Dipeptidyl Peptidase IV Inhibitor NVP-DPP728 Ameliorates Early Insulin Response and Glucose Tolerance in Aged Rats but Not in Aged Fischer 344 Rats Lacking Its Enzyme Activity

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ABSTRACT—The aim of this study was to investigate the effects of aging on glucose metabolism after oral glucose challenge in aged dipeptidyl peptidase IV (DPP-IV) positive (+) Fischer 344 (F344), DPP-IV deficient (−) F344 and DPP-IV(+) Wistar rats and to determine the effect of a DPP-IV inhibitor NVP-DPP728 (1-{2-[(5-cyanopyridin-2-yl)amino]ethylamino}acetyl-2-cyano-\((S)\)-pyrrolidine monohydrochloride salt) on glucose tolerance in aged rats. Aging caused a decrease in early insulin response after an oral glucose challenge in aged Wistar or DPP-IV(+) F344 rats, but not in aged DPP-IV(−) F344 rats, compared with young control groups. Glucose tolerance after an oral glucose challenge in aged DPP-IV(−) F344 rats was better than in aged DPP-IV(+) F344 and Wistar rats associated with the preservation of the early insulin response. NVP-DPP728 improved the glucose tolerance after an oral glucose challenge by potentiating the early insulin response throughout the inhibition of plasma DPP-IV activity in aged DPP-IV(+) Wistar and F344 rats. In contrast, NVP-DPP728 did not affect the glucose tolerance after an oral glucose challenge in aged DPP-IV(−) F344 rats. These results indicate that treatment with NVP-DPP728 ameliorated glucose tolerance in aged rats by the direct inhibition of plasma DPP-IV activity and presumably the subsequent increase in endogenous incretin action.

Keywords: Dipeptidyl peptidase IV, Fischer 344 rat, Glucose tolerance, Aging, Diabetes

Glucagon-like peptide-1 (GLP-1) is one of the important insulin-releasing hormones (incretins) and is postprandially released by the enteroglucagon-producing L cells in the lower gut, i.e., the ileum (1 – 3). GLP-1 regulates not only blood glucose via stimulation of glucose-dependent insulin secretion, but also the inhibition of gastric emptying and glucagon secretion. In addition, GLP-1 may regulate food intake in the central nervous system and glycogen synthesis in adipose tissue and muscle. Recent studies have shown that disruption of the GLP-1 receptor gene results in fasting hyperglycaemia and abnormal glycemic excursions after glucose challenge together with reduced levels of glucose-stimulated insulin (4), emphasizing the essential role of GLP-1 in the control of blood glucose.

Importantly, intravenous or subcutaneous administration of GLP-1 has been potently effective in patients with diabetes, normalizing hyperglycemia in moderate-to-severe type 2 diabetes subjects (5 – 8), raising the possibility of its use as a therapeutic agent. However, GLP-1 is metabolically unstable, having a plasma half-life of only 1 – 2 min in vivo (9, 10). It has been suggested that the circulating level of active GLP-1 in humans and rats (9, 11) is primarily regulated by dipeptidyl peptidase IV (EC 3.4.14.5; DPP-IV), a serine aminopeptidase. DPP-IV is identified as the enzyme that inactivates GLP-1 from an active GLP-1(7 – 36) amide to the GLP-1(9 – 36) amide by cleaving the N-terminal His-Ala dipeptide fragment in vivo (9). The difficulty of the development of GLP-1 as a therapeutic agent has been due to the lack of oral bioavailability of GLP-1 and rapid degradation to GLP-1(9 – 36), which can antagonize the effects of GLP-1(7 – 36) amide (12).

Recent studies in anesthetised pigs have demonstrated that acute DPP-IV inhibition by the specific DPP-IV inhibitor, valine-pyrrolidide, potentiated the insulinotropic effect of intravenously administered GLP-1 by inhibiting the NH\(_2\) terminal degradation of GLP-1 (11). Acute oral administration of the DPP-IV inhibitor has augmented insulin responses to oral glucose challenge and enhanced glucose clearance in insulin resistant obese Zucker rats (14, 15) and high-fat diet-fed C57BL/6J mice (16). These results indicate that DPP-IV inhibitors have potential as new...
therapeutic agents for type 2 diabetes (17, 18).

In aged Wistar rats, impaired glucose tolerance and decrease in insulin secretion has been demonstrated, associated with the progressive decline in insulin mRNA in islets of Langerhans and a decline in total pancreatic insulin content (19, 20). Therefore, aged Wistar rats are thought to be one of the diabetic animal models. More interestingly, Wang et al. demonstrated that subcutaneously infused GLP-1 had beneficial effects, including not only improvement of glucose tolerance and glucose-stimulated insulin response but also the up-regulation of pancreatic insulin, glucose transporter type 2 (GLUT2), and glucokinase mRNA expression, which are critical genes in insulin secretion and glucose sensing (21).

Watanabe et al. (22) found that Japanese Fischer 344 (F344) rats lacked DPP-IV enzyme activity. DPP-IV deficient (–) rats contain mRNA transcripts for DPP-IV but reduced levels of DPP-IV protein due to the translation of abnormal isoforms that fail to be processed into the biologically active mature glycosylated enzyme (23, 24). These rats have reduced N-terminal degradation of GLP-1 by DPP-IV following infusion of GLP-1 (9). However, not much is known about the glucose metabolism after oral glucose challenge in DPP-IV(–) F344 rats in an aged condition. Also, the effect of long-term inhibition of DPP-IV activity by a DPP-IV inhibitor on glucose excursion remains unclear. Therefore, DPP-IV(–) rats are thought to be a useful model for investigating the effect of long-term depletion of DPP-IV activity in diabetic conditions.

The present study was designed to compare the glucose tolerance after oral glucose challenge in chronically cannulated aged DPP-IV(–) F344, DPP-IV positive (+) F344 and DPP-IV(+/-) Wistar rats, and to determine the effect of a DPP-IV inhibitor, NVP-DPP728 (25), on the glucose excursion after a glucose load in aged rats.

MATERIALS AND METHODS

Materials

NVP-DPP728 (1-{2-[(5-cyanopyridin-2-yl)amino]ethyl-amino}acetyl-2-cyano-(S)-pyrrolidine monohydrochloride salt) was synthesised in Novartis Pharma (Summit, NJ, USA) (25).

Animals and surgery

Young (3- to 4-month-old) and aged (16- to 18-month-old) Fischer 344 and Wistar rats were purchased from Charles River Japan (Osaka), Clea Japan (Tokyo) and SLC Japan (Shizuoka) and given standard rodent chow (CE-2, Clea Japan) and water ad libitum. Their food intake was measured for 1 to 3 weeks. The rats were cannulated in the right external jugular vein at least 5 days prior to the oral glucose tolerance test under sodium pentobarbital (50 mg/kg, i.p.; Abbot Laboratories, North Chicago, IL, USA) anesthesia (15).

Oral glucose tolerance test

The method for the oral glucose tolerance test has been described previously (15). Briefly, rats fasted overnight were transferred to cages in the experimental room. The cannula of each animal was connected to a sampling tube, which was filled with a saline solution containing 10 unit/ml heparin. After 1 to 2 h of cage acclimation, NVP-DPP728 (10 μmol·kg⁻¹·ml⁻¹ in water) was administered orally 30 min before the oral glucose challenge. Blood samples (about 300 μl) were obtained from the cannula before (−5 and 0) and at 3, 5, 10, 15, 20, 30, 45, 60, 75 and 90 min after the oral glucose challenge (1 g/kg in 5 ml water), and blood glucose levels were analysed immediately. Blood samples were centrifuged (1500 × g, 4°C, 10 min). The plasma was stored at −80°C until analysis of the plasma insulin concentration and DPP-IV activity.

Blood glucose and plasma insulin

Blood glucose concentrations were measured using a blood glucose analyzer (Antsense II®; Daikin, Osaka). Plasma insulin concentrations were measured by using an enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Shibayagi, Gunma).

Plasma DPP-IV activity

Plasma DPP-IV activity was determined by the cleavage rate of 7-amino-4-methylcoumarin (AMC; Sigma, St. Louis, MO, USA) from synthetic substrate, H-glycyl-proline-AMC (Gly-Pro-AMC; Calbiochem-Novabiochem, Laufelfingen, Switzerland), as described previously (15, 25). In brief, 15 μl of plasma was mixed with 135 μl of 150 μM Gly-Pro-AMC in an assay buffer which was composed of 25 mM tris(hydroxymethyl)-aminomethane·HCl (pH 7.4), 140 mM NaCl, 10 mM KCl and 0.1% bovine serum albumin (Sigma). After incubation at room temperature, the fluorescence was determined using a spectrophotometer (excitation at 380 nm and emission at 460 nm) (CytoFluor™ II; PerSeptive Biosystems, Framingham, MA, USA). DPP-IV activity in plasma was expressed as the amount of product (nmol) per minute per ml. The deficiency of plasma DPP-IV activity was confirmed in Charles River Japan’s F344 rats. All of the F344 (Charles River Japan) rats used in this study were verified to be deficient in plasma DPP-IV activity (data not shown).

Statistical analyses

Data were expressed as means ± S.E.M. To determine the integrated glucose and insulin response to oral glucose challenge, incremental areas under the curves (AUC) of blood glucose and plasma insulin levels were calculated.
by the trapezoidal rule. Statistical analysis of the data was performed by two-way analysis of variance for the time-course study and unpaired Student’s *t*-test (two-tailed). Statistical significance was accepted at *P*<0.05.

**RESULTS**

**Plasma DPP-IV activity**

The basal DPP-IV activity in young DPP-IV(+) F344 and young Wistar rats was 4.8 ± 0.3 (n = 7) and 4.9 ± 0.2 (n = 7) nmol·min⁻¹·ml⁻¹, respectively. The basal DPP-IV activity was significantly increased with aging in aged DPP-IV(+) F344 (5.9 ± 0.2 nmol·min⁻¹·ml⁻¹, n = 7) and aged Wistar rats (6.1 ± 0.5 nmol·min⁻¹·ml⁻¹, n = 7). On the other hand, the DPP-IV activity was not detectable in young and aged DPP-IV(−) F344 rats.

**Glucose metabolism in aged DPP-IV(+) and (−) rats**

Effect of aging on glucose metabolism was investigated in DPP-IV(+) Wistar, DPP-IV(+) F344, and DPP-IV(−) F344 rats. In the aged Wistar rat group, the early insulin response (Insulin AUC (0–15 min)) after oral glucose challenge tended to be increased (27%) and the late-phase insulin response (Insulin AUC (15–60 min)) was significantly increased (67%), compared with a young control group. The early insulin response in aged DPP-IV(+) Wistar and DPP-IV(+) F344 rats tended to be increased slightly with aging (Table 1).

To investigate the effect of long-term depletion of DPP-IV activity, glucose tolerance was compared in aged Wistar, DPP-IV(+) F344, and DPP-IV(−) F344 rats (Fig. 1). The fasting glucose level in aged DPP-IV(−) F344 rats was comparable with that in aged DPP-IV(+) F344 rats, but was significantly lower than that in aged DPP-IV(+) Wistar rats (Fig. 1). There is no difference in the fasting plasma insulin levels between the three groups (Fig. 1). The glucose concentrations after oral glucose challenge in DPP-IV(−) F344 rats were significantly lower than those in DPP-IV(+) F344 or Wistar rats (Fig. 1). Early insulin response in aged DPP-IV(−) F344 rats tended to be higher than that in aged DPP-IV(+) F344, but was significantly higher than that in aged Wistar rats (Fig. 1 and Table 1).

Food intake (feeding volume) and body weight in young and aged DPP-IV(−) F344 control rats was significantly lower than those in young and aged Wistar control rats. However, there were no significant differences between young and aged DPP-IV(+) and (−) F344 rats (Table 2).

**Effect of NVP-DPP728 on the glucose tolerance in aged rats**

Effect of oral administration of NVP-DPP728 (10 μmol/kg, 30 min before glucose challenge) on the glucose tolerance was determined in aged Wistar, DPP-IV(+) F344 and DPP-IV(−) F344 rats. Treatment with NVP-DPP728 significantly and potently inhibited plasma DPP-IV activity in aged DPP-IV(+) Wistar and DPP-IV(+) F344 rats during the oral glucose tolerance test (Fig. 2).

### Table 1. Glucose tolerance after oral glucose challenge in young and aged rats

| Strain    | Condition   | Glucose AUC 0–15 min (×100 mg/dl·15 min) | Glucose AUC 15–60 min (×100 mg/dl·45 min) | Insulin AUC 0–15 min (×1000 pg/ml·15 min) | Insulin AUC 15–60 min (×1000 pg/ml·45 min) |
|-----------|-------------|----------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| Wistar    | Young       | 6.6 ± 0.4                              | 21.5 ± 1.2                               | 40.9 ± 4.5                               | 60.4 ± 8.3                               |
| Wistar    | Aged        | 6.4 ± 0.3                              | 22.1 ± 2.5                               | 29.9 ± 4.9                               | 100.8 ± 14.6                             |
| Wistar    | Aged + NVP-DPP728 | 5.6 ± 0.4                            | 11.3 ± 1.4*                             | 48.7 ± 9.1                               | 94.5 ± 16.3                              |
| F344(+)   | Young       | 8.2 ± 0.5                              | 26.6 ± 1.4                               | 48.9 ± 6.1                               | 93.1 ± 18.5                              |
| F344(+)   | Aged        | 7.2 ± 0.6                              | 21.3 ± 1.9                               | 41.9 ± 3.0                               | 126.2 ± 15.3                             |
| F344(+)   | Aged + NVP-DPP728 | 5.5 ± 0.4*                            | 12.8 ± 1.4*                             | 67.8 ± 11.7*                             | 96.2 ± 21.6                              |
| F344(−)   | Young       | 7.0 ± 0.5                              | 22.5 ± 1.9                               | 54.7 ± 7.9                               | 91.7 ± 14.6                              |
| F344(−)   | Aged        | 6.7 ± 0.4                              | 17.7 ± 1.9                               | 55.7 ± 7.6*                              | 115.7 ± 24.2                             |
| F344(−)   | Aged + NVP-DPP728 | 7.0 ± 0.5*                            | 19.5 ± 2.5                               | 59.2 ± 4.0                               | 141.4 ± 21.5                             |

Blood glucose and plasma insulin concentrations were determined during an oral glucose tolerance test (1 g/kg, p.o., *t* = 0 min). NVP-DPP728 (DPP728, 10 μmol/kg, p.o.) was administered 30 min before the glucose challenge. Incremental areas under the curve (AUC) of blood glucose and plasma insulin concentrations were calculated by the trapezoidal rule in Wistar, DPP-IV positive (F344 (F344(+) F344(−))) and DPP-IV deficient (F344 (F344(−))) rats under the young or aged condition. Values are means ± S.E.M. of 6–7 animals. *P*<0.05, significantly different from a group in the same animal substrain. *P*<0.05, significantly different from the same animal substrain group in the same condition. *P*<0.05, significantly different from a different animal substrain in the same condition.
DPP-IV(+) Wistar rats, NVP-DPP728 treatment significantly improved glucose tolerance after oral glucose challenge, associated with an enhanced early insulin response, compared with the control groups (Fig. 3 and Table 1). NVP-DPP728 also significantly suppressed glucose excursion through the significant increase in early insulin response in aged DPP-IV(+) F344 rats (Fig. 4 and Table 1). On the other hand, NVP-DPP728 did not affect glucose tolerance after glucose challenge in aged DPP-IV(–) F344 rats (Fig. 5 and Table 1).

Table 2. Feeding volume and body weight in young and aged rats

| Condition | Strain     | Feeding volume (g/day) | Body weight (g) |
|-----------|------------|-----------------------|-----------------|
| Young     | Wistar(+)  | 17.0 ± 0.2            | 273.2 ± 0.8     |
| Young     | F344(+)    | 14.1 ± 0.6            | 252.8 ± 2.5     |
| Young     | F344(–)    | 12.9 ± 0.5           * | 262.1 ± 6.0    * |
| Aged      | Wistar(+)  | 21.5 ± 1.3           * | 500.9 ± 5.5     |
| Aged      | F344(+)    | 11.9 ± 0.9           * | 365.0 ± 7.7     |
| Aged      | F344(–)    | 10.8 ± 0.7           * | 361.6 ± 4.3    * |

Values are means ± S.E.M. of 6 – 7 animals. *P<0.05, significantly different from a different animal substrain in the same condition.

Fig. 1. Glucose tolerance in aged rats. Blood glucose (A) and plasma insulin (B) concentrations were determined during an oral glucose tolerance test (1 g/kg, p.o., t = 0 min) in DPP-IV positive (+) Wistar (closed circles) and DPP-IV deficient (–) F344 (open squares) rats. Values are means ± S.E.M. of 6 – 7 animals. *P<0.05, significantly different from the DPP-IV(–) group.

Fig. 2. Effects of NVP-DPP728 on plasma DPP-IV activity in aged DPP-IV positive (+) Wistar and F344 rats. Vehicle (open squares) or NVP-DPP728 (closed circles, 10 μmol/kg, p.o.) was administered 30 min before the glucose challenge. Plasma DPP-IV activity in rats was determined during an oral glucose tolerance test (1 g/kg, p.o., t = 0 min). Values are means ± S.E.M. of 6 – 7 animals. *P<0.05, significantly different from the vehicle group.
DISCUSSION

Our results showed that the early insulin response in DPP-IV(+) Wistar and F344 rats was decreased with aging, but aging did not affect the early insulin response in aged DPP-IV(−) F344 rats. These results indicate that the preservation of early insulin response in aged DPP-IV(−) F344 rats may contribute to the improved glucose tolerance after oral glucose challenge. It has been demonstrated that DPP-IV(−) F344 rats lack the enzyme activity of DPP-IV, but other peptidases such as aminopeptidase, neutral endopeptidase, and gamma-glutamyl transpeptidase are intact (22). A recent study has demonstrated that mice lacking DPP-IV/CD26 show improved glucose tolerance associated with the enhanced insulin secretion and the increase in intact insulino-tropic form of incretin levels (26). Therefore, it is suggested that our findings in DPP-IV(−) F344 rats may result from the lack of the DPP-IV activity and presumably subsequent increase in endogenous incretin bioactivity. Moreover, long-term DPP-IV inhibition might preserve glucose homeostasis through a protective effect on the development of impaired glucose tolerance with aging.

In the present study, there was a strain difference in the fasting blood glucose concentration between aged Wistar
and F344 rats, although the fasting insulin concentrations were comparable between in three groups. A previous study demonstrated the strain difference in ageing between SD and F344 rats, indicating that the decrease in insulin responsiveness in models of the aged SD rat may be due to changes in body composition; i.e., weight gain and fat mass (27). In the present study, the body weight of aged Wistar rats was significantly higher than that of aged F344 rats. Thus, fasting hyperglycaemia in aged Wistar rats may be due to the differences in body composition.

Our study clearly demonstrated that the inhibition of plasma DPP-IV activity by treatment with a DPP-IV inhibitor NVP-DPP728 improved the glucose tolerance after glucose load in aged DPP-IV(+)/G2d F344 rats by potentiating early insulin release. These results were consistent with the previous results that DPP-IV inhibitor treatment improved glucose tolerance in other animal models, such as insulin resistant obese Zucker rats (14, 15), and high-fat diet-fed C57BL/6j mice (16). Moreover, NVP-DPP728 did not affect glucose-stimulated insulin release and glucose excursion in aged DPP-IV(-)/G2d F344 rats. These results directly proved that the glucose-lowering action of NVP-DPP728 after oral glucose challenge was due to the inhibition of plasma DPP-IV activity. Since the glucose metabolism in aged DPP-IV(-)/G2d F344 rat might mimic that in NVP-DPP728-treated aged DPP-IV(+)/G2d F344 rats, long-term treatment with DPP-IV inhibitors might be effective in improving glucose tolerance and possibly diabetes without tachyphylaxis.

Wang et al. (21) demonstrated that there was no difference in exogenously given GLP-1-stimulated insulin response between aged and young Wistar rats even though glucose tolerance and insulin secretion were decreased in aging Wistar rats. Additionally, in aged Wistar rats, GLP-1 infused subcutaneously had a beneficial effect including not only improvement of glucose tolerance and glucose-stimulated insulin response but also the up-regulation of pancreatic insulin, GLUT2, and glucokinase mRNA (21). These results may indicate that GLP-1 signalling and responses in Wistar rats were preserved under the aged condition. In the present study, the inhibition of DPP-IV activity by the treatment with NVP-DPP728 ameliorated glucose tolerance through the stimulation of early insulin response in aged DPP-IV(-)/G2d F344 rats. It has been demonstrated that DPP-IV activity inhibition by NVP-DPP728 increased plasma active GLP-1 concentrations during the oral glucose tolerance test in obese Zucker rats (15) and prevented inactivation of GLP-1 in normal rats (25). Taken together, NVP-DPP728 treatment might improve glucose tolerance through, at least in part, the increase in active GLP-1 concentrations in aged rats, although we did not measure GLP-1 concentrations in the present study.

It is well-known that DPP-IV plays a role in the inactivation of biologically active regulatory peptides, growth hormone-releasing hormone, neuropeptide Y, peptide YY, prolactin, and incretin (glucose-dependent insulinotropic polypeptide, GIP) (1–3, 17). Moreover, a recent finding demonstrated that DPP-IV inhibitor treatment improved glucose tolerance and enhanced glucose-stimulated insulin levels in mice lacking the GLP-1 receptor, associated with the increase in plasma intact GIP concentrations (26). Therefore, the possible effect by NVP-DPP728 on the degradation of other peptides as well as GLP-1 may not...
only contribute to, but also counteract the beneficial effects of NVP-DPP728 on glucose tolerance in aged rats.

In a clinical study, it has been demonstrated (28) that the plasma level of incretin, e.g., GLP-1 (total but not active form) after carbohydrate ingestion was increased in elderly healthy subjects (67.5 ± 4.1-year-old), compared with young subjects. One possible mechanism of the increase in GLP-1 level is an inappropriate accumulation due to impairment of incretin clearance by aging and the reflex of GLP-1 resistance caused by the failure of GLP-1 receptor signal transduction in pancreatic cells (28). Another conceivable explanation is that the contribution of endogenous incretin may increase with aging, but its physiological bioactivity would be very low because of the fast degradation by plasma DPP-IV (9, 10). Consistent with this hypothesis, recent clinical studies have demonstrated that intravenous administration of GLP-1 improved the fasting hyperglycemia and islet dysfunction in elderly patients (65- to 74-year-old) with Type 2 diabetes (29, 30).

In addition, our study demonstrated that the DPP-IV inhibitor NVP-DPP728 improved glucose tolerance in aged rats by inhibiting plasma DPP-IV activity, presumably in association with the endogenous incretin bioactivity. Taken together, these results indicate that treatment with DPP-IV inhibitor may provide a beneficial effect for elderly patients with type 2 diabetes.

In conclusion, our results suggest that the amelioration of glucose tolerance by treatment with NVP-DPP728 in aged DPP-IV(+) rats was directly due to the inhibition of plasma DPP-IV activity, presumably via the subsequent increase in endogenous incretins. In addition, the difference in glucose tolerance between DPP-IV(+) and (−) rats, especially under aged conditions, indicates the possibility that plasma enzyme activity of DPP-IV limits the resistance to the development of impaired glucose tolerance. These data strongly support a therapeutic approach using a drug, to improve basal and prandial glycemic controls in elderly patients with type 2 diabetes and impaired glucose tolerance.

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