Introduction

Type 2 diabetes is closely related to a high incidence of accelerated atherosclerosis, as both acute glucose fluctuations, from peak to nadirs, and chronic sustained hyperglycemia augment vascular oxidative stress (1–3). In particular, the augmentation of vascular oxidative stress has been suggested to be more sensitive to glucose fluctuations than chronic sustained hyperglycemia (4). Therefore, decreased glucose fluctuations may represent a strategy for preventing atherosclerosis via attenuation of vascular oxidative stress.

Dipeptidyl peptidase (DPP)-4 is a serine protease and cleaves X-proline dipeptides from the N-terminus of polypeptides (5). DPP-4 is responsible for rapid degradation of the incretin hormones, glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP). Both GLP-1 and GIP are secreted from the epithelium of the small intestine following food intake and act to lower blood glucose by increasing insulin secretion from β-cells in the pancreas (6). On the other hand, DPP-4 inhibitors prolong the half-life of GLP-1 and GIP in blood and augment glucose-dependent insulin secretion, resulting in the attenuation of blood glucose levels following food intake (7). Although the mechanism of DPP-4 inhibitor–mediated attenuation of blood glucose after food intake is known to be dependent on DPP-4 inhibition in blood, DPP-4 is expressed in many organs such as heart, kidney, liver, and vascular tissues (8). In fact, the DPP-4 inhibitor vildagliptin significantly reduces plasma DPP-4 activity and significantly increases plasma GLP-1 levels in Zucker diabetic fatty (ZDF) rats (9). In previous studies, GLP-1 has been shown to induce vascular dilatation via improved...
endothelial function and improved vascular oxidative stress (9–11). However, it is unclear whether vascular DPP-4 inhibition contributes to improvements in vascular dysfunction in diabetes.

In the present study, we used sitagliptin and linagliptin to study the effects of DPP-4 inhibition. Linagliptin is more tissue-penetrative than sitagliptin, and the apparent volume of distribution of linagliptin and sitagliptin was 1110–3060 l and 198 ± 30 l, respectively (12). In our preliminary study, 10 mg/kg of sitagliptin and 3 mg/kg of linagliptin produced equivalent reductions in glucose concentrations in an oral glucose tolerance test (OGTT) in ZDF rats. However, a significantly greater inhibition of vascular DPP-4 was observed in linagliptin-treated rats compared with sitagliptin-treated rats. Using these doses of DPP-4 inhibitors, we aimed to clarify the role of vascular DPP-4 inhibition on vascular dysfunction in a rodent diabetes model.

Materials and Methods

Animals

Six-week-old male ZDF (ZDF-Lepr<sup>bb</sup>/Crj) rats and lean (ZDF-Lepr<sup>bb</sup>+/+) rats were obtained from Charles River Laboratories Japan (Yokohama). ZDF rats were orally administered placebo (n = 6), sitagliptin (Sequia, Pangbourne, UK; 10 mg/kg per day; n = 6), or linagliptin (Boehringer Ingelheim Pharma GmbH and Co. KG, Biberach an der Riss, Germany; 3 mg/kg per day; n = 6) by gavage, once a day for 4 weeks. The placebo-treated rats were administered an equivalent volume of vehicle (0.1% carboxymethylcellulose in water). OGTT was carried out 1 h after the first and last drug administration. Blood samples for measuring plasma DPP-4 activity were obtained 0.5 and 24 h after the first and last drug administrations. Following the final blood sample collection, obtained under anesthesia with isoflurane 24 h after the last drug administration, the carotid artery and aorta were obtained for the evaluation of vascular DPP-4 activity and MDA levels. Using these doses of DPP-4 inhibitors, we aimed to clarify the role of vascular DPP-4 inhibition on vascular dysfunction in a rodent diabetes model.

OGTT assay

After fasting for 18 h, the rats were orally administered a glucose solution (2 g/kg). Blood samples were collected from the tail vein at 0 (just before the glucose administration), 30, 60, and 120 min after glucose administration. Blood glucose levels were determined using a standard glucometer (Antosense II; Daikin Industries, Osaka). Plasma insulin levels were determined using a Shibayagi Rat Insulin ELISA kit (Shibayagi, Gunma).

DPP-4 activity and MDA levels

DPP-4 activity in vascular extracts was measured for 1 h at 37°C with 1 mM glyceryl-prolyl-7-amino-4-methyl coumarin (Gly-Pro-MCA) in 25 mM N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid (HEPES), 140 mM NaCl, 80 mM MgCl<sub>2</sub>, and 1% bovine serum albumin, pH 7.8 (14).

The levels of MDA, which is a product of lipid peroxidation in tissue extracts, were measured by incubating vascular extracts for 1 h at 60°C with 20 mM thiobarbituric acid in 0.3 mM phosphoric acid (15). The reaction was terminated by cooling on ice, and the mixture was subsequently centrifuged at 15,000 × g for 5 min at 4°C. The absorbance of the supernatant was recorded at 532 nm.

Protein concentrations were assayed using BCA Protein Assay Reagents (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Acetylcholine-induced vascular relaxation

Isolated rat carotid arteries were cut into 10 × 1.0 mm helical strips and mounted on a myograph under a resting tension of 1.0 g in Tyrode’s solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1.1 mM MgCl<sub>2</sub>, 0.42 mM NaH<sub>2</sub>PO<sub>4</sub>, 12 mM NaHCO<sub>3</sub>, and 5.7 mM glucose, pH 7.4) at 37°C and continuously bubbled with 5% CO<sub>2</sub> / 95% O<sub>2</sub> (15). The strips were initially vasoconstricted with 50 mM KCl, followed by a wash-out of the bathing medium. Relaxation induced by acetylcholine (1 μM) was assessed after contraction to a steady-state tension using 1 μM noradrenaline.

Real-time reverse-transcribed polymerase chain reaction (RT-PCR)

Aortic total RNA (1 μg) was transcribed into cDNA using Superscript III reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA, USA) (15). The mRNA levels of p22<sub>phox</sub>, a subunit of NADPH oxidase, monocyte chemoattractant protein (MCP)-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were mea-
sured by real-time RT-PCR on a LightCycler with specialized software (Roche Diagnostics, Tokyo) using TaqMan fluorogenic probes. All primers and probes for p22\textsuperscript{phox}, MCP-1, and GAPDH were designed by Roche Diagnostics. The primers were as follows: 5’-gcacctgctgagtgtgatcta-3’ (forward) and 5’-aatgggagtccactgctcac-3’ (reverse) for p22\textsuperscript{phox}, 5’-agcatccacgtgctgtctc-3’ (forward) and 5’-gatcatcttgccagtgaatgagt-3’ (reverse) for MCP-1, and 5’-aatgtatccgttgtggatctga-3’ (forward) and 5’-gcttcacacctctggatgt-3’ (reverse) for GAPDH. The mRNA levels of p22\textsuperscript{phox} and MCP-1 were normalized to that of GAPDH.

**Statistical analysis**

Data are expressed as the mean ± standard error of the mean (S.E.M.). In Figs 1 and 2, temporal data were assessed using a repeated measure two-way ANOVA followed by Bonferroni/Dunn post hoc test. Significant differences in mean values between the lean and placebo-treated groups were evaluated using Student’s t-test for unpaired data. Significant differences between the mean values of placebo-, sitagliptin-, and linagliptin-treated groups were evaluated using a one-way ANOVA followed by Fisher’s protected least significant difference test. The difference was considered to be significant if the P-value was less than 0.05.

**Results**

**Body weight**

Prior to treatment, the average body weights of rats in lean, placebo-, sitagliptin-, and linagliptin-treated groups were 155 ± 4.1 g, 221 ± 3.9 g, 222 ± 4.4 g, and 221 ± 3.3 g, respectively. The lean rats weighed significantly less than the ZDF rats in the other groups. No significant differences in body weight were observed among placebo-, sitagliptin-, and linagliptin-treated ZDF groups throughout the experiment (Fig. 1).

**Blood glucose concentration and plasma insulin**

The changes in glucose concentrations in the OGTT were performed on all 4 groups 1 h after the first and the last treatments (Fig. 2: A and C). Although the difference was slight after the first treatment, blood glucose concentrations were significantly higher in placebo-treated ZDF rats than in lean rats at 0 min (just prior to glucose administration) (Fig. 2A). After the last treatment, plasma glucose levels were also significantly higher in placebo-treated ZDF rats than in lean rats at 0 min (Fig. 2C). Blood glucose concentrations in placebo-treated ZDF rats were significantly higher than in lean rats at 30, 60, and 120 min; and they were significantly attenuated by treatment with sitagliptin or linagliptin (Fig. 2: A and C). However, there was no significant difference between the sitagliptin- and linagliptin-treated groups (Fig. 2: A and C).

After the first treatment, plasma insulin concentrations were not different among all the groups at 0 min (Fig. 2B). At 30 min, plasma insulin concentration was
significantly higher in the placebo-treated group than in the lean group, but there was no significant difference at 30 and 60 min. On the other hand, plasma insulin concentrations were significantly higher in both sitagliptin- and linagliptin-treated groups, compared to the placebo-treated group at 30, 60, and 120 min (Fig. 2B). After the last treatment, plasma insulin concentrations were significantly higher in the placebo-treated group than in the lean group at 0 min (Fig. 2D). At 30 and 60 min, insulin concentrations in both sitagliptin- and linagliptin-treated groups were significantly higher than in the placebo-treated group, but no significant difference between these two groups was observed (Fig. 2D).

**DPP-4 activity in plasma**

Plasma DPP-4 activity was not significantly different among all groups before treatment (Fig. 3). Plasma DPP-4 activity was almost completely eliminated 0.5 h after the first treatment with sitagliptin and linagliptin, but no significant difference was observed (Fig. 3). Although significant reductions of DPP-4 activity were also observed 24 h after the first treatments with both drugs, the degree of reduction was significantly stronger in linagliptin treatment than in sitagliptin treatment (Fig. 3). Plasma DPP-4 activity was significantly higher in the placebo-treated group than in the lean group 0.5 h and 24 h after the last treatment (Fig. 3). Plasma DPP-4 activity in both sitagliptin- and linagliptin-treated groups was significantly lower than in the placebo-treated group 0.5 h and 24 h after the last treatment, and significant reductions were observed in the linagliptin-treated ZDF group compared to the sitagliptin-treated group both at 0.5 h and 24 h (Fig. 3).

**Acetylcholine-induced vascular relaxation**

In isolated carotid arteries, acetylcholine-induced vascular relaxation was significantly weaker in the placebo-treated group than in the lean group at the end of the experiment (Fig. 4). However, vascular relaxation was significantly stronger in both the sitagliptin- and linagliptin-treated groups than in the placebo-treated ZDF rats (Fig. 4). Furthermore, vascular relaxation was significantly stronger in the linagliptin-treated group than in the sitagliptin-treated group (Fig. 4).

**DPP-4 activity and MDA levels in vascular tissues**

Vascular DPP-4 activity was not significantly different between lean and placebo-treated groups after 4 weeks of treatment (Fig. 5A). However, DPP-4 activity was significantly reduced in both sitagliptin- and linagliptin-treated groups compared with the placebo-treated group. Furthermore, DPP-4 activity was significantly lower in the linagliptin-treated group than the sitagliptin-treated group (Fig. 5A).

Vascular MDA levels were significantly elevated in the placebo-treated group compared with the lean group.
Preliminary data (Fig. 5B). However, MDA levels were significantly attenuated by treatment with sitagliptin and linagliptin, with linagliptin producing significantly greater attenuation than sitagliptin (Fig. 5B).

**mRNA levels of p22phyox and MCP-1 in vascular tissue**

Vascular mRNA levels of the NADPH oxidase subunit p22phyox in the placebo-treated group were significantly higher than in the lean group. However, the levels were significantly attenuated by treatment with sitagliptin or linagliptin, and a further significant attenuation was observed in linagliptin compared with sitagliptin (Fig. 6A).

Vascular mRNA levels of MCP-1 in the placebo-treated group were significantly higher than in the lean group (Fig. 6B). However, significant attenuation was observed only with linagliptin treatment (Fig. 6B).

**Discussion**

The ZDF rat is an established animal model of diabetes with obesity (16, 17). Blood glucose concentrations in all ZDF groups were significantly higher than lean rats prior to drug treatment. After both the first and last treatments, a significant augmentation of blood glucose concentrations in placebo-treated ZDF rats was observed during OGTT, with sitagliptin and linagliptin producing equivalent reductions in blood glucose concentrations. In contrast, plasma insulin concentrations were significantly augmented by treatment with both sitagliptin and linagliptin, with no significant difference observed. On the other hand, plasma DPP-4 inhibition by linagliptin was significantly stronger than that of sitagliptin. Although the degree of sitagliptin- and linagliptin-induced attenuation of plasma DPP-4 activity differed, sitagliptin reduced plasma DPP-4 activity by greater than 70% at all points. Therefore, it may not be possible to observe differences between the effect of these drugs on circulating glucose and insulin levels, although the inhibitory effect of linagliptin on plasma DPP-4 activity was stronger than sitagliptin. However, acetylcholine-induced vascular relaxation of isolated arteries was significantly stronger in linagliptin-treated rats than in sitagliptin-treated rats. These observations suggest that the vascular protection by linagliptin may be dependent on other mechanisms in addition to the regulation of blood glucose and plasma insulin levels by inhibition of circulating DPP-4.

Linagliptin demonstrates very potent and tight binding to DPP-4 and shows very slow dissociation from its target; therefore linagliptin has the highest apparent volume of distribution among DPP-4 inhibitors and consequently exhibits widespread tissue penetration (12). Tissue-penetration of agents is strongly influenced by both lipophilicity and protein binding, and a high degree of binding to plasma and tissue proteins contributed to the highest tissue penetration of linagliptin (12). In the present study, the inhibitory effect of linagliptin on vascular DPP-4 activity was significantly stronger than that of sitagliptin. To the best of our knowledge, inhibition of tissue DPP-4 activity has not been compared using different DPP-4 inhibitors. Previously, we compared the inhibitory effect of four angiotensin-converting enzyme (ACE) inhibitors with differing degrees of lipophilicity in a hypertensive rat model (18). In that study, hypotensive effects and plasma ACE inhibition were equivalent among the ACE inhibitors investigated. However, highly tissue-penetrative ACE inhibitors exhibited stronger vascular ACE inhibition than poorly tissue-penetrative ACE inhibitors (18). Moreover, the most tissue-penetrative angiotensin II–receptor blocker (ARB), telmisartan, produced significantly greater augmentation of acetylcholine-induced vascular relaxation compared to the poorly tissue-penetrative ARB losartan (19). This effect was due to stronger blockade of angiotensin II receptors in vascular tissues, although blood pressure was reduced equivalently by telmisartan and losartan in hypertensive rats (19). In that study, vascular p22phyox gene expression was also significantly attenuated by telmisartan (19). These reports suggest that more tissue-penetrative agents result in powerful vascular protection via strong blockade of angiotensin II function. In the present study, strong inhibition of vascular DPP-4 activity by linagliptin may contribute to the observed powerful vascular protection.

DPP-4 inhibitors augment circulating GLP-1 levels, which are secreted from the epithelium of the small intestine after feeding (9, 20). In the present study, circulating GLP-1 levels may be augmented during OGTT, but we could not collect sufficient blood sample volumes to permit the detection of GLP-1 due to our requirement...
for measurements of blood glucose and plasma insulin levels. GLP-1 acts through GLP-1 receptors, which are abundant in the gastrointestinal tract and have also been detected in vascular smooth muscle and endothelial cells (21, 22). GLP-1 has been reported to protect vascular endothelial cells from various insults such as oxidative stress and inflammation (9, 23 – 25). In cultured endothelial cells, GLP-1 and GLP-1 analogues exhibited anti-inflammatory effects via reduction of oxidative stress and suppression of plasminogen activator inhibitor type-1 and vascular adhesion molecules (9, 23). GLP-1 also attenuated proinflammatory cytokines, interleukin-1β and tumor nuclear factor-α (TNF-α), and MCP-1 in lipopolysaccharide-stimulated cultured human macrophages (24). In a mouse atherosclerosis model, a GLP-1 agonist significantly attenuated macrophage adhesion to endothelial cells via suppression of TNF-α and MCP-1 gene expression (25). In this mouse model, both GLP-1 levels and GLP-1 receptors were significantly reduced in macrophages, and a GLP-1 analogue significantly suppressed macrophage foam cell formation, resulting in reduced atherosclerotic lesions (26). These results suggest a significant role for GLP-1 in protecting vascular endothelial cells, despite postprandial circulating GLP-1 levels increasing by only about 2-fold (9, 20). On the other hand, GLP-1 analogues continue to activate GLP-1 receptors by several-fold, compared with circulating GLP-1 level augmented by DPP-4 inhibitors after feeding (20). Therefore, in addition to the augmentation of circulating GLP-1 levels, the mechanism of vascular protection by DPP-4 inhibitor may be dependent on other factors related to vascular DPP-4 inhibition.

In a rat LPS-induced sepsis model, linagliptin augmented acetylcholine-induced vascular relaxation in isolated arteries by reducing MDA levels, which reflects tissue oxidative stress as well as attenuated blood glucose levels (27). In that study, aortic gene expression of an NADPH oxidase subunit and infiltration of inflammatory cells were also attenuated by linagliptin (27). In the present study, blood glucose levels were not different between linagliptin and sitagliptin, and the superior vascular protection by linagliptin is unlikely to be dependent on glucose regulation. On the other hand, linagliptin produced greater inhibition of vascular DPP-4 activity than did sitagliptin. Furthermore, linagliptin-induced augmentation of acetylcholine-induced vascular relaxation was superior to that by sitagliptin, and linagliptin reduced MDA levels and gene expression of the NADPH oxidase subunit p22phox and MCP-1. Shah et al. (28) demonstrated that the DPP-4 inhibitor alogliptin mediated vascular relaxation through a GLP-1–independent pathway. Although we could not determine whether or not the observed vascular protection was dependent on GLP-1, our findings suggest that powerful inhibition of vascular DPP-4 by linagliptin might play an important role in vascular protection.

Although sitagliptin weakly inhibited vascular DPP-4 activity, the inhibitory effect of sitagliptin on p22phox gene expression and MDA levels were clearly observed in vascular tissues. On the other hand, the attenuation of glucose levels was similarly controlled by sitagliptin and linagliptin. Hyperglycemia induces endothelial dysfunction via the reduction of nitric oxide formation and the augmentation of reactive oxygen stress and contributes to the pathogenesis of vascular complications in diabetes (29). In porcine coronary artery, the stimulation of high glucose levels induces the formation of superoxide anion via augmentation of p22phox gene expression (30). Therefore, the obvious attenuation of p22phox gene expression and MDA levels in vascular tissues induced by sitagliptin may be dependent on the reduction of blood glucose levels, although the degree of inhibition of vascular DPP-4 was weak.

The superior inhibitory effect of linagliptin, compared with that of sitagliptin, was observed not only in vascular tissue but also in plasma. In monocytes, DPP-4 expression was detected, and its expression was significantly augmented in patients with obesity or both obesity and diabetes (31). Although glucose and insulin levels in plasma were not significantly different between sitagliptin and linagliptin in this study, DPP-4 activity in monocytes, as in plasma, may differ between sitagliptin and linagliptin. Furthermore, a previous report demonstrated that DPP-4 inhibition resulted in reduced monocyte adhesion on fibronectin-coated culture plates (32). We did not determine the influence of inhibitors of DPP-4 activity in monocytes; however, the effects on monocytes may contribute to the protection of vascular endothelial cells. On the other hand, linagliptin has been reported to exert pleiotropic effects on not only endothelial cells but also vascular smooth muscle cells (SMCs) (27). In the present study, we did not examine the effects of agents that induce vascular relaxation, such as nitroprusside, on SMC function. However, altered SMC function may contribute to the effects of linagliptin on attenuating vascular dysfunction. Further studies are needed to clarify the mechanism of vascular protection by linagliptin.

We also performed a preliminary study on the effects of a DPP-4 inhibitor in lean rats. In the preliminary study, DPP-4 activity in plasma and arteries was significantly inhibited in lean rats following 4 weeks of treatment with 3 mg of linagliptin compared with placebo-treated lean rats, similar to the data reported in the present study. However, enhanced acetylcholine-induced vascular
relaxation in isolated arteries was not observed in linagliptin-treated lean rats compared with placebo-treated lean rats. Acetylcholine-induced relaxation was nearly 100%, even in placebo-treated lean rats. Therefore, the effect of linagliptin on vascular function may be limited in lean rats, and the beneficial effect of DPP-4 inhibition on vascular endothelial function may not be observable in lean rats, unlike the observed effects in ZDF rats seen described in the present study. To clarify the effect of DPP-4 inhibition on lean rats, it may be necessary to conduct very long duration studies.

In conclusion, the highly tissue-penetrative DPP-4 inhibitor linagliptin produced powerful vascular DPP-4 inhibition and this effect may play an important role in vascular protection via attenuation of oxidative stress.

References


