Original

Involvement of Vascular Endothelial Cells in the Anti-atherogenic Effects of Liraglutide in Diabetic Apolipoprotein E-null Mice

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Abstract : Glucagon-like peptide 1 receptor agonists (GLP-1RAs) have been shown to exert anti-atherosclerotic effects via multiple mechanisms on different types of cells. However, it is unclear which of these mechanisms are crucial. We investigated the role of vascular endothelial cells (VECs) in the anti-atherogenic effects of the GLP-1RA liraglutide in a mouse model of atherosclerosis. Streptozotocin-induced diabetic apolipoprotein E-null mice were randomly assigned to treatment with either vehicle (saline) or liraglutide (107 nmol/kg/day), and were subjected to femoral artery wire injury to remove VECs. After 4 weeks, vessel samples were collected for analysis. Streptozotocin-injected mice had fasting plasma glucose levels of ＞300 mg/dl and hemoglobin A1c levels of ＞9%, indicating that the injections had induced severe hyperglycemia. However, there were no differences in metabolic characteristics such as levels of hemoglobin A1c, fasting plasma glucose, total cholesterol, and triglycerides between the vehicle and liraglutide groups. Analysis of atherosclerotic plaque formation revealed that liraglutide treatment significantly suppressed plaque formation in the aorta. In addition, liraglutide treatment reduced plaque volume and intra-plaque macrophage accumulation at the aortic sinus. Furthermore, liraglutide treatment suppressed vascular expression of pro-inflammatory cytokines. In uninjured femoral arteries, no plaques were observed ; however, severe plaque formation occurred in femoral arteries that had been injured by wire insertion to remove VECs. Unlike in the uninjured aorta, liraglutide treatment did not affect plaque volume or arterial remodeling (intimal and medial thinning, and arterial dilation) in wire-injured femoral arteries. Of the various cells that liraglutide affects, VECs play a central role in liraglutide's anti-atherogenic effects in diabetic mice.

Key words : atherosclerosis, vascular endothelial cell, glucagon-like peptide-1 receptor agonist

Introduction

Glucagon-like peptide 1 (GLP-1) is a gut peptide hormone that is secreted from intestinal L

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cells in response to digestion[1,2]. In pancreatic β cells, which express GLP-1 receptors, GLP-1 stimulates insulin secretion in a glucose concentration-dependent manner, which enables GLP-1 to induce potent glucose-lowering effects without increasing the risk of hypoglycemia. In addition, GLP-1 exerts favorable metabolic effects such as reduction of body weight and amelioration of hypertriglyceridemia. Due to the very short half-life of endogenous GLP-1, GLP-1 receptor agonists (GLP-1RAs) that are resistant to degradation have been developed and are widely used for the treatment of type 2 diabetes.

In addition to pancreatic β cells, GLP-1 receptors are expressed in a variety of other cells, including cells in the cardiovascular system[3]. Numerous preclinical studies have demonstrated that GLP-1 and GLP-1RAs exert direct anti-atherogenic effects in animal models, and they induce favorable changes against atherosclerosis in various cells such as vascular endothelial cells (VECs), vascular smooth muscle cells (VSMCs), and macrophages[4-10]. Large-scale clinical trials have consistently demonstrated that treatment with GLP-1RAs reduces the incidence of adverse cardiovascular events compared with placebo in patients with type 2 diabetes[11-13]. Therefore, GLP-1RAs are recommended for the prevention of atherosclerotic cardiovascular disease in patients with type 2 diabetes[14]. However, the underlying mechanisms of this benefit are not yet fully understood.

In the present study, we sought to determine the role of VECs in the anti-atherogenic effects of the GLP-1RA liraglutide by comparing atherosclerotic plaque formation in endothelium-intact and endothelium-denuded vessels of mice.

Materials and methods

Treatment of mice

The study design was approved by the Animal Care Committee of Showa University School of Medicine (approval number : 07005). Six-week-old male apolipoprotein E-null (ApoE $^{-/-}$) mice (BALB/c, KOR/StmSlc-Apoeshl) were purchased from Sankyo Labo Service (Tokyo, Japan). ApoE $^{-/-}$ mice were maintained on standard rodent chow with free access to chow and water in a specific pathogen-free room in the Division of Animal Experimentation of Showa University School of Medicine. Diabetes was induced by injecting streptozotocin (Sigma-Aldrich Japan, Tokyo, Japan) as previously reported[15,16]. Briefly, ApoE $^{-/-}$ mice received intra-peritoneal injections of streptozotocin at a dose of 100 mg/kg/day for five consecutive days at 15 weeks of age, and additional injections at a dose of 50 mg/kg/day for five consecutive days at 18 weeks of age. At 20 weeks of age, development of diabetes was defined as random plasma glucose levels > 200 mg/dl, and diabetic mice were randomly assigned to one of two groups : treatment with vehicle (saline) or treatment with liraglutide (107 nmol/kg/day)[4]. Also, diet was switched at 20 weeks from standard rodent chow to western diet (30% fat and 0.15% cholesterol ; Oriental Yeast, Tokyo, Japan)[4,5,15,16]. Liraglutide treatment was delivered by osmotic pumps that were implanted under the dorsal skin (mini pump model 1002 ; Alzet, Cupertino, CA, USA). Two days after treatment initiation, mice were subjected to left femoral artery wire injury to remove VECs from the lumen, as previously established by Sata *et al*[17]. Briefly, a straight spring

guidewire was inserted into the femoral artery in a retrograde direction, via a small cut on the branch artery, and carefully withdrawn after 1 minute. After 4 weeks of treatment, mice were euthanized by isoflurane overdose, and blood and vessel samples were collected.

Assessment of plasma glucose, HbA1c and lipid levels

Blood samples were collected from the inferior vena cava after 6 hours of fasting. Plasma glucose levels were measured using a dextrometer (Glutest Sensor NeoSuper, Sanwa Kagaku, Aichi, Japan). Hemoglobin A1c (HbA1c) levels were determined using a latex-enhanced immunoassay and plasma lipid levels were determined using an enzymatic colorimetric assay, both performed using a Cobas analyzer (Roche Diagnostics Japan, Tokyo, Japan).

Blood pressure and pulse rate measurements

Blood pressure and pulse rate were measured using the tail-cuff method, as previously described (Model MK-2000ST ; Muromachi Kikai, Tokyo, Japan) [18].

Assessment of atherosclerosis

The aortas and femoral arteries were carefully removed from surrounding connective tissue. Atherosclerotic plaque formation was evaluated as previously described [4, 5, 15, 16]. Briefly, cryosections of the aortic sinus and femoral artery were stained with Elastica van Gieson and/or oil red O. The aorta was longitudinally dissected and stained with oil red O. Assessment of atherosclerosis was conducted by an investigator blinded to the treatment, using Image J software (National Institutes of Health, Bethesda, MD, USA).

Assessment of intra-plaque macrophage accumulation

Intra-plaque macrophage accumulation was assessed in cryosections of the aortic sinus by immunohistochemistry, using anti-MOMA2 antibody (1 : 60 ; RRID, AB_776518 ; Catalogue ID, ab33451 ; Abcam Japan, Tokyo, Japan) [4, 5].

Assessment of gene expression

Total RNA was extracted from the brachiocephalic arteries using Isogen (Nippon Gene, Tokyo, Japan), and used to synthesize cDNA using ReverTra Ace (Toyobo, Osaka, Japan) as previously described [18]. Gene expression was assessed by real-time reverse transcription PCR using the TaqMan gene expression assay and a sequence detection system (StepOne ; Life Technologies Japan, Tokyo, Japan). The following pre-designed TaqMan probe sets were used : interleukin-1β (*IL-1β*), Mm00434228 m1 ; *IL-6*, Mm00446190 m1 ; monocyte chemoattractant protein-1 (*Mcp-1*), Mm00441242 m1 ; tumor necrosis factor α (*TNFα*), Mm00443258 m1 ; 18S ribosomal RNA (*18sRNA*), Mm03928990 g [1].

Statistical analysis

Comparisons were conducted using an unpaired t-test with JMP software (version 13 ; SAS

Institute Inc., Cary, NC, USA）. Significance was defined as $p < 0.05$.

Results

Characteristics of the two groups were similar

The physiological and biochemical characteristics of diabetic mice treated with vehicle and those treated with liraglutide are shown in Table 1. Vehicle-treated mice had fasting plasma glucose levels of ＞300 mg/dl and HbA1c levels of ＞9%, indicating that the streptozotocin injections had induced severe hyperglycemia. However, there were no differences in physiological or biochemical characteristics, including fasting plasma glucose and HbA1c levels, between the vehicle-treated and liraglutide-treated groups.

Liraglutide treatment suppressed atherosclerotic plaque formation

Assessment of atherosclerosis in the aortas of the diabetic mice revealed that liraglutide treatment significantly reduced atherosclerotic plaque area as determined by oil red O staining on the aortic surface （Fig. 1A and D）. Consistent with this finding, liraglutide treatment reduced atherosclerotic plaque volume measured at the aortic sinus （Fig. 1B and E）. Furthermore, liraglutide treatment decreased intra-plaque macrophage accumulation, which is involved in atherosclerotic plaque progression and rupture （Fig. 1C and F）[19, 20].

Liraglutide treatment reduced pro-inflammatory cytokine expression

The effects of liraglutide on vascular inflammation, which plays an essential role in atherosclerotic plaque formation[21], were examined next. Pro-inflammatory cytokine expression was assessed in the brachiocephalic arteries, sites that are prone to atherosclerosis[22]. Liraglutide treatment significantly reduced expression of *IL-1β*, *IL-6*, and *Mcp-1* （Fig. 2A–C）, and resulted in a non-significant reduction in *TNFα* expression （$p = 0.15$, Fig. 2D）.

Table 1. Physiological and biochemical characteristics of diabetic ApoE$^{-/-}$ mice treated with vehicle or liraglutide*

	Vehicle （n＝9）	Liraglutide （n＝6）
Food intake （g/day）	3.8±0.2	3.7±0.3
Water intake （g/day）	16.5±1.4	20.0±1.1
Final body weight （g）	21.8±1.5	24.4±1.2
Pulse rate （beats/min）	642±7	619±13
Systolic BP （mmHg）	120±5	123±7
Plasma glucose （mg/dl）	345±41	383±24
Hemoglobin A1c （%）	9.1±0.9	9.1±0.3
Total cholesterol （mg/dl）	494±59	502±62
Triglycerides （mg/dl）	298±81	292±82
HDL-C （mg/dl）	71±6	63±3

*Values are mean±standard error of mean.

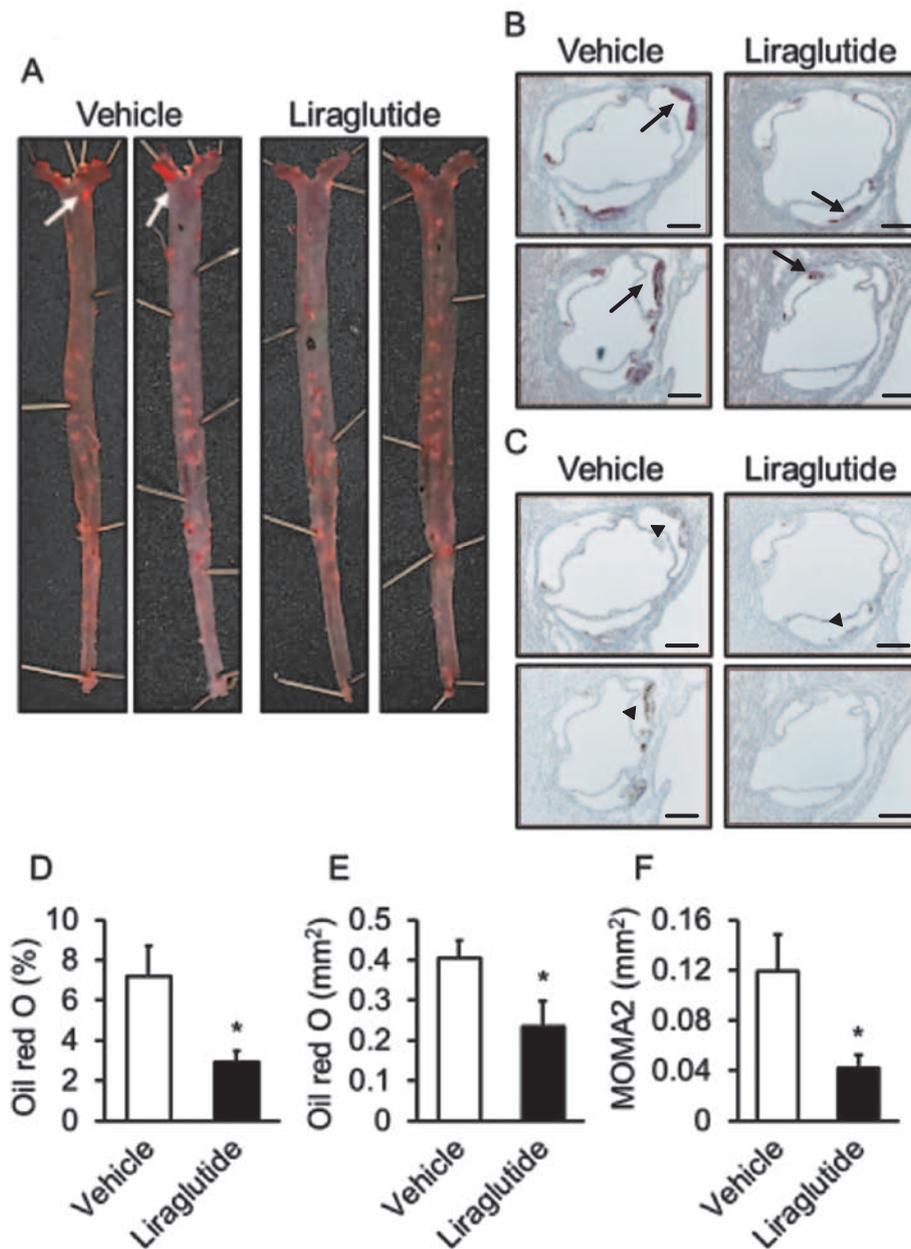 BP, blood pressure ; HDL-C, high-density lipoprotein cholesterol.

Fig. 1. Liraglutide treatment suppresses atherosclerotic plaque formation in diabetic ApoE $^{-/-}$ mice
Streptozotocin-induced diabetic mice were treated with vehicle or liraglutide for 4 weeks, and their aortas were collected for analysis of atherosclerosis. (A) Representative microscopic images of aortas stained with oil red O. White arrows indicate atherosclerotic plaques. (B, C) Representative images of the aortic sinus with oil red O staining and MOMA-2 immunostaining, respectively. Black arrows and arrowheads indicate atherosclerotic plaques and intra-plaque macrophage accumulation, respectively. Scale bars, 250 μm. (D) Atherosclerotic plaque area on the aortic surface ($^{*}p < 0.05$). (E, F) Atherosclerotic plaque volume and intra-plaque macrophage accumulation, respectively, at the aortic sinus ($^{*}p < 0.05$). Graphed values are means, and each error bar indicates standard error of mean.

Anti-atherogenic effects of liraglutide were compromised by VEC removal

The role of VECs in the anti-atherogenic effects of liraglutide was examined by removing VECs from the left femoral artery lumen by inserting a guidewire. After 4 weeks, atheroscle-
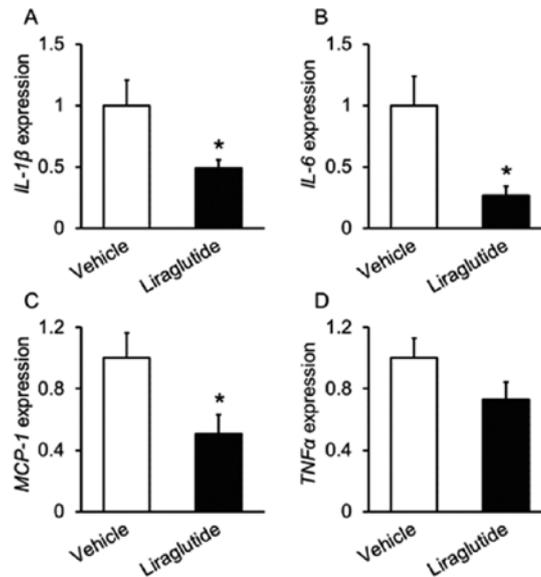
Fig. 2. Liraglutide suppresses expression of pro-inflammatory cytokines in the brachiocephalic artery
Streptozotocin-induced diabetic mice were treated with vehicle or liraglutide for 4 weeks, and their brachiocephalic arteries were collected for analysis of pro-inflammatory cytokine expression by real time reverse transcription PCR. (A-D) *IL-1β*, *IL-6*, *TNFα*, and *Mcp-1* expression levels, respectively. Target gene expression was normalized to that of the housekeeping gene *18sRNA* ($^*p < 0.05$). Graphed values are means, and each error bar indicates standard error of mean.

rotic lesions, accompanied by arterial remodeling (intimal and medial hyperplasia, and arterial dilation), formed in the wire-inserted left femoral arteries (Fig. 3A–E), whereas such changes were not observed in uninjured right femoral arteries. However, in contrast to our findings in uninjured aortas, liraglutide did not affect atherosclerotic lesion formation or arterial remodeling in wire-injured left femoral arteries (Fig. 3A–E).

Discussion

In the present study, we demonstrated that liraglutide treatment suppressed atherosclerotic plaque formation in the aortas of mice. However, the anti-atherogenic effects of liraglutide were not observed in femoral arteries in which VECs had been removed from the lumen surface by insertion of a guidewire. In the segment where a guidewire is inserted, VECs are almost completely removed by mechanical stress [17, 23]. Previous studies, including studies that we have conducted, have demonstrated anti-atherogenic effects of GLP-1 and GLP-1RAs in animal models [4–10], but these did not determine whether GLP-1RA treatment suppresses atherosclerosis in the femoral artery after wire injury. Our findings from the present study indicate that the anti-atherogenic effects of liraglutide are compromised by the removal of VECs.

Previous studies have demonstrated that GLP-1RAs induce anti-atherogenic changes in VECs, such as enhancement of nitric oxide (NO) production and suppression of cell adhesion molecule expression [5, 7, 18, 24]. Extensive research has shown the importance of NO not only in maintaining normal vessel conditions but also in preventing atherosclerosis and vascular remodeling [25, 26]. NO exerts multiple actions in various cells that are involved in the process of atherosclerotic
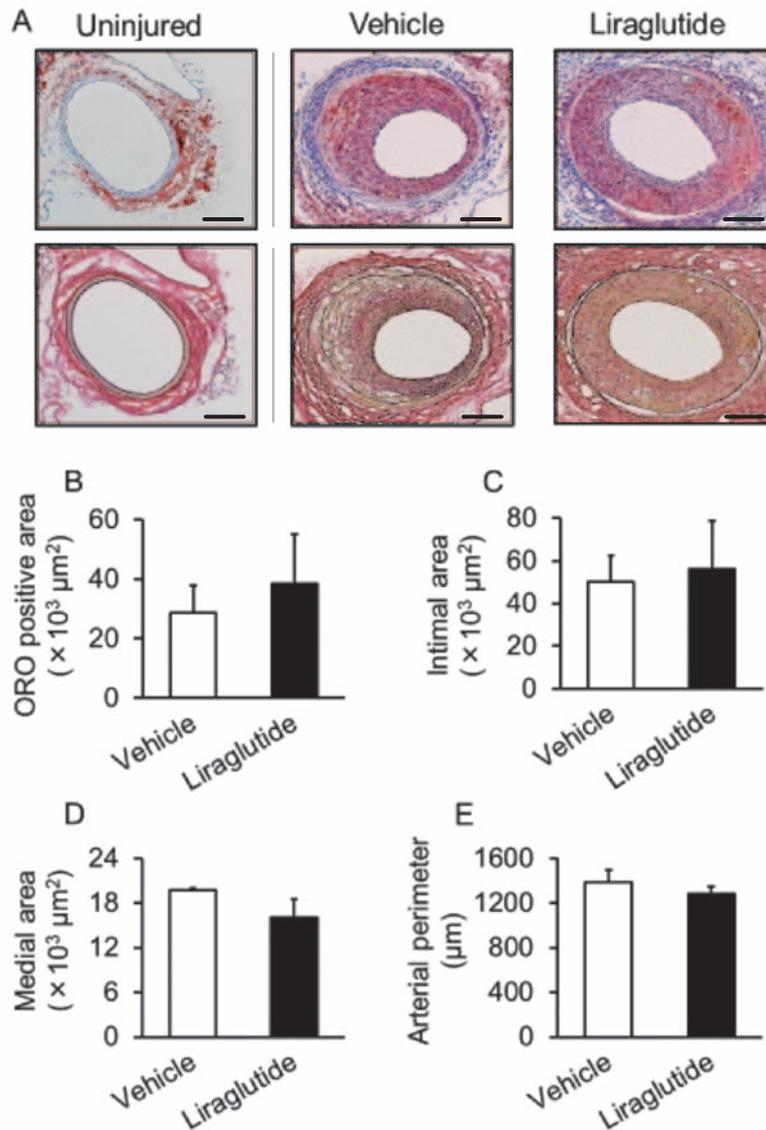
Fig. 3. Anti-atherogenic effects of liraglutide are compromised by removal of VECs
Streptozotocin-induced diabetic mice were treated with vehicle or liraglutide for 4 weeks, VECs were removed from the lumen surface of their left femoral arteries by wire injury (2 days after treatment initiation), and both femoral arteries were collected for analysis of atherosclerosis. (A) Representative images of the femoral arteries were stained with oil red O (upper panels) and Elastica van Gieson (lower panels). Scale bars, 100 μm. (B) Atherosclerotic plaque volume. (C) Intimal area. (D) Medial area. (E) Arterial perimeter. Graphed values are means, and each error bar indicates standard error of mean. ORO, oil red O.

plaque formation. For example, in VECs, NO prevents expression of pro-inflammatory cytokines and cell adhesion molecules via suppression of transcription factors (eg, nuclear factor κ B) and oxidative stress[27, 28]. Also, NO inhibits proliferation and migration of VSMCs — processes that contribute to the development of atherosclerotic plaques[29]. We previously reported that liraglutide treatment increases NO levels in plasma and phosphorylated endothelial NO synthase levels in the aorta[18]. In addition, liraglutide treatment suppresses peripheral arterial remodeling in mouse models, and this effect is completely abolished by inhibiting NO synthase. These find-

ings indicate that enhancement of NO production in VECs is a possible mechanism of the VEC-dependent anti-atherogenic effects of liraglutide.

Previous studies employing non-diabetic animals have shown that GLP-1 and GLP-1RAs suppress phenotypic changes and inflammatory responses in macrophages [5, 7, 30], which are involved in atherosclerotic plaque progression [19, 20]. Consistent with these findings, we found that liraglutide treatment suppressed intra-plaque macrophage accumulation and reduced vascular expression of pro-inflammatory cytokines in diabetic mice, suggesting that anti-inflammatory effects of liraglutide are preserved under hyperglycemic conditions. However, liraglutide treatment failed to suppress atherosclerosis formation in arteries in which VECs had been removed. These findings imply that the anti-inflammatory effects of liraglutide are not the main mechanisms by which liraglutide suppresses atherosclerosis.

The present study has several limitations. First, anti-atherogenic effects of liraglutide were compared in different vessels — endothelium-intact aortas and endothelium-denuded femoral arteries. As shown in Fig. 3A, atherosclerotic plaque is rarely formed in uninjured femoral arteries, which makes it difficult to directly compare the anti-atherogenic effects of liraglutide between uninjured and injured arteries. In addition, removal of VECs from the aorta is technically challenging in mice. Thus, the role of VECs in the anti-atherogenic effects of liraglutide needs to be further evaluated in bilateral arteries of larger animals such as rabbits or pigs [31]. For example, more robust conclusions could be obtained by comparing the anti-atherogenic effects of GLP-1RA in the left and right iliac arteries of cholesterol-fed rabbits following unilateral arterial injury.

In conclusion, although liraglutide has multiple effects on various cells, our data show that VECs are essential for liraglutide to suppress atherosclerosis in diabetic ApoE$^{-/-}$ mice.

Conflict of interest disclosure

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