

## Effects of Food Restriction on Pancreatic Islets in Spontaneously Diabetic Torii Fatty Rats

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**ABSTRACT.** The Spontaneously Diabetic Torii (SDT) fatty rat, established by introducing the *fa* allele of the Zucker fatty rat into the SDT rat genome, is a new model of obesity/type 2 diabetes. The present study investigated effects of food restriction on metabolic and endocrinological function in SDT fatty rats. SDT fatty rats were pair-fed with SDT rats from 7 to 21 weeks of age. The SDT fatty rats were already hyperinsulinemic and hyperlipidemic at 7 weeks of age. After 7 weeks of age, SDT fatty rats showed age-dependently increasing serum glucose levels associated with decreasing serum insulin levels. However, in pair-fed SDT fatty rats, hyperglycemia and hyperinsulinemia were attenuated at 9 weeks of age. After 9 weeks of age, the serum insulin levels unexpectedly increased in the pair-fed SDT fatty rats. Glucose tolerance was also improved, and the pancreatic insulin contents were increased in these rats. Pancreatic islets were hypertrophied in pair-fed SDT fatty rats compared with *ad lib*-fed SDT fatty rats, which were comparable to SDT rats. This study showed that, in SDT fatty rats, calorie restriction by paired-feeding with SDT rats attenuated hyperglycemia and hyperinsulinemia for the first 2 weeks. Thereafter, the serum insulin levels and pancreatic insulin contents were increased, though the restriction was continued. Hypertrophic pancreatic islets were also remarkable, indicating increased beta cell proliferation. The activated pancreatic beta cell functions might be due to rapid food ingestion, a change of feeding behavior resulting from increasing the fasting period, which was indispensable for calorie restriction.

**KEY WORDS:** beta cell, diabetes, pair-feeding, pancreas, SDT fatty rat.

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Both energy intake and energy expenditure are important in regulation of body weight [8]. Energy intake in excess of energy expenditure induces increase in body weight. Obesity increases the risks of certain diseases, such as hypertension, hypercholesterolemia, hypertriglyceridemia and increased insulin resistance [1, 23]. On the other hand, either reduced calorie intake or increased energy expenditure can reduce the body weight, which leads to disappearance of metabolic dysfunctions associated with obesity [2]. In rodents, it is also known that food restriction or increased energy expenditure by exercise extends lifespan by exerting beneficial effects on metabolism [11, 17, 22].

In Spontaneously Diabetic Torii (SDT) rats, a model for non-obese diabetes, blood glucose levels moderately increased with advancing age, and hyperglycemia was established after 16–20 weeks of age [12, 24, 25]. Development of hyperglycemia in SDT rats was accompanied by hypoinsulinemia. Age-dependent degenerative changes of pancreatic islets were decreased production, secretion of insulin and atrophy of islets. Early pathological changes of the pancreatic islets, such as congestion and hemorrhage, were observed from 8–10 weeks of age [14].

The SDT fatty rat was established by introducing the *fa* allele of the Zucker fatty rat into the SDT rat genome [13].

Male SDT-*fa/fa* (SDT fatty) rats exhibited hyperphagia/obesity associated with hyperglycemia, hyperinsulinemia and hyperlipidemia from 6 weeks of age [16]. The early onset of hyperglycemia in SDT fatty rats is considered to be caused by obesity and insulin resistance due to hyperphagia. Female SDT rats also exhibited the onset of hyperglycemia at 6 weeks of age, as observed in male SDT fatty rats, though the magnitude of hyperglycemia was lower than in males [5].

Food or calorie restriction of SDT fatty rats has been shown to temporarily improve hyperglycemia and hypertriglyceridemia and markedly reduce progression of diabetic complications [18]. It has been reported that food restriction exhibited beneficial effects on adipose tissue, such as increased insulin sensitivity and decreased tissue weight.

As the metabolic responses to restriction of calorie intake in Zucker Diabetic Fatty (ZDF) rats, amelioration of insulin requirement by decreasing insulin resistance led to prevention of hyperglycemia and hypertrophy pancreatic islet [20]. In db/db mice, a beneficial effect of calorie restriction was also observed: hyperglycemia and hyperinsulinemia were suppressed, and renal vascular changes were completely prevented after food restriction [7].

In the present study, we investigated effects of food restriction on SDT fatty rats. In addition to the effects of metabolism, insulin content, islet size, and mRNA expression of enzymes and receptors were examined.

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## MATERIALS AND METHODS

**Animals:** This study was conducted in compliance with the Guidelines for Animal Experimentation of Japan Tobacco Biological/Pharmacological Research Laboratories. Male SDT-*fa/fa* (SDT fatty) rats and age-matched SDT-*+/+* (SDT) rats from our colonies were used (Japan Tobacco Inc., Central Pharmaceutical Research Institute, Takatsuki, Japan). The SDT-*fa/fa* rat was produced by crossing heterozygous male SDT-*fa/+* rats with heterozygous female SDT-*fa/+* rats. The SDT-*fa/fa* rats and littermate SDT-*+/+* rats were selected by genotyping the *fa* locus. Genotyping of the *fa* locus was performed according to a PCR-restriction fragment length polymorphism (RFLP) method. SDT fatty rats at 6 weeks of age were divided into two groups: one group was allowed to feed (CRF-1, Charles River Japan, Yokohama, Japan) *ad libitum*, and the other group was on pair-fed the amount of food consumed by age-matched SDT rats from 7 to 21 weeks of age. Food consumption of the pair-fed SDT fatty rats (Fatty-PF Group) was about 50–60% of that of the *ad lib*-fed rats (Fatty Group) throughout the experimental period. As a control, age-matched SDT rats were used (SDT Group). A satellite group of each of the three groups was made to evaluate the islet size and mRNA expression in the pancreas at 9 and 13 weeks of age. The rats were housed individually in suspended bracket cages in a climate-controlled room with a temperature of  $23 \pm 3^\circ\text{C}$ , humidity of  $55 \pm 15\%$  and a 12-hr lighting cycle and had free access to water.

**Biological parameters:** Body weight, food intake and biochemical parameters [serum glucose, triglyceride (TG) and insulin levels] were measured at 6 and 7 weeks of age in the non-fasting state and thereafter at 2-week intervals from 7 to 21 weeks of age. Blood samples were collected from the tail vein of the rats. Serum glucose and TG were measured using commercial kits (glucose, glucose kit; TG, triglyceride kit; Roche Diagnostics, Basel, Switzerland) in an automatic analyzer (Hitachi 7180; Hitachi, Tokyo, Japan). Serum insulin levels were measured with a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mori-naga Institute of Biological Science, Yokohama, Japan).

**Glucose tolerance test:** A glucose tolerance test was conducted at 9, 15 and 21 weeks of age. Glucose solution (2 g/kg body weight) was orally administered to 4 hr-fasted animals, and blood samples were taken before glucose loading (0 min) and 10, 30, 60 and 120 min after glucose loading for determination of serum glucose and insulin levels.

**Measurements of insulin content in the pancreas:** Measurement of insulin content in the pancreas was conducted at 9 weeks of age. Pancreatic insulin was extracted by the acid/ethanol extraction method. Briefly, animals were sacrificed by exsanguination under light ether anesthesia. The pancreas was removed and promptly homogenized in a cold acid/ethanol mixture (75% ethanol, 23.5% distilled water, 1.5% 2 N hydrochloric acid) to extract insulin. The levels of insulin in the extract were measured as described above.

**Measurements of pancreatic islet size:** Measurement of

pancreatic islet size was conducted at 9 weeks of age. Animals were sacrificed by exsanguination under light ether anesthesia. The pancreas was removed promptly and fixed in 10% neutral buffered formalin. The tissue was paraffin-embedded by standard techniques and cut into thin sections (3 to 5  $\mu\text{m}$ ). The sections were treated with hematoxylin and eosin (HE) staining. The islet size on one slide per animal was examined histopathologically in a blind manner. All islets on slides were circled for image analysis, and islet size was calculated using the Win ROOF Ver. 5.01 software (Mitani Corporation, Fukui, Japan).

**Quantification of mRNA with real-time quantitative PCR:** Total RNA was extracted from the pancreas of the satellite group rats at 9 weeks of age. RNA was transcribed into cDNA using M-MLV reverse transcriptase and random primers (Invitrogen, Carlsbad, CA, U.S.A.). The reaction mixture was incubated for 10 min at  $25^\circ\text{C}$ , 1 hr at  $37^\circ\text{C}$  and 5 min at  $95^\circ\text{C}$ . Real-time PCR quantification was performed in a 50- $\mu\text{l}$  reaction mixture with an automated sequence detector combined with the ABI Prism 7700 Sequence Detection System software (Applied Biosystems, Foster City, CA, U.S.A.). The reaction mixture contained 50 ng of synthesized cDNA, 3.5 mM  $\text{MgCl}_2$ , 0.3  $\mu\text{M}$  primers, 0.1  $\mu\text{M}$  probes and 1.25 units of Ampli Taq Gold. The cycle parameters were 10 min at  $95^\circ\text{C}$ , followed by 40 cycles of 15 sec at  $95^\circ\text{C}$  and 60 sec at  $60^\circ\text{C}$ . The following primers and FAM-conjugated probes were designed using the Primer Express software (Applied Biosystems): glucose transporter 2 (GLUT2) (forward, GTCTGCAATTTTCAT-CATCGCC; reverse, AAAGGAAGAACACGTAAG-GCCC; probe, TCTGCTTCCAGTACATTGCGGACT-TCCT), IRS-2 (forward, CCCCAGTGTCCCCATCCT; reverse, TTTCCTGAGAGAGACGTTTTTCCA; probe, TGCTCACAATTCCAAGCGCCACAAT), Glucokinase (forward, AGCAGATCCACAACATCCTAAGC; reverse, TCCTGCGGAGCACATATGG; probe, CGACCCTCTGT-CACCGACTGC), Insulin (forward, GCCCAGGCTTTTG TCAAACAG; reverse, TTCCCCACACACCAGGTACAGA; probe, ACCTTTGTGGTCCCTCACCT) and 18s rRNA (purchased from Applied Biosystems).

**Statistical analysis:** The results of biological parameters are expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis of differences between mean values was performed using an F-test followed by the Student's *t*-test or Aspin-Welch's *t*-test. Differences were considered significant at  $P < 0.05$ .

## RESULTS

**Food intake and body weight:** The Fatty Group showed hyperphagia immediately after weaning; the mean value of food intake at 7 weeks of age was significantly higher in the Fatty Group compared with the SDT Group (46.8 g/day for the Fatty Group, 26 g/day for the SDT Group). Hyperphagia in the Fatty Group was sustained throughout the experimental period (Fig. 1A). Body weights were significantly higher in the Fatty Group as compared with in the SDT Group from

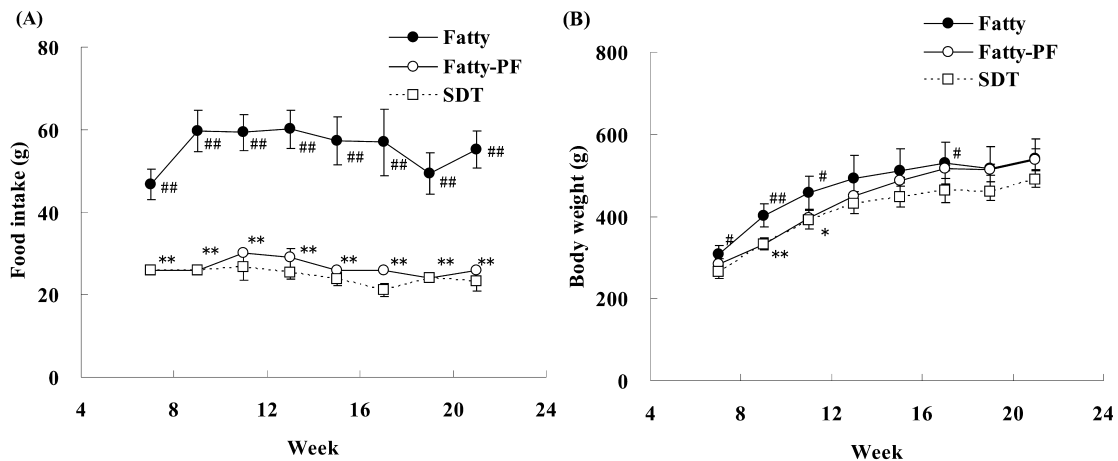


Fig. 1. Changes in food intake and body weight (A. Food intake; B. Body weight) in the Fatty, Fatty-PF and SDT Groups. Data shown as means  $\pm$  SD ( $n=5-6$ ). ##  $P<0.01$ , #  $P<0.05$ : significantly different from the SDT Group. \*\*  $P<0.01$ , \*  $P<0.05$ : significantly different from the Fatty Group.

7 to 17 weeks of age (Fig. 1B), while body weights were almost comparable between the Fatty-PF and SDT Groups from 7 to 13 weeks of age.

**Biochemical parameters:** The non-fasting serum glucose levels in the Fatty Group continued to increase throughout the experimental period, from  $534 \pm 138$  mg/dl at 7 weeks of age to  $773 \pm 80$  mg/dl at 21 weeks of age. The non-fasting serum glucose levels in the Fatty-PF Group were decreased immediately 2 weeks after pair-feeding ( $543 \pm 173$  mg/dl at 7 weeks of age;  $148 \pm 23$  mg/dl at 9 weeks of age) and thereafter were maintained at similar levels to those in the SDT rats until 15 weeks of age (Fig. 2A). After 15 weeks of age, the glucose levels were gradually increased in the Fatty-PF Group. In the Fatty Group, the non-fasting serum insulin levels were markedly elevated at 7 weeks of age and thereafter gradually decreased to the levels of the SDT Group (Fig. 2B). In the Fatty-PF Group, the non-fasting serum insulin levels were transiently decreased by initiation of pair-feeding (from  $13.57 \pm 4.63$  ng/ml to  $5.29 \pm 1.43$  ng/ml), but thereafter, the insulin levels were sharply elevated even under the pair-feeding condition. The non-fasting serum TG levels were significantly higher in the Fatty Group than those in the SDT Group at 7 weeks of age, and the high levels were maintained until 21 weeks of age (Fig. 2C). In the Fatty-PF Group, the non-fasting serum TG levels were decreased immediately by the dietary manipulation and gradually increased after 13 weeks of age, as was observed in the case of the insulin levels.

**Glucose tolerance test:** In the glucose tolerance test conducted at 9 weeks of age, the Fatty Group showed higher serum glucose levels after glucose loading without any significant response of plasma insulin compared with the other two groups (Fig. 3A and 3B). On the other hand, the Fatty-PF Group was comparable to the SDT Group in glucose tolerance, but the plasma insulin level response to glucose was significantly elevated. At 15 weeks of age, the Fatty Group glucose tolerance became more deteriorated, and insulin

response to glucose was completely disappeared (Fig. 3C and 3D). An age-related change in tolerance was not observed in the Fatty-PF Group as observed in the Fatty Group, but the insulin response to glucose completely disappeared despite markedly elevated basal plasma insulin levels (9 weeks of age,  $2.94 \pm 1.22$  ng/ml; 15 weeks of age,  $9.24 \pm 3.71$  ng/ml). At 21 weeks of age, there was no marked difference in glucose or insulin response to glucose loading in the Fatty Group compared with the values at 15 weeks of age (Fig. 3E and 3F). On the other hand, the Fatty-PF Group showed more deteriorated glucose tolerance and further increased basal insulin levels compared with those at 15 weeks of age.

**Pancreatic insulin content and islet size:** Pancreatic insulin contents were measured at 9 weeks of age. The insulin content was markedly lower in the Fatty Group compared with the other two groups (Table 1). The insulin content of the Fatty-PF Group was higher than those of the SDT Group (Table 1).

The size of the pancreatic islets of the Fatty Group was unexpectedly comparable to that of the SDT Group at 9 weeks of age (Table 1). Differing from the case of other *fa*-carrying rats, hypertrophy of islets was not detected at all in the SDT fatty rats. Furthermore, considering the decreased pancreatic insulin content, insulin synthesis was likely to be as decreased in the Fatty Group as in the SDT Group despite the existence of a beta cell mass. The size was larger in the Fatty-PF Group than in the other two groups. The pair-feeding unexpectedly induced hypertrophy of islets (hyperplasia of beta cells).

**Pancreas mRNA expression:** The mRNA levels of GLUT2, insulin, glucokinase and IRS-2 were measured in pancreatic extracts from the three groups at 9 weeks of age. No significant difference in the mRNA expression of the Fatty-PF Group was observed in the pancreas (Table 2). Although the mRNA levels of expression in the Fatty-PF Group showed a large deviation, the mRNA levels of

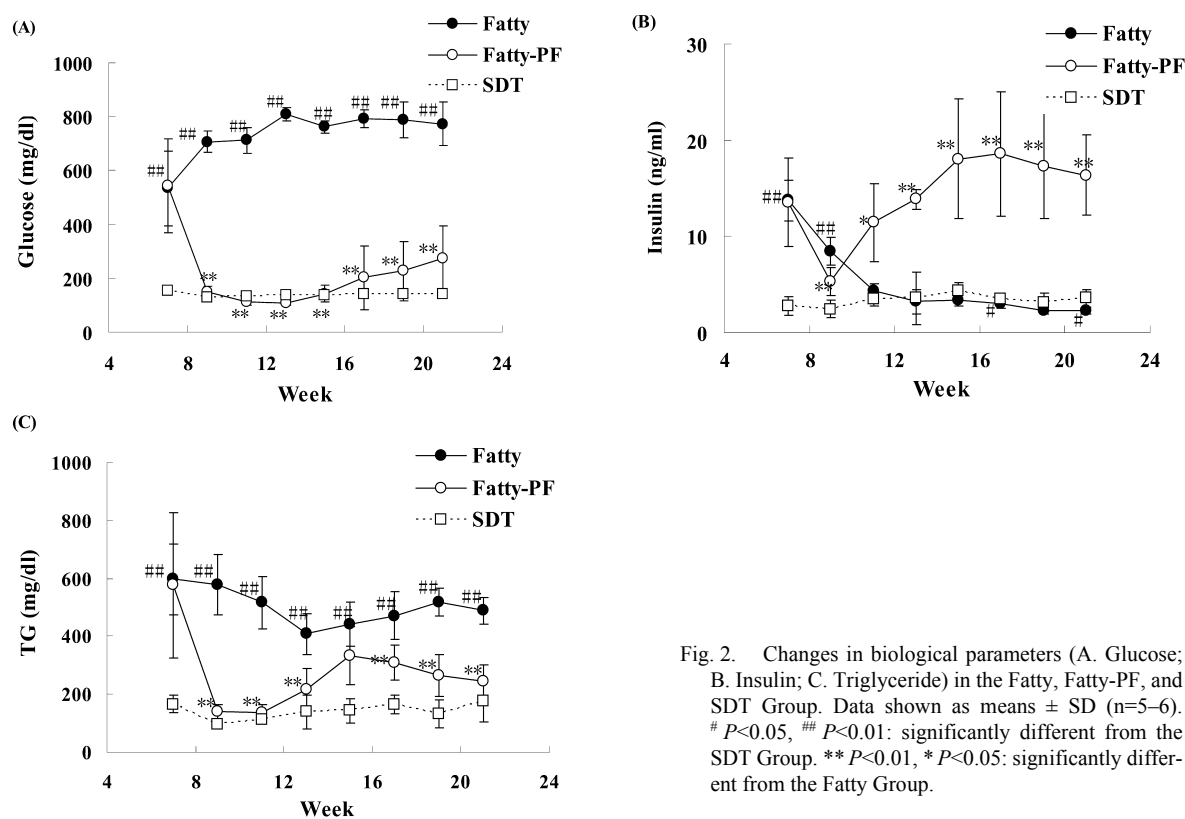


Fig. 2. Changes in biological parameters (A. Glucose; B. Insulin; C. Triglyceride) in the Fatty, Fatty-PF, and SDT Group. Data shown as means  $\pm$  SD ( $n=5-6$ ). #  $P < 0.05$ , ##  $P < 0.01$ : significantly different from the SDT Group. \*\*  $P < 0.01$ , \*  $P < 0.05$ : significantly different from the Fatty Group.

GLUT2 were lower by 75% in the Fatty Group and comparable in the Fatty-PF Group as compared with the SDT Group (Table 2). The mRNA levels of insulin were lower by 76% in the Fatty Group and by 40% in the Fatty-PF Group compared with the SDT Group (Table 2). The mRNA levels of glucokinase were comparable in the Fatty Group and approximately 59-fold higher in the Fatty-PF Group as compared with the SDT Group. The IRS-2 mRNA levels were comparable in the Fatty and Fatty-PF Groups as compared with the SDT Group.

## DISCUSSION

The diabetic condition of SDT rats is characterized by hyperglycemia independent of obesity or hyperinsulinemia [19, 21, 25]. Hyperglycemia in SDT rats is caused by age-dependent degeneration of pancreatic beta cells. Male SDT fatty rats showed hyperglycemia at younger ages than male SDT rats [15, 16], and the early onset of diabetes was likely due to insulin resistance increased by obesity having led to exhaustion of pancreatic beta cells. The purpose of this study was to investigate effects of food restriction on the diabetic condition, including pancreas functions, in SDT fatty rats. Due to the extreme change of plasma insulin levels before and after 9 weeks of age (2 weeks of pair-feeding), the pancreatic insulin content and size of pancreatic islet, mRNA expression in the pancreas was investigated at 9 weeks of age.

In the present study, the Fatty Group showed hyperglycemia associated with increased body weight and hyperinsulinemia, which was followed by severe hyperglycemia accompanied by hypoinsulinemia. The Fatty-PF Group, food intake of which was paired with that of SDT rats, was comparable to the SDT Group in body weight, serum TG, glucose and insulin levels for the first 2 weeks of pair-feeding. In surprising contrast, thereafter, the serum insulin and TG levels in the Fatty-PF Group unexpectedly began to increase age-dependently, though their body weight and serum glucose were still at the same levels as the SDT Group.

Focusing on the effect of pair-feeding on the serum insulin levels of the SDT fatty rats, some complex mechanisms involved in spontaneous impairment of pancreatic beta cells should be considered. The decrease in serum insulin levels in the Fatty-PF Group observed for the first 2 weeks of pair-feeding could simply be accepted as the effect of calorie restriction (pair-feeding). However, the serum insulin levels were increasing after 9 weeks of age (after 2 weeks of pair-feeding), while in the Fatty Group, the insulin levels were further decreasing with advancing age. This finding suggested prevention of spontaneous degeneration of beta cells by pair-feeding. The preventing effect was evidenced by an increase in both the pancreatic insulin content and size of the pancreatic islets. The effect was also reflected on elevated basal insulin levels and marked insulin response to glucose (glucose-stimulated insulin secretion) of the Fatty-

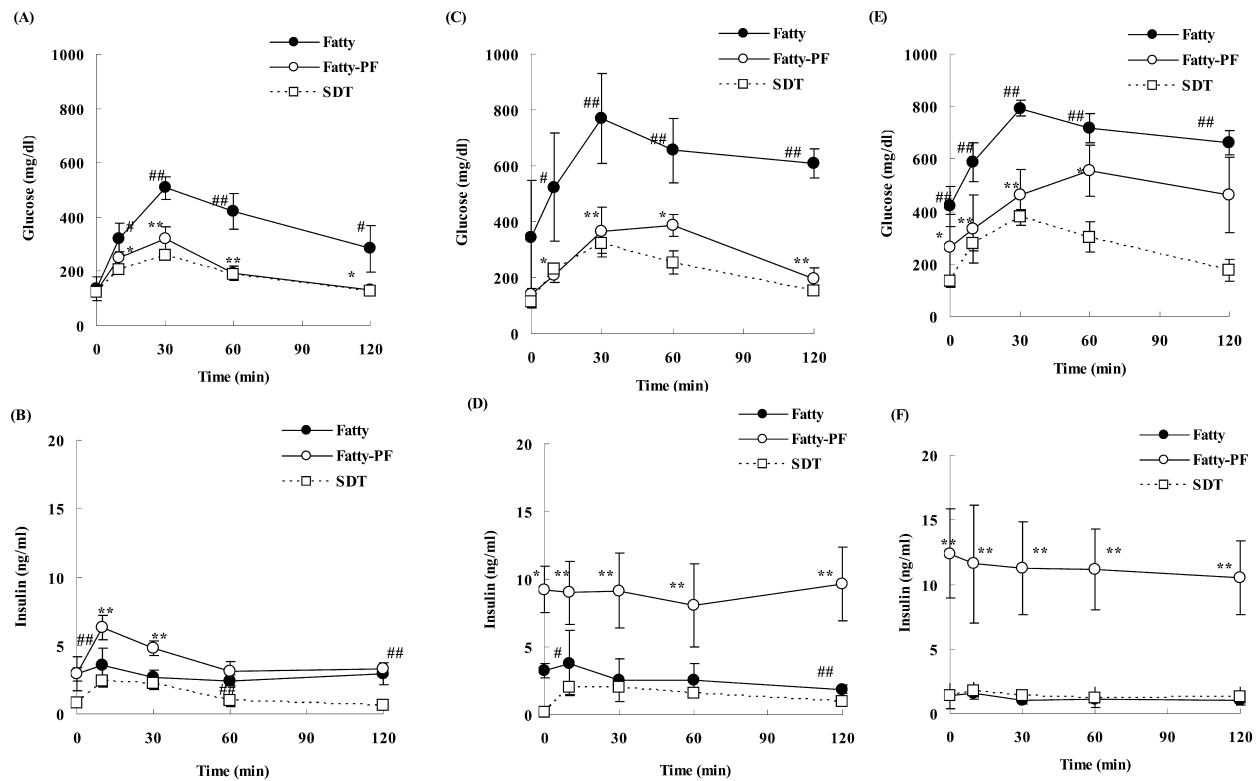


Fig. 3. Glucose tolerance tests in the Fatty, Fatty-PF and SDT Group. A glucose tolerance test was conducted at 9, 15 and 21 weeks of age. Glucose (2 g/kg body weight) was administered orally after 4 hr of fasting, and the plasma glucose and insulin levels were measured at the indicated time points. Data shown as means  $\pm$  SD (n=5–6). #  $P<0.05$ , ##  $P<0.01$ : significantly different from the SDT Group. \*  $P<0.05$ , \*\*  $P<0.01$ : significantly different from the Fatty Group.

Table 1. Insulin content and islet size at 9 weeks of age

	Fatty	Fatty-PF	SDT
Insulin content ( $\mu\text{g/g}$ pancreas)	$17.3 \pm 2.4^{##}$	$73.1 \pm 9.8^*$	$53.8 \pm 2.1$
Islet size ( $\mu\text{m}^2$ )	$16,733 \pm 2,242$	$24,589 \pm 1,219^*$	$14,562 \pm 1,935$

Data shown as means  $\pm$  SD (n=4), ##  $P<0.01$ : significantly different from the SDT Group. \*  $P<0.05$ ; significantly different from the Fatty Group.

Table 2. GLUT2, insulin, glucokinase and IRS-2 mRNA levels in the pancreas at 9 weeks of age

		Fatty	Fatty-PF	SDT
mRNA (copy/100,000 copy 18s rRNA)	GLUT2	$0.79 \pm 1.10$	$4.28 \pm 6.34$	$3.17 \pm 3.20$
	Insulin	$247.0 \pm 374.8$	$604.6 \pm 937.7$	$1,009.8 \pm 995.6$
	Glucokinase	$3.53 \pm 2.75$	$95.6 \pm 206.5$	$1.62 \pm 1.16$
	IRS-2	$5.78 \pm 3.84$	$5.25 \pm 3.34$	$5.53 \pm 2.73$

The expressions of mRNA were measured by a real-time quantitative PCR method. The mRNA levels were corrected with 18s rRNA. Data represent means  $\pm$  SD (n=5–6).

PF Group in the glucose tolerance test conducted at 9 weeks of age.

In ZDF rats, restricted calorie intake was confirmed to prevent glucose-stimulated insulin reaction and degenerative changes of pancreatic beta cells [20] and to suppress the hypertrophy of pancreatic islets (our preliminary data).

Restriction of food or calorie intake resulted in sustained or stable suppression of hyperinsulinemia in ZF and OLETF rats [6, 9, 10]. Based on these findings in the obese animals in the Fatty-PF Group, the time-dependent increase in serum insulin levels after 9 weeks of age appeared to run in clear contradiction to the general concept that reduction of food

or calorie intake decreases the insulin requirement through amelioration of insulin sensitivity by decreasing body weight or body fat mass.

The strange response of the SDT-fatty rats to food restriction could be explained not by calorie restriction, but by changes in feeding behavior that accompanied pair-feeding. The Fatty-PF Group was allowed to consume only about 50% of the diet consumed by the *ad lib*-fed rats every day. Under this feeding condition, the Fatty-PF Group had to be exposed to fasting longer than the *ad lib*-fed rats. It is easy to expect that pair-feeding leads to rapid ingestion, as observed in fasting-feeding rats or restriction of meal contact time. Indeed, in C57BL/6J mice, feeding behavior was changed drastically after food restriction (70% of *ad lib* levels); food-restricted mice consumed all the diet for a day within 60 min after they were given the diet, while control mice nibbled throughout the day [3]. Rapid food ingestion after fasting is easily expected to accelerate the rate of food absorption, which leads to stimulation of insulin secretion, in turn resulting in increased food efficiency.

The change in feeding behavior appeared to stimulate pancreatic beta cell functions, as indicated by the elevated glucose-stimulated insulin secretion (GSIS) and hypertrophy of pancreatic islets in the Fatty-PF Group observed rats after 2 weeks of pair-feeding. However, the reasons why the pancreatic beta cells of the SDT fatty rats are more sensitive to the change in feeding behavior than the calorie restriction per se remains to be clarified.

Focusing on the molecular basis of the mechanism, glucokinase is known to participate in not only glucose metabolism but also proliferation of beta cells. In haplo insufficiency of beta cell-specific glucokinase (*Gck*<sup>+/-</sup>) mice, hypertrophy of pancreatic islets was not observed when the mice were fed a high fat diet [27]. However, no significant difference in the expression of glucokinase mRNA was observed in beta cells after food restriction in the Fatty-PF Group. IRS-2 is also known to participate in proliferation of beta cells [26, 27]. However, increased IRS-2 mRNA expression was not found in the Fatty-PF Group either. Further studies are needed to examine the response of pancreatic beta cells related to the food restriction in SDT rats.

The Glut-2 mRNA expression was higher in the Fatty-PF Group than in the Fatty Group. The increased Glut-2 mRNA expression could lead to improvement of insulin secretion after glucose load in the Fatty-PF Group because homozygous Glut-2-deficient mice showed hyperglycemia and hypoinsulinemia associated with decreased insulin response to glucose loading [4].

In conclusion, the restriction of food intake in the SDT fatty rats attenuated diabetic conditions through prevention of obesity for 2 weeks after initiation of pair-feeding. After 2 weeks of pair-feeding, a rapid elevation of serum insulin levels was observed, while the insulin levels were further decreased with advancing age in the SDT fatty rats on *ad lib* feeding, a specific characteristic of this strain. In the pair-fed SDT fatty rats, both increased pancreatic insulin content

and hypertrophy of pancreatic islets were observed, suggesting that the hypertrophy of pancreatic cells was stimulated by pair-feeding. The unexpected response of pancreatic beta cells to food restriction might be attributed to altered feeding behavior by reduction of meal contact time.

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