAM-1 protein expression levels were measured by western blot. DEHP-induced ICAM-1 and VCAM-1 expression was strongly suppressed in the presence of 100 nM of GLP1R agonist in VSMCs compared with that in DEHP-treated cells in the absence of GLP1R agonist (Fig. 4).

GLP1R agonist reduces DEHP-induced activation of the Akt and ERK1/2 signaling pathways in VSMCs

To identify the signaling pathways involved in the effects of the GLP1R agonist, we examined the phosphorylation of Akt and ERK1/2 by western blot. As shown in Fig. 5, the treatment with 100 nM GLP1R

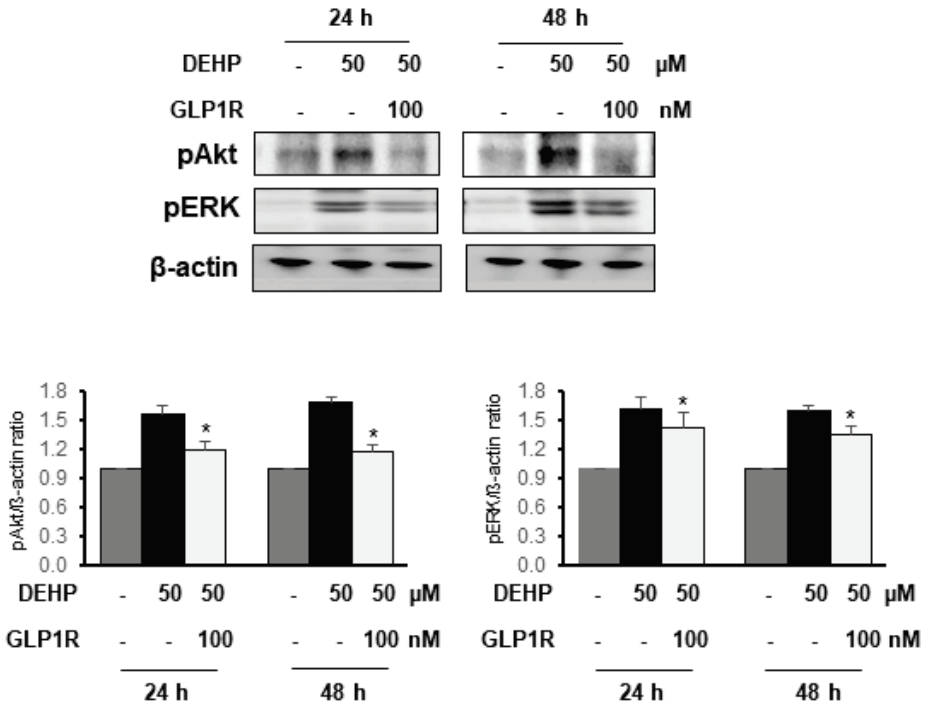

Fig. 5. GLP1R agonist reduces the phosphorylation of Akt and ERK1/2 in VSMCs. DEHP-induced Akt and ERK1/2 phosphorylation was markedly reduced in the presence of GLP1R agonist in VSMCs when compared with DEHP-stimulated cells in the absence of GLP1R agonist (means ± SEM; **P*<0.05).

agonist significantly suppressed DEHP-induced phosphorylation of Akt and ERK1/2 in VSMCs. The protein levels of p-JNK and p-p38 were not affected by the above treatments (data not shown).

Discussion

In this study, we investigated whether GLP1R agonist exerts suppressive effects on DEHP-treated VSMCs. Although the molecular mechanisms underlying the impact of DEHP are not fully understood, our data show that GLP1R agonist downregulates DEHP-induced expression of the inflammatory markers MMP-2 and MMP-9, as well as the adhesion molecules ICAM-1 and VCAM-1, in VSMCs. Moreover, the results of this study show that the inhibition of factors involved in inflammatory and atherosclerotic processes following GLP1R agonist therapy occur via the Akt and ERK1/2 pathways. This is the first study showing that GLP1R agonist is effective against DEHP-induced vascular complications.

DEHP has been associated with breast cancer, obesity, and atherosclerosis (Güven *et al.* 2005, Lin *et al*. 2011). Moreover, DEHP was linked to atherosclerosis-related CVD complications through the induction of inflammatory cytokines (Zhao *et al*. 2016). Atherosclerosis is associated with chronic inflammatory disorders involving monocytes, macrophages, endothelial cells, and smooth muscle cells (Ilhan and Kalkanli 2015). Increased proliferation and migration of VSMCs are involved in the formation of vascular diseases and finally lead to the development of atherosclerosis (Jawien *et al.* 1992). DEHP-induced proliferation of VSMCs involves the onset of vascular injury and atherosclerosis (Zhao *et al*. 2016). A recent study showed that GLP1 treatment effectively inhibited the proliferation of VSMCs via the AMPK signaling pathway (Teruo *et al.* 2017). In the present study, we found that 100 nM of GLP1R agonist inhibited the proliferation of VSMCs in response to DEHP.

Atherosclerosis is a major cause of the increased expression of inflammation and adhesion molecules in VSMCs following injury (Rolfe *et al*. 2000). The expression of MMPs, a family of zinc-dependent proteolytic enzymes, in VSMCs is associated with the degradation of connective tissue proteins and increased risk of developing atherosclerotic plaques (Newby 2005). Also, MMPs are critical for the development of arterial lesions through the regulation of proliferation and migration of smooth muscle cells (Cho and Reidy 2002). The expression of ICAM-1 and VCAM-1 on intimal smooth muscle cells is promoted in the atherosclerotic vascular wall (Jang et al. 1994). Several studies have investigated that VSMCs increased the ICAM-1 and VCAM-1 expression in atherosclerosis (Newby and Zaltsman 2000, Senior *et al*. 1991). Notably, GLP1 induced advantageous anti-inflammatory effects on human umbilical vein endothelial cells, human vascular

endothelial cells, and vascular diseases, including atherosclerosis and CVDs, in vivo and in vitro (Lee and Jun 2016). Our results showed that the GLP1R agonist decreased the expression of MMP-2, MMP-9, ICAM-1, and VCAM-1, indicating that decreased inflammation may be associated with the improvement of atherosclerosis. Thus, the GLP1R agonist seems to exhibit anti-inflammatory effects, which in turn may be beneficial in the prevention of atherosclerosis.

Akt and MAPK signaling pathways play a crucial role in the regulation of several diseases. Moreover, Akt and MAP/ERK1/2 signaling pathways play an important role in VSMC proliferation and migration (Rudijanto 2007, Seo *et al*. 2013, Qin *et al*. 2014). Previous studies have reported that GLP1R protects cardiomyocytes, endothelial cells, and VSMCs via Akt and ERK1/2 signaling cascades (Campbell and Trimble 2005, Wang 2002, Li *et al*. 2013, Igarashi *et al.* 2007). In view of these data, we investigated whether GLP1R agonist affected Akt and ERK1/2 activation. GLP1R agonist significantly reduced DEHP-induced Akt and ERK1/2 phosphorylation in VSMCs, suggesting the therapeutic applicability of GLP1R agonist in the prevention of atherosclerosis. Consequently, GLP1R agonist could be a more effective therapeutic agent against the DEHP-induced vascular complications.

In conclusion, the present study expands our understanding of the effects of the GLP1R agonist in VSMCs, revealing its potential for preventing the development of atherosclerosis. Thus, other or chemically modified GLP1R agonists may represent promising candidates for the treatment of atherosclerosis. Our study highlights the protective effect of the GLP1R agonist that significantly decreased cell viability and migration of DEHP-stimulated VSMCs through the inhibition of tissue remodeling and adhesion factors and the atherosclerosis-associated Akt and ERK1/2 pathways.

Conflict of Interest

There is no conflict of interest.

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