EXPERIMENTAL STUDY

Vildagliptin, a DPP-4 Inhibitor, Attenuates Endothelial Dysfunction and Atherogenesis in Nondiabetic Apolipoprotein E-Deficient Mice

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Summary

Dipeptidyl peptidase-4 (DPP-4) inhibitors are novel antidiabetic agents with possible vascular protection effects. Endothelial dysfunction is an initiation step in atherogenesis. The purpose of this study was to investigate whether vildagliptin (Vilda) attenuates the development of endothelial dysfunction and atherosclerotic lesions in nondiabetic apolipoprotein E-deficient (ApoE$^{-/-}$) mice. Eight-week-old nondiabetic ApoE$^{-/-}$ mice fed a Western-type diet received Vilda (50 mg/kg/day) for 20 weeks or 8 weeks. After 20 weeks of treatment, Vilda administration reduced atherogenesis in the aortic arch as determined by *en face* Sudan IV staining compared with the vehicle group ($P < 0.05$). Vilda also reduced lipid accumulation ($P < 0.05$) and vascular cell adhesion molecule-1 (VCAM-1) expression ($P < 0.05$) and tended to decrease macrophage infiltration ($P = 0.05$) into atherosclerotic plaques compared with vehicle. After 8 weeks of treatment, endothelium-dependent vascular reactivity was examined. Vilda administration significantly attenuated the impairment of endothelial function in nondiabetic ApoE$^{-/-}$ mice compared with the vehicle group ($P < 0.05$). Vilda treatment did not alter metabolic parameters, including blood glucose level, in both study protocols. To investigate the mechanism, aortic segments obtained from wild-type mice were incubated with exendin-4 (Ex-4), a glucagon-like peptide-1 (GLP-1) analog, in the presence or absence of lipopolysaccharide (LPS). Ex-4 attenuated the impairment of endothelium-dependent vasodilation induced by LPS ($P < 0.01$). Furthermore, Ex-4 promoted phosphorylation of eNOS at Ser1177 which was decreased by LPS in human umbilical endothelial cells ($P < 0.05$). Vilda inhibited the development of endothelial dysfunction and prevented atherogenesis in nondiabetic ApoE$^{-/-}$ mice. Our results suggested that GLP-1-dependent amelioration of endothelial dysfunction is associated with the atheroprotective effects of Vilda.

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Key words: Diabetes mellitus, Endothelial function, Atherosclerosis, GLP-1

Recent animal and clinical studies have documented that the cardiovascular protective properties of dipeptidyl peptidase-4 (DPP-4) inhibitors are independent of their antidiabetic action.[1-4] The fundamental role of DPP-4 inhibitors is elevation of glucagon-like peptide-1 (GLP-1) level, which promotes insulin secretion from the pancreas,[5] whereas the receptor for GLP-1 is widely expressed in many cell types, including vascular cells and macrophages, suggesting pleiotropic and cardioprotective effects of DPP-4 inhibitors beyond their blood glucose-lowering effect.[6,7] Previous studies, including our own, have demonstrated that administration of DPP-4 inhibitors reduced the development of atherosclerotic plaques in normoglycemic animal models, with no alteration of metabolic parameters, including blood glucose and lipid levels.[8-12] Several studies have also reported cardioprotective effects of DPP-4 inhibitors in clinical situations.[13,14] The underlying mechanisms are not fully understood; however, several studies have demonstrated that inhibition of pro-inflammatory activation of vascular cells by GLP-1 contributes to the cardioprotective effects of DPP-4 inhibitors.[8,11]

Vildagliptin (Vilda), which is one of the most investigated DPP-4 inhibitors, shows a more stable glycemic

control profile in diabetic patients compared with other members of this class.[15,16] Previous studies have demonstrated that Vilda attenuated the progression of atherosclerosis in both diabetic and nondiabetic apolipoprotein E-deficient (ApoE^{−/−}) mice by reduction of pro-inflammatory activation of macrophages, an important cell type in atherogenesis.[11] Atherosclerosis is an inflammatory disease in which multiple cell types are involved. Vascular inflammation causes endothelial dysfunction, an initiator of atherosclerosis.[17] Endothelial dysfunction alters vascular responses, which stimulate the development of atherosclerosis.[18,19] Recent studies have suggested that endothelial dysfunction could be a therapeutic target for the inhibition of atherosclerotic disease.[20] However, the effects of Vilda on endothelial cell function have not been fully investigated. Therefore, in this study, we administered Vilda to nondiabetic ApoE^{−/−} mice and examined its effects on endothelial cell function and atherogenesis. Our findings demonstrated that Vilda reduces the development of atherosclerosis and ameliorates endothelial dysfunction in this mouse model. The results of *in vitro* and *ex vivo* experiments suggested that protective properties on endothelial cells which depend on GLP-1 at least partially contribute to these effects.

Methods

Animals and drug administration: ApoE^{−/−} (C57BL/6J background) mice were originally purchased from the Jackson Laboratory. Mice were maintained under a 12-hour light/dark cycle. Vilda was supplied by Novartis Pharma. To examine the effect of Vilda on the development of atherosclerosis, male ApoE^{−/−} mice were treated with Vilda 50 mg/kg/day from 8 weeks old by gavage for 20 weeks. To investigate the effect of Vilda on endothelial function at an earlier stage of atherosclerosis, the same dose of Vilda was administered to female ApoE^{−/−} mice for 8 weeks. A Western-type diet (WTD) was started from 8 weeks old in both experiments. Vilda was dissolved in 0.5% carboxymethyl cellulose (CMC) solution. The control group received an equal volume of CMC. All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. The protocol was reviewed and approved by our institutional ethics committee.

Blood pressure and laboratory data: Blood pressure (BP) of each mouse was measured using a tail-cuff system (BP-98A, Softron) as described in our previous paper.[21] The blood glucose level was measured from the tail vein using a glucometer (NIPRO StatStrip XP2, NIPRO) without fasting and with fasting in 8-week and 20-week treatments, respectively. 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℃ until required. Plasma total cholesterol, high density lipoprotein (HDL)-cholesterol, and triglyceride levels were measured at LSI Medience Corporation (Japan).

Quantification of atherosclerotic lesions: The development of atherosclerotic lesions in the aorta was determined by Sudan IV staining as described previously.[22] In brief, mice were sacrificed with an overdose of pentobarbital and perfused with 0.9% sodium chloride solution at a constant pressure. Both the heart and whole aorta were immediately removed. The thoracic aorta was opened longitudinally and fixed with 10% neutral buffered formalin. To quantify atherosclerotic lesions in the aortic arch, we performed *en face* Sudan IV staining. The percentage of Sudan IV-positive area in the aortic arch was calculated.

Histological and immunohistochemical analysis: The heart was cut along a horizontal plane between the lower tips of the left and right atria. The upper portion was snap-frozen in OCT compound (Tissue-Teck). Then, the aortic root was sectioned serially (at 5-μm intervals) from the point where the aortic valves appeared to the ascending aorta until the valve cusps were no longer visible. These frozen sections of the aortic root were used for histological and immunohistochemical analyses. Sections were stained with oil red O to detect lipid deposition. Also, sections were incubated with anti-monocyte/macrophage marker (MOMA-2) antibody (BioRad), anti-intercellular adhesion molecule-1 (ICAM-1) antibody, and anti-vascular cell adhesion molecule-1 (VCAM-1) antibody (Abcam). Sections were then incubated with biotinylated secondary antibody (VECTOR Laboratories, Inc.), followed by VECTASTAIN ABC-AP Kit (VECTOR Laboratories, Inc.), and stained using a VectorRed AP Substrate Kit (VECTOR Laboratories, Inc.). All sections were counterstained with hematoxylin. The ratio of positive area to plaque area was calculated in three valve lesions in the aortic root and used for comparison.[22]

Vascular reactivity assay: The descending thoracic aorta was cut into 2-mm rings with special care to preserve the endothelium and mounted in an organ bath filled with modified Krebs-Henseleit buffer (KHB; 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose) aerated with 95% O_2 and 5% CO_2 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 precontracted 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 10 ng/mL lipopolysaccharide (LPS) in the presence/absence of a GLP-1 analog, exendin-4 (Ex-4, Sigma-Aldrich), for 24 hours before analysis of vascular reactivity.

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 10 nM Ex-4 for 16 hours in EBM-2 containing 2% FBS and then stimulated with 10 ng/mL LPS for 30 minutes.

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 (Roche Life Science). Proteins were separated by SDS-PAGE and transferred onto polyvinilidine difluoride membranes (Hybond-P; GE Health-

care). After blocking with 5% bovine serum albumin, the membranes were incubated with primary antibody against either phosphorylated-eNOS[Ser 1177], eNOS (BD Biosciences), or β-actin (Sigma-Aldrich) overnight at 4℃. After blots were washed, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 hour. Antibody distribution was visualized with ECL-Plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-1000, Fuji Film).

Statistical analysis: All results are expressed as mean ± SEM. Comparison of parameters between two groups was performed using unpaired Student's *t*-test. Comparisons of dose-response curves between groups were made by two-factor repeated measures ANOVA, followed by Tukey's *post hoc* test. A value of *P* < 0.05 was considered significant.

Results

Vilda inhibited the development of atherosclerosis in nondiabetic ApoE^{−/−} mice: To examine the effect of Vilda on the progression of atherogenesis, ApoE^{−/−} mice were treated with Vilda or vehicle for 20 weeks. Vilda attenuated atherosclerotic lesion progression in the aortic arch as determined by *en face* Sudan IV staining compared with vehicle (*P* < 0.05) (Figure 1). Administration of Vilda to nondiabetic ApoE^{−/−} mice did not alter metabolic parameters, including blood glucose and lipid levels, as shown in Table I. The result of oil red O staining demonstrated that Vilda significantly reduced lipid deposition in atherosclerotic plaques (*P* < 0.05) (Figure 2A). The result of immunostaining demonstrated that Vilda significantly reduced VCAM-1 expression (*P* < 0.05) and tended to decrease macrophage accumulation (*P* = 0.05) in atherosclerotic plaques (Figure 2B and C).

Vilda improved endothelial function in nondiabetic ApoE^{−/−} mice: To investigate the effect of Vilda on endothelial function in a nondiabetic condition, vascular response was examined in wild-type and ApoE^{−/−} mice. After WTD feeding for 8 weeks, endothelium-dependent vasodilation in response to Ach was significantly impaired in ApoE^{−/−} mice compared with that in age- and sex-matched wild-type mice. However, treatment with Vilda for 8 weeks significantly improved the impairment of endothelium-dependent vasodilation in ApoE^{−/−} mice compared with vehicle administration (*P* < 0.05) (Figure 3A). On the other hand, endothelium-independent vasorelaxation in response to SNP did not differ between the Vilda and vehicle groups (Figure 3B). Metabolic parameters, including blood glucose level, did not differ between the Vilda-treated group and vehicle-treated group (Table II).

Ex-4 attenuated endothelial dysfunction induced by LPS: To investigate whether increased GLP-1 level is associated with improvement of endothelium-dependent vascular function, Ach-induced vasorelaxation was examined using aortic rings obtained from wild-type mice. Inflammatory stimulation with LPS impaired vasorelaxation in response to Ach, although Ex-4, a GLP-1 analog, significantly ameliorated this response (*P* < 0.01) (Figure 4A). Neither LPS nor Ex-4 affected endothelium-independent

vasorelaxation in response to SNP (Figure 4B). To investigate the underlying mechanism by which Ex-4 attenuated impairment of endothelium-dependent vasorelaxation induced by LPS, we examined the phosphorylation of eNOS[Ser1177] in HUVEC. The results of western blotting demonstrated that phosphorylation of eNOS[Ser1177] was promoted by the presence of Ex-4 in LPS-treated HUVEC (*P* < 0.05) (Figure 5).

Discussion

In this study, we found that Vilda attenuated endothelial dysfunction and reduced atherosclerotic lesions in nondiabetic ApoE^{−/−} mice. Vilda also reduced VCAM-1 expression and tended to decrease macrophage accumulation in atherosclerotic plaques. The results of an *ex vivo* experiment using aortic rings demonstrated that Ex-4, a GLP-1 analog, ameliorated endothelial dysfunction induced by LPS. Also, an *in vitro* experiment using HUVEC showed that Ex-4 increased eNOS[Ser1177] phosphorylation, which was deteriorated by LPS. Recent studies demonstrated protective effects of DPP-4 inhibitors on endothelial function. However, only a few studies have examined the effects of DPP-4 inhibitors on endothelial function and atherogenesis in a normoglycemic atherosclerotic mouse model.[23,24] Also, we demonstrated that GLP-1 attenuated endothelial dysfunction in HUVEC stimulated with LPS, which plays an important role in the process of atherogenesis.[25] The results of our study suggest that the elevated GLP-1 level caused by DPP-4 inhibition by Vilda contributes, at least partially, to the improvement of endothelial function and reduction of atherosclerotic lesion development.

Previous studies have demonstrated that DPP-4 inhibitors attenuate atherogenesis in diabetic ApoE^{−/−} mice.[11,26] Atherosclerosis is the most serious manifestation in patients with diabetes. Blood glucose-lowering treatment with DPP-4 inhibitors suppresses multiple cellular and molecular mechanisms that stimulate atherogenesis. In fact, several clinical studies reported that DPP-4 inhibitors, including Vilda, improved endothelial dysfunction, an initiation step in the development of atherosclerosis in diabetic patients.[14,27] On the other hand, accumulating evidence suggests that DPP-4 inhibitors prevent atherogenesis independent of their blood glucose-lowering effect.[8-12] One of the underlying mechanisms is the suppression of inflammatory activation of immune cells, such as macrophages.[10,28-30] In addition to the activation of inflammatory cells, endothelial dysfunction plays a pivotal role in the initiation of atherogenesis.[31] Therefore, in this study, we examined the effect of Vilda on the development of endothelial dysfunction and atherosclerosis in nondiabetic ApoE^{−/−} mice. We found that Vilda-treated animals had reduced atherosclerotic lesions in the aortic arch and less accumulation of lipid and macrophages and inflammatory molecule expressions such as VCAM-1 in atherosclerotic plaques compared with vehicle-treated mice. These results are consistent with previous studies which reported atheroprotective effects of this class of antidiabetic drug.

Impaired endothelial function causes atherosclerosis.[20] Endothelium-derived nitric oxide (NO) plays a cru-
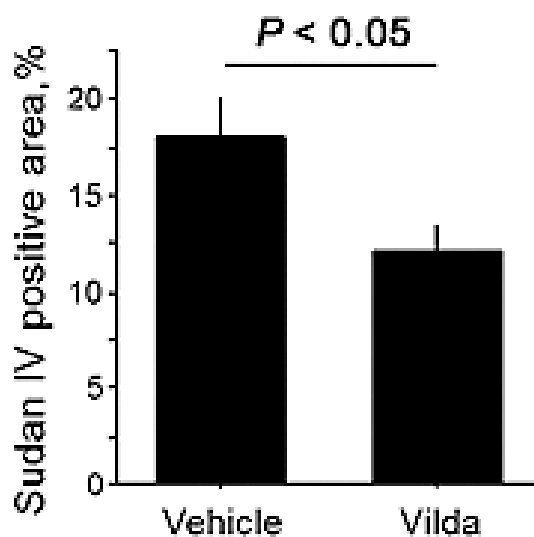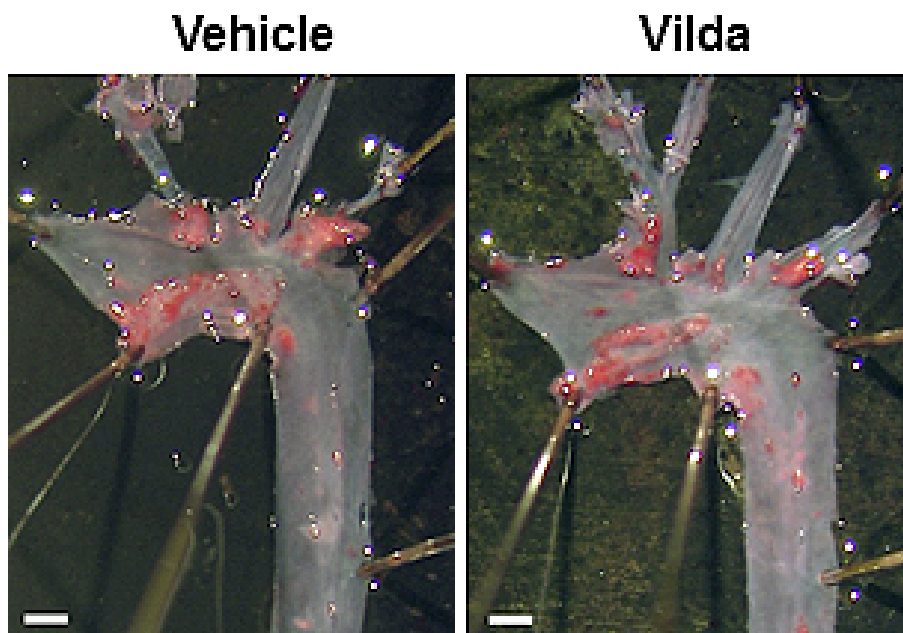
Figure 1. Vilda inhibited the development of atherosclerosis in nondiabetic ApoE$^{-/-}$ mice. *En face* Sudan IV staining of the aortic arch showed that Vilda administration for 20 weeks significantly reduced the progression of atherosclerotic lesions in the aortic arch compared with the vehicle group (n = 10-12, per group). Scale bar: 1 mm.

Table I. Effect of Vilda on Metabolic Parameters after 20-Week Treatment

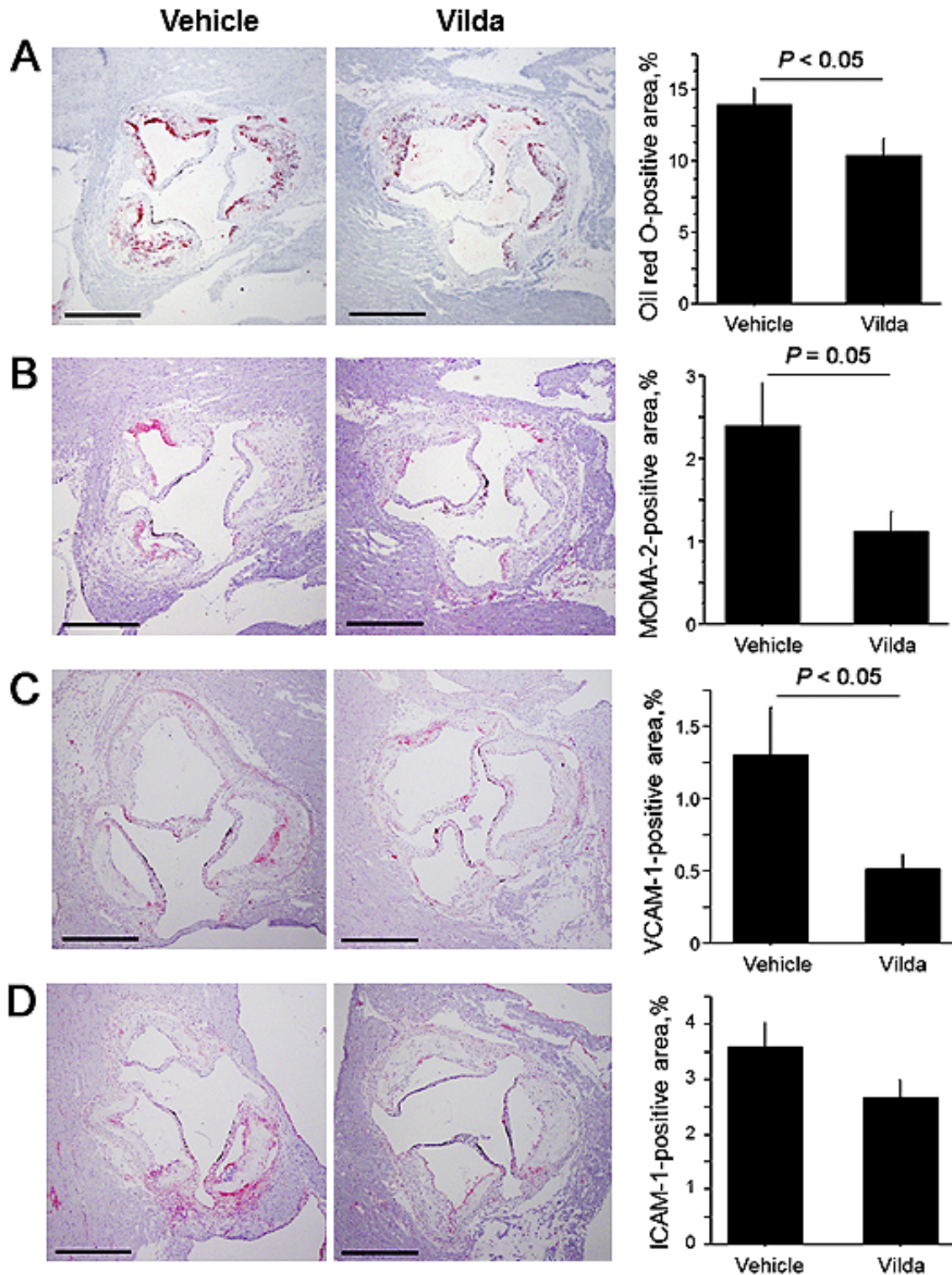|	Vehicle (n = 12)	Vilda (n = 10)	P-value
Body weight, g	29.2 ± 2.2	35.7 ± 4.5	0.19
Blood glucose (with fasting), mg/dL	82.8 ± 5.7	96.0 ± 5.2	0.11
Total cholesterol, mg/dL	705.1 ± 98.0	799.2 ± 100.1	0.51
Triglyceride, mg/dL	68.8 ± 13.8	79.2 ± 14.1	0.61
HDL-cholesterol, mg/dL	14.5 ± 2.0	21.7 ± 3.4	0.08
Heart rate, bpm	605 ± 35	609 ± 45	0.95
Systolic BP, mmHg	96.1 ± 3.2	94.6 ± 4.1	0.78
Diastolic BP, mmHg	78.4 ± 1.9	76.9 ± 4.0	0.75

Figure 2. Effects of Vilda on the characteristics of atherosclerotic plaques. **A:** Oil red O staining demonstrated that Vilda reduced lipid deposition in atherosclerotic plaques in the aortic root compared with vehicle (*n* = 10-12, per group). **B-D:** Immunostaining against MOMA-2 (**B**), VCAM-1 (**C**), and ICAM-1 (**D**) demonstrated that Vilda reduced VCAM-1 expression and tended to decrease macrophage accumulation in atherosclerotic plaques in the aortic root compared with vehicle (*n* = 10-12, per group). Scale bar: 500 μm.

cial role in vascular homeostasis, whereas reduction of production and/or bioavailability of NO contributes to the

development of atherosclerosis.[32,33] NO is produced in the endothelium *via* nitric oxide synthase (NOS); atheroscle-
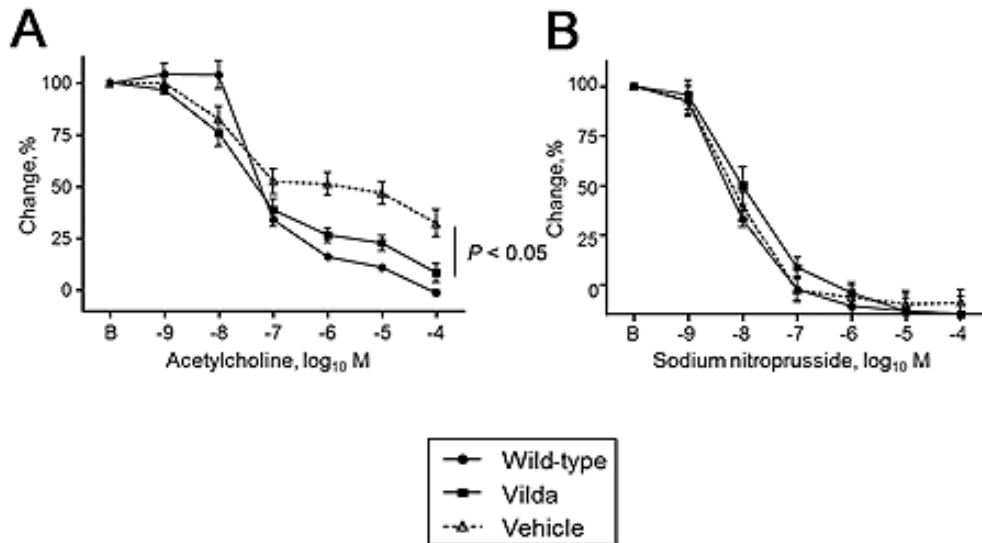
Figure 3. Vilda attenuated endothelial dysfunction in nondiabetic ApoE$^{-/-}$ mice. Vascular reactivity to Ach or SNP was determined using aortic rings isolated from Vilda- or vehicle-administered ApoE$^{-/-}$ mice and age- and sex-matched wild-type mice (n = 4-11, per group). ApoE$^{-/-}$ mice treated with vehicle showed impairment of endothelial function compared with wild-type mice. Vilda administration for 8 weeks ameliorated endothelium-dependent vasodilation in response to Ach compared with the vehicle group in ApoE$^{-/-}$ mice (**A**). Vasorelaxation in response to SNP did not differ among the three groups (**B**).

Table II. Effect of Vilda on Metabolic Parameters after 8-Week Treatment

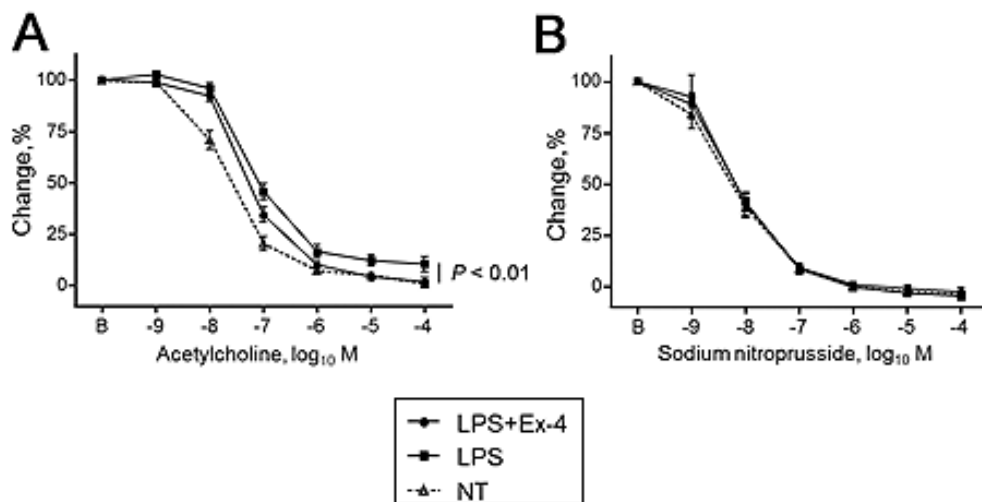|	Vehicle (n = 7)	Vilda (n = 11)	P-value
Body weight, g	23.7 ± 0.5	24.1 ± 0.6	0.72
Blood glucose (without fasting), mg/dL	134.1 ± 9.3	126.7 ± 5.5	0.47
Total cholesterol, mg/dL	1443.9 ± 73.3	1267.4 ± 63.5	0.09
Triglyceride, mg/dL	78.6 ± 13.4	80.2 ± 10.3	0.93
HDL-cholesterol, mg/dL	16.0 ± 2.7	14.2 ± 1.5	0.53
Heart rate, bpm	668.4 ± 29.6	661.1 ± 21.6	0.84
Systolic BP, mmHg	109.7 ± 3.9	109.6 ± 4.3	0.98
Diastolic BP, mmHg	67.7 ± 3.2	67.1 ± 2.6	0.88

Figure 4. Ex-4 attenuated endothelial dysfunction induced by LPS. 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 wild-type mice (n = 8, per group). Aortic rings were incubated with LPS (10 ng/mL) in the presence or absence of Ex-4 (10 nM) for 24 hours. Ex-4 significantly improved endothelium-dependent vasodilation which was impaired by LPS (**A**). Neither Ex-4 nor LPS had an effect on endothelium-independent vasodilation (**B**).
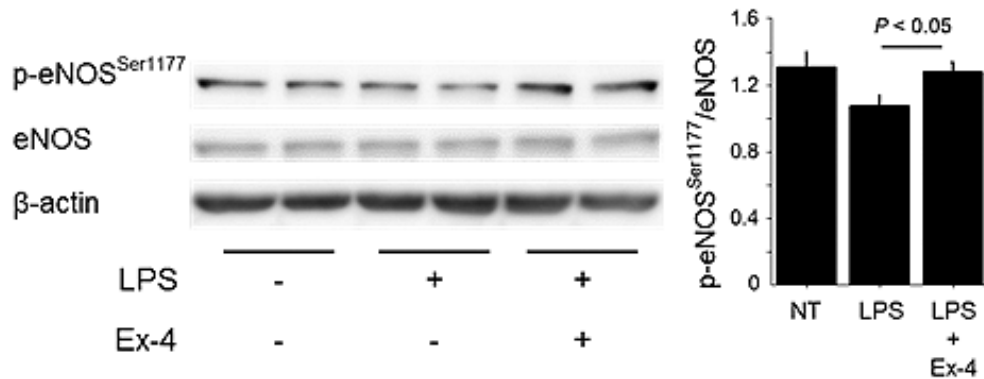
Figure 5. Ex-4 promoted phosphorylation of eNOS. To investigate the effect of Ex-4 on endothelial cells, phosphorylation of eNOS^{Ser1177} was examined in HUVEC. Western blotting demonstrated that Ex-4 promoted phosphorylation of eNOS^{Ser1177}, which was impaired in the presence of LPS ($n = 5$, per group).

rotic stimuli, such as hyperlipidemia and hyperglycemia, deteriorate this function. Recent studies have demonstrated that DPP-4 inhibitors increased NO production, leading to the improvement of endothelial function in human and animal studies.[14,27,34] Furthermore, the results of a clinical study which investigated the effect of sitagliptin, a DPP-4 inhibitor, on endothelial function in coronary artery disease and uncontrolled diabetic patients suggested that it ameliorated endothelial dysfunction without blood glucose alteration.[13] Also, previous studies have demonstrated that genetic deletion of eNOS by using eNOS-deficient mice or pharmacological blockade of eNOS by L-NG-nitroarginine methyl ester attenuated protective effects of DPP4 inhibitors on endothelial cells, including vascular relaxation and blood flow recovery.[35-37] Therefore, the results of our present study suggested that Vilda improved endothelial cell function, especially in the early stages of atherosclerosis, by the activation of eNOS in nondiabetic animals independently of blood glucose.

In this study, we examined the effect of GLP-1 on endothelial function. Previous studies have demonstrated that GLP-1 has various protective effects on the endothelium.[23,38-40] The results of our *ex vivo* experiments demonstrated that endothelial function was impaired by LPS in aortic segments isolated from wild-type mice, although Ex-4, a GLP-1 analog, ameliorated this response. Also, the results of our *in vitro* experiments using HUVEC demonstrated that Ex-4 ameliorated LPS-induced impairment of eNOS^{Ser1177} phosphorylation. Increased eNOS phosphorylation at Ser1177 suggests elevated NO production in endothelial cells.[32,33] Previous studies have reported that LPS decreases eNOS activation[41,42] by the activation of inflammatory signaling pathways, such as p38 MAPK and NF-κB.[43,44] On the other hand, Ex-4 or a GLP-1 analog inhibit the activation of these inflammatory signaling pathways in many cell types, including endothelial cells.[45-48] Taken together, Ex-4 protects eNOS phosphorylation by the downregulation of these inflammatory signaling induced by LPS. Because other studies have suggested various links between GLP-1 and eNOS,[49] further studies are needed. Thus, these results suggest that increased GLP-1 level contributed, at least in part, to the improvement of endothelial function.

In conclusion, Vilda attenuated endothelial dysfunction and reduced atherosclerotic lesions in nondiabetic ApoE$^{-/-}$ mice without an alteration of the blood glucose level. This study increases the understanding of the mechanisms by which Vilda attenuates atherosclerosis. Effects of DPP-4 inhibitors independent of glucose lowering may provide an attractive therapeutic option for atherosclerosis.

Acknowledgments

Disclosure

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