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Dipeptidyl peptidase-4 inhibitor, linagliptin, ameliorates endothelial dysfunction and atherogenesis in normoglycemic apolipoprotein-E deficient mice



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ABSTRACT

Background: Dipeptidyl peptidase-4 (DPP-4) inhibitors have vasoprotective effects. This study investigated whether a recently approved DPP-4 inhibitor, linagliptin (Lina), suppresses atherogenesis in non-diabetic apolipoprotein-E deficient (*ApoE*^{−/−}) mice, and examined its effects on endothelial function.

Methods and results: Lina (10 mg/kg/day) was administered orally to *ApoE*^{−/−} mice for 20 weeks. Lina reduced atherogenesis without alteration of metabolic parameters including blood glucose level compared with control ($P < 0.05$). Results of immunohistochemical analyses and quantitative RT-PCR demonstrated that Lina significantly decreased inflammatory molecule expression and macrophage infiltration in the atherosclerotic aorta. Lina administration to *ApoE*^{−/−} mice for 9 weeks ameliorated endothelium-dependent vasodilation compared with that in untreated mice. Plasma active glucagon-like peptide-1 (GLP-1) level was significantly higher in the treated group ($P < 0.05$). Exendin-4 (Ex-4), a GLP-1 analog, ameliorated endothelium-dependent vasodilation impaired by palmitic acid (PA) in wild-type mouse aortic segments. Ex-4 promoted phosphorylation of eNOS^{Ser1177} and Akt, both of which were abrogated by PA, in human umbilical vein endothelial cells. In addition, Lina administration to *ApoE*^{−/−} mice decreased oxidative stress, as determined by urinary 8-OHdG secretion and NADPH oxidase subunit expression in the abdominal aorta.

Conclusion: Lina inhibited atherogenesis in non-diabetic *ApoE*^{−/−} mice. Amelioration of endothelial dysfunction associated with a reduction of oxidative stress by GLP-1 contributes to the atheroprotective effects of Lina.

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1. Introduction

Chronic inflammation plays a central role in the pathogenesis of atherosclerosis [1]. Endothelial dysfunction caused by cardiovascular risk factors is a key initiator of vascular inflammation [2]. Dysfunction

of endothelial cells alters vascular responses and induces the expression of adhesion molecules and chemokines which stimulate monocyte-endothelial cell interaction, leading to the development of atherosclerosis [3]. Therefore, abrogation of endothelial dysfunction is an attractive strategy for preventing vascular inflammation and atherosclerosis.

Dipeptidyl peptidase-4 (DPP-4) inhibitors are a new class of anti-diabetic drugs that improve glucose metabolism by raising the active concentration and duration of action of glucose-like peptide (GLP)-1, a gut hormone secreted in response to nutrient ingestion that stimulates glucose-dependent insulin secretion [4]. In addition to their anti-diabetic property, recent studies have suggested that DPP-4 inhibitors have anti-inflammatory effects independent of their glucose-lowering effect [5–9]. The GLP-1 receptor mediates major function of GLP-1. The GLP-1 receptor is known to be expressed in pancreatic β cells, although recent studies demonstrated its expression in endothelial cells, suggesting vasoprotective effects independent of blood glucose level [10,11].

Abbreviations: DPP-4, dipeptidyl peptidase-4; Lina, linagliptin; *ApoE*^{−/−}, apolipoprotein-E deficient; GLP-1, glucagon-like peptide-1; Ex-4, exendin-4; PA, palmitic acid; WTD, western-type diet; CMC, carboxymethyl cellulose; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cellular adhesion molecule-1; Mac-3, macrophage antigen-3; Ach, acetylcholine; SNP, sodium nitroprusside; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; qPCR, quantitative real-time PCR; ROS, reactive oxygen species.

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Table 1
Effects of Lina on metabolic parameters after 20-week treatment.

	Ctrl (N = 14)	Lina (N = 16)	P-value
Body weight, g	37.3 ± 2.0	34.2 ± 2.2	0.33
Blood glucose, mg/dL	92.9 ± 6.2	99.5 ± 6.0	0.45
Insulin, ng/mL	4.8 ± 0.7	4.2 ± 0.8	0.57
Total cholesterol, mg/dL	690.0 ± 49.8	554.6 ± 72.6	0.15
Triglyceride, mg/dL	73.5 ± 5.3	84.8 ± 10.8	0.39
HDL cholesterol, mg/dL	12.5 ± 1.3	11.5 ± 1.8	0.67
Heart rate, bpm	680.0 ± 20.8	687.7 ± 24.8	0.83
Systolic BP, mm Hg	97.7 ± 3.6	93.0 ± 1.5	0.19
Diastolic BP, mm Hg	74.1 ± 3.9	67.4 ± 2.1	0.12

BP; blood pressure, Ctrl; control, Lina; linagliptin. All values are mean ± SEM.

Previously, we have reported that the GLP-1 analog, exendin-4 (Ex-4), attenuated neointima formation after mechanical vascular injury by inhibiting macrophage activation at least partially in normoglycemic mice [12]. Also several studies have demonstrated that DPP-4 inhibitors suppressed the development of atherosclerosis even in non-diabetic atherosclerotic models [13–16]. However, the effects of DPP-4 inhibitors on endothelial cell function, associated with atherogenesis, in normoglycemic animals have not been fully investigated. Therefore, in this study, we administered linagliptin (Lina), which was recently approved as an oral glucose-lowering drug [17], to normoglycemic apolipoprotein E-deficient (*ApoE*^{-/-}) mice and examined the effects of Lina on endothelial cell function and atherogenesis. Our findings demonstrated that Lina reduced the development of atherosclerosis and ameliorated endothelial dysfunction in this mouse model. Results of in vitro and ex vivo experiments suggested that the antioxidant effect of GLP-1 contributed, at least partially, to these results.

2. Materials and methods

2.1. Animals and drug administration

ApoE^{-/-} (C57BL/6J background) mice were originally purchased from The Jackson Laboratory. The *ApoE*^{-/-} mouse, which exhibits severe hypercholesterolemia, is a widely used mouse model of atherosclerosis [18]. All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. Lina was supplied by Boehringer Ingelheim, Japan. To examine the effect of Lina on atherogenesis, 8-week-old male *ApoE*^{-/-} mice receiving a western-type diet (WTD) were treated with Lina 10 mg/kg/day by gavage for 20 weeks. To examine the effect of Lina on endothelial function at earlier stage of atherosclerosis, the same dose of Lina was administered to 7-week-old female *ApoE*^{-/-} for 9 weeks. WTD was started from 8 weeks of age. Lina was suspended in 0.5% carboxymethyl cellulose (CMC) solution. The control group received an equivalent volume of CMC. Mice were maintained under a 12 h light/dark cycle.

2.2. Blood pressure and laboratory data

The blood pressure of each mouse was measured by a tail-cuff system (BP-98A, Softron) as described previously. At the time of sacrifice, blood was collected from the heart into EDTA-containing tubes. After blood samples were centrifuged, plasma was stored at -80 °C until required. Plasma total cholesterol, HDL-cholesterol, and triglyceride levels were measured at LSI Medience Corporation (Japan). Plasma levels of insulin and active GLP-1 were measured using commercially available kits (Shibayagi Co., Ltd. and Immuno-Biological Laboratories Co., Ltd., respectively). Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration in a 16 h urine collection was determined using a commercially available kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd.) and corrected by creatinine level.

2.3. Quantification of atherosclerotic lesions

The severity of atherosclerotic lesions in the aortas was assessed as previously described [19]. In brief, mice were sacrificed with an overdose of pentobarbital, and perfused with 0.9% sodium chloride solution at a constant pressure via the left ventricle. Both the heart and whole aorta were immediately removed. The thoracic aorta was excised, opened longitudinally, and fixed with 4% paraformaldehyde. To quantify atherosclerotic lesions in the aortic arch, we performed en face Sudan IV staining. The percentage of Sudan IV-positive area was measured. The abdominal aorta was removed and snap-frozen in liquid nitrogen for gene expression analysis.

2.4. Histological and immunohistochemical analyses

Frozen sections of the aortic root (at 5- μ m intervals) were obtained as described previously [19]. The sections were stained with Oil red O to detect lipid deposition. Monocyte chemoattractant protein-1 (MCP-1), vascular cellular adhesion molecule-1 (VCAM-1), and macrophage antigen-3 (Mac-3) expression were detected using an anti-MCP-1 (BD Pharmingen), anti-VCAM-1 (Abcam) or anti-Mac3 (BD Biosciences) antibody followed by the avidin-biotin complex technique and stained using a Vector Red substrate kit (Vector). Each section was counterstained with hematoxylin.

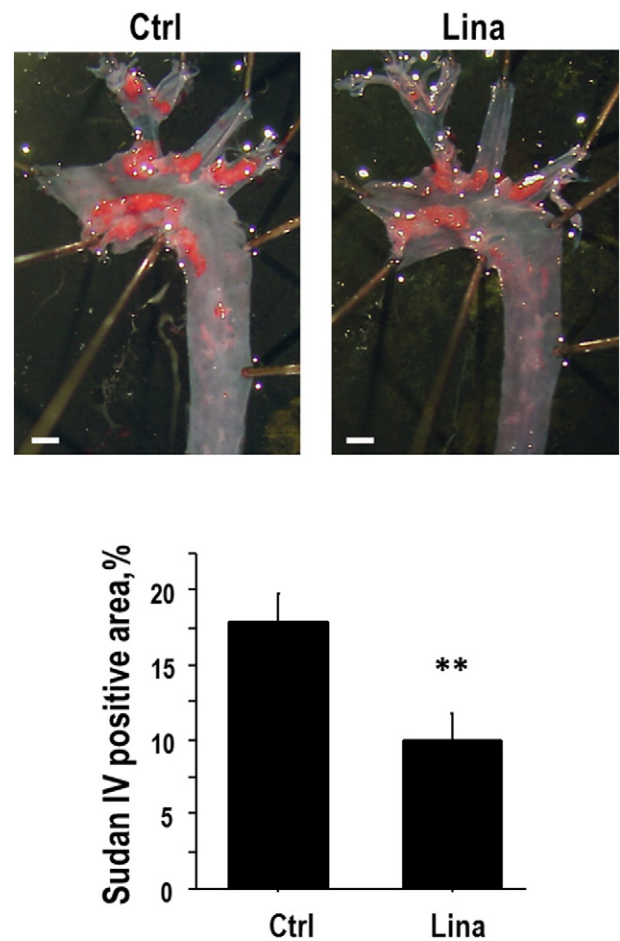


Fig. 1. Effect of Lina on atherosclerotic lesion progression in normoglycemic *ApoE*^{-/-} mice. En face Sudan IV staining of the aortic arch showed that Lina administration for 20 weeks significantly reduced the progression of atherosclerotic lesions compared with vehicle (n = 12, per group). Bar: 1 mm. **: P < 0.01. All values are mean ± SEM.

2.5. Vascular reactivity assay

Analysis of vascular reactivity was performed as described previously [20]. In brief, the descending thoracic aortas from mice were cut into 2-mm rings with special care to preserve the endothelium, and mounted in organ baths filled with modified Krebs–Henseleit buffer (KHB; 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11.1 mM glucose) aerated with 95% O₂ and 5% CO₂ at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were primed with 31.4 mM KCl, and then pre-contracted with phenylephrine, producing submaximal (60% of maximum) contraction. After the plateau was attained, the rings were exposed to increasing

concentrations of acetylcholine (ACh; 10⁻⁹ to 10⁻⁴ M) and sodium nitroprusside (SNP; 10⁻⁹ to 10⁻⁴ M) to obtain cumulative concentration–response curves. In some experiments, aortic segments were incubated with 200 μM palmitic acid (PA, Chem Service) in the presence/absence of Ex-4 for 4 h before analysis of vascular reactivity.

2.6. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Life Technologies and cultured in EGM-2 (Lonza). HUVEC (passages 4–6) were treated with 1 or 10 nM Ex-4 (Sigma-Aldrich) in EBM-2 (Lonza). For inflammatory activation, cells were stimulated

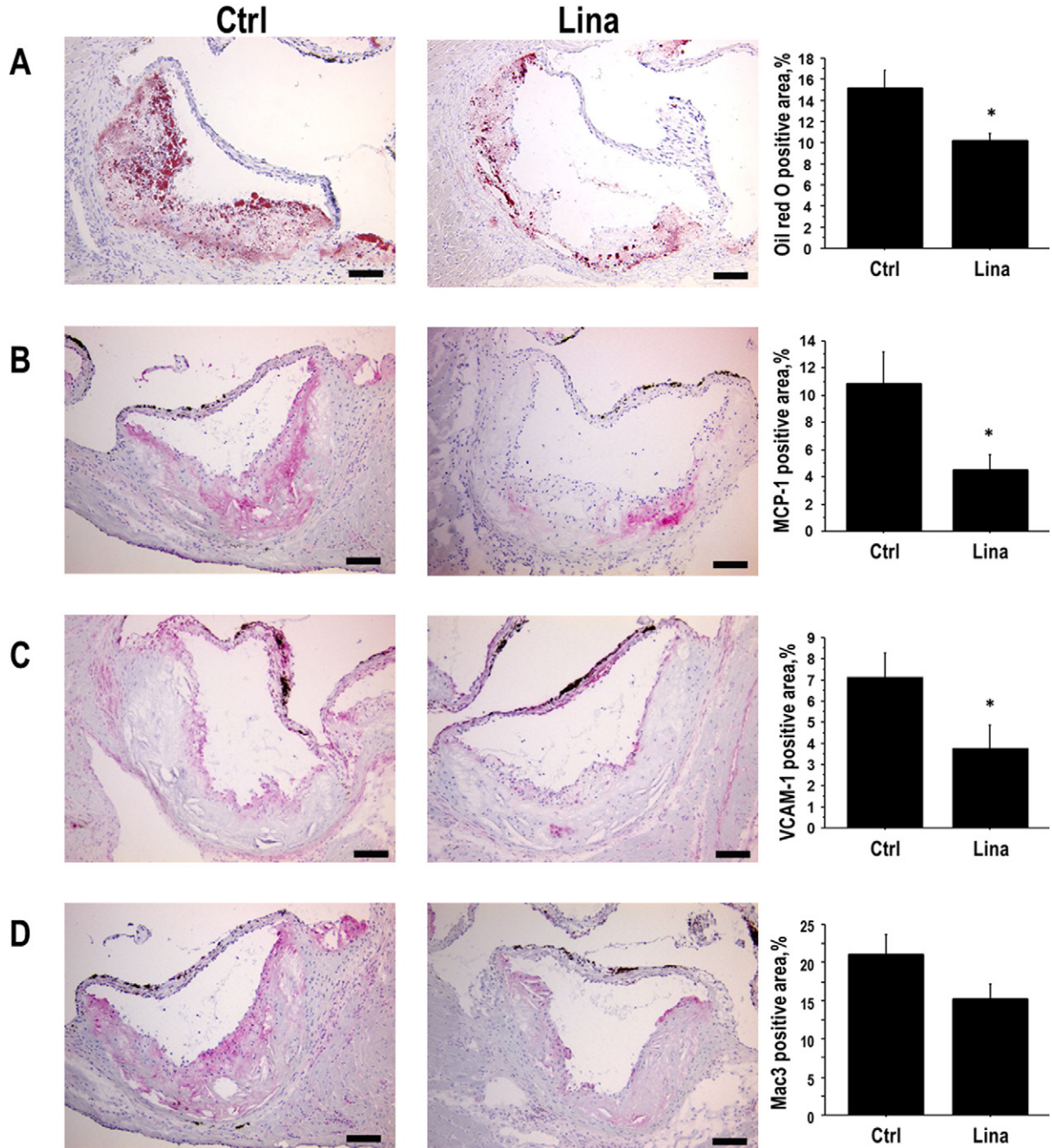


Fig. 2. Effect of Lina on characteristics of atherosclerotic plaques. (A) Representative Oil red O staining of aortic root. Lina significantly decreased lipid deposition in plaques. (B and C) Representative MCP-1 and VCAM-1 staining of atherosclerotic plaques. Lina significantly reduced the expression of MCP-1 (B) and VCAM-1 (C) in atherosclerotic lesions. (D) Representative Mac3 staining of atherosclerotic plaques. Lina tended to decrease macrophage accumulation in plaques. (n = 11–12, per group). Bar: 100 μm. *; P < 0.05. All values are mean ± SEM.

with 200 μ M PA. Phosphorylation of Akt and eNOS^{Ser1177} was induced by vascular endothelial growth factor (VEGF; 10 ng/mL) for 5 min.

2.7. Western blot analysis

Cell lysates were prepared using RIPA buffer (Wako Pure Chemical Industries, Ltd.) containing a protease inhibitor cocktail (Takara Bio Inc.) and phosphatase inhibitors. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare). After blocking with 5% bovine serum albumin, the membranes were incubated with primary antibody against either phosphorylated-eNOS^{Ser1177}, Akt, phosphorylated-Akt^{Ser473} (Cell Signaling Technology), eNOS (BD Biosciences), or β -actin (Sigma) overnight at 4 °C. Horseradish peroxidase-conjugated anti-mouse Ig (Cell Signaling Technology) or anti-rabbit Ig (Chemicon) antibody was then used as a secondary antibody. Antibody distribution was visualized with ECL-plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-1000, Fuji Film).

2.8. Real-time polymerase chain reaction

Total RNA was extracted from the aorta using an illustra RNAspin RNA Isolation Kit (GE Healthcare). cDNA was synthesized from 100 ng of total RNA using a QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR (qPCR) was performed on an Mx3000P (Agilent Technologies) using Power SYBR Green PCR Master Mix (Applied Biosystems). Mouse PCR primers were as follows: F4/80, sense 5'-TGCATCTAGCAATGGACAGC-3' and antisense 5'-GCCTTCTGGA TCCATTGAA-3'; VCAM-1, sense 5'-CCCGTCATTGAGGATATTGG-3' and antisense 5'-GGCATTGTACAGACCAC-3'; MCP-1, sense 5'-CCACTC ACCTGCTGCTACTCAT-3' and antisense 5'-TGGTGATCCTCTTGTAGCTC TCC-3'; p47^{phox}, sense 5'-ACCTGTCGGAGAAGGTGGT-3' and antisense 5'-TAGGTCTGAAGGATGATGGG-3'; Nox2, sense 5'-ACTCCTGGGTCAG CACTGG-3' and antisense 5'-GTTCTGTCCAGTTGTCTTCG-3'; β -actin, sense 5'-CCTGAGCGCAAGTACTCT GTGT-3' and antisense 5'-GCTGAT CCACATCTGCTGGAA-3'. Human PCR primers were as follows: VCAM-1, sense 5'-ATGAATTGAAACCCAAACA-3' and antisense 5'-CCTGGCTC AAGCATTGTCATA-3'; MCP-1, sense 5'-CCCCAGTCAACCGCTGTTAT-3' and antisense 5'-AGATCTCCTTGGCCACAATG-3'; GAPDH; sense 5'-TGGGTGTGAACCATGAGAAG-3' and antisense 5'-GCTAAGCAGTTGGT GGTGC-3'. Data are expressed in arbitrary units that were normalized by β -actin or GAPDH.

2.9. Statistical analysis

All results are expressed as mean \pm SEM. Comparison of parameters between two groups was performed with unpaired Student's *t*-test. Comparisons of dose–response curves were made by two-factor repeated-measures ANOVA, followed by Tukey's post hoc test for comparison between groups. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effects of Lina on atherosclerotic lesion progression in ApoE^{-/-} mice

To investigate the effect of Lina on atherogenesis, ApoE^{-/-} mice were treated with Lina or vehicle for 20 weeks. Administration of Lina to normoglycemic ApoE^{-/-} mice did not alter metabolic parameters including blood glucose level, as shown in Table 1. Lina decreased the development of atherosclerotic lesions in the aortic arch as determined by en face Sudan IV staining compared with control (17.9 ± 1.8 vs. $10.0 \pm 1.7\%$, $P < 0.01$) (Fig. 1). Oil red O staining demonstrated that Lina decreased lipid deposition in plaques in the aortic root (15.2 ± 1.7 vs. $10.2 \pm 0.7\%$, $P < 0.05$) (Fig. 2A). Immunostaining revealed that Lina significantly reduced the expression of MCP-1 (10.9 ± 2.3 vs. $4.5 \pm 1.1\%$, $P < 0.05$) and VCAM-1 (7.1 ± 1.1 vs. $3.8 \pm 1.1\%$, $P < 0.05$) in

atherosclerotic plaques (Fig. 2B and C). Lina tended to decrease accumulation of macrophages in plaques as determined by immunostaining against Mac3 (21.1 ± 2.5 vs. $15.3 \pm 1.9\%$, $P = 0.09$) (Fig. 2D).

3.2. Lina reduced expression of inflammatory molecules in aorta

We also examined the effects of Lina on the expression of inflammatory molecules in the atherosclerotic aorta using qPCR. Lina decreased mRNA expression of MCP-1 ($P = 0.06$) and VCAM-1 ($P < 0.05$) in the abdominal aorta compared with control (Fig. 3A). Also, the expression of a macrophage marker, F4/80, in Lina-treated mice was lower than that in control mice ($P < 0.05$). These results were consistent with the immunohistochemical results, and suggested that Lina inhibits the expression of chemokines and adhesion molecules, leading to attenuated monocyte/macrophage infiltration into the plaque. Furthermore, Lina reduced the expression of NADPH oxidase subunits, p47^{phox} and Nox2 in atherosclerotic aorta (Fig. 3B).

3.3. Lina ameliorated endothelial dysfunction in ApoE^{-/-} mice

Impairment of endothelial function is the initial step in vascular inflammation. Therefore, to investigate the effect of Lina on endothelial function in a normoglycemic condition, endothelium-dependent vasodilation was examined in wild-type and ApoE^{-/-} mice. Endothelium-dependent vasodilation in response to Ach was significantly impaired in ApoE^{-/-} mice after 8-week WTD feeding compared with that in age-sex-matched wild-type mice; however, Lina administration for 9 weeks

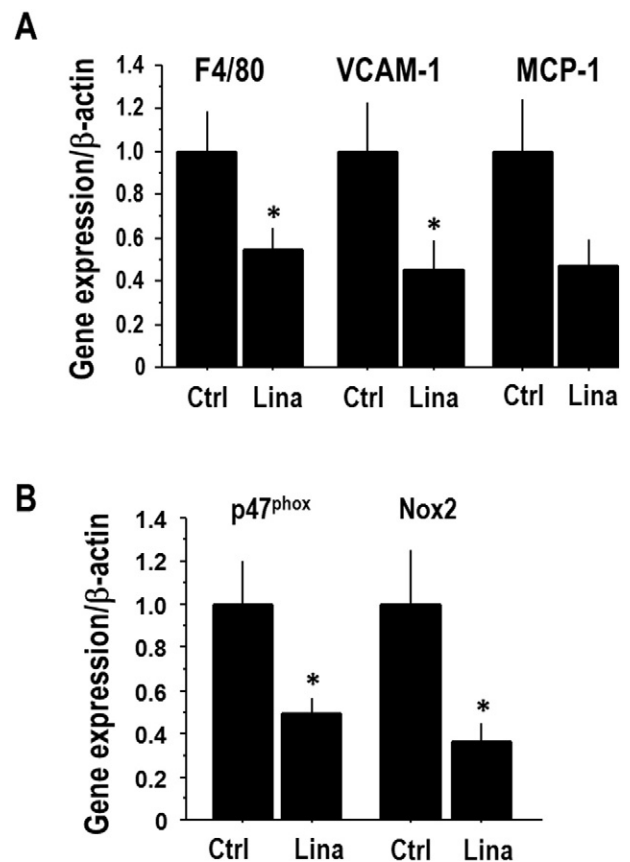


Fig. 3. Effect of Lina on expression of inflammatory molecules in abdominal aorta. (A) Results of qPCR demonstrated that Lina administration reduced RNA expression of VCAM-1 and MCP-1 in atherosclerotic aorta. Lina also decreased the expression of F4/80, suggesting reduction of macrophage accumulation in atherosclerotic aorta. (B) Results of qPCR demonstrated that Lina administration reduced RNA expression of NADPH oxidase subunits, p47^{phox} and Nox2, in atherosclerotic aorta. ($n = 12$, per group). *; $P < 0.05$. All values are mean \pm SEM.

ameliorated the impairment of endothelium-dependent vasodilation (Fig. 4A). Metabolic parameters did not differ between vehicle and Lina treated *ApoE*^{-/-} mice (Table 2). On the other hand, endothelium-independent relaxation in response to SNP did not differ between the Lina and control groups (Fig. 4B). In this experiment, Lina administration to *ApoE*^{-/-} mice significantly elevated the plasma level of active GLP-1 compared with control (11.8 ± 0.5 vs. 17.7 ± 1.3 pmol/L, P < 0.01), as shown in Fig. 4C. Furthermore, Lina administration significantly decreased urinary excretion of 8-OHdG in *ApoE*^{-/-} mice compared to control (Fig. 4D).

3.4. Ex-4, a GLP-1 analog, attenuated endothelial dysfunction caused by PA

To investigate whether increased GLP-1 level is associated with improvement of endothelial function, endothelium-dependent vasodilation was examined using aortic rings obtained from wild-type mice. Ex-4, a GLP-1 analog, significantly improved vasodilation in response to Ach, which was impaired by incubation with PA, a common free fatty acid found in hyperlipidemic animals (Fig. 5A). Ex-4 did not affect endothelium-independent relaxation in response to SNP (Fig. 5B). We further investigated the molecular mechanism by which Ex-4 attenuated impaired endothelium-dependent vasodilation induced by PA. Results of western blotting demonstrated that Ex-4 significantly promoted phosphorylation of Akt and eNOS^{Ser1177}, which were reduced in the presence of PA in HUVECs (Fig. 5C). In addition, we examined whether GLP-1 attenuated the expression of inflammatory molecules related to monocyte/macrophage recruitment in endothelial

Table 2
Effects of Lina on metabolic parameters after 9-week treatment.

	Ctrl (N = 12)	Lina (N = 12)	P-value
Body weight, g	25.6 ± 0.5	24.8 ± 0.5	0.30
Blood glucose, mg/dL	121.0 ± 2.6	116.0 ± 3.9	0.30
Insulin, ng/ml	0.9 ± 0.1	1.1 ± 0.1	0.52
Total cholesterol, mg/dL	881.8 ± 26.7	864.0 ± 37.1	0.71
Triglyceride, mg/dL	45.6 ± 4.2	50.7 ± 5.4	0.47
HDL cholesterol, mg/dL	9.5 ± 0.9	11.9 ± 0.9	0.06

Ctrl; control, Lina; linagliptin. All values are mean ± SEM.

cells. Ex-4 significantly attenuated the expression of VCAM-1 and MCP-1 in HUVECs treated with PA (Fig. 6A and B).

4. Discussion

Previous studies have reported that DPP-4 inhibitors reduced the development of atherosclerosis in both hyperglycemic and normoglycemic atherosclerosis models by inhibiting vascular inflammation [21]. Most of those studies have demonstrated that DPP-4 inhibitors reduce vascular inflammation via suppression of pro-inflammatory activation of monocytes/macrophages independent of blood glucose level [22–24]. Endothelial dysfunction, as well as inflammatory cell activation, is a pivotal factor for the initiation of atherogenesis [25]. Therefore, in this study, we focused on the effect of a DPP-4 inhibitor on endothelial function associated with the development of atherosclerosis in *ApoE*^{-/-} mice. Corresponding to the previous studies, we demonstrated that Lina reduced atherosclerotic lesion progression and the expression of inflammatory molecules in the

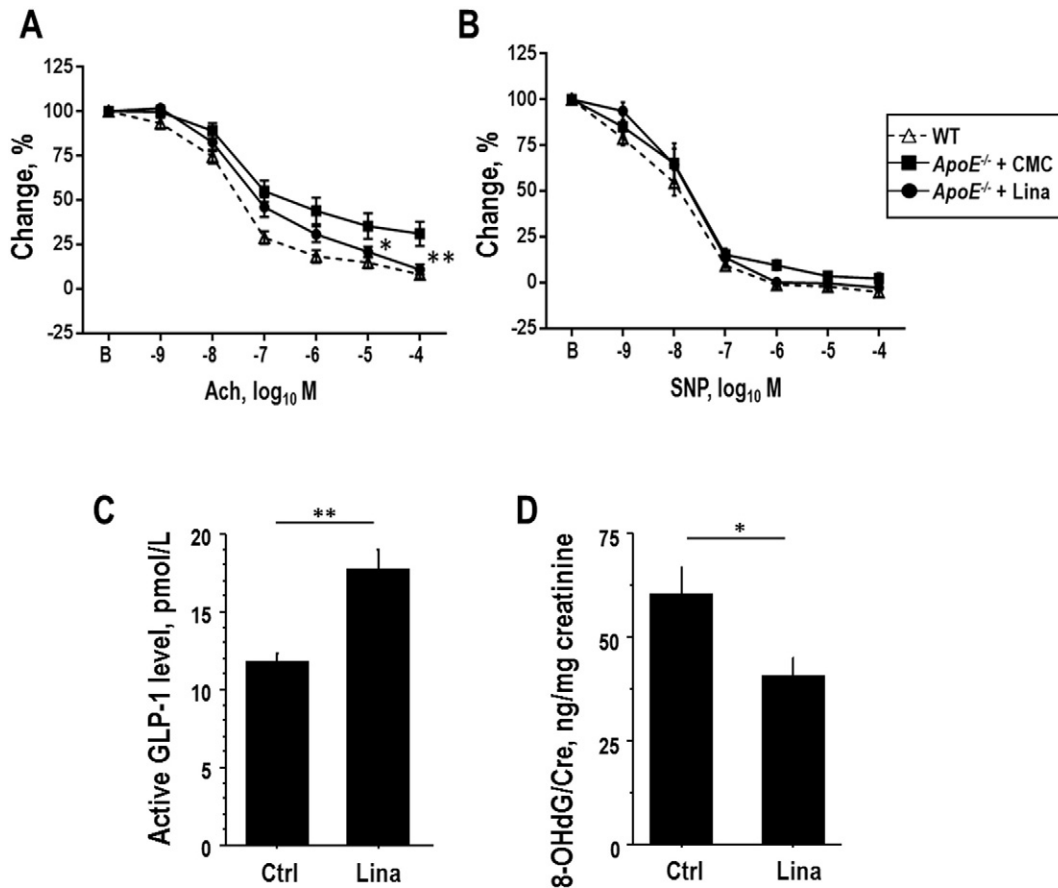


Fig. 4. Lina improved endothelial function in normoglycemic *ApoE*^{-/-} mice. (A and B) Vascular reactivity to Ach or SNP was determined using aortic rings isolated from Lina- or CMC-administered *ApoE*^{-/-} mice and age-sex-matched WT mice. Lina administration for 9 weeks ameliorated endothelium-dependent vasodilation in response to Ach compared with CMC in *ApoE*^{-/-} mice at earlier stage of atherosclerosis (A). Vasorelaxation in response to SNP (B) did not differ among the three groups. (n = 10–12, per group) *; P < 0.05 and **; P < 0.01 compared with CMC group. (C) Lina administration significantly increased plasma level of active GLP-1 in *ApoE*^{-/-} mice. (D) Lina administration significantly decreased urine secretion of 8-OHdG in *ApoE*^{-/-} mice. (n = 5–7, per group). *; P < 0.05 and **; P < 0.01. Data represent mean ± SEM.

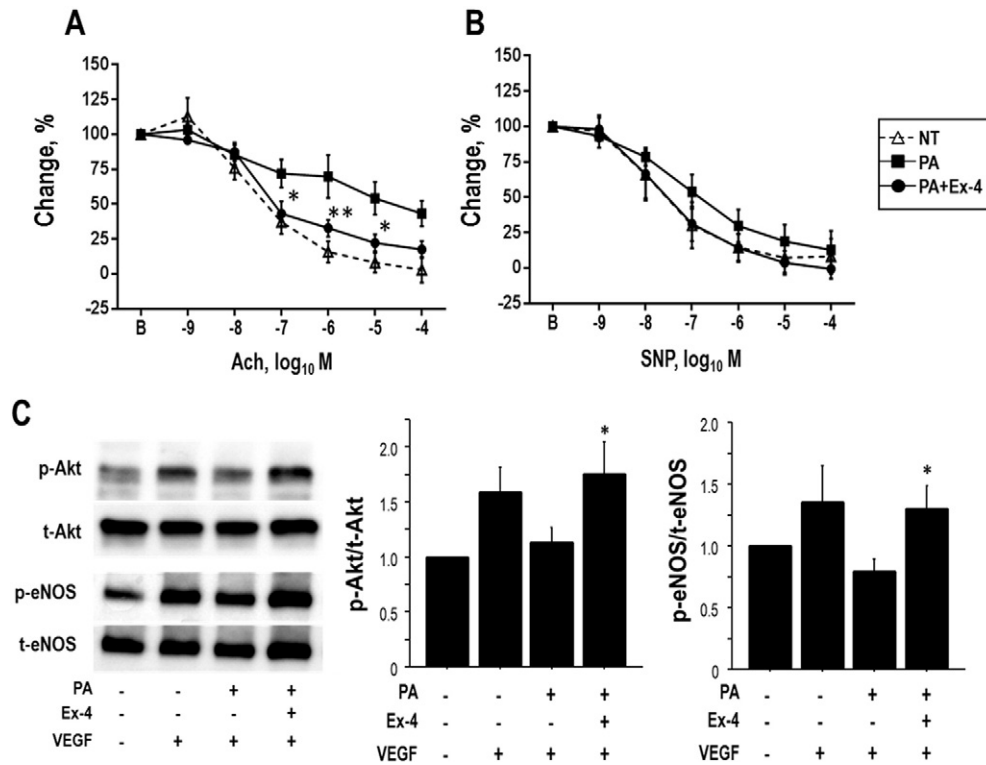


Fig. 5. Ex-4 attenuated endothelial dysfunction induced by PA. (A and B) To investigate the effects of Ex-4, a GLP-1 analog, on endothelial function, vascular reactivity to ACh or SNP was examined using aortic rings isolated from WT mice. Aortic rings were incubated with PA (200 μM) in the presence or absence of Ex-4 for 4 h. Ex-4 significantly improved endothelium-dependent vasodilation impaired by PA (A). On the other hand, EX-4 did not affect endothelium-independent vasodilation (B) (n = 8–9, per group). (C) To investigate the effects of Ex-4 on endothelial cells, phosphorylation of Akt^{Ser473} and eNOS^{Ser1177} in response to VEGF was examined in HUVEC. Results of western blotting demonstrated that Ex-4 promoted the phosphorylation of Akt and eNOS, which was impaired in the presence of PA (n = 5–6, per group). *; P < 0.05 and **; P < 0.01 compared with PA. All values are mean ± SEM.

atherosclerotic aorta in normoglycemic *ApoE*^{-/-} mice in this study. Furthermore, our results demonstrated that elevated GLP-1 level contributed to the improvement of endothelial function. Reduction of oxidative stress by Lina administration might have roles in the protection of endothelial function and in suppression of atherogenesis, at least in part.

It is a widely accepted view that endothelial damage disturbs homeostasis and initiates the atherosclerotic process including endothelial permeability, platelet aggregation, leukocyte adhesion, and generation of cytokines [26]. Therefore, endothelial dysfunction is considered to be an early marker of atherosclerosis and a target for the prevention of atherosclerosis [3]. Cardiovascular risk factors cause endothelial dysfunction. Especially, type 2 diabetes associates with endothelial

dysfunction, which contributes to the development of its micro- and macrovascular complications. Reactive oxygen species (ROS) generated under a hyperglycemic condition play a causal role in endothelial dysfunction in type 2 diabetes [27]. Previous studies demonstrated that GLP-1 regulates vascular tone and endothelial function [11,28,29]. In fact, GLP-1-mimetics and DPP-4 inhibitors, including Lina, attenuated endothelial dysfunction in a hyperglycemic condition through NO production in human and animal experiments [11,30–35]. Also, several studies showed that Lina improved vascular function in an LPS-induced sepsis model [36] and hypertension [37], although few studies have investigated the effects of Lina on endothelial function, associated with the development of atherosclerosis in a hyperlipidemic model. The

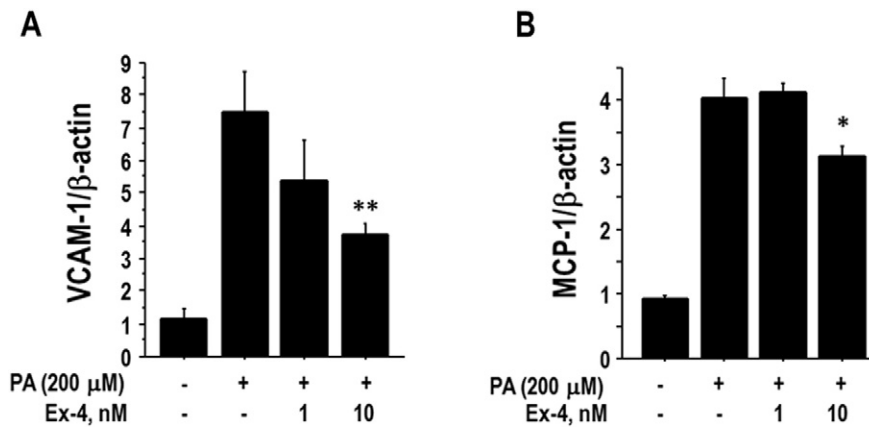


Fig. 6. Ex-4 attenuated expression of inflammatory molecules in endothelial cells. (A and B) To investigate the effects of Ex-4, a GLP-1 analog, on the expression of inflammatory molecules related to monocyte/macrophage recruitment in endothelial cells, HUVECs were incubated with 200 μM PA for 4 h in the presence or absence of Ex-4. Results of qPCR analysis demonstrated that Ex-4 significantly reduced the expression of VCAM-1 (A) and MCP-1 (B). (n = 4, per group). *; P < 0.05 and **; P < 0.01 compared with PA. All values are mean ± SEM.

results of our present study demonstrated that endothelium-dependent vasodilation was improved in Lina-treated *ApoE*^{-/-} mice, indicating its protective effects on endothelial cell function in a hyperlipidemic condition, one of the major cardiovascular risk factors.

Lina elevated plasma active GLP-1 concentration in *ApoE*^{-/-} mice. Therefore, we investigated the effect of GLP-1 on endothelial function. Our ex vivo experiment using aortic rings obtained from WT mice demonstrated that Ex-4, a GLP-1 analog, improved endothelium-dependent vasodilation which was impaired by the presence of PA, one of the most common free fatty acids found in hyperlipidemic animals [38,39]. Previous studies have shown that PA-induced oxidative stress contributes to endothelial dysfunction and atherogenesis [40,41]. Our in vitro experiments using HUVEC demonstrated that Ex-4 increased phosphorylation of eNOS^{Ser1177} and its upstream kinase, Akt^{Ser473}, in response to VEGF [42], which was affected by the presence of PA. This result corresponds to previous studies that reported Akt/eNOS activation by Ex-4 [43,44]. Phosphorylation eNOS at Ser1177 increases NO production and mediates endothelial function [20]. These results suggested that the antioxidant property of GLP-1 attenuated endothelial dysfunction caused by hyperlipidemia. In addition, Lina administration decreased ROS generation as determined by urinary excretion of 8-OHdG and the expression of NADPH oxidase subunits Nox2 and p47^{phox} in the aorta. The expression of Nox2 and p47^{phox} is associated with vascular oxidative stress, leading to the development of atherosclerosis. Especially, several studies have shown that Nox2-derived oxidative stress has a pathogenic role in the functional changes of the arterial wall, including endothelial cell function, in hyperlipidemia [45–47]. In fact, Lina-treated mice showed lower expression of inflammatory molecules in the abdominal aorta in our study. The result of in vitro experiments using HUVEC which demonstrated inhibitory effect of EX-4 on inflammatory molecule expression supported this in vivo data. These results suggest that the antioxidative property of Lina, which is derived from elevated GLP-1 level, contributes at least partially to the reduction of endothelial damage and atherogenesis caused by hyperlipidemia. Recent evidence suggests the cardioprotective effects of DPP-4 inhibitors independent of incretins [48]. DPP-4 inhibitors attenuate degradation of stromal cell-derived factor-1 and substance P as well as incretins, which may contribute to vasoprotection at least in part [49]. Also, recent studies suggest direct effects of DPP-4 inhibitors on vascular cells [48,50,51]. Further studies are needed to elucidate these effects of Lina, however those studies will help clarify its vasoprotective effects and underlying mechanisms.

In conclusion, our results demonstrated that Lina inhibited the development atherosclerotic lesions in *ApoE*^{-/-} mice, independently of blood glucose level. The beneficial effect of Lina was associated with attenuation of endothelial dysfunction, at least in part by a reduction of oxidative stress. Taken together with the results of previous studies, our results show that DPP-4 inhibitors have anti-inflammatory effects on various cell types in the vasculature. Our present study supports the results of recent studies that suggested atheroprotective effects of DPP-4 inhibitors. These non-glycemic properties of DPP-4 inhibitors may provide attractive therapeutic options for atherosclerosis.

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Disclosures

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