Enteroinsular Axis of *db/db* Mice and Efficacy of Dipeptidyl Peptidase IV Inhibition

Tadashi Nagakura, Nobuyuki Yasuda, Kazuto Yamazaki, Hironori Ikuta, and Isao Tanaka

In type 2 diabetic patients, the administration of glucagon-like peptide-1 (GLP-1), known as an incretin, exerts antidiabetic effects. However, GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPPIV) after its release. DPPIV inhibition is thought to be a rational strategy to treat type 2 diabetes. In this study, using C57BLKS/J-*db/db* (*db/db*) mice as a model of type 2 diabetes, we examined the effect of acute DPPIV inhibition on glucose tolerance at the early and later stages of diabetes, determining plasma active GLP-1 and insulin levels. In addition, we investigated changes of plasma DPPIV activity. Compared with normal C57BL6/J (B6) and *db/+* mice, significantly increased plasma DPPIV activities were observed in *db/db* mice. Expression of the proglucagon gene encoding GLP-1 was significantly upregulated in the colon of *db/db* mice. The administration of valine-pyrrolidide, a DPPIV inhibitor, resulted in potentiated insulin secretion mediated by increased endogenous GLP-1 action, leading to improved glucose tolerance in *db/db* mice at 6 weeks of age. However, although acute DPPIV inhibition with valine-pyrrolidide resulted in higher plasma active GLP-1 and insulin levels in *db/db* mice at 23 weeks of age, it did not improve glucose tolerance. The function of the enteroinsular axis is preserved in both stage of diabetes and the DPPIV inhibitor potentiated it, but the progression of insulin resistance appeared to block the improvement of glucose tolerance through DPPIV inhibition. Our results suggest that DPPIV inhibition is a suitable approach for treatment of impaired glucose tolerance (IGT), and type 2 diabetes in the early stage.

TYPE 2 DIABETES is characterized by hyperglycemia, insulin resistance, absolute or relative insulin deficiency, increased hepatic glucose production, and, frequently, accelerated gastric emptying and obesity. Glucagon-like peptide-1 [GLP-1 or GLP-1-(7-36)amide] is the most important insulin-releasing hormone (incretin) involved in the enteroinsular axis, and inhibits glucagon secretion, hepatic glucose production, gastric emptying, and appetite.[1-6] Furthermore, GLP-1 has trophic effect on pancreatic β cells.[7,8] Because of these actions of GLP-1, it is a good candidate for the treatment of metabolic disturbances in type 2 diabetes.[4-6]

In patients with type 2 diabetes, the administration of GLP-1 itself exerts antidiabetic effects.[9-13] However, the active form of GLP-1 is rapidly eliminated from the circulation. The enzyme responsible for the degradation of GLP-1 is dipeptidyl peptidase IV (DPPIV or CD26, EC 3.4.14.5), a serine protease that exists in plasma and on the surface of various types of cells, particularly in the liver, kidney and small intestine.[14-18] In addition to the use of GLP-1 and a DPPIV-resistant GLP-1 receptor agonist, exendin-4,[19-21] DPPIV inhibition is another possible strategy for treatment of subjects with type 2 diabetes, since it should extend the half-life of GLP-1.[22,23] Valine-pyrrolidide,[24,25] NVP-DPP728,[26,27] and P32/98[28,29] have been reported as DPPIV inhibitors.

In considering the application of DPPIV inhibition for type 2 diabetes treatment, the following points are important: (1) whether the enteroinsular axis functions normally in patients, (2) whether the effectiveness of DPPIV inhibition is influenced by the diabetic condition, and (3) whether DPPIV activity is changed in the disease. In the present study, we examined the enteroinsular axis in *db/db* mice as a type 2 diabetes model at early and relatively late stages of diabetes, and also measured plasma DPPIV activity. In addition, we examined whether a DPPIV inhibitor might improve glucose tolerance in *db/db* mice.

MATERIALS AND METHODS

Chemicals

Valine-pyrrolidide was synthesized in our laboratories.

Animals

Male + $Lepr^{db}$/+ $Lepr^{db}$ (*db/db*) and *m* +/+ $Lepr^{db}$ (*db/+*) mice of C57BLKS/J-*m* +/+ $Lepr^{db}$ strain, and male C57BL6/J (B6) mice as wild-type homozygous controls, aged 5 weeks, were purchased from Japan Clea (Tokyo, Japan). The mice were provided with a commercial diet (MF, Oriental Yeast, Tokyo, Japan) and water ad libitum and were kept under conventional conditions with controlled temperature, humidity and lighting (22 ± 2°C, 55 ± 5% and a 12-hour light/dark cycle with lights on at 7 AM). All procedures were conducted according to the Eisai Animal Care Committee's guideline.

Experiment 1: Determination of Plasma DPPIV Activity at Different Ages in db/db Mice and Nondiabetic Littermates, and in Wild-Type Mice

Plasma DPPIV activity of *db/db* and *db/+* mice, and of B6 mice was measured in the fed condition at 6, 8, 10, 14, and 23 weeks of age (n = 10 or 11). Blood (50 μL) was drawn from the caudal vein with a heparinized capillary tube, and plasma was obtained by centrifugation. Blood glucose was also determined at the same ages.

Experiment 2: Determination of Effects of Acute DPPIV Inhibition on Glucose Profiles During a Glucose Tolerance Test in db/db Mice and Wild-Type Mice

To examine the effects of DPPIV inhibition on glucose tolerance, we performed an oral glucose tolerance test (OGTT) using *db/db* mice and B6 mice at 6 and 23 weeks of age (n = 5). Mice were fasted for 18 hours and given glucose orally at a dose of 2 g/kg body weight together with valine-pyrrolidide at a dose of 30 mg/kg body weight, or vehicle (distilled water), via a gastric tube at 10 AM. This dose was enough to

From Tsukuba Research Laboratories, Eisai Co, Ltd, Ibaraki, Japan.

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Address reprint requests to Kazuto Yamazaki, PhD, Tsukuba Research Laboratories, Eisai Co, Ltd, 5-1-3, Tokodai, Tsukuba, Ibaraki 300-2635, Japan.

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induce obvious in vivo effect.[25] Blood (10 μL) was taken from the caudal vein 0, 30, 60, and 120 minutes after the oral glucose administration, and used for the measurement of blood glucose levels.

Experiment 3: Determination of Effects of Acute DPPIV Inhibition on Insulin and GLP-1 Profiles in db/db Mice and Wild-type Mice

We evaluated the effects of DPPIV inhibition on glucose-stimulated GLP-1 and insulin secretion in mild and severe type 2 diabetic mice. Five *db/db* mice and 5 B6 mice were used at 7 and 24 weeks of age. After an 18-hour fast, mice were given glucose orally at a dose of 2 g/kg body weight, with valine-pyrrolidide at a dose of 30 mg/kg body weight or vehicle, via a gastric tube. Blood (250 μL) was taken from the orbital sinus 15 minutes after the administration.

Experiment 4: Measurement of Proglucagon Gene Expression in Colons of db/db Mice and Wild-Type Mice

Five 24-week-old *db/db* mice and 5 B6 mice were killed by cervical dislocation and ~5 cm of the upper colon was excised (from the connection between the cecum and colon). The tissue was rinsed in saline, soaked in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted with TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Twenty micrograms of total RNA was separated on 1.2 % agarose gels containing 17 % formaldehyde, transferred to nylon membranes (GeneScreen Plus, NEN Life Science Products, Boston, MA), and ultraviolet-crosslinked. Rat proglucagon cDNA fragment was used as a probe, generated by using the following primers: 5′-atgaagaccgtttacatcgtg-3′ and 5′-gatcttggtttgaatcagcc-3′. Mouse β-actin fragment was used as an internal control, which was amplified with the following primers: 5′-ggacgaactggagaaaatctggca-3′ and 5′-ggagcaatgatcttgatcttcattgt-3′. These polymerase chain reaction (PCR) products were radiolabeled with [α-^{32}P]dCTP using a BcaBEST labeling kit (Takara, Otsu, Japan), and hybridized to the membrane. Northern blot hybridization was conducted at 65°C for 3 to 4 hours in PerfectHyb Plus hybridization solution (Sigma, St Louis, MO) with the labeled probe. After the hybridization, the filter membrane was washed in 2× saline-sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 65°C for 5 minutes, 0.2× SSC/0.1% SDS at 50°C for 15 minutes, and finally in 0.1× SSC/0.1% SDS at 40°C for 5 minutes. The membrane was exposed to an imaging plate for 2 hours, and the radioactivity was visualized and quantified with a Bioimaging analyzer, BAS2000 System (Fuji Photo Film, Tokyo, Japan). The proglucagon intensity was normalized with respect to β-actin intensity.

Assay

For determination of DPPIV activity, 5 μL of plasma was incubated with 145 μL of phosphate-buffered saline (PBS) containing 0.4 mmol/L Gly-Pro-*p*-nitroaniline (Gly-Pro-pNA, Peptide Institute, Minoh, Japan) for 20 minutes at room temperature, and the absorption was determined at 405 nm with a spectrophotometer (Spectra MAX, Molecular Devices, Sunnyvale, CA). The DPPIV activity is expressed as mU/mL. One enzyme unit was defined as the amount of the enzyme required for the formation of 1 μmol of pNA per minute. For measurement of blood glucose, 10 μL of blood was collected from the caudal vein for blood glucose determination. Blood glucose was determined with Glu CII-test (Wako, Osaka, Japan). Plasma immunoreactive insulin concentrations were determined with an insulin enzyme-liked imunosorbent assay (ELISA) kit using rat insulin as a standard (Morinaga, Yokohama, Japan). Plasma immunoreactive intact GLP-1 levels were measured with a Glucagon-Like Peptide (Active) ELISA kit (Linco Research, St Charles, MO)

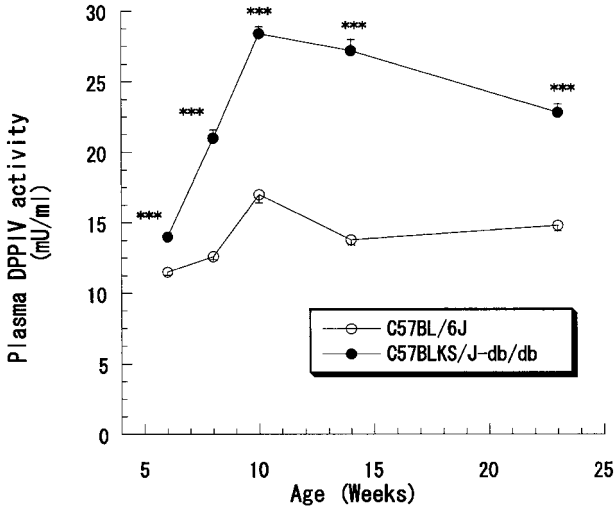

Fig 1. Changes of plasma DPPIV activities in C57BL/6J (B6) mice and C57BLKS/J-*db/db* mice from 6 to 24 weeks of age. Significantly higher DPPIV activities are observed in *db/db* mice, compared with those of age-matched B6 mice. B6 mice (○) and *db/db* mice (●). Data are expressed as means ± SEM; n = 10-11. $^{***}P < .001$.

Statistics

Data are expressed as means ± SEM. Statistical analysis was conducted by use of the *F* test, followed by Student's *t* test (when $P \geq .05$ in *F* test) or Mann-Whitney's *U* test (when $P < .05$ in *F* test) (StatView Version 4.0, Abacus Concepts, Cary, CA). We considered a *P* value less than .05 to be statistically significant.

RESULTS

Change of Plasma DPPIV Activity

Figure 1 indicates the changes of plasma DPPIV activities of *db/db* mice and B6 mice in the fed state from 6 to 23 weeks of age. A slight, but significant difference of plasma DPPIV activities between these mice had already appeared at 6 weeks of age (14.0 ± 0.3 and 11.5 ± 0.2 mU/mL, respectively; $P < .001$). The significantly higher DPPIV activities of *db/db* mice were maintained throughout this examination ($P < .001$ at the other ages). Blood glucose and body weight of *db/db* mice and B6 mice were as follows: blood glucose, 268.3 ± 11.3 versus 153.1 ± 6.7 mg/dL, 407.8 ± 10.6 versus 141.4 ± 3.4 mg/dL, and 501.3 ± 14.9 versus 154.5 ± 3.0 mg/dL at 6, 10, and 23 weeks of age, respectively, $P < .001$ at all ages; body weight, 31.9 ± 0.2 versus 22.0 ± 0.2 g, 45.3 ± 0.2 versus 27.1 ± 0.5 g, and 45.3 ± 0.7 versus 31.4 ± 0.9 g at 6, 10, and 23 weeks of age, respectively, $P < .001$ at all ages. Similarly, the plasma DPPIV activities of *db/db* mice were significantly increased by 1.2- to 1.5-fold, compared with those of *db/+* mice (data not shown).

Effects of Acute DPPIV Inhibition on Glucose Tolerance

We performed OGTT using 6- and 23-week-old *db/db* mice and B6 mice to compare the effects of DPPIV inhibition on glucose tolerance at the 2 different stages of diabetes. In 6-week-old B6 mice, significantly lower blood glucose levels were seen 30 and 60 minutes after the oral glucose load in the DPPIV inhibitor–treated group, in comparison with those in the vehicle-treated group ($P < .05$ and $P < .01$, respectively) (Fig

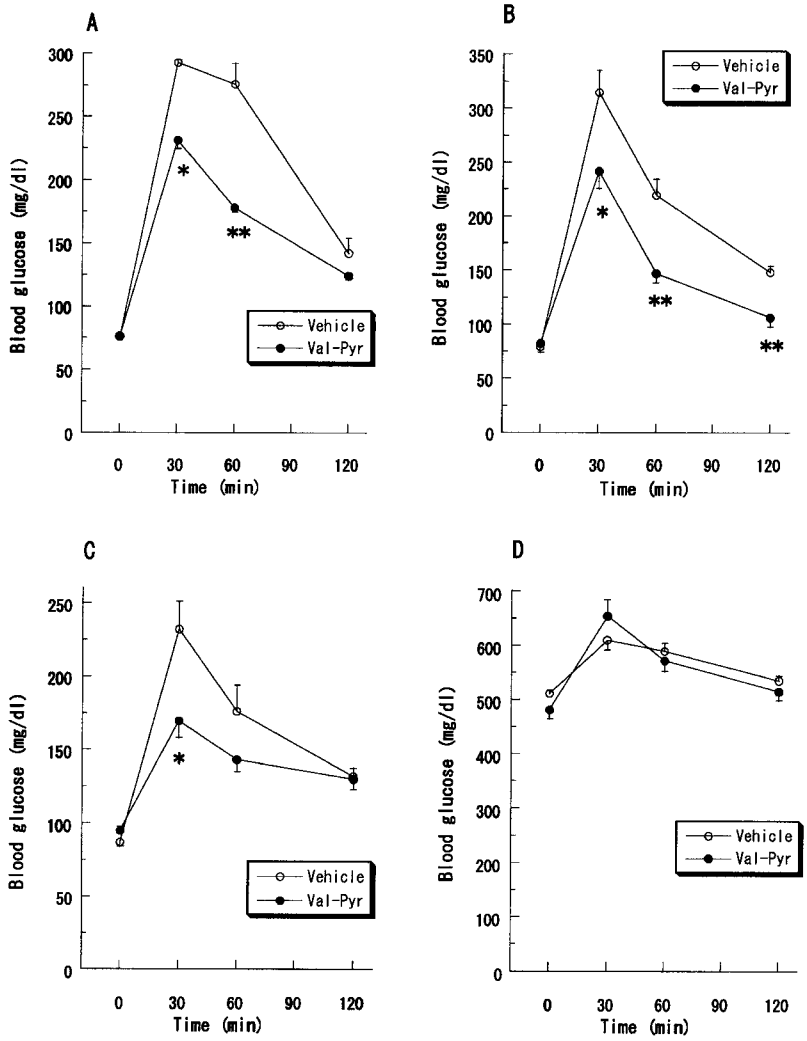

Fig 2. Oral glucose tolerance with or without valine-pyrrolidide in C57BL/6J (B6) mice (A and C) and C57BLKS/J-*db/db* mice (B and D) at 6 weeks of age (A and B) and at 23 weeks of age (C and D). Glucose (2 g/kg body weight) and valine-pyrrolidide (30 mg/mg body weight) were simultaneously administered at time 0. Vehicle (○) and valine-pyrrolidide treatment (●). Data are expressed as means ± SEM; n = 5. $^{*}P < .05$; $^{**}P < .01$.

2A). Blood glucose of the 2 groups reached similar levels at 120 minutes. In addition to the significant reduction at 30 and 60 minutes ($P < .05$ and $P < .01$, respectively), the valine-pyrrolidide treatment significantly lowered blood glucose 120 mintues after oral glucose challenge in 6-week-old *db/db* mice ($P < .01$) (Fig 2B). Fasting blood glucose levels were not significantly different between B6 mice and *db/db* mice at this age (75.9 ± 1.0 and 80.4 ± 2.6 mg/dL, respectively; n = 10). Glucose tolerance was also improved by the DPPIV inhibitor in B6 mice at 23 weeks of age (Fig 2C). A significant decrease of blood glucose was detected at 30 minutes ($P < .05$). On the other hand, the valine-pyrrolidide treatment showed no effect on glucose tolerance in *db/db* mice at any time (Fig 2D). Fasting blood glucose of *db/db* mice was extremely high at this age (495.5 ± 10.0 *v* 90.5 ± 2.4 mg/dL; n = 10, $P < .001$).

Plasma Insulin and Intact GLP-1 Concentrations After Oral Glucose Challenge

We measured plasma insulin and active GLP-1 levels after the oral glucose challenge of *db/db* mice and B6 mice at 7 and 24 weeks of age. At 7 weeks of age, vehicle-treated *db/db* mice showed 9-fold higher plasma insulin levels than B6 mice (10.8 ± 1.2 *v* 1.2 ±0.1 ng/mL). The administration of valine-pyrrolidide significantly increased insulin levels in both *db/db* mice ($P < .01$) and B6 mice ($P < .05$) (Fig 3A). For plasma intact GLP-1, the concentrations were almost the same in vehicle-treated *db/db* mice and B6 mice (1.29 ±0.08 and 1.02 ± 0.04 pmol/L, respectively). The DPPIV inhibition caused significant and extreme elevation of plasma active GLP-1 levels in *db/db* mice ($P < .05$) but not in B6 mice (Fig 3B). At 24 weeks of age, plasma insulin levels of the vehicle-treated *db/db* mice were lower than those of vehicle-treated B6 mice, being different from those at the younger age (1.4 ± 0.2 *v* 2.0 ± 0.1 ng/mL) (Fig 3C). There was a tendency to increased plasma insulin levels in DPPIV inhibitor-treated *db/db* mice (3-fold; $P = .076$). Plasma active GLP-1 levels in the vehicle-treated *db/db* mice and B6 mice were 7.9 ± 0.9 and 4.9 ± 0.6 pmol/L, respectively. A significant increase of plasma active GLP-1 levels was induced by DPPIV inhibition in *db/db* mice ($P < .01$) but not in B6 mice (Fig 3D).

Change of Proglucagon Expression in the Diabetic State

We examined whether proglucagon gene expression in the large intestine is affected by the diabetic condition. Northern

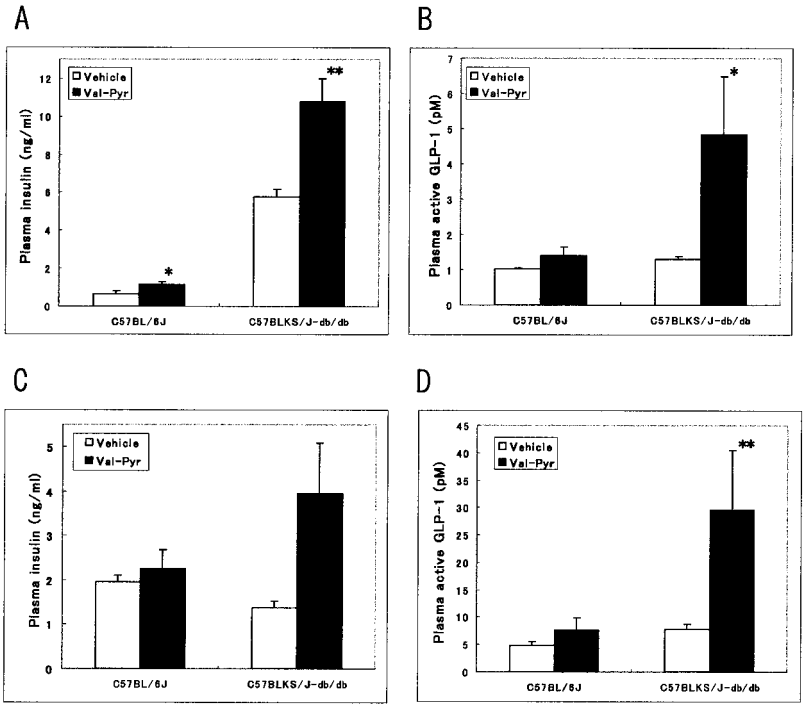

Fig 3. Plasma imunoreactive insulin (A and C) and active GLP-1 levels (B and D) after oral glucose challenge with or without valine-pyrrolidide in C57BL/6J (B6) mice and C57BLKS/J-*db/db* mice at 7 weeks of age (A and C) and at 24 weeks of age (B and D). Blood was drawn from the orbital sinus at 15 minutes after the administration. Vehicle (□) and valine-pyrrolidide treatment (■). Data are expressed as means ± SEM; n = 5. $^{*}P < .05$; $^{**}P < .01$.

blot analysis revealed that proglucagon expression in the proximal colon was significantly upregulated by 1.2-fold in *db/db* mice ($P < .01$) (Fig 4).

DISCUSSION

We examined the effects of acute DPPIV inhibition with valine-pyrrolidide on glucose tolerance at different stages of diabetes. OGTT was performed using *db/db* mice as a type 2 diabetes rodent model, at 6 and 23 weeks of age. Fasting blood glucose levels were similar in B6 mice and *db/db* mice at 6 weeks of age, but they became very much higher in *db/db* mice than in B6 mice at 23 weeks of age. Thus, the progress of diabetes was obvious in *db/db* mice at 23 weeks of age. Acute DPPIV inhibition was effective on glucose tolerance in B6 mice at both 6 and 23 weeks of age, but it was effective in *db/db* mice only at 6 weeks of age, suggesting that acute treatment of DPPIV inhibitor improved glucose tolerance only in the early stage of type 2 diabetes. Several studies have shown the glucose-lowering effect of exogenous GLP-1 or GLP-1 analogs in *db/db* mice.[30-32] DPPIV inhibitors presumably increase endogenous GLP-1 action on the pancreas to improve glucose tolerance in *db/db* mice at the early stage of diabetes.

To investigate the condition of the enteroinsular axis in type 2 diabetes, we measured plasma insulin and active GLP-1 levels of *db/db* mice after oral glucose challenge, with or without valine-pyrrolidide. At 7 weeks of age, vehicle-treated *db/db* mice showed higher insulin levels than vehicle-treated B6 mice, indicating that hyperinsulinemia owing to lowered insulin sensitivity exists in *db/db* mice at this age. We confirmed that acute DPPIV inhibition maintains high levels of plasma active GLP-1 and enhances insulin secretion in 7-week-old *db/db* mice. These results indicate that the DPPIV inhibi-

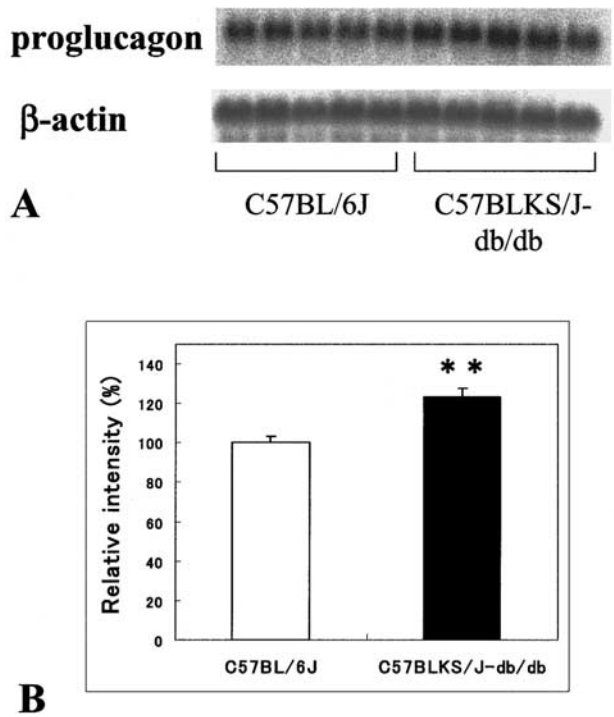

Fig 4. Northern blot analysis of proglucagon gene expression in C57BL/6J (B6) mice and C57BLKS/J-*db/db* mice. (A) Proglucagon and β-actin signals in the proximal colon. (B) The relative proglucagon intensity in B6 mice (□) and *db/db* mice (■). Data are expressed as means ± SEM; n = 5. $^{**}P < .01$.

tion enhances insulin secretion by increasing the half-life of plasma active GLP-1, and that the increased insulin levels overcome insulin resistance leading to improvement of glucose tolerance at the early stage of diabetes. Though acute DPPIV inhibition also increased plasma insulin and active GLP-1 levels in 24-week-old *db/db* mice, glucose tolerance was not improved in these mice. Vehicle-treated *db/db* mice at this age manifested lower insulin levels than their B6 counterparts, suggesting that pancreatic β-cell functions are impaired and insulin resistance is progressed in 24-week-old *db/db* mice, but the enteroinsular axis seems to work even at a relatively late stage of diabetes.

Further, we investigated the relation between plasma DPPIV activity and diabetic condition. We observed that *db/db* mice showed significantly higher plasma DPPIV activities than either B6 mice or *db/+* mice did. The difference of plasma DPPIV activities between *db/db* mice and B6 mice was small at the early stage of diabetes, but significant, being about 1.2-fold. However, it became larger with age, suggesting that the progress of the diabetic condition in mice affected plasma DPPIV activity. On the other hand, it was reported that plasma DPPIV activities were less in both middle-aged and elderly patients with diabetes.[33] The reason for the discrepancy is unknown, but the high DPPIV activity in *db/db* mice may be specific to a leptin receptor mutant.

GLP-1 is encoded by the proglucagon gene, which is most highly expressed in L cells in the large intestine. Northern blot analysis revealed that proglucagon gene expression in the proximal colon was significantly upregulated in *db/db* mice, in comparison with B6 mice. Expression of GLP-1 was examined in Zucker diabetic fatty (ZDF) rats, which are a model for type 2 diabetes. Compared with lean nondiabetic controls, ZDF rats showed increased expression of the proglucagon gene in the colon. Moreover, basal GLP-1 levels in plasma were elevated in ZDF rats.[34] Ørskov et al found that plasma GLP-1 levels were slightly elevated in obese type 2 diabetic patients, but not in mildly diabetic non-obese patients.[35] Thus, in combination with our observations, it appears that GLP-1 expression is activated under obese, fatty hyperglycemic conditions. It is possible that the upregulation of proglucagon gene expression is a consequence of chronic hyperglycemia in the diabetic condition. In comparison with B6 mice, plasma active GLP-1 levels of *db/db* mice were remarkably increased by DPPIV inhibitor treatment at 7 and 24 weeks of age. This may be a result of enhancement of GLP-1 production and inhibition of elevated plasma DPPIV activity in the diabetic state.

The administration of GLP-1 lowers blood glucose levels in type 2 diabetic patients, and may be therapeutically useful for treatment of the diabetes.[4,6] This is mainly based on its potentiation of glucose-stimulated insulin secretion from pancreatic β cells. Although the benefit of GLP-1 treatment has been demonstrated by many studies, the short half-life of active GLP-1 in the circulation limits its feasibility.[3] In the present study, inhibition of DPPIV activity increased the half-life of active GLP-1, leading to a high circulating concentration after nutrient consumption, even at a relatively late stage of diabetes. However, the following factors are important for the efficacy of DPPIV inhibitor and GLP-1 itself in type 2 diabetes treatment: (1) the extent to which β-cell function is preserved, and (2) how insulin resistance progresses. Acute administration of DPPIV inhibitor does not improve glucose tolerance in the state of progressed insulin resistance, in spite of preserved reaction of the enteroinsular axis. However, single administration can do, with potentiation of endogenous GLP-1 action, in the early stage of diabetes, where insulin resistance is not so severe and pancreatic β cells function well. Accordingly, it is expected that chronic administration of DPPIV inhibitor from the early stage is a promising treatment for impaired glucose tolerance (IGT), preventing the progression from IGT to type 2 diabetes. Recent study documented this hypothesis: long-term treatment with P32/98 causes sustained improvement in glucose tolerance and hyperinsulinemia in Zucker *fa/fa* rats.[36] In addition, when responsiveness of β cells to GLP-1 is preserved to some degree in progressed type 2 diabetes as observed in *db/db* mice, a DPPIV inhibitor may be useful in combination therapy with other antidiabetic drugs improving insulin resistance, such as thiazolidinediones.

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