Interrelationship of dipeptidyl peptidase IV (DPP4) with the development of diabetes, dyslipidaemia and nephropathy: a streptozotocin-induced model using wild-type and DPP4-deficient rats

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Abstract

We examined the role of dipeptidyl peptidase IV (DPP4) in the development of diabetes, dyslipidaemia and renal dysfunction induced by streptozotocin (STZ). F344/DuCrIj rats, which lack DPP4 activity, and wild-type rats were treated with STZ. Plasma DPP4 activity and biochemical parameters were measured until 42 days after STZ treatment. At the end of the experiment, renal function and DPP4 expressions of the kidney, liver, pancreas and adipose tissues were determined. Increases in blood glucose, cholesterol and triglycerides were evoked by STZ in both rat strains; however, the onset of hyperglycaemia was delayed in DPP4-deficient rats as compared with wild-type rats. By contrast, more severe dyslipidaemia was observed in DPP4-deficient rats than in wild-type rats after STZ treatment. Plasma DPP4 activity increased progressively with time after

STZ treatment in wild-type rats. The kidney of wild-type rats showed decreased DPP4 activity with increased *Dpp4* mRNA after STZ treatment. In addition, kidney weight, serum creatinine and excreted amounts of urinary protein, glucose and DPP4 enzyme were enhanced by STZ. DPP4-deficient rats showed increased serum creatinine in accordance with decreased creatinine clearance as compared with wild-type rats after STZ treatment. In conclusion, plasma DPP4 activity increased after STZ treatment, positively correlating to blood glucose. DPP4-deficient rats were resistant to developing diabetes, while susceptible to dyslipidaemia and reduction of glomerular filtration rate by STZ. DPP4 activation may be responsible for hyperglycaemia, lipid metabolism and preservation of renal function.

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Introduction

Dipeptidyl peptidase IV (DPP4, EC3.4.14.5) exists on the surface of various types of cells, such as kidney, liver, small intestine, pancreas, and in a soluble form in plasma (Mentlein 1999). DPP4 is a serine protease, which cleaves the penultimate *L*-proline or *L*-alanine at the N-terminus of several polypeptides, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP; De Meester *et al.* 2000). The major incretin hormones, GLP-1 and GIP, are gastrointestinal peptides involved in the regulation of postprandial nutrient homeostasis (Green *et al.* 2004a, Holst 2006). These peptides augment nutrient-induced insulin release from pancreatic β -cells in a glucose-dependent fashion (Green *et al.* 2004a,b, Holst 2006). These are also reported to be associated with insulin biosynthesis, proliferation of pancreatic β -cells and inhibition of food intake (Pospisilik *et al.* 2003, Holst 2006). Abundant studies in both human and animal models have established DPP4 inhibition, followed by GLP-1 elevation, as a promising therapeutic strategy for the

treatment of diabetes (Deacon *et al.* 2002, Pospisilik *et al.* 2003, Flock *et al.* 2007, Fonseca *et al.* 2007, Mikhail 2008). The Food and Drug Administration and the European Medicines Agency recently approved a DPP4 inhibitor for use in type 2 diabetic patients. However, it remains unclear whether DPP4 activity is correlated to the onset or severity of diabetes and diabetic complications or not. In an *in vitro* study, DPP4 activity and mRNA expression were enhanced by exposure of human glomerular endothelial cells to high glucose (Pala *et al.* 2003). On the other hand, in various published clinical reports over the last decade, circulating DPP4 activity has been reported to be both increased (Mannucci *et al.* 2005, Ryskjaer *et al.* 2006) and decreased (Meneilly *et al.* 2000, McKillop *et al.* 2008) in diabetic patients. Additionally, it has been reported that the degree of plasma DPP4 activity was associated with obesity (Lugari *et al.* 2004), gender (Durinx *et al.* 2001) and aging (Meneilly *et al.* 2000, Durinx *et al.* 2001, Ryskjaer *et al.* 2006). Moreover, several reports, including clinical studies, have confirmed that widely used anti-diabetic agents, metformin (Lenhard *et al.* 2004, Lindsay *et al.* 2005, Green *et al.* 2006) and pioglitazone

(Lenhard *et al.* 2004), reduced the levels of circulating DPP4 activity *in vivo*. Considering these clinical findings, it may be complicated to define the correlation of DPP4 activity to the severity of hyperglycaemia because of the diverse clinical background; therefore, experimental diabetic models with unified conditions can support to identify whether DPP4 activation is physiologically involved or not.

F344/DuCrCrJ rats, which are a substrain of the inbred Fischer 344 (F344) strain maintained at Charles River Japan (Osaka, Japan) lack DPP4 enzyme activity (Thompson *et al.* 1991, Erickson *et al.* 1992, Mitani *et al.* 2002, Yasuda *et al.* 2002). F344/DuCrCrJ rats express *Dpp4* mRNA but lack DPP4 activity due to the translation of abnormal isoforms that fail to be processed to the biologically active mature glycosylated enzyme (Thompson *et al.* 1991, Erickson *et al.* 1992). F344/DuCrCrJ rats have reduced GLP-1 degradation ability, which leads to higher levels of plasma active GLP-1, as compared with wild-type F344/Jcl rats (Yasuda *et al.* 2002). Accordingly, F344/DuCrCrJ rats, compared with wild-type F344/Jcl rats, can efficiently assess the responsibility of DPP4 activity for the development of diabetes and diabetic complications such as nephropathy. A couple of studies have previously reported that high-fat diet-induced insulin resistance and glucose intolerance were less prevalent in F344/DuCrCrJ rats than wild-type F344/Jcl rats (Mitani *et al.* 2002, Yasuda *et al.* 2002); however, no data have determined the significance of DPP4 activity in insulin-dependent diabetes, dyslipidaemia and diabetic renal dysfunction.

In the present study, the changes in DPP4 expressions of plasma and several tissues (kidney, liver, pancreas and adipose tissue) were determined in a streptozotocin (STZ)-induced diabetic model. In addition, differences in the levels of blood glucose, insulin, cholesterol, triglycerides, creatinine and urinary parameters were determined between F344/Jcl (wild-type) and F344/DuCrCrJ (DPP4-deficient) rats treated with or without STZ, to assess the importance of DPP4 activity in hyperglycaemia, lipid metabolism and renal function.

Materials and Methods

Experimental design

Male F344/Jcl (wild-type) and F344/DuCrCrJ (DPP4-deficient) rats aged 8 weeks were purchased from Clea Japan (Tokyo, Japan) and Charles River Japan (Osaka, Japan) respectively. All rats were housed in an air-conditioned room at $23 \pm 2^\circ\text{C}$ with $50 \pm 10\%$ humidity under controlled lighting conditions (12h light: 12h darkness cycle). The rats were fed a commercial diet (MF; Oriental Yeast, Tokyo, Japan) and had access to tap water *ad libitum*. At 9 weeks of age, rats were randomly divided into the following four groups: wild-type (control), $n=6$; wild-type (STZ), $n=10$; DPP4-deficient (control), $n=6$; and DPP4-deficient (STZ), $n=10$. Experimental diabetes was induced by a single i.p. injection of STZ (30 mg/kg; Wako Pure Chemical, Osaka,

Japan) in 0.05 M citrate buffer (pH 4.5) after an overnight fast, and then the rats were re-fed. Control rats received an equivalent amount of citrate buffer. Before and 6 h, 1, 3, 14, 28 and 42 days after injection of STZ or citrate buffer, blood samples were obtained from tail veins. Over a 24-hour period between days 35 and 37, the rat was placed in a metabolic cage containing a urine collection funnel to measure urine excretion. All rats had access to food and water *ad libitum* while in the cages. Urine was collected for exactly 24 h and the volume was measured. Urine samples were stored at -20°C until the measurement of several parameters. At the end of the experimental period, the right kidney, liver, pancreas and epididymal fat were immediately collected and weighed after the rats were killed under anaesthesia with urethane (5 g/kg, intraperitoneally; Sigma). The collected tissues for DPP4 enzyme assay and the pancreas for insulin assay were immediately frozen and stored at -80°C . For purification of RNA from each tissue, the harvested samples were immediately cut into slices less than 5 mm thick, and the slices were stored in RNAlater RNA Stabilization Reagent (Qiagen) at -20°C . All animal care and treatments were conducted in accordance with the guidelines of the animal use and care committee of the University of Tokushima.

Measurement of blood glucose, plasma insulin and lipid parameters

After blood samples were obtained from tail veins, glucose concentrations were immediately measured using a glucose analyser (Glucose Pilot; Avenitir Biotech, Carlsbad, CA, USA). The collected blood samples were immediately centrifuged to separate plasma. Plasma insulin concentration was measured by ELISA using an Ultra-Sensitive Rat Insulin Kit (Morinaga Institute of Biological Science, Yokohama, Japan; Iwakura *et al.* 2005). The levels of cholesterol and triglycerides were measured using reagents manufactured by Wako Pure Chemical Industries.

DPP4 enzyme assay in plasma

DPP4 activity was determined by the cleavage rate of 7-amino-4-methylcoumarin (AMC) from the synthetic substrate *H*-glycyl-prolyl-AMC (Gly-Pro-AMC; Sigma), as described previously (Villhauer *et al.* 2003), with some modifications. Briefly, 5 μl sample was mixed with 35 μl assay buffer (25 mmol/l HEPES, 140 mmol/l NaCl, 80 mmol/l MgCl_2 , 1% (w/v) BSA (pH 7.8)). After 5-min preincubation at room temperature, the reaction was initiated by the addition of 40 μl assay buffer containing 0.1 mmol/l substrate Gly-Pro-AMC. After incubation for 20 min, fluorescence was determined using a spectrofluorometer (Tecan Infinite M200, Tecan Japan, Yokohama; excitation 380 nm/emission 460 nm). The standard curve of free AMC was generated using 0–50 $\mu\text{mol/l}$ solutions of AMC (Sigma). DPP4 activity in plasma was expressed as the amount of cleaved AMC per minute per ml (nmol/min/ml).

Immunohistochemistry

The pancreas was dissected from surrounding tissue, fixed overnight in 10% (v/v) formaldehyde and embedded in paraffin. Immunostaining was conducted according to the standard peroxidase reaction method. Briefly, sections were incubated with 3% (v/v) H₂O₂ for 10 min to avoid non-specific peroxidase reactions. Histological sections were immunostained for insulin using monoclonal anti-insulin (1:1000 dilution; Sigma) as the primary antibody and peroxidase-conjugated rabbit anti-mouse IgG (1:200 dilution; Sigma) as the secondary antibody. The sections were then incubated with 3,3'-diaminobenzidine tetrahydrochloride solution for 15 min. After immunostaining, the sections were lightly counterstained with Mayer's hematoxylin, and immunoreactive cells were observed under a light microscope.

Pancreatic insulin content

Pancreatic insulin was extracted with the acid ethanol method (0.18 mmol/l hydrochloric acid in 70% (v/v) ethanol; Zhou *et al.* 2003), and then the insulin concentrations were determined (Ultra Sensitive Rat Insulin Kit; Morinaga Institute of Biological Science). Data were normalized by the protein concentration (Bradford Protein Assay; Bio-Rad Laboratories).

DPP4 enzyme assay in kidney, liver, pancreas and epididymal fat

Frozen tissues were homogenized in cold buffer (25 mmol/l HEPES, 140 mmol/l NaCl, 80 mmol/l MgCl₂ (pH 7.8)) containing 1% (v/v) Triton X-100. Following homogenization, the samples were centrifuged at 1000 *g* for 10 min at 4 °C. The supernatants were collected and centrifuged twice at 20 000 *g* for 10 min at 4 °C. The final supernatants were immediately used for DPP4 enzyme assay, which was described above. DPP4 activity in each tissue was expressed as the amount of cleaved AMC per minute per tissue weight (nmol/min/g tissue).

Quantitative real-time PCR

Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen), according to the manufacturer's methods. cDNA was synthesized using 1 µg total RNA and SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed using SYBR Premix Ex Taq (Takara, Tokyo, Japan) on the AB 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling profile: initial denaturation at 95 °C for 10 s followed by 40 cycles of amplification (denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and extension at 72 °C for 34 s) and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used in real-time PCR were the following: rat

DPP4 sense 5'-CTCCAGAGGACAACCTTGAC-3', antisense 5'-GGACAAGTGTGCTCTTGAGT-3'; rat GAPDH sense 5'-CTGAGAATGGGAAGCTGGTCAT-3', antisense 5'-TGGTGCAGGATGCATTGCT-3'.

Urinary parameters and renal function

Proteinuria was assessed by the Bradford method (Bradford Protein Assay Kit; Bio-Rad Laboratories). Urinary glucose concentrations were determined using a glucose analyser (Glucose Pilot; Avenitir Biotech). DPP4 activity in urine was determined using the same method as for the plasma DPP4 assay. Serum and urinary creatinine were measured by the Jaffé method using a commercial reagent (Wako Pure Chemical). Data are shown as urinary excretion (ml/24h), protein excretion (mg/24 h), glucose excretion (nmol/24 h) and DPP4 excretion (nmol/min/24 h). Creatinine clearance (Ccr: ml/min/kg) was used to estimate glomerular filtration rate (GFR).

Data analysis

All values are expressed as the means $\pm$ S.E.M. Statistical significance was evaluated by Student's unpaired *t*-test for comparison between two groups using JMP (release 7.0; SAS Institute, Cary, NC, USA).

Results

Body weight, food intake, blood glucose, plasma insulin, cholesterol, triglycerides and DPP4 activity

No significant differences in body weight, food intake, blood glucose, insulin, cholesterol and triglycerides were found between F344/Jcl (wild-type) and F344/DuCrIcrJ (DPP4-deficient) rats at 0 days under fasting (Fig. 1). The levels of blood glucose, cholesterol and triglycerides were not significantly different between these rat strains in the control group (not treated with STZ) at any points of the experiment (Fig. 1). However, the levels of plasma insulin in DPP4-deficient (control) rats were significantly lower than that in the wild-type (control) 6 h to 42 days after feeding (Fig. 1e and f). In STZ-treated groups, limited body weight gain (Fig. 1a) and increased food intake (Fig. 1b) were observed as compared with the control in both rat strains. No significant difference in body weight was found between wild-type and DPP4-deficient rats treated with or without STZ, but food intake 7 days after STZ treatment in DPP4-deficient rats was significantly less than that in wild-type rats (Fig. 1b). Although marked hyperglycaemia and hypoinsulinaemia occurred after STZ injection in both rat strains, DPP4-deficient rats exhibited relatively lower blood glucose than wild-type rats in the early phase (6 h to 28 days) after STZ treatment despite no differences in insulin levels (Fig. 1c-f). Blood glucose levels in both rat strains reached a plateau (about 30 mmol/l) 42 days after STZ treatment. Regarding the results of lipid parameters, STZ elevated the

Figure 1 Changes in (a) body weight, (b) food intake, (c–d) blood glucose, (e–f) plasma insulin, (g) cholesterol, (h) triglycerides and (i–j) plasma DPP4 activity in F344/Jcl (wild-type) and F344/DuCrIj (DPP4-deficient) rats after i.p. injection with or without STZ (30 mg/kg). Data are shown as the means $\pm$ S.E.M. * P <0.05, ** P <0.01 and *** P <0.001 for differences between wild-type (control) and wild-type (STZ). # P <0.01 and ## P <0.001 for differences between DPP4-deficient (control) and DPP4-deficient (STZ). † P <0.05 and †† P <0.001 for differences between wild-type (control) and DPP4-deficient (control). § P <0.05, §§ P <0.01 and §§§ P <0.001 for differences between wild-type (STZ) and DPP4-deficient (STZ). *** and ### in Fig. 1a are the values from 1 to 42 days respectively. *** and ### in Fig. 1b are the values from 7 to 42 days respectively. Open circles: wild-type (control) n =10; closed circles: wild-type (STZ) n =10; open triangles: DPP4-deficient (control) n =6; closed triangles: DPP4-deficient (STZ) n =10.

levels of cholesterol (Fig. 1g) and triglycerides (Fig. 1h) to some extent in wild-type rats, exhibiting significant differences in cholesterol levels at 28–42 days and triglycerides levels at 1–3 days respectively. In DPP4-deficient rats, however, both cholesterol and triglycerides were considerably elevated by STZ from 3 days to the end of the experiment, which were significantly higher than those in wild-type (STZ), as shown in Fig. 1g and h. For further assessment of the significance of DPP4 activity in diabetes and dyslipidaemia, changes in DPP4 activity were monitored in the process of developing metabolic abnormalities. Plasma DPP4 activity increased progressively with time after STZ treatment in wild-type rats (Fig. 1i and j). Significant increases in plasma DPP4 activity of wild-type (STZ) were observed from 6 h to the end as compared with wild-type (control). Next, we investigated the correlation of plasma DPP4 activity with blood glucose or insulin in wild-type F344/Jcl rats exposed to STZ or vehicle. As a result, the degree of plasma DPP4 activity was positively correlated to the levels of blood glucose (Fig. 2a) and negatively correlated to plasma insulin

Figure 2 The correlation of plasma DPP4 activity to the levels of (a) blood glucose, (b) plasma insulin in F344/Jcl (wild-type) rats treated with or without STZ (30 mg/kg). (a) r^2 =0.6584 (P <0.001); (b) r^2 =0.6778 (P <0.001).

Figure 3 Histological study for insulin content of the pancreas in F344/Jcl (wild-type) and F344/DuCrIcrIj (DPP4-deficient) rats 42 days after i.p. injection with or without STZ (30 mg/kg). (a) Wild-type (control); (b) wild-type (STZ); (c) DPP4-deficient (control); (d) DPP4-deficient (STZ).

(Fig. 2b) in wild-type rats respectively. Plasma DPP4 activity in F344/DuCrIcrIj rats was not detected at any point in the experiment.

Histological and quantitative studies of insulin content in the pancreas

Insulin content in the pancreas was measured to assess the effects of DPP4 deficiency on STZ-induced islet destruction. In control groups, there were no differences in visual islet β -cell number per unit area (Fig. 3a and c) and total pancreatic insulin content (Table 1) between wild-type and DPP4-deficient rats. At 42 days after STZ treatment, immunostained insulin was little observed in both wild-type rats (Fig. 3b) and DPP4-deficient rats (Fig. 3d). Quantitative study also showed that STZ caused a marked reduction of

pancreatic insulin content in both rat strains, and STZ-induced depletion of pancreatic insulin was not significantly different between these rat strains (Table 1).

Tissue weight and DPP4 expressions in tissues 42 days after STZ or vehicle treatment

There were no differences in kidney weight and epididymal fat per body weight between wild-type and DPP4-deficient rats in the control, but the liver and pancreas in DPP4-deficient rats were significantly smaller than in wild-type rats in the control (Table 2). In STZ-treated groups, the weight (g/kg body weight) of the kidney, liver and pancreas significantly increased, while the weight of epididymal fat markedly decreased as compared with control groups. DPP4 activity was detected in each tissue of wild-type rats (Table 2). DPP4 activity of the kidney significantly

Table 1 Total pancreatic insulin content 42 days after treatment with or without streptozotocin (STZ). Data are shown as the means $\pm$ S.E.M.

	Wild-type rats		DPP4-deficient rats	
	Control	STZ	Control	STZ
Total pancreatic insulin (pmol/mg protein)	114.4 $\pm$ 16.4	0.8 $\pm$ 0.2*	107.5 $\pm$ 37.0	0.7 $\pm$ 0.1 [†]

* $P < 0.001$ versus wild-type (control). [†] $P < 0.001$ versus DPP4-deficient (control). Wild-type (control) $n = 6$; wild-type (STZ) $n = 10$; DPP4-deficient (control) $n = 6$; DPP4-deficient (STZ) $n = 10$.

Table 2 Tissue weight and DPP4 expressions 42 days after treatment with or without streptozotocin (STZ). Data are shown as the means ± S.E.M.

	Wild-type rats		DPP4-deficient rats	
	Control	STZ	Control	STZ
Tissue weight (g/kg body weight)				
Kidney	3.0±0.1	5.5±0.1 [‡]	3.0±0.1	5.4±0.2
Liver	36.0±0.5	43.3±0.8 [‡]	30.8±0.5 ^a	40.5±1.0
Pancreas	2.0±0.2	2.8±0.1 [‡]	1.4±0.1 [¶]	2.6±0.1
Epididymal fat	23.3±1.2	5.1±0.8 [‡]	23.3±0.7	6.0±1.3
DPP4 activity (nmol min ⁻¹ g tissue ⁻¹)				
Kidney	1460.8±54.9	1206.2±55.4 [‡]	ND	ND
Liver	119.7±9.6	295.8±58.1 [‡]	ND	ND
Pancreas	11.2±0.8	14.4±1.6	ND	ND
Epididymal fat	19.7±2.8	38.0±2.7 [‡]	ND	ND
<i>Dpp4/Gapdh</i> mRNA				
Kidney	1.94±0.04	2.16±0.04 [‡]	2.36±0.06 ^b	2.27±0.05
Liver	1.72±0.08	2.31±0.12 [‡]	2.20±0.12 [¶]	2.46±0.13
Pancreas	0.68±0.07	1.12±0.12 [*]	0.75±0.15	1.17±0.11 [§]

**P*<0.05, [‡]*P*<0.01, [‡]*P*<0.001 versus wild-type (control). [§]*P*<0.05, ^{||}*P*<0.001 versus DPP4-deficient (control). [¶]*P*<0.05, ^a*P*<0.01, ^b*P*<0.001 versus wild-type (control). Wild-type (control) *n*=6; wild-type (STZ) *n*=10; DPP4-deficient (control) *n*=6; DPP4-deficient (STZ) *n*=10. ND, not detected.

decreased after STZ treatment in wild-type rats. By contrast, DPP4 activity of the liver and epididymal fat was significantly enhanced by STZ in wild-type rats. In F344/DuCrIrlj rats, DPP4 activity in each tissue was not detected. On the other hand, quantitative real-time PCR showed that F344/DuCrIrlj rats contained significantly larger amounts of *Dpp4* mRNA in the kidney and liver than in wild-type F344/Jcl rats in the control (Table 2). In STZ-treated groups, STZ significantly up-regulated *Dpp4* mRNA expressions of the kidney and liver in F344/Jcl rats and the pancreas in both rat strains. RNA in epididymal fat could not be extracted in this experiment.

Urinary parameters and renal function

Table 3 shows the changes in urinary parameters in wild-type and DPP4-deficient rats exposed to STZ or vehicle. Urinary excretion, protein excretion and glucose excretion for 24 h were not different between these rat strains in the control. These parameters were significantly elevated by STZ in both wild-type and DPP4-deficient rats. There were no significant differences in these parameters between rat strains treated with STZ. DPP4 activity in urine was detected in wild-type F344/Jcl rats but not

F344/DuCrIrlj rats. DPP4 excretion for 24 h was significantly enhanced by STZ treatment. Figure 4 shows the importance of DPP4 activity in renal function. No significant differences in serum creatinine levels were found between wild-type and DPP4-deficient rats before STZ treatment. After STZ treatment, serum creatinine levels significantly increased at 1–28 days in wild-type rats. In DPP4-deficient rats, however, serum creatinine levels were considerably elevated by STZ from 1 day to the end of the experiment, and were significantly higher than that in the wild-type (STZ), as shown in Fig. 4a. In agreement with this, creatinine clearance (Ccr) in DPP4-deficient (STZ) was significantly lower than that in wild-type (STZ), as shown in Fig. 4b.

Discussion

STZ is commonly used to induce type 1 and late-phase type 2 diabetic models by selective β-cell destruction in small rodents (Pospisilik *et al.* 2003, Srinivasan *et al.* 2005). In order to examine the roles of DPP4 in metabolic abnormality and renal dysfunction, we produced type 1 diabetic rats using STZ,

Table 3 Urinary parameters in wild-type and DPP4-deficient rats treated with or without streptozotocin (STZ). Data are shown as the means ± S.E.M.

	Wild-type rats		DPP4-deficient rats	
	Control	STZ	Control	STZ
Urinary excretion (ml/24 h)	5.2±0.9	97.5±11.2 [*]	5.8±0.7	83.8±8.0 [‡]
Protein excretion (mg/24 h)	11.1±1.8	50.2±9.8 [*]	11.2±1.2	33.9±4.1 [‡]
Glucose excretion (nmol/24 h)	0.09±0.1	12.7±1.9 [*]	0.08±0.01	12.0±1.9 [‡]
DPP4 excretion (nmol/min per 24 h)	11.3±4.8	72.4±12.4 [*]	ND	ND

**P*<0.001 versus wild-type (control). [‡]*P*<0.001 versus DPP4-deficient (control). Wild-type (control) *n*=6; wild-type (STZ) *n*=10; DPP4-deficient (control) *n*=6; DPP4-deficient (STZ) *n*=10. ND, not detected.

Figure 4 Interrelationship between DPP4 activity and renal function. (a) Change in serum creatinine levels in F344/Jcl (wild-type) and F344/DuCrIcrIj (DPP4-deficient) rats after i.p. injection with or without STZ (30 mg/kg). Data are the means $\pm$ S.E.M. * P < 0.05 and ** P < 0.01 for differences between wild-type (control) and wild-type (STZ). ** P < 0.01 and *** P < 0.001 for differences between DPP4-deficient (control) and DPP4-deficient (STZ). * P < 0.05 and ** P < 0.01 for differences between wild-type (STZ) and DPP4-deficient (STZ). Open circles: wild-type (control) n = 6; closed circles: wild-type (STZ) n = 10; open triangles: DPP4-deficient (control) n = 6; closed triangles: DPP4-deficient (STZ) n = 10. (b) Difference in creatinine clearance (Ccr) between F344/Jcl (wild-type) and F344/DuCrIcrIj (DPP4-deficient) rats treated with STZ (30 mg/kg). ** P < 0.01 for difference between wild-type (STZ) and DPP4-deficient (STZ) n = 10.

with extensive islet destruction and depletion of both pancreatic and plasma insulin. In STZ-induced diabetic rats, dyslipidaemia and marked loss of adipose tissue mass, which are characteristics of insulin insufficiency, were also observed. Moreover, it was confirmed that STZ enhanced both expressions of *Dpp4* mRNA in all examined tissues and circulating DPP4 enzyme activity in wild-type rats, showing a positive correlation of plasma DPP4 activity to blood glucose. The main source of endogenous DPP4 has been proposed to be microvascular endothelial cells of some areas, including the kidney (Augustyns *et al.* 1999, Pala *et al.* 2003). An *in vitro* study determined that exposure to high glucose caused increases in *Dpp4* mRNA and enzyme activity in human glomerular endothelial cells (Pala *et al.* 2003). From these findings, it is suggested that the STZ-induced increase in plasma DPP4 activity is attributed to enhanced biosynthesis of DPP4 enzyme and its secretion in endothelial cells by high blood glucose. It may also be reasonable that hyperglycaemia-induced cell damage, including the kidney, caused DPP4 to leak into the circulation, since kidney DPP4 activity was reduced even though kidney *Dpp4* mRNA was up-regulated in STZ-induced diabetes. In addition, the increased activity of plasma DPP4 could still worsen hyperglycaemia since DPP4 activation may lead to decreases in the anti-diabetic effects of GLP-1 and GIP; therefore, it was supposed that DPP4 inhibition was effective in ameliorating STZ-induced metabolic abnormalities. A DPP4 inhibitor, P32/98, was reported to reduce the progressive severity of diabetes induced by STZ in Wistar rats, considering that DPP4 inhibition followed by increased GLP-1 and GIP prevented insulin-secretory capacity via enhanced islet neogenesis, β -cell survival and insulin biosynthesis (Pospisilik *et al.* 2003). Consistent with

this, mice lacking the gene encoding DPP4 were refractory to loss of β -cell mass and hyperglycaemia evoked by high-fat diet for 8 weeks followed by STZ injection (Conarello *et al.* 2003). In the present study, DPP4-deficient rats exhibited significantly lower levels of blood glucose and food intake than wild-type rats in the early phase after STZ treatment, even though STZ-induced insulin depletion occurred in DPP4-deficient rats as well as wild-type rats. The failure of DPP4 deficiency to prevent β -cell function from STZ exposure could be attributed to the severe islet destruction and depletion of pancreatic insulin in this experiment. In addition, in control groups, the levels of plasma insulin in DPP4-deficient rats were significantly lower than that in wild-type rats after feeding, which could indicate that DPP4-deficient rats were more sensitive to insulin than wild-type rats. Accordingly, the present study suggests that DPP4-deficient rats are resistant to developing hyperglycaemia owing to lower food intake and higher insulin sensitivity.

Contrary to our expectations, greater increases in circulating cholesterol and triglycerides were found in DPP4-deficient rats than wild-type rats after STZ treatment. These results imply that DPP4-deficient rats are resistant to developing hyperglycaemia, but susceptible to dyslipidaemia induced by STZ. Recent studies of obesity have proposed that GIP, a substrate of DPP4, plays an important role in lipid abnormality since GIP is a key element linking overnutrition to obesity through increased nutrient uptake and triglyceride accumulation in adipocytes (Miyawaki *et al.* 2002, McClean *et al.* 2007). However, the mechanisms underlying excessive increases in circulating lipid by deficiency of DPP4 activity are unknown in non-obese diabetes. In wild-type rats, hypertriglyceridaemia occurred significantly in only the early phase (1–3 days) after STZ treatment; thus, the elevation of triglycerides appears to be attenuated by increased plasma DPP4 activity after STZ treatment. Furthermore, STZ also enhanced liver DPP4 activity and mRNA expression. Although additional studies characterizing the roles of DPP4 activity in the liver or small intestine in lipid transport, biosynthesis and secretion are needed, DPP4 activation may enable lipid metabolism in STZ-induced non-obese diabetes.

The importance of DPP4 in renal function was assessed in this model. As a result, decreased DPP4 enzyme activity and increased *Dpp4* mRNA expression were detected in the kidney of wild-type rats after STZ treatment. Moreover, the ratio of kidney weight to body weight, serum creatinine levels and excreted amounts of urinary protein, glucose and DPP4 enzyme were significantly enhanced by STZ. Increases in kidney weight, serum creatinine and urinary excretion of protein and glucose have been detected in clinical and experimental diabetic nephropathy. The reasons for decreased kidney DPP4 activity and increased urinary DPP4 excretion by STZ remain uncertain; however, they may occur as a result of renal damage, which causes the leakage of microvascular endothelial DPP4 enzyme of the kidney, as suggested by previous reports (Scherberich *et al.* 1992, Augustyns *et al.* 1999, Pala *et al.* 2003). Of note, this comparative study of wild-type

and DPP4-deficient rats determined that DPP4-deficient rats were more sensitive to GFR reduction induced by STZ. This finding indicates that DPP4 activity in plasma and/or kidney may be closely related to protection against renal dysfunction. DPP4 is most expressed in the kidney; however, the role of DPP4 activity in renal function is poorly understood. A recent study reported that the inhibition of DPP4 activity in kidney augmented the ability of endogenous neuropeptide Y_{1–36} released from renal sympathetic nerves to enhance angiotensin II-mediated renal vasoconstriction *in vitro*, since neuropeptide Y_{1–36} is a substrate for DPP4 (Jackson & Mi 2008). This could explain the reduction in GFR in DPP4-deficient rats. Another possibility is that dyslipidaemia is one of the risk factors of glomerular sclerosis, proteinuria and GFR reduction (Molitch 2006, Fogo 2007). In the present study, however, the time course of serum creatinine shows that the GFR reduction in DPP4-deficient rats occurred rapidly (within days) after STZ treatment. Therefore, it is unlikely that the increased circulating lipid in DPP4-deficient rats could alter renal function within this time frame. Moreover, there were no significant differences in urinary protein excretion and kidney weight (renal hypertrophy) between wild-type and DPP4-deficient rats after STZ treatment. Considering all of the above, it is strongly presumed that the adverse effect of DPP4 deficiency on the kidney is functional rather than structural. However, it is possible that a long-term blockade of DPP4 accompanied by chronic dyslipidaemic state will lead to renal failure structurally as well as functionally. In addition, chronic renal disease, especially with nephrotic syndrome, has been reported to develop dyslipidaemia (Farbakhsh & Kasiske 2005, Molitch 2006), which suggests that a close relationship exists among dyslipidaemia, renal dysfunction and DPP4 in late-stage diabetes, but further studies are required.

In conclusion, DPP4 deficiency delayed the onset of diabetes, but worsened dyslipidaemia and renal dysfunction induced by STZ. This study could be the basis of additional research into the advantages and disadvantages of DPP4 inhibition for insulin-dependent diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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