

Increased Plasma Dipeptidyl Peptidase IV (DPP IV) Activity and Decreased DPP IV Activity of Visceral But Not Subcutaneous Adipose Tissue in Impaired Glucose Tolerance Rats Induced by High-Fat or High-Sucrose Diet

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Several studies have investigated whether dipeptidyl peptidase IV (DPP IV) activity is correlated to the severity of diabetes; however, it remains unclear. To investigate the roles of DPP IV activity in metabolic abnormalities, impaired glucose tolerance rats were produced using a high-fat (HF) or high-sucrose (HS) diet. HF diet-fed rats obviously exhibited impaired glucose tolerance, with increases in subcutaneous and epididymal fat mass, insulin resistance and dyslipidaemia. In rats fed a HS diet rather than a normal diet, lower body weight and fasting blood glucose were observed temporarily in the early period after HS diet feeding; however, impaired glucose tolerance was evoked to some extent with an increase in epididymal fat mass. Both HF and HS diet-fed rats showed significantly higher plasma DPP IV activity than normal diet-fed rats, in the order of HF diet > HS diet > normal diet. HF and HS diets did not significantly affect DPP IV activity and mRNA expression in the kidney. On the other hand, HF, but not HS, diet caused a significant decrease in DPP IV activity in the liver as compared to the control. Of note, both HF and HS diets caused a significant decrease in DPP IV activity in epididymal fat, even though they did not change DPP IV activity in subcutaneous fat. In conclusion, HF or HS diet-induced impaired glucose tolerance with visceral fat accumulation may be interrelated with increased plasma DPP IV activity and decreased DPP IV activity of visceral but not subcutaneous adipose tissue.

Key words dipeptidyl peptidase IV; impaired glucose tolerance; visceral adipose tissue; high-fat diet; high-sucrose diet; insulin resistance

Dipeptidyl peptidase IV (DPP IV, EC3.4.14.5) exists on the surface of various types of cells, particularly the kidney, liver, small intestine, and in a soluble form in plasma.¹⁾ DPP IV has been recognized to play an important role in the cleavage and inactivation of biologically active peptides with penultimate L-proline or L-alanine at the N-terminus, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP).²⁾ GLP-1 and GIP, incretin hormones, are responsible for postprandial glucose homeostasis, mainly through the regulation of nutrient-induced insulin release from pancreatic β cells in a glucose-dependent fashion.^{3,4)} These peptides are also reported to be involved in insulin biosynthesis, proliferation of pancreatic β cells and inhibition of food intake.^{4,5)} A variety of studies over the last decade have confirmed that DPP IV inhibition could exert beneficial effects on postprandial glucose intolerance as well as impaired insulin-secretory capacity through active GLP-1 elevation.^{6–11)} Recently, the Food and Drug Administration and the European Medicines Agency approved a DPP IV inhibitor, sitagliptin,^{10,11)} for use in type 2 diabetic patients. Accordingly, it has become more significant to determine the role of DPP IV activation in the process of glucose intolerance, type 2 diabetes and diabetic complications. However, it is still unclear whether DPP IV activation is correlated to the onset or severity of diabetes and diabetes-related diseases such as dyslipidaemia, obesity and nephropathy.

Overnutrition, especially a high-fat diet, is a well-known risk factor for obesity accompanied by insulin resistance and hepatic hypertrophy with fatty degeneration.^{6,12,13)} Several

studies in small rodents have reported that DPP IV inhibitors improved oral glucose tolerance in high-fat diet-fed animals.^{6,11)} Moreover, it has been confirmed that high-fat diet-induced insulin resistance and glucose intolerance were less prevalent in DPP IV-deficient rats than wild-type rats.^{6,14)} Considering these findings, DPP IV activity is supposed to be closely related to overnutrition-induced glucose intolerance; however, no data have determined changes in plasma DPP IV activity in the process of developing obesity, impaired glucose tolerance and dyslipidaemia evoked by a high-fat diet. In addition, the roles of DPP IV activity in subcutaneous and visceral adipose tissues in obesity and diabetes have not been investigated yet.

In the present study, differences in DPP IV expressions of plasma and tissues (kidney, liver, subcutaneous and visceral adipose tissues) were determined among rats fed a normal, high-sucrose (HS; 54% sucrose) or high-fat (HF; 82% fat) diet, in order to investigate the interrelationship of DPP IV activity with impaired glucose tolerance and visceral fat accumulation. The HF diet-fed rat was used as a typical impaired glucose tolerance model with obesity and dyslipidaemia. By contrast, the HS diet-fed rat was considered as a milder impaired glucose tolerance model with increases in visceral fat mass but not subcutaneous fat mass, body weight or lipid parameters.

MATERIALS AND METHODS

Animals Male F344/Jcl rats, 4 weeks old, were pur-

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chased from Clea Japan (Tokyo, Japan). 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 (12:12-h light–dark cycle). The rats were given free access to diet and water, and then acclimatized for one week on a standard diet (MF: Oriental Yeast, Tokyo, Japan) before use. All animal care and treatments were conducted in accordance with the guidelines of the animal use and care committee of the University of Tokushima.

Chow The following diet was used in this experiment: normal diet (MF: 3.6 kcal/g, 13% fat, 60% carbohydrate, 26% protein); HS diet (F2HScD: 3.7 kcal/g, 6% fat, 80% carbohydrate, 13% protein) and HF diet (F2HFD2: 6.4 kcal/g, 82% fat, 3% carbohydrate, 15% protein). These were purchased from Oriental Yeast.

Experimental Design At 5 weeks of age, rats were randomly divided into the following three groups: normal diet, $n=8$; HS diet, $n=9$ and HF diet, $n=9$. Every two weeks from the start of the experiment, blood samples were obtained from tail veins at 9:00–10:00 a.m. and, 10 weeks after the start of the experiment, an oral glucose tolerance test (OGTT) was performed. At the end (14 weeks) of the experiment, the right kidney, liver, abdominal subcutaneous fat and epididymal fat were immediately collected and weighed after the rats had been killed under anesthesia with urethane (5 g/kg, intraperitoneally; Sigma, St. Louis, MO, U.S.A.). The collected tissues for DPP IV enzyme 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 stored in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) at -20°C .

Measurement of Blood Biochemical Parameters After blood samples were collected, fasting blood glucose (FBG) concentrations were immediately measured using a glucose analyzer (Glucose Pilot; Avenitir Biotech, Carlsbad, CA, U.S.A.). Fasting plasma insulin (FPI) was determined by enzyme-linked immunosorbent assay using an Ultra-Sensitive Rat Insulin Kit (Morinaga Institute of Biological Science, Yokohama, Japan).¹⁵ Homeostasis model assessment–insulin resistance (HOMA-IR) and HOMA- β were calculated from the values of FBG and FPI using the standard formulae to estimate insulin resistance and insulin-secretory capacity, respectively.¹⁶ The levels of triglycerides (TG), total cholesterol (T-CHO), high density lipoprotein (HDL)-cholesterol and creatinine were measured using commercial reagents according to the manufacturer's methods (Wako Pure Chemical Industries, Osaka, Japan).

DPP IV Enzyme Assay in Plasma DPP IV 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,¹⁷ with some modifications. Briefly, 5 μl of sample was mixed with 35 μl of assay buffer (25 mmol/l *N*-(2-hydroxyethyl)piperazine *N'*-2-ethanesulfonic acid (HEPES), 140 mmol/l NaCl, 80 mmol/l MgCl_2 , 1% BSA, pH 7.8). After 5-min preincubation at room temperature, the reaction was initiated by the addition of 40 μl of assay buffer containing 0.1 mmol/l substrate Gly-Pro-AMC. After incubation for 20 min, fluorescence was determined using a spectrofluorometer (Tecan InfiniteTM 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). DPP IV activity in plasma was expressed as the amount of cleaved AMC per minute per ml (nmol/min/ml).

Oral Glucose Tolerance Test (OGTT) After rats were fasted for 18 h, glucose was orally administered (1 g/kg). Blood samples were obtained before and 30, 60, 90 and 120 min after glucose load. Blood glucose concentrations were measured immediately, and data were quantified by calculating the area under the curve ($AUC_{0-120\text{min}}$) using the trapezoidal rule.

DPP IV Enzyme Assay in Kidney, Liver, Abdominal Subcutaneous Fat and Epididymal Fat Frozen tissues were homogenized in cold buffer (25 mmol/l HEPES, 140 mmol/l NaCl, 80 mmol/l MgCl_2 , pH 7.8) containing 1% 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 20000 *g* for 10 min at 4°C . The final supernatants were immediately used for DPP IV enzyme assay, as described above. DPP IV activity in each tissue was normalized by protein concentration (Bradford Protein Assay; Bio-Rad Laboratories, Hercules, CA, U.S.A.), and then expressed as the amount of cleaved AMC per minute (nmol/min/ng protein).

Quantitative Real-Time Polymerase Chain Reaction (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, Carlsbad, CA, U.S.A.). 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, U.S.A.) 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 the real-time PCR were as follows: rat DPP4 sense 5'-CTCCAGAGGACAACCTTGAC-3', antisense 5'-GGACAAGTGTGCTCTTGAGT-3'; rat GAPDH sense 5'-CTGAGAATGGGAAGCTGGTCAT-3', antisense 5'-TGGTGCAGGATGCATTGCT-3'.

Data Analysis All values are expressed as the means \pm S.E.M. One-way ANOVA followed by Dunnett's multiple comparison was performed to evaluate differences among the three groups. Student's unpaired *t*-test was used for comparison between two groups. Differences were considered significant at $p < 0.05$.

RESULTS

Body Weight, Food Intake and Blood Biochemistry In the HS diet group, there were no significant differences in food intake (Fig. 1b), FPI (Fig. 1d) and HOMA-IR (Fig. 1e) compared to the control (normal diet group); however, significantly lower levels of body weight (Fig. 1a) were observed at 1 to 9 weeks. In addition, decreased FBG (Fig. 1c) and increased HOMA- β (Fig. 1f) were found at only 2 weeks in rats fed the HS diet compared with the normal diet. In the HF diet group, significant increases in body weight and calorie intake were found compared to the control. Moreover, HF

Table 1. Blood Biochemistry in Rats Fed a Normal, HS or HF Diet for 12 Weeks

	Normal diet	HS diet	HF diet
Triglycerides (mg/dl)	190.1±14.1	173.0±10.3	483.4±45.0***
Total cholesterol (mg/dl)	59.1±3.8	48.9±3.0	63.8±2.3
HDL-cholesterol (mg/dl)	42.2±2.5	36.9±1.2	26.5±1.1***
T-CHO/HDL	1.4±0.1	1.3±0.1	2.4±0.1***
Creatinine (mg/dl)	0.82±0.05	0.81±0.06	0.89±0.08

Data are shown as the means±S.E.M. ****p*<0.001 vs. normal diet (Dunnett's multiple comparison test). Normal diet: *n*=8; HS diet: *n*=9; HF diet: *n*=9. T-CHO: total cholesterol.

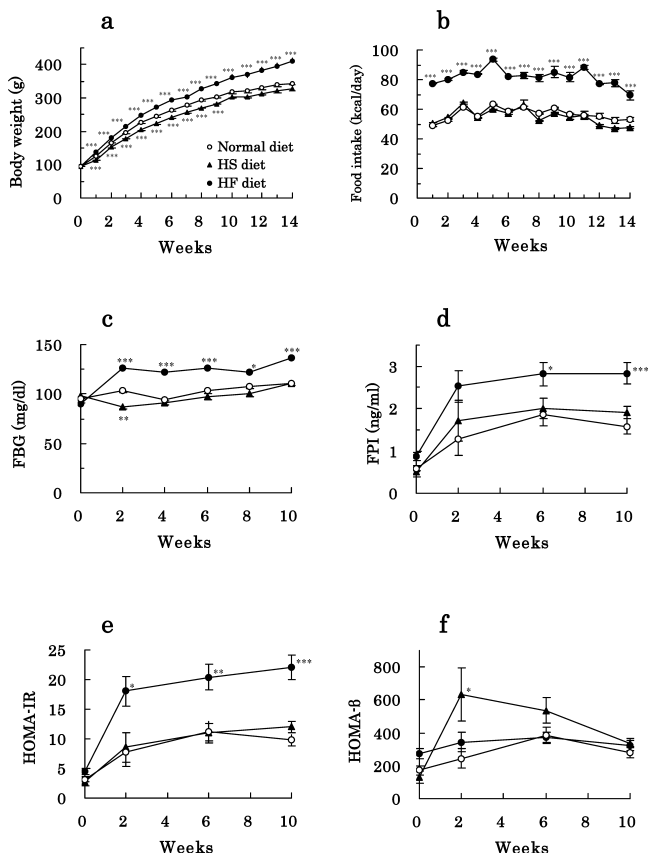


Fig. 1. Body Weight (a), Food Intake (b), FBG (c), FPI (d), HOMA-IR (e) and HOMA-β (f) in Rats Fed a Normal, HS or HF Diet

Data are shown as the means±S.E.M. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. normal diet (Dunnett's multiple comparison test). Open circles: normal diet (*n*=8); closed triangles: HS diet (*n*=9); closed circles: HF diet (*n*=9).

diet-fed rats showed significant increases in FBG and HOMA-IR at 2 to 10 weeks and FPI at 6 to 10 weeks, respectively. HOMA-β in HF diet-fed rats was not significantly different from that in the control. Table 1 shows the differences in blood biochemical parameters (lipid and creatinine) among the groups at 12 weeks. In HS diet-fed rats, there were no significant differences in TG, T-CHO, HDL-cholesterol and T-CHO/HDL compared to the control. By contrast, HF diet-fed rats showed significant increases in TG and T-CHO/HDL and decreased HDL-cholesterol compared to the control. No significant difference was found in serum creatinine among these groups.

Plasma DPP IV Activity To assess the association of DPP IV with the development of glucose intolerance, differences in plasma DPP IV activity among the groups were determined (Fig. 2). As a result, in normal diet-fed rats, plasma DPP IV activity decreased progressively with time up to 6

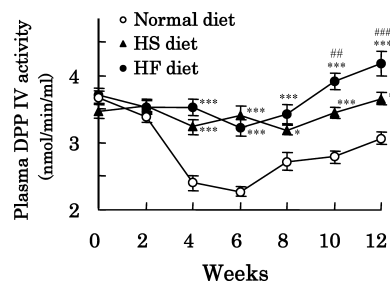


Fig. 2. Plasma DPP IV Activity in Rats Fed a Normal, HS or HF Diet

Data are shown as the means±S.E.M. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. normal diet (Dunnett's multiple comparison test). ##*p*<0.01, ###*p*<0.001 vs. HS diet (Student's unpaired *t*-test). Open circles: normal diet (*n*=8); closed triangles: HS diet (*n*=9); closed circles: HF diet (*n*=9).

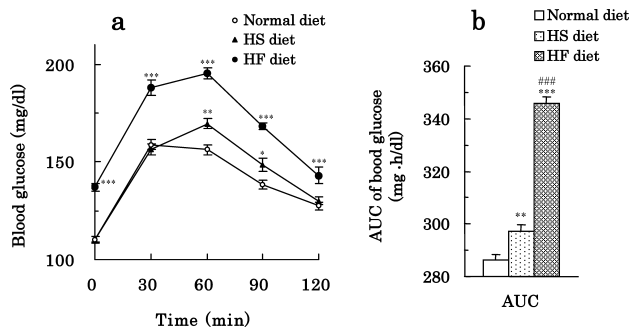


Fig. 3. OGTT in Rats Fed a Normal, HS or HF Diet

Blood glucose levels (a) and the $AUC_{0-120min}$ of blood glucose (b) are shown. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. normal diet (Dunnett's multiple comparison test). ###*p*<0.001 vs. HS diet (Student's unpaired *t*-test). Normal diet (*n*=8), HS diet (*n*=9), HF diet (*n*=9).

weeks, and subsequently partially recovered. On the other hand, plasma DPP IV activity in both HS and HF diet-fed rats did not decrease but slightly increased, exhibiting significantly higher levels of plasma DPP IV activity than in normal diet-fed rats from 4 weeks to the end of the experiment. In the comparison of HS diet- and HF diet-fed rats, plasma DPP IV activity was significantly higher from 10 weeks to the end in rats fed the HF diet than the HS diet.

OGTT To determine differences in glucose tolerance among groups, OGTT was performed at 10 weeks. As a result, HS diet-fed rats showed a significantly greater increase in blood glucose than normal diet-fed rats at 60 and 90 min after glucose load (Fig. 3a). In HF diet-fed rats, blood glucose at every point was significantly higher than in the control (Fig. 3a). $AUC_{0-120min}$ of blood glucose in HS and HF diet-fed rats was significantly higher than in control rats, and a greater increase in the $AUC_{0-120min}$ of blood glucose was observed in rats fed the HF diet than HS diet (Fig. 3b).

Tissue Weight In rats fed the HS diet compared with the

Table 2. Tissue Weight in Rats Fed a Normal, HS or HF Diet for 14 Weeks

	Normal diet	HS diet	HF diet
Tissue weight (g)			
Kidney	1.04±0.04	0.88±0.03*	1.07±0.04
Liver	10.2±0.2	10.8±0.3	11.9±0.2***
Subcutaneous fat	1.8±0.1	2.2±0.2	8.6±0.3***
Epididymal fat	8.1±0.3	9.9±0.4*	18.8±0.6***,###

Data are shown as the means±S.E.M. * $p<0.05$, *** $p<0.001$ vs. normal diet (Dunnett's multiple comparison test). ### $p<0.001$ vs. HS diet (Student's unpaired *t*-test). Normal diet: $n=8$; HS diet: $n=9$; HF diet: $n=9$.

normal diet, smaller kidneys and increased epididymal fat mass were significantly observed at 14 weeks (Table 2). In HF diet-fed rats, significant increases in liver weight, abdominal subcutaneous and epididymal fat mass were found as compared to the control at 14 weeks (Table 2). In addition, fatty livers were observed in all rats fed the HF diet for 14 weeks (data not shown).

DPP IV Activity and mRNA Expressions in Tissues

The influences of HS and HF diets on DPP IV expressions of these tissues were investigated at 14 weeks. As shown in Fig. 4a, HS and HF diets did not significantly affect DPP IV activity of the kidney. On the other hand, HF, but not HS, diet caused a significant decrease in DPP IV activity in the liver compared to the control (Fig. 4b). Regarding the results for adipose tissues (Fig. 4c), there was no significant difference in DPP IV activity in abdominal subcutaneous fat among these groups, whereas both HS and HF diets caused a significant decrease in DPP IV activity in epididymal fat. There were no significant differences in DPP IV mRNA expressions in the kidney and liver among normal, HS and HF diet groups (Table 3). RNA in subcutaneous and epididymal fat could not be extracted in this experiment.

DISCUSSION

Several studies have investigated whether DPP IV activity is correlated to the severity of diabetes; however, this is controversial. In an *in vitro* study, DPP IV activity and mRNA expression were enhanced by exposure of human glomerular endothelial cells to high glucose.¹⁸⁾ On the other hand, in various published clinical reports over the last decade, circulating DPP IV activity has been reported to be both increased^{19,20)} and decreased^{21,22)} in diabetic patients. Additionally, it has been reported that the degree of plasma DPP IV activity was associated with obesity,²³⁾ gender²⁴⁾ and aging.^{20,24)} Moreover, several reports, including clinical studies, have confirmed that widely used anti-diabetic agents, metformin^{25–27)} and pioglitazone,²⁷⁾ reduced the levels of circulating DPP IV activity *in vivo*. Considering these clinical findings, it may be complicated to define the correlation of DPP IV activity with the severity of hyperglycaemia because of the diverse clinical background. Accordingly, we produced an impaired glucose tolerance model with simple conditions (normal, HS or HF diet) to identify whether DPP IV activation is physiologically involved.

In the present study, HF diet-fed rats obviously exhibited impaired glucose tolerance, with hyperglycaemia, dyslipidaemia, obesity and insulin resistance, but did not impair in-

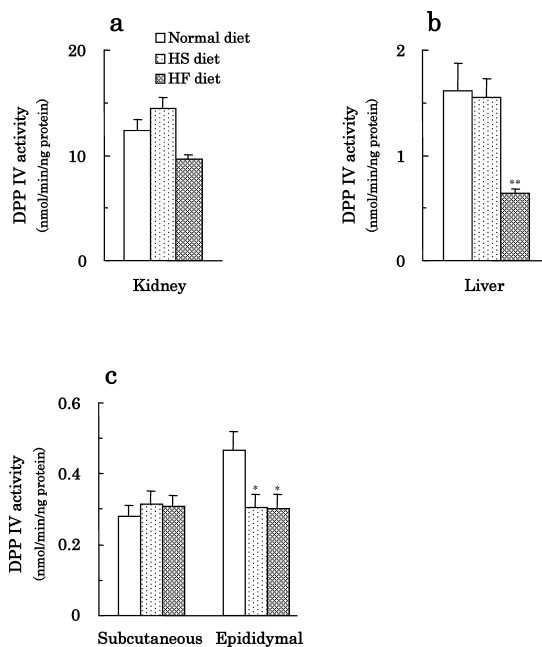


Fig. 4. DPP IV Activity of the Kidney (a), Liver (b), Abdominal Subcutaneous Fat and Epididymal Fat (c) in Rats Fed a Normal, HS or HF Diet for 14 Weeks

Data are shown as the means±S.E.M. * $p<0.05$, ** $p<0.01$ vs. normal diet (Dunnett's multiple comparison test). Normal diet ($n=8$), HS diet ($n=9$), HF diet ($n=9$).

Table 3. DPP IV mRNA Expressions in Rats Fed a Normal, HS or HF Diet for 14 Weeks

	Normal diet	HS diet	HF diet
DPP IV/GAPDH mRNA			
Kidney	1.16±0.03	1.21±0.05	1.11±0.04
Liver	1.16±0.06	1.07±0.09	1.26±0.09

Data are shown as the means±S.E.M. Normal diet: $n=8$; HS diet: $n=9$; HF diet: $n=9$.

sulin-secretory capacity or renal function. On the other hand, HS diet-fed rats had comparatively milder glucose intolerance with increases in visceral fat mass but not subcutaneous fat mass, body weight, FBG or FPI. In rats fed the HS diet rather than the normal diet, lower levels of body weight, FBG and kidney weight were found, probably due to the insufficient nutrition of protein and fat in the HS diet during the growth period. Regarding the results of DPP IV activity, plasma DPP IV activity in normal diet-fed rats decreased progressively with time up to 6 weeks after the start of the experiment, implying that the alteration in plasma DPP IV activity may be involved in body growth or aging. This interpretation is supported by some clinical reports that suggest the association of plasma DPP IV activity with aging.^{20,24)} By contrast, HF and HS diet-fed rats showed higher levels of plasma DPP IV activity than normal diet-fed rats. The rank order of plasma DPP IV activity was HF diet>HS diet>normal diet; therefore, the degree of plasma DPP IV activity seems to be consistent with the severity of impaired glucose tolerance. Pala *et al.*¹⁸⁾ have indicated that exposure to high glucose enhanced biosynthesis of DPP IV enzyme in vascular endothelial cells and its secretion into systemic circulation. Accordingly, the present study suggests that postprandial blood glucose elevation *via* the accumulation of visceral

fat may be responsible for the increase in plasma DPP IV activity in both HF and HS diet groups. Moreover, the increased activity of plasma DPP IV could still worsen glucose intolerance since DPP IV activation may lead to decreases in the anti-diabetic effects of incretin hormones, GLP-1 and GIP. Considering all of the above, it is likely that a close relationship exists between the development of impaired glucose tolerance and elevation of plasma DPP IV activity. In addition, HF diet-fed rats had visible fatty livers with decreased DPP IV activity, most likely causing hepatic hypertrophy with fatty degeneration, which impairs insulin sensitivity. The reasons for and the significance of decreased DPP IV activity in the liver remain uncertain; however, it may be reasonable that hepatic cell damage caused DPP IV to leak into the circulation, since microvascular endothelial cells in some areas, including the kidney and liver, have been supposed to be the main source of endogenous DPP IV.^{18,27)}

Of note, both HS and HF diets caused a significant decrease in DPP IV activity of epididymal but not subcutaneous adipose tissue. This is the first study to show that DPP IV activity of adipose tissues changes differently between subcutaneous and visceral fat in the development of impaired glucose tolerance. To date, the roles of DPP IV activity in subcutaneous and visceral adipose tissues in obesity and diabetes have not been investigated; however, decreased DPP IV activity in adipose tissue is potentially meaningful in the risk of developing obesity since GIP, a substrate for DPP IV, is a key element linking overnutrition to obesity through increased nutrient uptake and triglyceride accumulation in adipocytes.²⁸⁾ The decreased DPP IV activity of visceral adipose tissue, followed by active GIP elevation in the surroundings, may enable lipid accumulation in adipocytes, but further studies are required.

In conclusion, HF or HS diet-induced impaired glucose tolerance with visceral fat accumulation may be closely associated with increased plasma DPP IV activity and decreased DPP IV activity of visceral but not subcutaneous adipose tissue. In addition, the HF diet caused hepatic hypertrophy with decreased hepatic DPP IV activity, which may worsen insulin resistance.

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