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K579, a slow-binding inhibitor of dipeptidyl peptidase IV, is a long-acting hypoglycemic agent

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Abstract

Dipeptidyl peptidase IV inhibitors are expected to be categorized in a new type of antidiabetic drugs. We had developed a long-acting dipeptidyl peptidase IV inhibitor, K579 [(*S*)-1-[4-methyl-1-(2-pyrimidinyl)-4-piperidylamino]acetyl-2-pyrrolidinecarbonitrile]. The aim of present study was to characterize the pharmacological profiles of K579. In normal rats, K579 suppressed the blood glucose elevation after an oral glucose tolerance test with the increment of plasma insulin and active forms of glucagon-like peptide-1 (GLP-1). During repetitive glucose loading using Zucker fatty rats, pretreatment with K579 attenuated the glucose excursion after the second glucose loading as well as the first glucose loading without inducing hypoglycemia. The kinetic study using cell extract revealed that K579 was a more potent and slower binding inhibitor than the existing dipeptidyl peptidase IV inhibitor (NVP-DPP728, 1-[[[2-[(5-cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(*S*)-pyrrolidine). These profiles of K579 might be advantageous over the existing dipeptidyl peptidase IV inhibitor with respect to less dosing frequency.

Keywords: Antihyperglycemic effect; Dipeptidyl peptidase IV; GLP-1; Insulin; (Rat)

1. Introduction

Impaired insulin secretion in type 2 diabetes is often characterized by a decreased first-phase insulin response (Polonsky et al., 1988; Porte, 1991; Taylor et al., 1994) which leads to postprandial hyperglycemia (Kosaka et al., 1994). Sulfonylureas, which are widely used as potent hypoglycemic agents for type 2 diabetes, strongly inhibit the ATP-sensitive K^+channel activity by binding to the high-affinity receptor protein (sulfonylurea receptor) in the pancreatic β cell (Panten et al., 1992). Sulfonylureas stimulate insulin secretion irrespective of blood glucose levels, and thus, cause hypoglycemia which is a common undesirable side effect of sulfonylurea treatment (Asplund et al., 1983; Binder and Bendtson, 1992; Stahl and Berger, 1999). Control of the matters such as incretins, which potentiate physiological glucose-dependent insulin release, would be preferred for the treatment of insulin secretory disorders.

Although several gastrointestinal regulatory peptides have been proposed as incretins, only glucose-dependent insulinotropic poly-peptide-1 (GIP) and glucagonlike peptide 1 (GLP-1) appear to fulfill the requirements to be considered as physiological stimulants of postprandial insulin release (Holst, 1997). Both of these peptides stimulate glucose-induced insulin secretion with the cyclic AMP accumulation in the β cell (Lu et al., 1993). Several reports conclude that GIP and GLP-1 may play a physiological role in maintaining glucose tolerance. Each antagonist of GIP and GLP-1 inhibits glucose-induced insulin secretion in rats (Tseng et al., 1996, 1999). In addition, the postprandial GLP-1 response is reduced in type 2 diabetes patients compared to healthy subjects (Vilsboll et al., 2001). The continuous infusion of GLP-1 in type 2 diabetes patients suppresses the plasma glucose level (Larsen et al., 2001; Toft-Nielsen et al., 1999). However, incretins have short duration of action due to enzymatic degradation in vivo by dipeptidyl peptidase IV (Deacon et al., 1995; Hansen et al., 1999; Kieffer et al., 1995).

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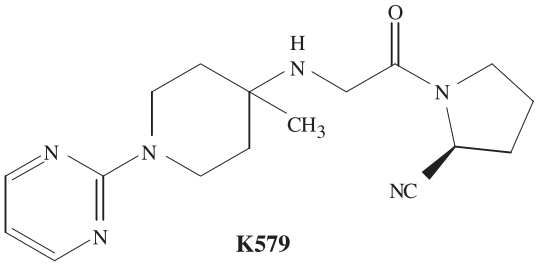

Fig. 1. Structure of K579.

Recently, dipeptidyl peptidase IV inhibitors, which protect incretins from enzymatic degradation, have been noticed as new oral therapeutic tools for type 2 diabetes (Drucker, 2001; Holst and Deacon, 1998). Dipeptidyl peptidase IV inhibitors would correct the postprandial glucose excursion in type 2 diabetes patients by prolonging the action of postprandial incretins and insulin. In fact, dipeptidyl peptidase IV inhibitors attenuate the glucose excursion after glucose loading with the elevation of plasma GLP-1 and insulin levels in Zucker fatty rats (Balkan et al., 1999; Sudre et al., 2002; Villhauer et al., 2003) which are the characterized model of obesity and insulin resistance, and in type 2 diabetes (Ahren et al., 2002).

We had identified K579 [(*S*)-1-[4-methyl-1-(2-pyrimidinyl)-4-piperidylamino]acetyl-2-pyrrolidinecarbonitrile] (Fig. 1), a cyanopyrrolidine derivative, as a compound that had an apparently longer duration of plasma dipeptidyl peptidase IV inhibitory activity in normal rats compared with NVP-DPP728 (1-{2-[(5-cyanopyridin-2-yl)amino]ethylamino}acetyl-2-cyano-(*S*)-pyrrolidine) which is also a cyanopyrrolidine derivative inhibiting dipeptidyl peptidase IV by a slow-binding mechanism (Hughes et al., 1999). K579 inhibits rat, canine, monkey and human dipeptidyl peptidase IV with half-maximal inhibitory concentration (IC_{50}) values of 3, 8, 8 and 5 nmol/l, respectively (Nakajima et al., 2002).

In the present study, we investigated the effects of the newly developed dipeptidyl peptidase IV inhibitor, K579, on the plasma GLP-1, insulin and glucose levels after glucose loading in Wistar rats. In addition, we compared the effects of K579 on glucose excursion during the repetitive oral glucose tolerance test in Zucker fatty rats with data generated with NVP-DPP728. Finally, we analyzed inhibitory mechanism of K579 and compared with that of NVP-DPP728.

2. Materials and methods

2.1. Chemicals

K579 and NVP-DPP728 were synthesized at Pharmaceutical Research Institute, Kyowa Hakko Kogyo (Shizuoka, Japan). For the in vivo experiments, each compound was suspended in 0.5% methylcellulose 400 cP (Wako, Osaka, Japan) and orally administered at a volume of 5 ml/kg.

2.2. Animals

Male Wistar rats and obese (fa/fa) male Zucker fatty rats were purchased from Charles River Japan (Kanagawa, Japan). The animals received standard laboratory chow, FR-2 (Funabashi Farms, Chiba, Japan) and water ad libitum. They were housed in a temperature (19–25 °C)-, humidity (30–70%)- and light (diurnal time; 0700–1900)-controlled room. Wistar rats were cannulated in the left carotid artery for more than 7 days prior to the oral glucose tolerance test under sodium pentobarbital anesthesia (50 mg/kg, i.p., Tokyo Kasei Kogyo, Tokyo, Japan). The free ends of the catheters were tunneled subcutaneously around the side of the neck to the middle of both shoulder blades where they were taken to the exterior through an incision in the skin. The catheters were filled with polyvinylpyrrollidon (Wako; 0.125 g/ml) in saline containing 500 U/ml heparin to maintain patency. The protocol was approved by the Bioethical Committee of Pharmaceutical Research Institutes, Kyowa Hakko Kogyo.

2.3. Effects of K579 and NVP-DPP728 on glucose, GLP-1 and insulin levels, and plasma dipeptidyl peptidase IV activity during oral glucose tolerance test in Wistar rats

After 24 h fasting, Wistar rats at the age of 9 weeks were transferred from the holding room to the laboratory and were left to be acclimatized to the new conditions for at least 1 h before the experiments. Approximately 1000 U/ml of sodium heparin was administered via the carotid line to confirm patency and to prevent coagulation during the study. After collection of baseline blood samples via the carotid line, the rats were divided into experimental groups according to plasma glucose level. Thirty minutes after the administration of test compound, 2 g/kg glucose was given orally to the rats. Additional samples were withdrawn prior to glucose loading (0 min), and 7, 15 and 30 min after glucose loading. Blood samples were collected in chilled Eppendorf tubes where dipeptidyl peptidase IV inhibitor (Linco Research, St. Charles, MO, USA) had been added to achieve final concentration of 100 μmol/l. Blood samples were centrifuged, and separated plasma was stored at −20 °C until analyses.

2.4. Effects of K579 and NVP-DPP728 on plasma dipeptidyl peptidase IV activity in Zucker fatty rats

After 24 h fasting and a period of acclimatization in the laboratory, a tail blood sample was taken from each male Zucker fatty rat at the age of 12–14 weeks, and a test compound or vehicle was administered orally. Blood samples were collected prior to, and 0.5, 1, 2, 4 and 6 h after administration of the test compounds in chilled Eppendorf tubes where dipeptidyl peptidase IV inhibitor (Linco Research) had been added to achieve final concentration of 100 μmol/l. Blood was centrifuged immediately

to obtain plasma and the dipeptidyl peptidase IV activity was determined.

2.5. Effects of K579 and NVP-DPP728 on glucose excursion during repetitive oral glucose tolerance test in Zucker fatty rats

Zucker fatty rats at the age of 9–12 weeks were divided into experimental groups according to their glucose profiles. Briefly, 4–7 days before the experiments, the fasting (24 h) rats were orally administered 2 g/kg of glucose. Plasma glucose levels were measured prior to glucose loading, and 0.5, 1 and 2 h after glucose loading.

Baseline fasting (24 h) plasma glucose was measured in Zucker fatty rats as described above. The first and second glucose loading (2 g/kg each) was done 0.5 and 4.5 h after the administration of test compounds, respectively. Plasma glucose levels were measured before compound administration (at −0.5 h), prior to the first glucose loading (at 0 h), and 0.5, 1, 2, 4 (prior to the second glucose loading), 4.5, 5 and 6 h after the first glucose loading (at 0.5, 1, 2, 4, 4.5, 5 and 6 h).

2.6. Plasma analysis

Plasma glucose concentration was assayed by using a standard glucose–oxidase technique (Katayama Kagaku Kogyo, Osaka, Japan). Plasma insulin concentration was determined through an enzyme-linked immunosorbent assay using a commercial kit for rat insulin (Morinaga Institute of Biological Science, Kanagawa, Japan). Plasma GLP-1 concentration was measured by an enzyme-linked immunosorbent assay technique that measured the biologically active GLP-1 forms, GLP-1-(7-36) and GLP-1-(7-37) amide, in plasma (Linco Research).

Plasma dipeptidyl peptidase IV activity was measured using the cleavage rate of 7-amino-4-methylcoumarin (AMC; Peptide Institute, Osaka, Japan) from a substrate, Gly-Pro-AMC (Peptide Institute), based on a modified method described previously (Deacon et al., 1998; Kubota et al., 1992). Briefly, aliquots of plasma were incubated with the substrate in an assay buffer which was composed of 25 mmol/l HEPES (nacalai tesque, Kyoto, Japan), 140 mmol/l NaCl (Kokusan Chemical, Tokyo, Japan) and 1% bovine serum albumin (Seikagaku, Tokyo, Japan). After incubation at room temperature, free AMC generated in proportion to dipeptidyl peptidase IV activity was determined using a spectrofluorometer (excitation at 390 nm and emission at 460 nm; Wallac 1420 ARVOsx, Wallac Oy, Türku, Finland). Catalytic dipeptidyl peptidase IV activity in plasma was expressed as the amount of product (nmol) per min per ml.

2.7. Preparation of human dipeptidyl peptidase IV

Human dipeptidyl peptidase IV was prepared from the human colonic carcinoma cell line, Caco-2 cells, according to the procedure described previously (Darmoul et al., 1992; Reisher et al., 1993) with some modifications. In brief, Caco-2 cells were routinely maintained in Dulbecco's modified Eagle's essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% inactivated fetal calf serum (JRH, KS) and 1% non essential amino acids (Gibco Brl, NY), with medium changes every 2 days. Preparation of human dipeptidyl peptidase IV from Caco-2 cells was performed as described previously (Hughes et al., 1999) with some modifications. In brief, after washing the cells with cold phosphate-buffered saline (ICN, OH) three times, they were solubilized in ice-cold Tris buffer (10 mmol/l Tris–HCl, 150 mmol/l NaCl, 5 μg/ml aprotinin, nonidet-P40, pH 8.0). Cell lysates were left on ice for 30 min before separating the soluble fraction from cell debris by centrifugation at 40,000 × g at 4 °C for 30 min. The preparations contained approximately 58 mU dipeptidyl peptidase IV/mg protein (1 unit cleaves 1 μmol of Gly-Pro-AMC/min). Gly-Pro-AMC was added in the diluted extract protein (2 μg) with assay buffer (25 mmol/l Tris–HCl buffer (pH 7.4) containing 140 mmol/l NaCl, 10 mmol/l KCl, 1 wt.% bovine serum albumin) to make up to 0.1 ml final volume. Reaction progress was monitored using a spectrofluorometer (excitation at 390 nm and emission at 460 nm) (Wallac 1420 ARVOsx, Wallac Oy). Under these conditions, K_m for Gly-Pro-AMC was 51 μmol/l.

2.8. Kinetics of inhibition of dipeptidyl peptidase IV

Association rate constants of the indicated compounds and human dipeptidyl peptidase IV were determined under pseudo-first-order conditions based on a modified method described previously (Hughes et al., 1999). The substrate of Gly-Pro-AMC, at 95 μmol/l as the final concentration in assay buffer, was preincubated with the compounds for 5 min. Inhibitory reactions were initiated by adding 1 μg of enzyme stock. Total elapsed time between the enzyme addition and the initiation of data collection was less than 30 s. Readings were taken every 10 s for a total of 1200 s, and initial (blank) fluorescence values were subtracted from the data prior to subsequent calculations. Data were exported to Microsoft Excel and were fitted to the integrated rate equation for slow binding inhibition (Eq. (1)) through a nonlinear regression analysis according to the method described by Williams and Morrison (1979).

$$P = v_s t + (v_0 - v_s)(1 - \exp(-k' t))/k' \quad (1)$$

$$k' = k_{off} + k_{on}' [I_0] \quad (2)$$

$$k_{on} = k_{on}' (1 + [S_0]/K_m) \quad (3)$$

Values for v_0 (initial rate), v_s (final steady-state rate) and k' (apparent rate constant for the transition from v_0 to v_s) were obtained for each progress curve. Values for k' were plotted against the inhibitor concentration of $[I_0]$. A linear

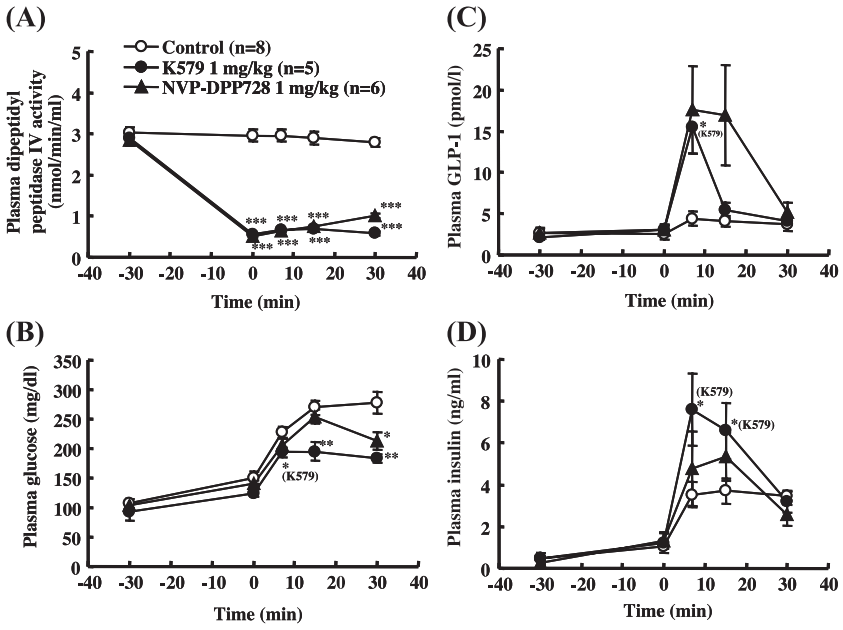

Fig. 2. Effects of K579 and NVP-DPP728 on plasma dipeptidyl peptidase IV activity (A), glucose (B), GLP-1 (C) and insulin (D) levels after glucose loading (2 g/kg) in Wistar rats. Oral glucose tolerance test (2 g/kg) was performed 30 min after administration of K579 or NVP-DPP728. Data represent mean ± S.E.M. ($n = 5–8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from the control by Student's t-test or Aspin–Welch test.

dependency between $[I_0]$ and k' was obtained and fitted (Eq. (2)) to obtain estimates of k_{on}' and k_{off} (dissociation rate constant). The rate constant of k_{on}' was subsequently corrected for the competition of the substrate by using Eq. (3), where $[S_0]$ was the concentration of the substrate, and K_m was the separately determined Michaelis–Menten con-

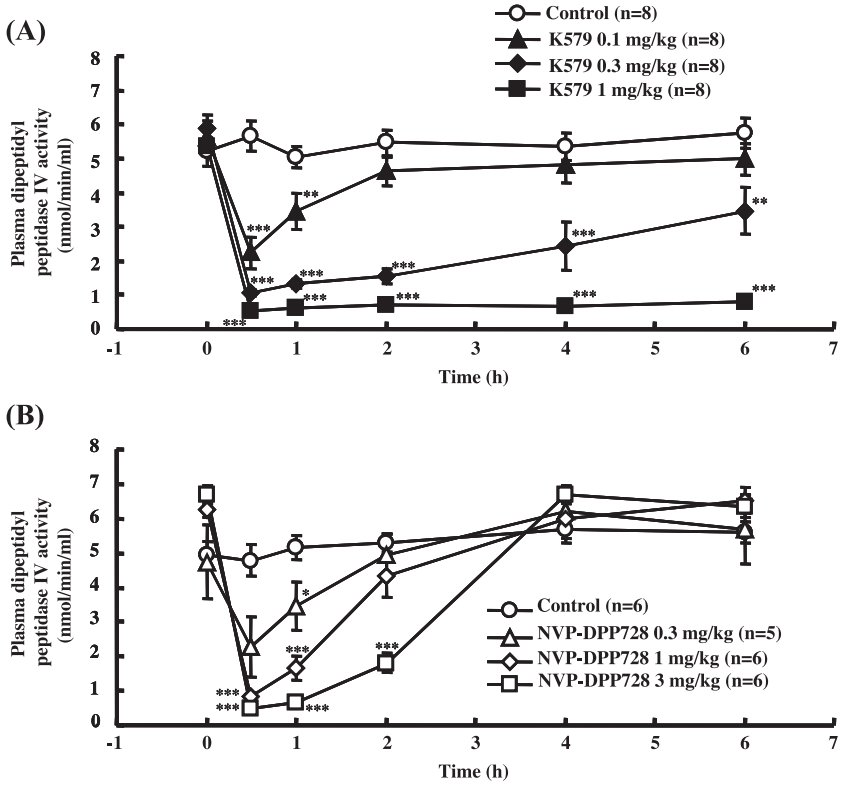

Fig. 3. Effects of K579 (A) and NVP-DPP728 (B) on plasma dipeptidyl peptidase IV activity in Zucker fatty rats. Each compound was orally administered to rats at 0 h. Data are expressed as mean ± S.E.M. ($n = 5–8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from the control by one-way analysis of variance and further post hoc Dunnett test.

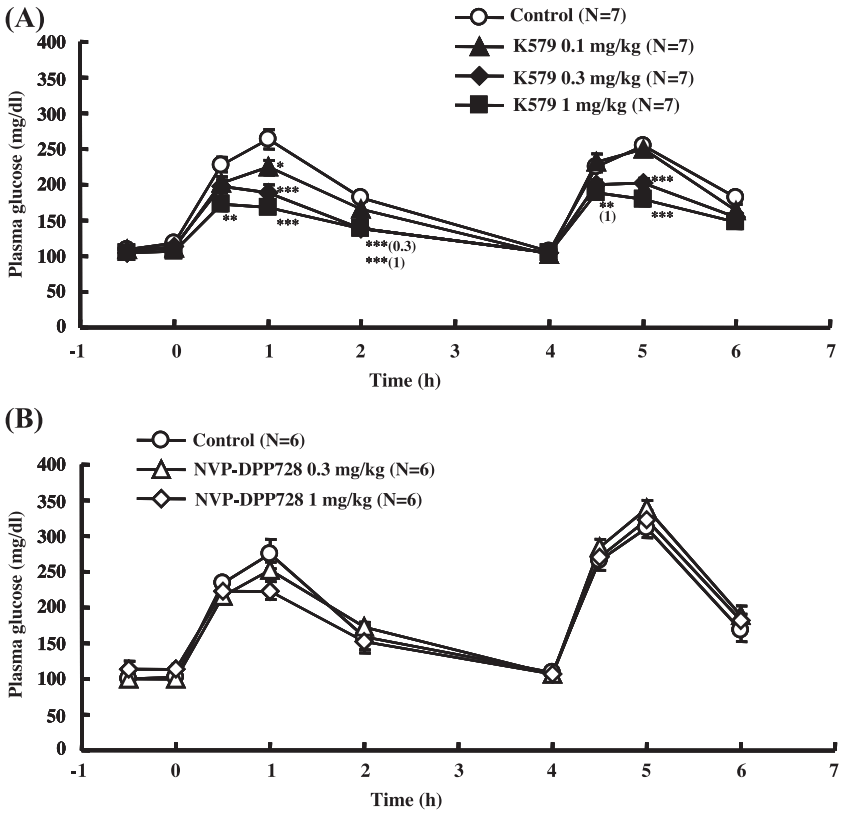

Fig. 4. Effects of K579 (A) and NVP-DPP728 (B) on glucose excursion during repetitive oral glucose tolerance test in Zucker fatty rats. All rats received 2 g/kg glucose orally both at 0 and 4 h. Each compound was orally administered to rats at −0.5 h. Data are expressed as mean ± S.E.M. ($n = 6$ or 7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from the control by one-way analysis of variance and further post hoc Dunnett test.

stant. K_i was calculated from k_{off}/k_{on}. The half-life of enzyme-inhibitor complex (EI half-life) was calculated from $0.693/k_{off}$.

2.9. Statistical analyses

Data were expressed as the means ± S.E.M. Statistical analyses were performed using SAS (Release 8.2, SAS Institute, Cary, NC, USA) for Windows. Statistical significance within each group was estimated using *F*-test followed by Student's *t*-test or Aspin–Welch test. When experimental series involved more than two groups, statistical analysis was done by one-way analysis of variance and further post hoc Dunnett test. $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effects of K579 and NVP-DPP728 on glucose, GLP-1 and insulin levels, and plasma dipeptidyl peptidase IV activity during oral glucose tolerance test in Wistar rats

After oral administration to fasted Wistar rats, K579 and NVP-DPP728 inhibited almost totally the plasma dipeptidyl peptidase IV activity for 1 h (Fig. 2A). Both of K579 and NVP-DPP728 suppressed hyperglycemia after glucose loading (Fig. 2B) with transient elevation of the active forms of GLP-1 (Fig. 2C) and insulin (Fig. 2D) secretion but did not cause hypoglycemia.

3.2. Effects of K579 and NVP-DPP728 on plasma dipeptidyl peptidase IV activity in Zucker fatty rats

In Zucker fatty rats, K579 inhibited the plasma dipeptidyl peptidase IV activity dose dependently (Fig. 3A). Treatment with K579 at a dose of 1 mg/kg notably inhibited the plasma dipeptidyl peptidase IV activity even 8 h after the administration. NVP-DPP728 also inhibited the plasma dipeptidyl peptidase IV activity dose dependently (Fig. 3B). However, the duration of the inhibitory activity of NVP-DPP728 was shorter than that of K579 when comparing the duration at a same dose.

3.3. Effects of K579 and NVP-DPP728 on glucose excursion during repetitive oral glucose tolerance test in Zucker fatty rats

Pretreatment with 0.1 mg/kg or higher of K579 significantly attenuated the glucose excursion in Zucker fatty rats after the second glucose loading as well as the first glucose loading (Fig. 4A). On the other hand, 0.3 or 1 mg/kg of

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f K579 (Fig. 5A) and N[illegible]
lear time-dependent app[illegible]
s a characteristic of s[illegible]
inhibition constants fo[illegible]
n in Table 1. Although [illegible]
e for K579 and NVP-D[illegible]
same order (i.e., 2.7 and 1[illegible]
d dissociation (k_{off}) rate co[illegible]
at of NVP-DPP728, which[illegible]
579/dipeptidyl peptidase IV[illegible]
K579/dipeptidyl peptidase [illegible]
hose of NVP-DPP728. The[illegible]

Table 1
Kinetic constants for dipeptidyl peptidase IV inhibi[illegible]
DPP728

	k_{on} [10^6 s^{-1} $(mol/l)^{-1}$]	k_{off} (10^{-3} s^{-1})
K579	1.06	2.57
NVP-DPP728	0.22	0.3[illegible]

Reactions were performed as des[illegible]
curves, such as those depicted i[illegible] Fig. 5A and B
Eq. (1). EI half-life valu[illegible]
mean ± S.E.M. of two to[illegible]

enzyme-in[illegible]
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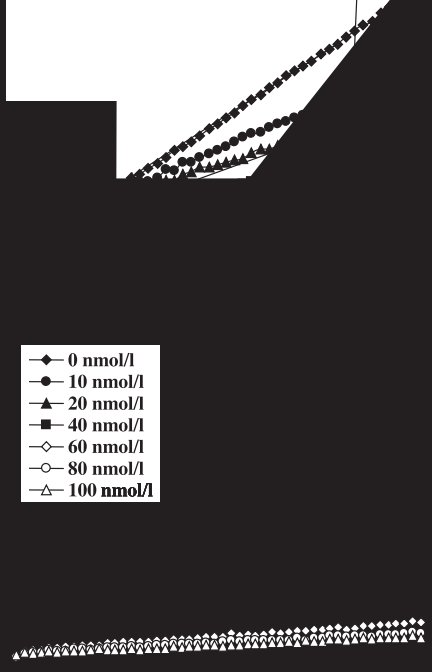

The improvement of insulin secretion and efficacy by dipeptidyl peptidase IV inhibitors observed in this study could have been mediated by enhanced GLP-1 secretion. These results were consistent with the previous reports showing that dipeptidyl peptidase IV inhibitor inhibited plasma dipeptidyl peptidase IV activity, preserved the endogenously secreted active forms of GLP-1, augmented the insulin response and ameliorated the glucose excursion during oral glucose tolerance test in normal and obese Zucker rats (Balkan et al., 1999; Sudre et al., 2002). Although the effects of NVP-DPP728 on the active forms of GLP-1 or the insulin response were not significant in this study, we confirmed that, in the other study under the conditions which were same as those in this experiment, NVP-DPP728 significantly increased the active forms of GLP-1 and the insulin secretion and subsequently suppressed the glucose excursion. Under continued inhibition of plasma dipeptidyl peptidase IV, the potentiation of the insulin response to an oral glucose challenge occurred without the additional administration of K579 in obese Zucker rats (Figs. 3A and 4A). In contrast, the inhibitory effect of NVP-DPP728 on the plasma dipeptidyl peptidase IV hardly remained before the second glucose loading which might contribute the loss of effect on the glucose fluctuation during the second oral glucose tolerance test. These results suggested that K579 should require less dosing frequency for correcting the postprandial glucose excursion than NVP-DPP728. NVP-DPP728 suppressed, however not significantly, the glucose excursion after the first glucose loading. In the other study of investigating data reproducibility using Zucker fatty rats, we confirmed that 1 mg/kg of NVP-DPP728 attenuated the glucose excursion after only the first glucose loading and did not affect the glucose fluctuation after the second glucose loading. The potency and duration of dipeptidyl peptidase IV inhibition by K579 were reduced when the dose of K579 was lowered, which suggested that K579 was a reversible dipeptidyl peptidase IV inhibitor. Moreover, in a preliminary assay, we confirmed that K579 was dissociated from the enzyme-inhibitor complex in a time-dependent manner when enzyme-inhibitor complex was 20-fold diluted in substrate (data not shown).

The results of present in vivo study demonstrated that K579 was effective in rats after oral administration, and that it acted by stimulating insulin secretion in a glucose concentration-dependent manner. This property as a dipeptidyl peptidase IV inhibitor was also indicated by previous reports showing that the existing dipeptidyl peptidase IV inhibitors enhanced physiological insulin secretion in response to glucose loading (Balkan et al., 1999; Miyake et al., 2002; Nagakura et al., 2003; Pospisilik et al., 2002; Sudre et al., 2002; Villhauer et al., 2003). These dependencies on glucose concentration are mainly due to the profile of GLP-1 as an incretin (Drucker, 1998; Goke et al., 1993; Holst, 1997). Glucose concentration dependency for insulin secretion and reduction of plasma glucose indicated that K579 had a lower risk of inducing hypoglycemia than conventional sulfonylureas.

In conclusion, K579 was a slower binding dipeptidyl peptidase IV inhibitor in vitro than NVP-DPP728. The in vivo effects of K579 (i.e., inhibition of dipeptidyl peptidase IV in plasma and resulting suppression of blood glucose elevation) lasted longer than those of NVP-DPP728 which might be advantageous of K579 over the existing dipeptidyl peptidase IV inhibitors with the potential to require less frequent dosing for controlling hyperglycemia.

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