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Exendin-4, a glucagon-like peptide-1 receptor agonist, reduces intimal thickening after vascular injury

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ABSTRACT

Glucagon-like peptide-1 is a hormone secreted by L cells of the small intestine and stimulates glucose-dependent insulin response. Glucagon-like peptide-1 receptor agonists such as exendin-4 are currently used in type 2 diabetes, and considered to have beneficial effects on the cardiovascular system. To further elucidate the effect of glucagon-like peptide-1 receptor agonists on cardiovascular diseases, we investigated the effects of exendin-4 on intimal thickening after endothelial injury. Under continuous infusion of exendin-4 at 24 nmol/kg/day, C57BL/6 mice were subjected to endothelial denudation injury of the femoral artery. Treatment of mice with exendin-4 reduced neointimal formation at 4 weeks after arterial injury without altering body weight or various metabolic parameters. In addition, *in vitro* studies of isolated murine, rat and human aortic vascular smooth muscle cells showed the expression of GLP-1 receptor. The addition of 10 nM exendin-4 to cultured smooth muscle cells significantly reduced their proliferation induced by platelet-derived growth factor. Our results suggested that exendin-4 reduced intimal thickening after vascular injury at least in part by the suppression of platelet-derived growth factor-induced smooth muscle cells proliferation.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a hormone secreted from L cells of the small intestine and stimulates glucose-dependent insulin response [1–3]. In addition, GLP-1 has other effects that may improve diabetes, such as suppression of glucagon secretion [2], inhibition of gastrointestinal secretion and motility [4], and inhibition of food intake [5]. Accordingly, enhancement of GLP-1 action appears to have ideal profiles to fill the unmet needs of treatment of type 2 diabetes mellitus. However, a single administration of GLP-1 is not an effective treatment for diabetes, because the peptide is rapidly metabolized to the inactive form by dipeptidyl peptidase-4 (DPP-4). Thus, GLP-1 receptor (GLP-1R) agonists that are resistant to DPP-4, such as exendin-4 and DPP-4 inhibitors are currently used for the treatment of type 2 diabetes.

Given that one of the main purposes of treatment of type 2 diabetes is to prevent the onset of cardiovascular diseases, GLP-1R agonists have beneficial effects on several biomarkers of the cardiovascular system such as blood pressure and serum lipids with body weight reduction and blood glucose lowering effects [6]. Also, GLP-1R agonists have multiple direct effects on the cardiovascular system. For example, GLP-1R agonists are reported to reduce infarction size in *in vivo* models of myocardial ischemia [7,8] and improve myocardial contractility in various settings [9,10]. Regarding the effect on vascular function, GLP-1 stimulates vasodilation [11,12], inhibits the expression of tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor type-1, and vascular cell adhesion molecule-1 [13]. Indeed, GLP-1 is known to ameliorate endothelial dysfunction in type 2 diabetic patients with established coronary artery disease without affecting whole-body glucose uptake [14]. Furthermore, we recently found that GLP-1R

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; BrdU, bromodeoxyuridine; CREB, cAMP response element binding protein; DMEM, Dulbecco's Modified Eagle Medium; DPP-4, dipeptidyl peptidase-4; ELISA, enzyme-linked immunosorbent assay; Ex-4, exendin-4; FBS, fetal bovine serum; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; MAPK, Mitogen-activated protein kinase; PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TNF-α, tumor necrosis factor-α.

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agonists reduce the expression of TNF-α and monocyte chemotactic protein-1 induced by lipopolysaccharide in macrophages and inhibit monocyte adhesion to the endothelium and attenuate atheroma formation in ApoE knock-out mice [15]. Thus, GLP-1R agonists seem to have direct effects on the cells involved in the progression of atherosclerosis, such as endothelial cells or macrophages.

While the progression of atherosclerosis involves multiple complex processes, proliferation of smooth muscle cells (SMCs) is regarded as one of the most important processes in both early and late pathogenic processes [16]. In addition, proliferation of SMCs is a fundamental process in the pathogenesis of restenosis after angioplasty [17]. Especially, diabetic patients are at higher risk for developing post-angioplasty restenosis and more than 2-fold higher post-angioplasty mortality, compared with non-diabetic subjects [18]. In our previous study, we detected GLP-1R protein expression not only in endothelial cells and monocytes/macrophages but also in murine SMCs, suggesting that GLP-1R agonists may have direct effects on SMCs during neointimal formation. However, to our knowledge, there is no information on the effects of GLP-1 on neointimal formation and SMCs proliferation. In the present study, we investigated the effects of exendin-4 (Ex4), a GLP-1R agonist, on intimal thickening after vascular injury using the model of endovascular femoral artery guide wire injury.

2. Materials and methods

2.1. Animals

The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University. Six-week-old male C57BL/6 mice were purchased from Oriental yeast (Tokyo, Japan). All mice were housed in a polycarbonate cage with a wooden chip mat on the floor. Water was available *ad libitum*. All mice were fed normal chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber, total: 356 kcal/100 g) (CRF-1, Charles River Japan, Yokohama, Japan). The animal room was kept on a 12 h light/dark cycle at a constant temperature (22 ± 1 °C) and relative humidity of 55 ± 5% throughout the experimental period.

2.2. Experimental protocol

C57BL/6 mice were divided into the saline- (n = 10) and Ex4- (n = 10) treated groups. At the age of 8 weeks, a mini-osmotic pump (ALZEST, model 1004; DURECT, Cupertino, CA) was implanted under the skin of the back of each mouse after local anesthesia. The skin incision was closed with wound clip. Saline or 24 nmol/kg/day exendin-4 (Sigma–Aldrich, Tokyo) was infused through the osmotic pump that delivered the solution continuously for up to 4 weeks. Endothelial denudation injuries were induced in the femoral artery at the age of 9 weeks, followed by evaluation of neointimal formation at the age of 13 weeks.

2.3. Laboratory data

Blood samples were collected at euthanasia. The plasma glucose level was measured by glucose oxidase method using a compact glucose analyzer (One Touch Ultra Glucose Meter, Life Scan, Tokyo). Plasma insulin was measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga, Kanagawa, Japan). Total cholesterol, HDL, LDL, and triglycerides were measured by autoanalyzer. (Hitachi, Hitachi, Japan).

2.4. Cell culture

Rat and murine aortic SMCs were isolated and cultured as described previously [19]. Human primary coronary artery SMCs were commercially obtained (Lonza Inc., Allendale, NJ) and maintained in SmGM2 medium according to the instructions supplied by the manufacturer. Rat aortic SMCs were obtained from Cell Application Inc. (San Diego, CA). Mouse and rat aortic SMCs were maintained in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Cells were used between passages three and seven for the experiments and individual experiments were repeated at least three times with different preparations of cells.

2.5. Cell proliferation assay

To evaluate the growth response of murine SMCs to platelet-derived growth factor (PDGF), the bromodeoxyuridine (BrdU) incorporation assay was performed using Cell Proliferation ELISA kit (1647229, Roche Applied Science, Germany) according to the instructions provided by the manufacturer. Briefly, murine SMCs were plated at a density of 3000 cells/well in 96-well culture plates with complete media. After growth to 60–70% confluence and culture in DMEM with 0.3% FBS for 12 h, the murine SMCs were pretreated with 10 nM Ex4 or saline for 12 h and then stimulated with PDGF (25 ng/ml, Sigma–Aldrich, St Louis, MO) for 24 h. BrdU solution (10 μM) was added during the last 4 h of DNA synthesis. After stimulation, the cells were dried and fixed, and the cellular DNA was denatured with FixDenat solution (Roche Applied Science, Mannheim, Germany) for 30 min at room temperature. A mouse anti-BrdU monoclonal antibody conjugated with peroxidase (Roche Applied Science) was added to the culture plates and incubated again at room temperature for 90 min. Finally, tetramethylbenzidine was added for 15 min at room temperature and absorbance of the samples was measured by a microplate reader at 370 nm.

2.6. Endovascular femoral artery guide wire injury

Mouse femoral artery endothelial denudation injury was induced in C57BL/6 mice of the saline (n = 10) and Ex4 (n = 10) groups at the age of 9 weeks as described previously [20]. Briefly, endovascular injury was induced by four passages of a 0.25 mm SilverSpeed-10 hydrophilic guide wire (Micro Therapeutics Inc., Irvine, CA) into the left femoral artery. Sham surgery without injury was performed on the contralateral right side. Mice were euthanized 4 weeks after injury and the femoral arteries were isolated for tissue analysis.

2.7. Tissue preparation and morphometry

Following sacrifice, mice were perfused via a cannula in the left ventricle with phosphate-buffered saline for 5 min, followed by 4% paraformaldehyde for 30 min at 100 cm H_2O. The femoral arteries were embedded in paraffin and cut into 5 μm sections for further analysis. Serial sections of 1.5 mm proximal region from the incise on site for the wire insertion were evaluated by staining with Elastica van Gieson stain kit (4033–4037, Muto Pure Chemicals Co, Tokyo) to visualize the internal elastic lamina. Specimens were viewed under a microscope (E800; Nikon, Tokyo) connected to an XYZ controller and a digital camera (Sony, Tokyo). The intimal and medial areas were measured by computerized morphometry using the software Image-Pro Plus. Intimal hyperplasia was defined as the formation of a neointimal layer medial to the internal elastic lamina. The medial area represented the area between the

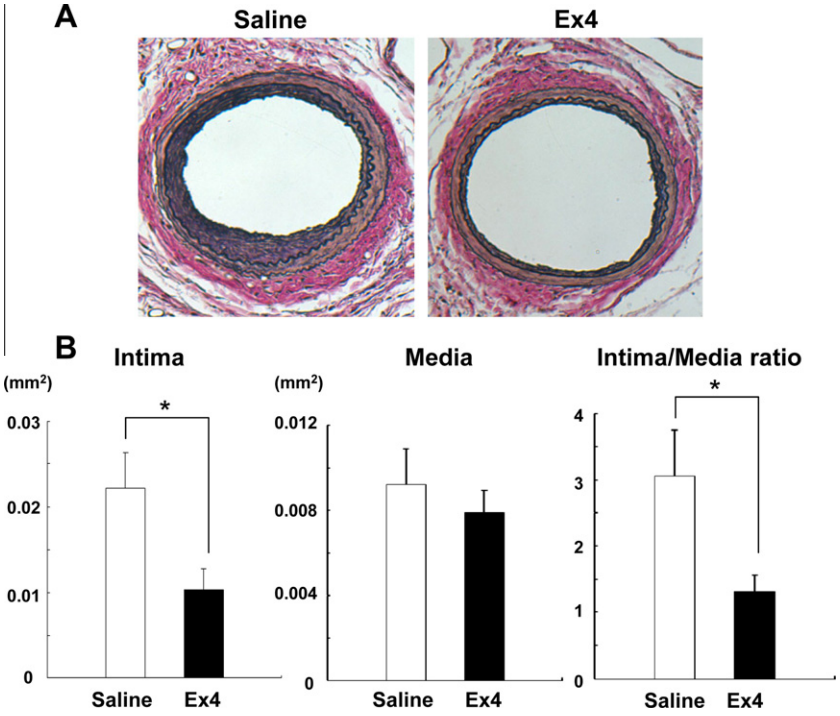

Fig. 1. Exendin-4 (Ex4) suppresses neointima formation in mice. Endothelial denudation injury was induced in the left femoral artery of mice treated with Ex4 ($n = 10$) or saline ($n = 10$). (A) Tissues were harvested after 28 days and stained with Elastica van Gieson to visualize the internal elastic lamina (magnification 10×). (B) The area of intima, media, and intima/media ratio was calculated in each group. Data are mean ± SEM. $^{*}P < 0.01$ vs. saline treated group.

external elastic lamina and the internal elastic lamina. The intima-to-media ratio was calculated as the intimal area divided by the media area.

2.8. Western blot analysis

Western blotting was performed as described previously [19]. Primary antibodies were obtained from the following suppliers: GLP-1R (Abcam, Cambridge, UK), phosphor-p44/42 Mitogen-activated Protein Kinase (MAPK), p44/42MAPK, phosphor-Akt, Akt, phosphor- cAMP response element binding protein (CREB), CREB, phosphor-P70S6kinase, P70S6kinase (Cell Signaling, Danver, MA), CyclinD (Millipore, Billerica, MA), CyclinE (Upstate, Temecula, CA), CyclinA, P27 (Santa Cruz Biotechnology, Santa Cruz, CA), P21 (Becton Dickinson, Franklin Lakes, NJ) and GAPDH (Cell Signaling, Danver, MA).

2.9. Measurement of cAMP level

Murine SMCs were plated in 96-well plates at a density of 5×10^3 cells per well and cultured overnight. After 24 h of serum deprivation with 0.3% FBS, SMCs were incubated with Ex4 (10 nM) or forskolin (30 μM) for 30 min. After incubation, the medium was aspirated and a lysis buffer was added. Intracellular cAMP concentration ($[cAMP]_i$) was determined using the cAMP EIA kit

Table 1
Laboratory data of C57BL/6 mice after treatment with saline or exendin-4 (Ex4).

	Saline ($n = 10$)	Ex4 ($n = 10$)
Body weight (g)	22.16 ± 0.97	21.72 ± 0.88
Blood glucose (mg/dl)	89.8 ± 25.0	89.5 ± 3.1
Insulin (ng/ml)	0.218 ± 0.04	0.211 ± 0.06
Total cholesterol (mg/dl)	74.1 ± 4.9	73.6 ± 4.5
LDL cholesterol (mg/dl)	3.2 ± 0.79	3.2 ± 0.67
HDL cholesterol (mg/dl)	42.1 ± 2.1	42.6 ± 3.8
Triglyceride (mg/dl)	34.4 ± 9.2	32.2 ± 7.2

Blood samples were collected from saline- and Ex4-treated mice at 4 weeks after endothelial injury. Each sample was obtained in the fasting state. There were no differences in each parameter between the two groups. Data are mean ± SEM.

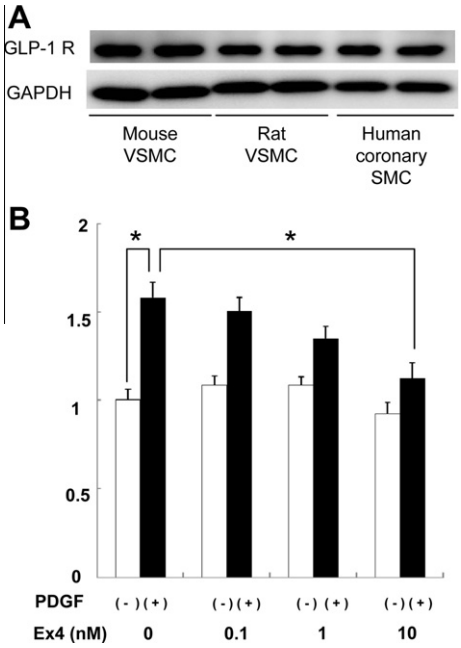

Fig. 2. Ex4 attenuates PDGF-induced SMC proliferation. (A) Cell lysates of cultured coronary SMCs of rat, mouse and human were harvested and subjected to Western blotting to detect GLP-1R and GAPDH. (B) Primary aortic murine SMCs were incubated with PDGF. The cells were harvested before or 24 h after the addition of PDGF, and BrdU contents were measured. BrdU contents in cultured SMCs without the addition of PDGF and Ex4 was set at 1.0 and results are presented as the relative BrdU contents. Data are the mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs control.

(Amersham Biosciences) according to the instructions supplied by the manufacturer.

2.10. Statistical analysis

ANOVA and paired or unpaired *t*-tests were performed as appropriate. *P* values <0.05 were considered statistically significant. Results are expressed as mean ± SEM.

3. Results

3.1. Ex4 treatment attenuates formation of neointimal

C57BL/6 mice were treated with saline or 24 nmol/kg/day Ex4 from the age of 8 weeks to 12 weeks. Mouse femoral artery endothelial denudation injuries were performed at the age of 9 weeks in both groups, then the neointimal formation was evaluated at the age of 13 weeks. As depicted in Fig. 1A, endothelial denudation injury in saline-treated control mice resulted in a considerable neointimal formation. In contrast, this formation was substantially suppressed in Ex4-treated mice. Quantitative analysis showed 58.3% reduction in neointimal formation in Ex4-treated mice compared to saline treated mice (Saline: 0.0216 ± 0.0028, Ex4: 0.0090 ± 0.0014 mm^2; $P < 0.01$), although the areas of the media layer were comparable (Saline: 0.0092 ± 0.0017, Ex4: 0.0079 ± 0.0010 mm^2). Accordingly, the intima/media (I/M) ratio was significantly reduced by Ex4 (Saline: 3.0 ± 0.7, Ex4: 1.2 ± 0.2; $P < 0.01$) (Fig. 1B). Regarding the contralateral femoral arteries where sham surgery was performed, the intima-media thickness was comparable in the saline- and Ex4-treated C57BL/6 mice (data not shown). These results indicate that Ex4 attenuates neointimal formation after vascular injury.

3.2. Ex4 does not alter body weight or glucose metabolism

We reported previously that 24 nmol/kg/day exendin-4 has only a modest effect on metabolic parameters in non-obese and non-diabetic C57BL/6 mice [15,21]. In the present study, body weight, blood glucose, serum insulin and blood lipids were also comparable between the two groups at the end of experiments (Table 1). These results indicate that Ex4 reduces neointimal formation independent of metabolic parameters.

3.3. Ex4 attenuates PDGF-induced SMC proliferation

We reported previously the expression of GLP-1R in murine SMCs [15]. In the present study, GLP-1R was found to be expressed abundantly in SMC isolated from mouse aorta, rat aorta, and human coronary arteries (Fig. 2A). To investigate the direct effect of Ex4 on SMC proliferation, we employed primary aortic SMC isolated from C57BL/6 mice and assessed the effects of Ex4 on PDGF-induced cell proliferation. The proliferation of SMCs was sig-

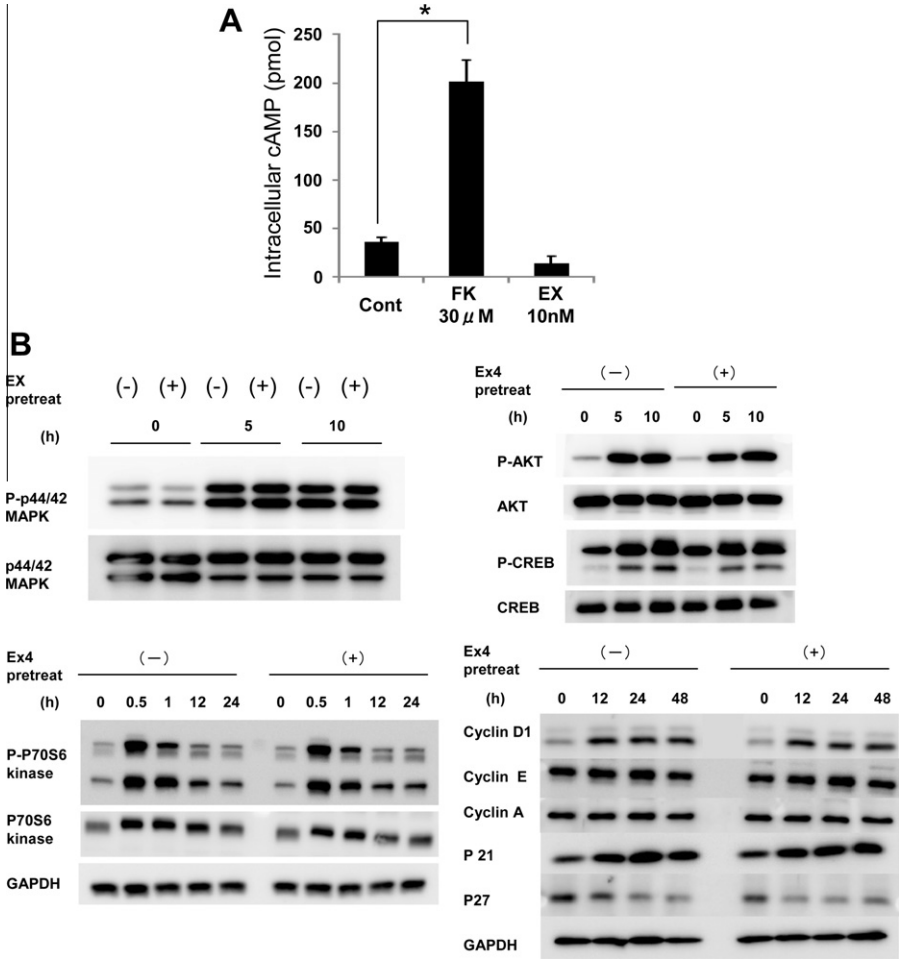

Fig. 3. Ex4 does not modify PDGF-induced downstream signaling in cultured SMCs. (A) Intracellular cAMP concentration of murine SMCs was determined after the addition of 30 μM Forskolin (FK) or 10 nM Ex4. Data are mean ± SEM of five independent experiments. *$P < 0.05$ vs control. (B) After serum-deprivation, murine SMCs were pre-treated with 10 nM Ex4 or saline for 12 h and then stimulated with PDGF for the indicated time. Cell lysates were harvested and subjected to Western blotting to assess PDGF-induced downstream signaling including P-p44/42 MAPK, P-Akt, P-CREB, P-P70S6kinase, and cell cycle modulators.

nificantly augmented by PDGF treatment. Ex4 did not alter cell proliferation in the absence of PDGF, however, Ex4 attenuated PDGF-induced SMC proliferation in a dose-dependent manner (Fig. 2B). These findings suggest that Ex4 can stimulate GLP-1R signal cascade in SMCs.

3.4. Ex4 does not activate canonical GLP-1R signaling pathway in SMCs

Next, we searched for the mechanism of the effect of Ex4 on PDGF-induced proliferation of SMC. The GLP-1R is Gs protein coupled receptor, and its activation increases $[cAMP]_i$ typically in pancreatic β cells [22]. However, we could not identify a significant increase in $[cAMP]_i$ after the addition of Ex4 to SMCs (Fig. 3A). We then investigated the effect of Ex4 on PDGF-induced signaling pathway such as phosphorylation of p44/42 MAPK, Akt, CREB and P70S6kinase, and the expression of cell cycle modulators in cultured SMCs. However, Ex4 did not alter the activation or expression of these proteins in murine SMCs (Fig. 3B).

4. Discussion

In the present study, we provided evidence that GLP-1R agonists reduced intimal thickening after vascular injury. This effect was not associated with changes in metabolic parameters, thus it was not mediated by improvement in metabolic parameters. On the other hand, the *in vitro* experiments showed the expression of GLP-1 receptor in aortic vascular smooth muscle cells and that the addition of 10 nM of Ex4 significantly decreased PDGF-induced proliferation of cultured SMCs. These results suggest that GLP-1R agonists attenuate neointimal formation after vascular injury at least in part through their direct action on SMCs.

GLP-1 is believed to exert its actions mainly through a Gs protein-coupled receptor. In pancreatic β cells, GLP-1-induced increase in intracellular cAMP plays an important role in increased insulin secretion. Based on this finding, we examined $[cAMP]_i$ in cultured SMCs after the addition of Ex4. Although GLP-1R is abundantly expressed in SMCs, no significant increase in $[cAMP]_i$ was observed in cultured SMCs after the treatment. Thus, the direct effect of Ex4 on PDGF-induced proliferation of SMCs seems to be independent of the canonical GLP-1R signal pathway. In the present study, we tried to identify the signal(s) induced by PDGF that is(are) altered by Ex4, however, no significant differences were observed in the activation of various proteins involved in cell proliferation or in the expression levels of cell cycle regulators that were investigated. Recently, Hattori et al. found that GLP-1R induces the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) independent of the activity of adenylate cyclase in endothelial cells [23]. Therefore, we investigated the phosphorylation of AMPK by the addition of Ex4 to cultured murine SMCs and found that Ex4 induces phosphorylation of AMPK in murine SMCs (H.G. and H.W. unpublished observation). Thus, AMPK activation by uncanonical GLP-1 signal transduction pathway might be involved in the inhibition of SMC proliferation. Further studies are required to explore this mechanism of action.

Our results showed that Ex4 reduced PDGF-induced SMC proliferation and inhibited neointimal formation after vascular injury. However, some potential effects of Ex4 on other vascular cells may contribute to the attenuation of neointimal formation. Since depletion of macrophages reduces neointimal formation after vascular injury [24], macrophage activation is a crucial factor in SMC proliferation. Indeed, we reported previously that Ex4 inhibited the inflammatory response in macrophages [15]. Thus, in addition to its effect on SMCs, the anti-inflammatory effects of Ex4 may play a role in the attenuation of neointimal formation.

In conclusion, our data suggest that Ex4, a GLP-1R agonist, significantly reduced PDGF-induced SMC proliferation and inhibited neointimal formation after vascular injury. Extrapolation of our data to the clinical setting suggests that Ex4 could be potentially suitable for prevention of restenosis after angioplasty. This unique effect of GLP-1R agonists may help design new therapies for cardiovascular disease in patients with type 2 diabetes.

Duality of interest

The authors declare no duality of interest in relation to this manuscript.

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