Which Is the Primary Etiologic Event in Otsuka Long-Evans Tokushima Fatty Rats, a Model of Spontaneous Non–Insulin-Dependent Diabetes Mellitus, Insulin Resistance, or Impaired Insulin Secretion?

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To identify the primary disorder causing diabetes mellitus in a model rat (Otsuka Long-Evans Tokushima Fatty [OLETF]) with non–insulin-dependent diabetes mellitus (NIDDM), we studied the temporal relationship between insulin resistance and impairment of pancreatic β-cell function. Groups of 28 male OLETF rats and male nondiabetic control Long-Evans Tokushima Otsuka (LETO) rats were given an intravenous (IV) glucose and glucagon tolerance test (IVGTT) and hyperinsulinemic euglycemic clamp tests at 10, 16, 24, and 40 weeks of age. After the euglycemic clamp test, abdominal fat was measured and the pancreas was examined histologically. At 16 weeks of age, insulin-mediated whole-body glucose uptake as measured by the hyperinsulinemic euglycemic clamp technique was significantly reduced in OLETF rats (glucose infusion rate [GIR], 40.9 ± 4.2 μmol/kg · min) as compared with LETO rats (78.4 ± 6.9). On the other hand, plasma insulin responses to glucose and glucagon in OLETF rats were higher than those in LETO rats at 16 and 24 weeks of age, but clearly decreased at 40 weeks of age (Σimmunoreactive insulin [IRI] to glucagon, 8.81 ± 1.81 *v* 27.32 ± 4.59 nmol · min in OLETF and LETO rats, respectively, $P < .01$). Abdominal fat deposition was significantly greater in OLETF rats than in LETO rats at all ages tested except 10 weeks. Pancreatic islets of OLETF rats became enlarged and fibrotic. These results demonstrated that insulin resistance preceded impairment of pancreatic β-cell function in OLETF rats, and that insulin resistance seemed closely related to fat deposition in the abdominal cavity.

NON–INSULIN-DEPENDENT diabetes mellitus (NIDDM) model rats, Otsuka Long-Evans Tokushima Fatty (OLETF), established by Kawano et al[1] show glucose intolerance with hyperinsulinemia upon reaching adulthood. During the chronic course of diabetes mellitus, OLETF males eventually become hypoinsulinemic and develop insulin-dependent diabetes mellitus–like diabetes, requiring insulin therapy for survival. In nondiabetic obesity, the endocrine pancreas can respond to insulin resistance by an adequate increase in insulin secretion, but when the endocrine pancreas response becomes inadequate glucose intolerance becomes apparent. According to this view, the sequence of events would be primary insulin resistance, resulting in hyperinsulinemia with several metabolic consequences, and ultimate endocrine decompensation.[2]

There has been no detailed longitudinal study on the temporal relationship between insulin resistance and impairment of pancreatic β-cell function in OLETF rats. Therefore, we performed chronologic studies on the insulin resistance of peripheral tissues in these model rats, as evaluated by a hyperinsulinemia euglycemic clamp technique, and on the plasma insulin responses to glucose and glucagon.

MATERIALS AND METHODS

Animals

A spontaneous diabetic rat with polyuria, polydipsia, and slight obesity was discovered in an outbred colony of Long-Evans rats that had been purchased from Charles River Canada (St Constant, Canada) in 1983 and subsequently maintained at the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). By 20 generations of selective breeding, a diabetic strain (OLETF) was established in 1990. Cumulative incidences of diabetes in male rats of this strain are 67%, 78%,[3] and 81.2%[1] at 4, 6, and 10 months of age, respectively. Twenty-eight male OLETF rats were obtained from the Tokushima Research Institute, Otsuka Pharmaceutical. A nondiabetic strain (Long-Evans Tokushima Otsuka [LETO]) that has been maintained by sister-brother mating in our animal facilities under specific pathogen-free conditions (Institute for Animal Experimentation, University of Tokushima) was used as a nondiabetic control. Animals were fed rat chow (Oriental Yeast, Tokyo, Japan) ad libitum and kept at controlled temperature (21° ± 2°C), humidity (55% ± 5%), lighting (7 AM to 7 PM), and air conditioning.

Intravenous Glucose and Glucagon Test

At 10, 16, 24, and 40 weeks of age, an intravenous (IV) glucose and glucagon tolerance test (IVGTT) was performed after an overnight fast. In this test, 0.5 g glucose (500 g/L)/kg body weight and 0.2 mg glucagon were injected IV as a bolus just after withdrawing blood samples at 0 and 75 minutes, respectively, and blood was taken from a contralateral right cervical vein under anesthesia at 0, 3, 6, 75, 78, and 81 minutes for measurements of plasma glucose and insulin levels.

Measurement of In Vivo Glucose Disposal by Euglycemic Clamp Studies

Insulin-mediated whole-body glucose uptake was measured in anesthetized rats using a euglycemic clamp[4] within 2 weeks after the IVGTT. After an overnight fast, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg · kg^{-1}) and catheters were inserted into the jugular and femoral veins. Rats received an infusion of insulin at 70 pmol · kg^{-1} · min^{-1} for 1 hour. Infusion of 100-g/L glucose solution was started at time 0, and the rate was adjusted to clamp plasma glucose concentration at approximately 6.1 mmol/L. Plasma samples for determination of glucose levels were obtained at 2- to 5-minute intervals throughout the study. At the end of the 60-minute study, rats were treated with pentobarbital (60 mg · kg^{-1}), and the abdomen was quickly opened, blood was withdrawn from the aorta for determination of total

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cholesterol and triglyceride levels, and the pancreas was removed for morphologic study. Data on total-body glucose uptake represent mean values for the glucose infusion rate (GIR) during the last 20 minutes.

Assays

Plasma glucose levels were determined by the glucose oxidase method (Fuji Dri-Chem 2000, Fuji Medical System, Tokyo, or Toecho Super, Kyoto Daiichi Kagaku, Kyoto, Japan). Insulin levels were measured with a commercial kit (Daiichi Radioisotope, Tokyo, Japan) with rat insulin as a standard (Novo Nordisk, Bagsvaerd, Denmark). Total cholesterol and triglyceride levels were measured automatically by enzymatic techniques with a Hitachi Autoanalyzer (Type 736, Hitachi, Tokyo, Japan).

Histology

Paraffin sections (4 μm) of Formalin-fixed pancreata were obtained from widely separated regions of each pancreas and stained with hematoxylin-eosin. A minimum of 20 islets per individual were examined.

Statistical Analysis

Data are expressed as the mean ± SEM unless otherwise indicated. Significance was determined by ANOVA, followed by Tukey's test for individual comparisons of means. The χ^2 test was used for comparing frequencies.

RESULTS

During the IVGTT, one and two OLETF rats died accidentally at 10 and 40 weeks of age, respectively.

Body Weight and Abdominal Fat

Chronologic changes in body weight and abdominal fat in OLETF and LETO rats are listed in Table 1. There was a significant difference in body weight between OLETF and LETO rats from as early as 10 weeks of age, with OLETF rats being significantly heavier than LETO rats of the same age. Similarly, the amount of fat in the abdominal cavity was approximately three times greater in OLETF rats than in LETO rats of the same age. Fat deposition in the abdominal cavity increased with age in both strains, but the increase was greater in OLETF rats than in LETO rats.

Plasma Lipid Concentrations

Plasma lipid levels tended to increase with age. Plasma total cholesterol and triglyceride levels in OLETF rats at 40 weeks of age were significantly greater than those in any other groups (Table 1).

Plasma Glucose and Insulin Responses to IV Glucose and Glucagon

Chronologic changes in plasma glucose and immunoreactive insulin (IRI) responses to IV glucose and glucagon loads in OLETF and LETO rats are shown in Fig 1. At 10 weeks of age, basal plasma glucose concentration was significantly higher in OLETF rats than in LETO rats (6.2 ± 0.4 *v* 4.2 ± 0.2 mmol/L). It increased significantly with age in the former group, reaching a level of 10.4 ± 0.7 mmol/L at 24 weeks of age. After an IV glucose load, plasma glucose level increased, reaching a peak at 3 minutes except in one group (10-week-old OLETF), and then decreased to the basal level at 75 minutes in rats of all ages in both groups. Plasma glucose increased less after an IV glucagon load than after an IV glucose load. Plasma glucose during IVGTT was significantly higher at several points in OLETF rats than in LETO rats. Plasma IRI

Table 1. Chronologic Changes in Body Weight, Abdominal Fat Deposition, and Plasma Cholesterol and Triglyceride Levels in OLETF and LETO Rats

Group	No.	Body Weight (g)	Abdominal Fat (g)	Total Cholesterol (mmol/L)	Triglyceride (mmol/L)
10 weeks					
OLETF	6	396 ± 9.8	13.8 ± 1.05	1.50 ± 0.06	0.85 ± 0.09
LETO	7	304 ± 3.0*	6.4 ± 0.49	1.37 ± 0.04	0.32 ± 0.05
16 weeks					
OLETF	7	470 ± 8.8*	23.4 ± 1.21†	1.79 ± 0.09	1.36 ± 0.15
LETO	7	377 ± 8.8‡§	9.3 ± 1.02§	1.48 ± 0.05	0.38 ± 0.04§
24 weeks					
OLETF	7	564 ± 18.2*‖	36.9 ± 4.21*§	1.89 ± 0.06	1.65 ± 0.30*
LETO	7	417 ± 5.0‡#	14.3 ± 0.69‖#	1.76 ± 0.08†‡¶	0.51 ± 0.06†§#
40 weeks					
OLETF	5	559 ± 23.9*§	49.7 ± 4.24*§#	2.64 ± 0.21*§#	2.68 ± 0.34*§#
LETO	7	512 ± 4.6*‡¶**‡‡	23.5 ± 1.82†‡¶ #‡‡§§	1.92 ± 0.13§#††§§	0.72 ± 0.07‡¶#§§

*P < .01 *v* 10-week OLETF.
†P < .05 *v* 10-week LETO.
‡P < .01 *v* 10-week LETO.
§P < .01 *v* 16-week OLETF.
‖P < 0.05 *v* 16-week OLETF.
¶P < .01 *v* 16-week LETO.
#P < .01 *v* 24-week OLETF.
**P < .05 *v* 24-week OLETF.
††P < .01 *v* 24-week LETO.
‡‡P < .05 *v* 24-week LETO.
§§P < .01 *v* 40-week OLETF.

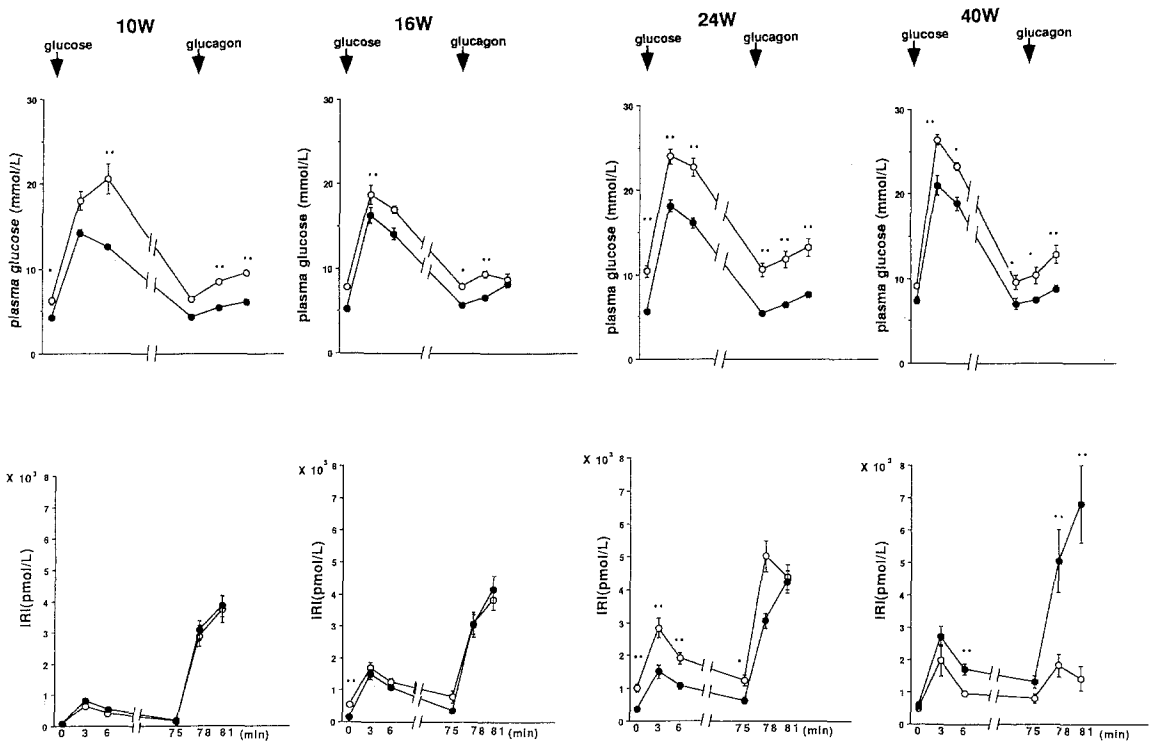

Fig 1. Chronologic changes in plasma glucose and IRI responses to glucose and glucagon in OLETF (○) and LETO (●) rats. Points and bars represent the mean ± SEM. *$P < .05$; **$P < .01$. W, weeks (age).

increased to a peak 3 minutes after IV glucose administration and then decreased to the basal level at 75 minutes. It increased again after IV glucagon administration. Maximum plasma IRI concentrations attained after IV glucose administration were significantly lower than those after IV glucagon administration in both groups of all ages except OLETF rats of at 40 weeks. There were no significant differences in the magnitudes of plasma IRI responses to either IV glucose or IV glucagon administration in OLETF and LETO rats at 10 weeks of age. At 16 weeks of age, basal plasma IRI concentration was higher in OLETF rats than in LETO rats, but concentrations induced by IV glucose and glucagon were not different from each other. At 24 weeks of age, basal insulin concentration and insulin secretion in response to IV glucose were significantly increased in OLETF rats versus LETO rats. The area under the curve of plasma IRI in response to IV glucose was not significantly different between both groups at 16 weeks of age (OLETF 7.78 ± 0.83 *v* LETO 6.32 ± 0.66 nmol · min), but it was significantly higher in OLETF rats (12.78 ± 1.23 nmol · min) than in LETO rats (6.64 ± 0.78) at 24 weeks of age. At 40 weeks of age, plasma IRI response to IV glucose administration was not exaggerated, but was instead suppressed in OLETF rats, as seen by the reduced concentration at 6 minutes compared with the corresponding value in LETO rats, although plasma glucose concentrations were higher in the former than in the latter. The reduced secretory function of pancreatic β cells in OLETF rats at 40 weeks was apparent in the plasma IRI response to IV glucagon administration as compared with that in LETO rats of the same age (Fig 1).

In Vivo Glucose Disposal

Insulin-stimulated glucose disposal in vivo was reduced 42% in OLETF rats at 16 weeks of age (GIR, 40.9 ± 4.2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) versus 10 weeks of age (70 ± 4.9); the latter value was similar to that in LETO rats of the same age (76.6 ± 8.1). Significant reductions of insulin-stimulated glucose disposal in vivo were also observed in OLETF rats at 24 and 40 weeks. Insulin-stimulated glucose disposal in vivo did not decrease with age in LETO rats (GIR in LETO rats at 40 weeks, 79.1 ± 10.4 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig 2).

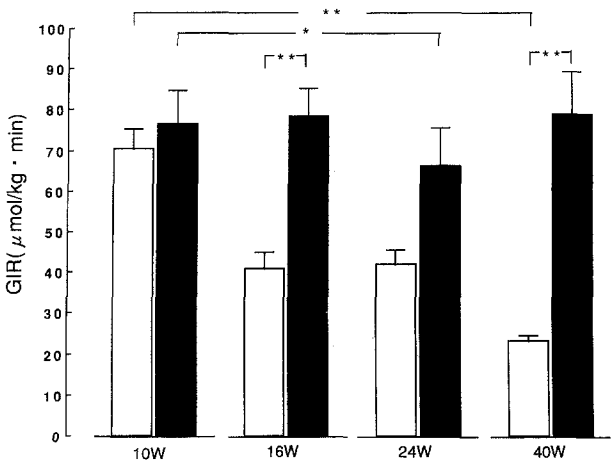

Fig 2. Chronologic changes in GIR of OLETF (□) and LETO (■) rats. GIR is the mean value in the last 20 minutes. Points and bars represent the mean ± SEM. *$P < .05$; **$P < .01$. W, weeks (age).

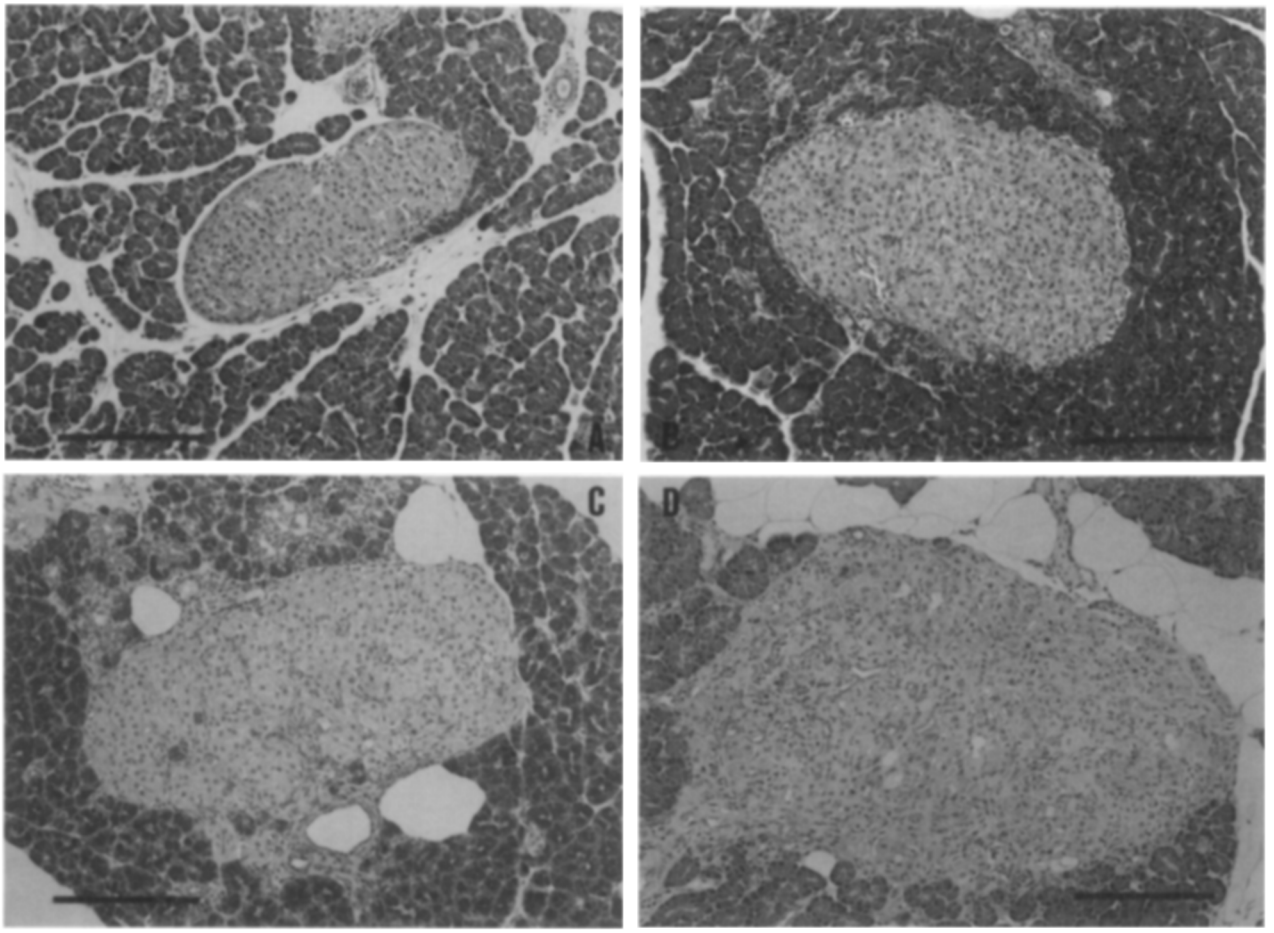

Fig 3. Typical light-microscopic features of islets from OLETF rats at ages 10 weeks (A), 16 weeks (B), 24 weeks (C), and 40 weeks (D). Note the changes in islet size and degree of connective tissue proliferation. Hematoxylin and eosin (original magnification ×100); bar, 200 μm.

Histologic Findings

Light-microscopic findings in random sections through the pancreata of OLETF rats at 10, 16, 24, and 40 weeks are shown in Fig 3A, B, C, and D, respectively. Islets of the 40-week-old rat shown in Fig 3D are clearly enlarged and have typical alterations. More than half the islets in individual rats were 250 to 500 μm in diameter, and islets of greater than 500 μm were observed in all five pancreata examined, as shown in Table 2. Various extents of connective tissue proliferation were seen in the enlarged islets, in which clusters of endocrine cells were widely separated from each other by traverse bands of connective tissue, resulting in a nodular appearance. Profound fibrotic change was noted in four of five pancreata of 40-week-old OLETF rats. In contrast, islets of the 10-week-old rat shown in Fig 3A appear normal with respect to size and proliferation of connective tissue. Histologic alterations of the islets of OLETF rats at 16 and 24 weeks were intermediate between those of animals at 10 and 40 weeks, although findings at 16 weeks resembled those at 10 weeks and findings at 24 weeks resembled those at 40 weeks most closely. The extent of increase in connective tissue was less than the extent of enlargement of islets in all groups when compared by age. The frequencies of various degrees of histopathologic changes differed significantly in various age groups of OLETF rats ($\chi^2 = 27.1$ and $P < .1$ for enlargement of islets, and $\chi^2 = 23.2$ and $P < .01$ for increase in connective tissue). No significant pathologic changes in islets were observed in LETO rats of various age groups; there was no enlargement of islets or proliferation of connective tissue in islets even in LETO rats at 40 weeks of age, the oldest ones examined.

Table 2. Variations in Islet Size and Structure in Pancreata From OLETF Rats of Various Ages

Age (wk)	Enlargement of Islets				Increase in Connective Tissue			
	—	±	1+	2+	—	±	1+	2+
10	4	1	1		6			
16	1	1	5		5	1	1	
24			3	4		1	2	4
40				5			1	4

NOTE. Values are the number of rats that showed a given degree of histologic change. Islet size: —, >95% of 100 to 250 μm in diameter; ±, between — and 1+; 1+, predominantly (>50%) 150 to 350 μm in diameter, with a few >500 μm (<2%); 2+, predominantly (>50%) 250 to 500 μm in diameter, >500 μm frequent (>10%). Connective tissue proliferation: —, no fibrosis; ±, between — and 1+; 1+, thin fibrous bundles in >50% of islets; 2+, thick fibrous bundles in >50% of islets.

DISCUSSION

We studied the temporal relationship between insulin resistance and impairment of pancreatic β-cell function in OLETF rats. Our findings showed that insulin resistance preceded insulin deficiency in OLETF rats. Insulin sensitivity as evaluated by the GIR was significantly lower in OLETF rats than in LETO rats at 16 weeks, and the impairment of pancreatic β-cell function became obvious in OLETF rats at 40 weeks of age: ie, reduced IRI responses to glucose and glucagon despite the presence of hyperglycemia (Fig 1). If the hyperinsulinemia is regarded as an indicator of the dysfunction of the pancreatic β cell, it begins at 16 weeks of age in OLETF rats, when insulin resistance becomes obvious, showing that we cannot say which is the primary etiologic event in this rat, insulin resistance or impaired insulin secretion. Furthermore, it can be said that this hyperinsulinemia is due to a primary β-cell abnormality that in turn causes peripheral insulin resistance. But this is not the case, because the magnitude of hyperinsulinemia was not comparable to that of insulin resistance at 16 weeks of age, and it became larger at 24 weeks of age even though the magnitude of insulin resistance at this age was similar to that at 16 weeks of age. The findings, ie, hyperglycemia in the presence of an exaggerated IRI response to IV glucose in OLETF rats at 24 weeks of age, can be explained by supposing that in OLETF rats insulin secretion was not sufficient to overcome the ineffectiveness of insulin action. If so, pancreatic β-cell function in OLETF rats may be impaired earlier than the stage of decrease in the plasma IRI response to stimuli, in terms of the absolute concentration. To compensate for insulin insensitivity, pancreatic β cells secrete more insulin but not enough to overcome insulin resistance in OLETF rats. At 40 weeks of age, plasma IRI responses to IV administration of glucose and glucagon were lower in OLETF rats than in LETO rats (Fig 1). In particular, the lack of an insulin response to IV glucagon administration indicated the inability of β cells to secrete insulin. This IRI secretion pattern of OLETF rats resembles that of patients with NIDDM: ie, an exaggerated response of plasma IRI to stimuli in an early stage and a lack of response in a later stage.[5]

Islets in OLETF rats changed morphologically with age, showing enlargement and connective tissue proliferation. Shino et al[6] reported similar changes in Zucker rats: they observed moderate islet hypertrophy as early as 5 weeks of age, which increased with age to a maximum at 24 weeks, when good correlations were found between the degrees of islet hypertrophy and obesity and the plasma insulin level. Hayek and Woodside[7] reported that the islet size in obese Zucker rats is closely associated with excess insulin secretion. Histologic changes in the pancreas of OLETF rats might be the result of overactivity of β cells in compensating for insulin insensitivity, which was supported by our previous findings[3] that the histologic changes described earlier, were not observed in the pancreata of exercise-trained OLETF rats in which insulin sensitivity was increased, probably due to a reduction of fat deposition, especially in the abdominal cavity, resulting in normalization of hyperinsulinemia.

The hyperglycemia associated with hyperinsulinemia observed in OLETF rats might also be due to the presence of a large amount of proinsulin in the plasma. Zucker and Antoniades[8] reported that a portion of serum IRI in Zucker "fatty" rats, which show obesity, an increased plasma concentration of IRI, and insulin resistance,[9] may represent a proinsulin-like immunoreactive material that is less active biologically than insulin. The insulin antibody used in our radioimmunoassay for insulin is not specific for insulin but cross-reacts with proinsulin to a certain extent, according to the information provided by the manufacturer. Possibly, proinsulin-like materials are included in our IRI value. If the plasma proinsulin level of OLETF rats is greater than that of LETO rats, as reported for Zucker "fatty" rats, the biological activity of IRI is decreased and hyperglycemia remains despite increased blood levels of IRI in OLETF rats. Plasma concentration of proinsulin in OLETF rats has to be determined.

What causes insulin resistance in this strain of rat? OLETF rats gain body weight faster than normal control LETO rats, with the difference gradually increasing with age (Table 1). Wistar Fatty rats, which were established by transferring the *fa* gene from Zucker rats to Wistar Kyoto rats, develop obesity and show diabetic symptoms such as hyperglycemia, polydipsia, and glycosuria.[10] So obesity is probably a potent diabetogenic factor. The weight of abdominal fat is also greater in OLETF rats than in LETO rats of the same age, and it increases with age (Table 1). Bolinder et al[11] reported that visceral adipocytes have lower sensitivity to insulin's antilipolytic effects than subcutaneous adipocytes. Moreover, Fujioka et al[12] reported that many patients with visceral fat obesity show glucose intolerance and that glucose tolerance improves markedly after decreasing the visceral fat volume as a result of substantial weight reduction. Judging from these findings, the high level of abdominal fat deposition is also considered a factor in the insulin insensitivity of OLETF rats, although the exact mechanism is not fully elucidated despite various hypotheses.[11,13-16]

Plasma triglyceride level in OLETF rats increased chronologically, with the level at 40 weeks being three times that at 10 weeks (Table 1). This increase might result from hyperinsulinemia. Insulin controls fat mobilization, principally by stimulating triglyceride synthesis in adipose tissue and the liver. In Zucker Fatty rats, hyperlipidemia has been explained by increased production of triglyceride in the liver, owing to hyperinsulinemia.[17] Hyperlipidemia is thought to induce insulin resistance in peripheral tissue by the Randle effect[18]: a high plasma free fatty acid (FFA) level tends to increase muscle FFA and decrease glucose uptake by muscle, and makes muscle resistant to insulin.[8] The FFA level of OLETF rats is also an interesting matter for investigation.

In conclusion, our data indicate that insulin resistance begins earlier than hyperinsulinemia in the spontaneous NIDDM model, OLETF rats. This insulin resistance is closely related to fat deposition, especially in the abdominal cavity.

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