Obesity Is Necessary But Not Sufficient for the Development of Diabetes Mellitus

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To investigate whether inheritance or obesity plays a more important role in the development of non–insulin-dependent diabetes mellitus (NIDDM), female Otsuka-Long-Evans-Tokushima Fatty (OLETF) rats, which possess the diabetogenic gene, ODB-1, and Long-Evans-Tokushima-Otsuka (LETO) rats, which have no ODB-1, were compared. Neither strain becomes obese and diabetic when bred ordinarily. Female OLETF rats and male and female LETO rats were assigned to two groups of 20 rats each. Obesity was induced in one group by feeding a high-energy "cafeteria" diet (group D), and the other group was given standard chow (group C). Twenty male OLETF rats were used as NIDDM positive controls. At 25 weeks of age, the mean body weight of group D male LETO and female OLETF rats increased at a rate similar to that of male OLETF rats; female LETO rats did not show increased body weight. The incidence of diabetes mellitus in obese female OLETF rats in group D and positive control male OLETF rats was the same (80%). Only 30% of obese male LETO rats in group D developed diabetes mellitus. The insulin response to intravenous glucose in group D female OLETF rats was the highest for all groups but not sufficient to decrease blood glucose levels. In female OLETF rats, glucose infusion rate (GIR) during a euglycemic-hyperinsulinemic clamp test in group D was decreased to 50% of the group C value and tissue glucose uptake as determined by ^{3}H-glucose infusion was significantly decreased in muscle. In male LETO rats, group D GIR was mildly decreased (80% of group C value) compared with the GIR of female OLETF rats. For obese group D female OLETF rats, abdominal fat increased more with obesity than in their male LETO counterparts. GIR was inversely correlated with the weight of abdominal fat when the data of all groups of animals were combined. The expression of GLUT4 mRNA and its protein level in adipose and muscle tissues and tumor necrosis factor alpha (TNF-α) protein in adipose tissue were not significantly different between group D and group C of both strains. In conclusion, the incidence of diabetes in female OLETF rats that possess the diabetogenic gene was significantly greater than in the LETO strains that do not possess the gene, in the presence of excess adiposity.

THE ISSUE OF OBESITY vis-a-vis inheritance (genetics) as a factor in the development of non–insulin-dependent diabetes mellitus (NIDDM) is a subject of considerable debate. It is well known that obesity induces a variety of geriatric diseases and that visceral obesity is an important component of the insulin resistance syndrome (syndrome X).[1] Conversely, there is also a body of epidemiologic evidence suggesting that genetic factors are involved in the development of NIDDM. For example, concordance of the prevalence of NIDDM in identical twins approaches 90%.[2] There is no direct information on the relative importance of environmental and genetic factors in the etiology of NIDDM, since such a study is impossible to conduct using human subjects. To further study this issue, we used female Otsuka-Long-Evans-Tokushima Fatty (OLETF) rats, which have the diabetogenic gene, ODB-1[3] but do not become obese or develop diabetes mellitus when fed standard chow, and Long-Evans-Tokushima-Otsuka (LETO) rats, which have no ODB-1 gene and do not develop diabetes mellitus. Obesity was induced by feeding a high-energy "cafeteria" diet. Because insulin resistance constitutes the first diabetic step for OLETF rats,[4] the mechanism of insulin resistance was also investigated in these rats by measuring insulin sensitivity in various tissues.

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MATERIALS AND METHODS

Animals

A spontaneously 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 (St Constant, Canada) in 1983 and subsequently maintained at the Tokushima Research Institute of Otsuka Pharmaceutical (Tokushima, Japan). After 20 generations of selective breeding, the diabetic strain, OLETF, was established in 1990.[5] According to Kawano et al,[5] the cumulative incidence of diabetes in male and female OLETF rats at 23 weeks of age is 86.0% and 0%, respectively. A nondiabetic strain (LETO) was used as a nondiabetic control. Twenty male OLETF, 40 female OLETF, 40 male LETO, and 40 female LETO rats were obtained from Tokushima Research Institute (Otsuka Pharmaceutical). They were maintained in our animal facilities (Institute for Animal Experimentation, University of Tokushima, Tokushima, Japan) under specific pathogen-free conditions at controlled temperature (21° ± 2°C), humidity (55% ± 5%), and lighting (7 AM to 7 PM).

Experimental Design

Female OLETF and male and female LETO rats were randomly assigned to two groups of 20 rats each. One group (group D) of rats were given a palatable high-energy "cafeteria" diet composed of supermarket foods including cookies, chocolate bars, and rice crackers mixed with standard rat chow (Oriental Yeast, Tokyo, Japan) starting at 5 weeks of age. The other group (group C) of rats and positive control male OLETF rats were given standard chow only. Tap water was available ad libitum for both groups.

Oral Glucose Tolerance Test

At 25 weeks of age, 10 rats from each group were given an oral glucose tolerance test (OGTT) after an overnight fast. Glucose 2 g/kg (500 g/L) body weight was administered orally, and blood was taken from a tail vein without anesthesia at 0, 30, 60, and 120

minutes for measurement of blood glucose levels. Rats were diagnosed as diabetic if the peak blood glucose level was at least 16.7 mmol/L and 120-minute blood glucose level was at least 11.1 mmol/L.

Intravenous Glucose Tolerance Test

At 27 weeks of age, the same 10 rats from each group were given an intravenous glucose tolerance test (IVGTT). After an overnight fast, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg · kg^{-1}). Immediately after withdrawing blood samples at 0 minutes, rats were injected with glucose 0.5 g/kg (500 g/L) body weight via one jugular vein. Blood was taken from a contralateral right cervical vein for measurement of blood glucose levels at 0, 3, 6, 9, 12, and 15 minutes and for measurement of plasma insulin levels at 0, 3, and 6 minutes.

Measurement of In Vivo Glucose Disposal by a Euglycemic-Hyperinsulinemic Clamp Test

Insulin-mediated whole-body glucose uptake was measured in anesthetized rats using a euglycemic clamp technique[6] within 2 weeks after the IVGTT. After an overnight fast, six rats from each group were anesthetized by intraperitoneal injection of pentobarbital (50 mg · kg^{-1}) and catheters were inserted into the femoral vein. Rats received an infusion of insulin (Novo Nordisk, Bagsvaerd, Denmark) at a rate of 60 pmol · kg^{-1} · min^{-1} for 1 hour. An infusion of 100-g/L glucose solution was started at time zero, and the rate was adjusted to clamp plasma glucose at approximately 6.1 mmol/L. Blood samples for determination of glucose were obtained at 2- to 5-minute intervals throughout the study. Data on total-body glucose uptake represent the mean values for the glucose infusion rate (GIR) during the last 20 minutes.

Measurement of Hepatic Glucose Output

Hepatic glucose output (HGO) was measured in the same group of rats during the euglycemic-hyperinsulinemic clamp test. At time zero, 2.5 μCi (10 μCi/mL) D-[U-^{14}C]glucose (Amersham International Buckinghamshire, England) was infused as a bolus followed by continuous infusion at a rate of 10 μCi/h. Blood samples for determination of D-[U-^{14}C]glucose specific activity were obtained at 55 and 60 minutes. Blood was deproteinized in $Ba(OH)_2/ZnSO_4$ as described by Somogyi[7] and centrifuged (2 minutes at 16,000 × *g*). D-[U-^{14}C]Glucose in the supernatant was determined by liquid scintillation counting (LSC-700; Aloka, Tokyo, Japan). The glucose disappearance rate (Gd) and HGO were determined by Steel's method.[8] Gd and HGO were calculated as follows:

$$\text{Gd} = \frac{\text{blood glucose (mg/dL)} \times \text{flow rate (μL/min)} \times \text{total count/plasma count}}{\text{body weight (kg)}} \times 0.2 \times 10^{-4}$$

$$\text{Gd} - \text{GIR} = \text{HGO}.$$

The average HGO at 55 and 60 minutes was calculated.

Determination of Insulin-Stimulated Glucose Utilization Index in Adipose Tissue and Muscle Tissue

The glucose utilization index for retroperitoneal, mesenteric, and subcutaneous adipose tissues and skeletal muscle tissue was measured in the same group of animals during the euglycemic-hyperinsulinemic clamp test using the 2-deoxy-D-[1-^{3}H]glucose technique as described by Ferré et al[9] and James et al.[10] In brief, 2-deoxy-D-[1-^{3}H]Glucose (25 μCi, 1 mCi/mL; Amersham) was injected in 250 μL 0.9% NaCl as a bolus through the femoral vein. A blood sample (50 μL) for determination of the plasma tracer concentration was obtained 60 minutes after the bolus administration. At the end of the clamp test, rats were rapidly anesthetized with pentobarbital (60 mg · kg^{-1}), and retroperitoneal, mesenteric, and subcutaneous adipose tissues and skeletal muscle tissue were removed and frozen in liquid N_2. Blood was deproteinized in $Ba(OH)_2/ZnSO_4$ as described earlier, and the supernatant was used for determination of 2-deoxy-D-[1-^{3}H]glucose by liquid scintillation counting. Tissue samples were weighed and placed into 1 mol/L NaOH (2.5 mL/1 g tissue) and heated at 60°C for 45 minutes to totally digest the tissues, after which 1 mol/L HCl (2.5 mL/1 g tissue) was added. One milliliter of 6% $HClO_4$ was added to 200 μL neutralized solution, and 1 mL $Ba(OH)_2/ZnSO_4$ was added to another 200 μL solution. After centrifugation, supernatants (800 μL) of these solutions were used for determination of radioactivity after addition of 10 mL ATOMLIGHT scintillation solution (Biotechnology Systems, Boston, MA) by liquid scintillation counting. Because 2-deoxyglucose and 2-deoxyglucose 6-phosphate are both soluble in 6% $HClO_4$ and only 2-deoxyglucose is soluble in the Somogyi reagent [$Ba(OH)_2/ZnSO_4$], the content of 2-deoxy-D-[1-^{3}H]glucose 6-phosphate in each tissue was obtained by subtracting the radioactivity (dpm) in the $Ba(OH)_2/ZnSO_4$ supernatant from that in the $HClO_4$ supernatant. Tissue glucose uptake (defined as the glucose metabolic index, Rg′) was calculated using the following equation described by Kraegen et al[11]:

$$\text{Rg}'(\mu\text{mol}/100\,\text{g}/\text{min}) = \frac{\text{Cp} \times \text{Cm}^*(60)}{\int_0^{60} \text{Cp}^*(\text{t})\,\text{dt}}.$$

Cp is the steady-state plasma glucose concentration over a 60-minute period of observation (mmol/L); Cm* is tissue accumulation of [^{3}H]2-deoxyglucose 6-phosphate per unit mass at 60 minutes (dpm/mg wet weight); Cp*(t) is the plasma [^{3}H]2-deoxyglucose concentration (dpm/mL); and t = 0 when the tracer is administered as a bolus.

Assays

Plasma glucose and blood glucose were determined by the glucose oxidase method (Fuji Dri-Chem 2000, Fuji Medical Systems, 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).

Extraction of RNA and Northern Blotting for GLUT4 Detection

In another series of experiments, 10 rats in each group were fasted and anesthetized by intraperitoneal injection of pentobarbital (60 mg · kg^{-1}). Then retroperitoneal fat and mesenteric fat tissues and skeletal muscle tissue were removed, weighed, and immediately frozen in liquid N_2 for Northern and Western blot analyses. Total RNA was extracted from samples of retroperitoneal fat tissue and skeletal muscle tissue using ISOGEN (Nippon Gene, Toyama, Japan)[12] following the manufacturer's protocol with minor modifications. The tissues were homogenized in the reagent and centrifuged. After removing the top lipid layer (in the case of RNA preparation from fat tissue), the homogenized solution was mixed with chloroform and then centrifuged. Total RNA was precipitated from the aqueous phase by addition of isopropanol, washed with 70% ethanol, and dissolved in water. This RNA solution was then mixed with ISOGEN and chloroform, precipitated by isopropanol, washed, and redissolved in water. RNA (6 μg) was denatured in 50% formamide and 2.2 mol/L formaldehyde at 65°C for 10 minutes and electrophoresed in 1%

agarose gel containing 2.2 mol/L formaldehyde. The gel was blotted onto a Hybond-N nylon hybridization membrane (Amersham International). The membrane was hybridized with the [α-^{32}P]dCTP random-priming–labeled[13] rat GLUT4 cDNA probe (kindly provided by Dr Oka, University of Yamaguchi, Yamaguchi, Japan) and rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA, washed at a stringency of 0.2× SSC (1 × SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate) and 0.1% sodium dodecyl sulfate at 68°C, and exposed to x-ray film.[14] The amount of intact RNA in each lane of the gel was judged to be constant by ethidium bromide fluorescence, identifying specific bands of 18S and 28S RNA directly in the gel and after transferring the RNA to a nylon hybridization membrane. A Bio-image analyzer BAS2000 (Fuji Film Institution, Tokyo, Japan)[15] was used for quantification. After quantification of expression levels of GLUT4 and G3PDH mRNAs, normalization of GLUT4 to G3PDH mRNA was performed.

Preparation of Total Membrane Fractions and Western Blotting for GLUT4 Detection

Approximately 500 mg skeletal muscle or retroperitoneal fat was homogenized in 8 mL buffer (20 mmol Tris, pH 7.5/5 mmol EDTA/0.1 mmol DTT/20 μmol [*p*-amidinophenyl]-methane sulfonyl fluoride hydrochloride [WAKO Pure Chemical Industries, Osaka, Japan]/250 mmol sucrose). After centrifugation at 9,000 × *g* for 10 minutes at 4°C, the supernatant was recentrifuged at 217,000 × *g* for 30 minutes at 4°C to precipitate the membrane fraction. The pellets were then suspended in 50 μL buffer (50 mmol HEPES, pH 7.5/150 mmol NaCl/1% Triton X-100/10 μmol *p*-amidino PMSF/5 mmol EDTA/5 mmol EGTA/20 mmol sodium pyrophosphate/1 mmol orthovanadate/20 mmol NaF) and sonicated with four 15-second bursts. After incubation for 60 minutes at 4°C, the homogenate was centrifuged at 163,000 × *g* for 30 minutes at 4°C and the supernatant was used for determination of protein concentration with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). The membrane fractions were subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[16] The gel was electroblotted onto a nitrocellulose filter[17] and blocked with 5% skim milk (DIFCO, Detroit, MI). The filter was immunoblotted with rabbit IgG antibody against rat GLUT4 (TRI, Cambridge, MA) followed by an anti–rabbit IgG horseradish peroxidase–linked F(ab')$_2$ fragment (from donkey). Immunolabeled bands were detected using an enhanced chemiluminescence Western blotting system (Amersham) and exposed to x-ray film. The amount of GLUT4 protein in the total membrane fractions was then determined quantitatively by densitometric techniques.

Preparation of Total Proteins and Western Blotting for Tumor Necrosis Factor Alpha Detection

Approximately 500 mg mesenteric fat tissue was homogenized in 1 mL of the same buffer as used for the preparation of total membrane fractions, and then centrifuged at 6,800 × *g* for 10 minutes at 4°C. After removing the top lipid layer, the intermediate phase was again centrifuged at 6,800 × *g* for 10 minutes at 4°C, and the supernatant was used for determination of protein concentration. Western blotting was performed in a similar way for GLUT4 protein detection, except that rabbit anti–mouse tumor necrosis factor alpha (TNF-α) antibody (Genzyme, Cambridge, MA) was used as the first antibody. Recombinant murine TNF-α (GIBCO BRL, Gaithersburg, MD) was used as a positive control. Four samples from each group of rats were examined.

Statistical Analysis

The 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. Student's *t* test was used for comparing amounts of GLUT4 mRNA and protein and TNF-α protein between each group. Correlations between GIR, abdominal fat, and fasting blood glucose were determined by Spearman's test.

RESULTS

Body Weight and Abdominal Fat

The body weight of positive control male OLETF rats increased from 152.8 ± 3.6 g at 5 weeks of age to 626.9 ± 7.3 g at 25 weeks of age (Fig 1). The cafeteria diet successfully induced obesity in both D groups of female OLETF rats and male LETO rats, but not in female LETO rats. Body weights of both D groups of female OLETF rats (599.2 ± 5.5 g) and male LETO rats (634.3 ± 7.9 g) were not significantly different from those of positive control male OLETF rats at 25 weeks of age. However, the body weight of group D female LETO rats (355.2 ± 5.8 g) was much less than that of positive control male OLETF rats at 25 weeks of age. Abdominal fat deposits, which consist of mesenteric, retroperitoneal, and epididymal (in the case of the male) fat, in group D female OLETF rats (96.56 ± 3.86 g) were significantly higher than those in group C (42.75 ± 2.12 g) and positive control (71.74 ± 5.05 g) male OLETF rats. In group D male LETO rats, abdominal fat deposits were also significantly higher (60.02 ± 2.85 g) than in group C (23.96 ± 2.19 g). Abdominal fat deposits in group D female LETO rats (30.5 ± 1.75 g) were much smaller than in positive control male OLETF rats. Because obesity was not induced in female LETO rats, they were excluded from further experiments.

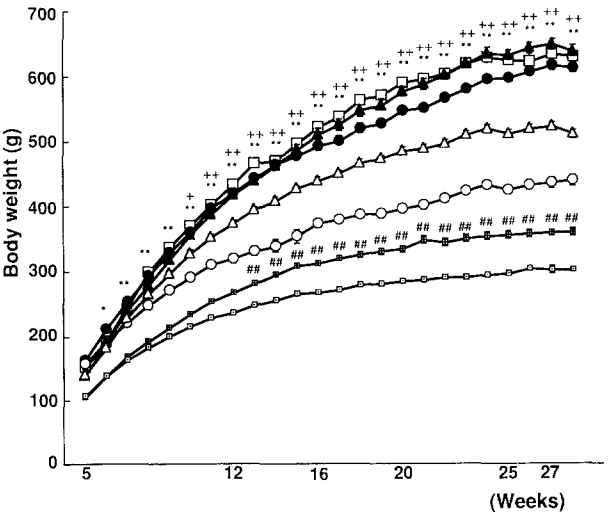

Fig 1. Chronological changes in body weight of positive control OLETF male (□), group D OLETF female (●), group C OLETF female (○), group D LETO male (▲), group C LETO male (△), group D LETO female (⊞), and group C LETO female (⊡) rats. Points and bars represent the mean ± SEM. *P < .05, **P < .01: *v* group C, OLETF female rats. ^{+}P < .05, ^{++}P < .01: *v* group C, LETO male rats. $^{\#\#}P$ < .01 *v* group C, LETO female rats.

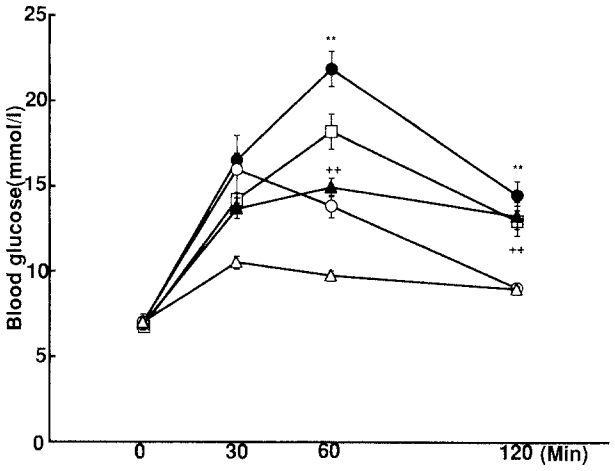

Fig 2. Blood glucose responses to an oral glucose load in positive control OLETF male (□), group D OLETF female (●), group C OLETF female (○), group D LETO male (▲), and group C LETO male (△) rats at 25 weeks of age. Points and bars represent the mean ± SEM. **$P < .01$ *v* group C OLETF female rats. $^{++}P < .01$ *v* group C LETO male rats.

OGTT and Incidences of Diabetes Mellitus

Blood glucose responses to an oral glucose load in five groups at 25 weeks of age are shown in Fig 2. At all time points after an oral glucose load, group D female OLETF rats showed significantly higher blood glucose levels than group C male LETO rats. The incidence of diabetes mellitus in group D female OLETF rats and positive control male OLETF rats was identical: 80%. However, only 30% of group D male LETO rats developed diabetes mellitus. No group C female OLETF and male LETO rats became diabetic.

IVGTT

Blood glucose and plasma immunoreactive insulin (IRI) responses to an intravenous glucose load at 27 weeks of age are shown in Fig 3. Basal blood glucose levels for group C male OLETF and group D female OLETF rats were higher than the respective basal blood glucose levels in the OGTT performed at 25 weeks of age. This suggests a possible deterioration of glucose tolerance in these rats as a function of age. Group D female OLETF rats showed the highest blood glucose and insulin levels of the five groups. The total IRI area after an intravenous glucose load for group D male LETO rats (7.29 ± 0.7 nmol/L · 6 min) was significantly higher than for group C (3.61 ± 0.29 nmol/L · 6 min), whereas for female OLETF rats the total IRI area for group D (9.48 ± 0.64 nmol/L · 6 min) was not significantly different from that for group C (7.90 ± 0.78 nmol/L · 6 min). The total IRI area for positive control male OLETF rats was 5.50 ± 0.58 nmol/L · 6 min.

Measurement of In Vivo Glucose Disposal by the Euglycemic-Hyperinsulinemic Clamp Test

The GIR, which represents total-body glucose uptake, was inversely correlated with the weight of abdominal fat ($r = -.79, P < .01$; Fig 4) when the data from all groups of animals were combined. This correlation was much higher than that between GIR and body weight ($r = -.38, P < .05$; data not shown). There was also a significant correlation between GIR and fasting blood glucose concentration ($r = -.48, P < .01$; Fig 5). GIR was reduced 50% in group D female OLETF rats compared with group C, whereas only a 20% decrease was observed for group D male LETO rats compared with group C (Fig 6).

Measurement of HGO

HGO levels are shown in Fig 6. In positive control male OLETF rats, HGO during the hyperinsulinemic-euglycemic clamp test was not inhibited but was significantly higher than that of normal male LETO rats. For male LETO rats, HGO for group D was higher than for group C, but the difference was not statistically significant.

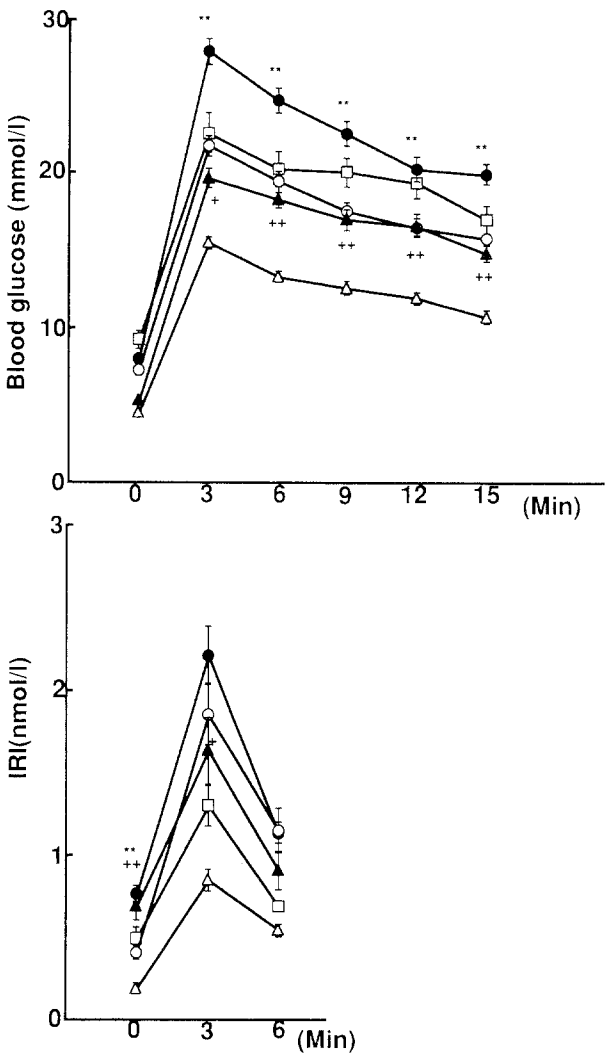

Fig 3. Blood glucose and IRI responses to intravenous glucose load in positive control OLETF male (□), group D OLETF female (●), group C OLETF female (○), group D LETO male (▲), and group C LETO male (△) rats at 27 weeks of age. Points and bars represent the mean ± SEM. **$P < .01$ *v* group C OLETF female rats. $^{+}P < .05$, $^{++}P < .01$: *v* group C LETO male rats.

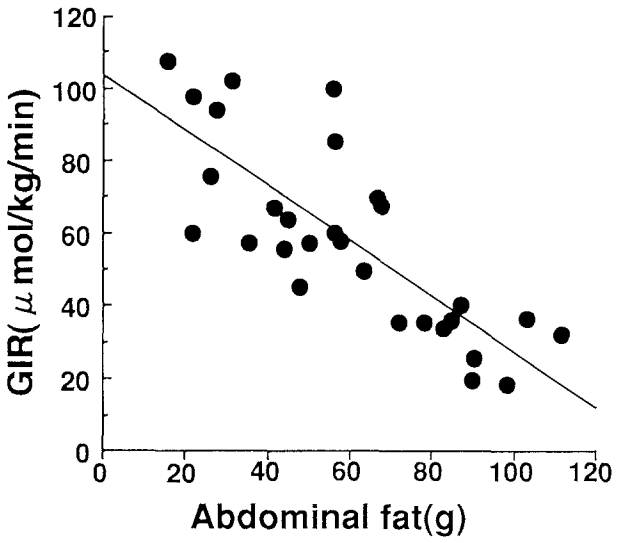

Fig 4. Correlation between GIR and weight of abdominal fat in male and female OLETF rats and male LETO rats at 30 weeks of age. GIR was inversely correlated with the weight of abdominal fat ($r = -.79, P < .01$).

Determination of Insulin-Stimulated Glucose Utilization Index in Adipose and Muscle Tissue

Figure 6 shows ^{3}H-glucose uptake in muscle and retroperitoneal fat during a euglycemic-hyperinsulinemic clamp test. In muscle, the glucose uptake of group D female OLETF rats was about 50% that of group C, whereas that of group D male LETO rats was higher than that of group C. In addition, glucose uptake in muscle of positive control male OLETF rats was significantly lower compared with that of normal controls, group C male LETO rats. In retroperitoneal fat, there was no significant difference between group D and group C in rats of either strain, except for a tendency for higher glucose uptake in male LETO versus male

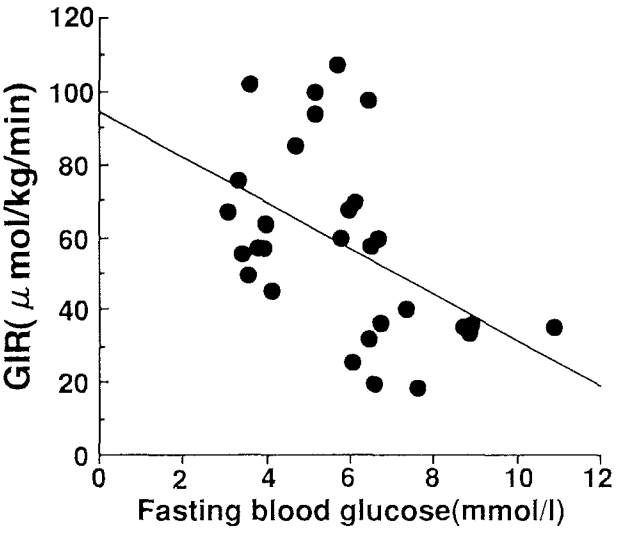

Fig 5. Correlation between GIR and fasting blood glucose in male and female OLETF rats and male LETO rats at 30 weeks of age. GIR was inversely correlated with fasting blood glucose ($r = -.48, P < .01$).

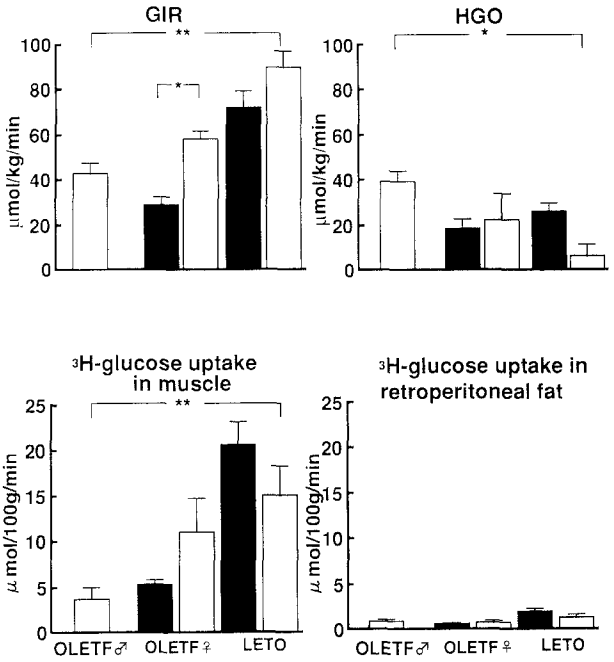

Fig 6. GIR, HGO, and ^{3}H-glucose uptake in muscle and retroperitoneal fat tissues during a euglycemic-hyperinsulinemic clamp test in male OLETF, female OLETF, and male LETO rats. After overnight fasting, rats were anesthetized and received an infusion of 100 g/L-glucose solution, insulin at 60 pmol · kg^{-1} · min^{-1} for 1 hour, ^{14}C-glucose at 10 μCi/h for 1 hour, and ^{3}H-glucose 25 μCi by bolus; infusion of 100-g/L glucose solution was adjusted to clamp blood glucose at approximately 6.1 mmol/L. GIR values are the mean for the final 20 minutes. (■) Group D rats; (□) group C rats. Bars represent the SEM. **$P < .01$, *$P < .05$: *v* other groups.

OLETF rats. The same was true for mesenteric and subcutaneous fat tissues (data not shown). The mean glucose uptake in subcutaneous, retroperitoneal, and mesenteric fat tissues ranged from 11% to 15% of that in muscle.

Northern Blot Analysis for GLUT4 mRNA and Western Blot Analysis for Total Membrane GLUT4 Protein in Adipose and Muscle Tissue

Table 1 shows expression levels of GLUT4 mRNA and total membrane GLUT4 protein in retroperitoneal fat and muscle tissues. There was no significant difference in these parameters between group D and C rats of either strain,

Table 1. Expression of GLUT4 mRNA and Total Membrane GLUT4 Protein in Muscle and Retroperitoneal Fat of Group C and D Rats

	GLUT4 mRNA/G3PDH (AU/mm²)		Total Membrane GLUT4 Protein (AU/mm²)	
Group	Muscle	Retroperitoneal Fat	Muscle	Retroperitoneal Fat
C OLETF male	0.40 ± 0.03	0.15 ± 0.02	0.35 ± 0.08	1.77 ± 0.83
D OLETF female	0.54 ± 0.08	0.27 ± 0.04*	0.35 ± 0.04	1.72 ± 0.61
C OLETF female	0.62 ± 0.09	0.11 ± 0.03	0.33 ± 0.03	2.65 ± 0.55
D LETO male	0.36 ± 0.06	0.38 ± 0.06	0.18 ± 0.07	2.06 ± 0.32
C LETO male	0.25 ± 0.02	0.24 ± 0.06	0.21 ± 0.04	1.49 ± 0.70

*$P < .05$ *v* group C OLETF female.

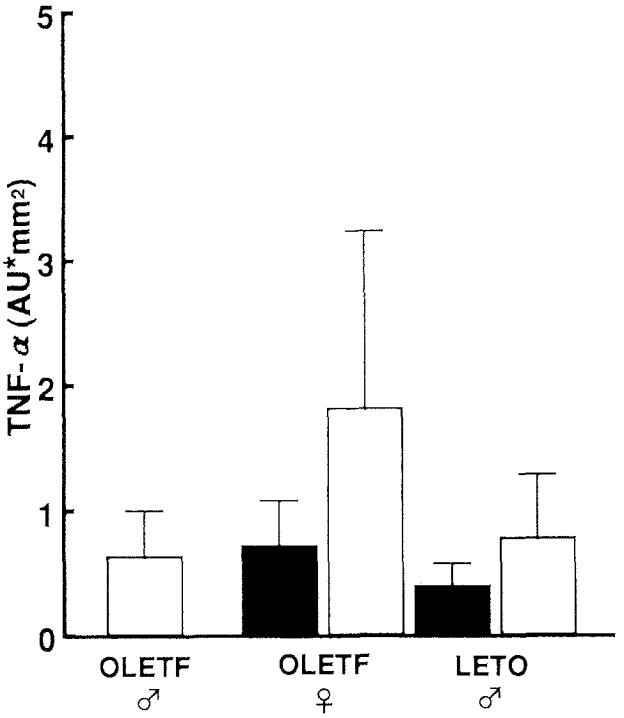

Fig 7. Expression level of TNF-α protein in mesenteric fat tissue by Western blot. (■) Group D rats; (□) group C rats. Four samples from each group of rats were examined. Bars represent the SEM.

except for mRNA levels of retroperitoneal fat in female OLETF rats.

Western Blot Analysis for TNF-α Protein in Adipose Tissue

Figure 7 shows expression levels of TNF-α protein in mesenteric fat. Expression levels of TNF-α protein in group D female OLETF and LETO rats tended to be lower than in the respective C groups. However, the difference was not statistically significant, because of large individual variations.

DISCUSSION

This study indicates that obesity alone is not sufficient for the development of insulin resistance and diabetes mellitus in the animals used. Female OLETF rats, which have a diabetogenic gene, ODB-1, and male LETO rats, which have no ODB-1, both became obese when fed a palatable high-energy cafeteria diet, as did positive control male OLETF rats (Fig 1). However, the prevalence of diabetes mellitus among them differed. Most female OLETF rats became diabetic, but only 30% of male LETO rats developed diabetes mellitus.

Abdominal fat deposits in group D female OLETF rats were larger than in group D male LETO rats, even though their body weight was similar at the end of the experiment. In a previous study, we[18] demonstrated that obese female OLETF rats fed a cafeteria diet had deposits of both abdominal (97.9 ± 4.7 g) and subcutaneous (119.3 ± 6.1 g) fat and that the percent body fat of obese female OLETF rats (36.8% ± 0.4%) was larger than that of lean counterparts (29.4% ± 2.1%). This suggests that total fat deposition increased in obese female OLETF rats. The data collected in this study clearly show that body weight and abdominal fat deposits were much smaller in group D female LETO rats versus group D female OLETF rats. These data suggest that the tendency to accumulate more fat in female OLETF rats is in part gender-dependent and also strain-specific. Shi et al[19] reported that castrated female OLETF rats had increased food intake, body weight, and abdominal fat deposits compared with sham-operated female OLETF rats. Estrogen was reported to cause a decrease in food intake.[20] Thus, fat deposits induced by a cafeteria diet in female OLETF rats could not be explained solely by a gender effect. Little or no expression of the CCK-A receptor gene, which is associated with food intake, was observed in the hypothalamus of OLETF rats.[21] A defect in the CCK-A receptor gene is one of the causes of overeating and obesity in OLETF rats. Based on these data, it can be presumed that female OLETF rats with a defect in the CCK-A receptor gene will overeat to the same degree as their male counterparts if estrogen is absent, but under ordinary conditions this impairment would be ameliorated by endogenous estrogen. Palatability of food easily overcomes the suppressive effect of estrogen on the appetite of female OLETF rats, which tend to overeat because of a CCK-A receptor defect, compared with their LETO counterparts.

According to Björntorp,[22] abdominal adipose tissue is a major site of insulin resistance, some degree of glucose intolerance, or both. In our treated rats, GIR and the weight of abdominal fat depots were inversely correlated to a significant degree (Fig 4). The correlation between the weight of abdominal fat and GIR is stronger than that between body weight and GIR. In fact, the GIR in group D male LETO rats is significantly higher than in positive control male OLETF rats and the weight of abdominal fat is much less in the former than in the latter, although body weight did not differ significantly for these groups. It appears that insulin resistance is associated with abdominal fat deposition rather than body weight gain, a fact consistent with the findings in African-Americans.[23] If the difference in insulin resistance between obese male LETO and obese female or male OLETF rats is only due to the difference in abdominal fat deposition, then the mechanism(s) by which abdominal fat depots differ from one another[24] will require further study.

Fasting blood glucose is increased with an increase in insulin resistance (Fig 5). Animals with insulin resistance can maintain glucose levels by increasing the insulin response. However, a failure of an adaptive increase in insulin secretion could result in hyperglycemia. Insulin secretory ability appeared to decrease in group D female OLETF rats. After an intravenous glucose load (Fig 2), total insulin secretion in group D male LETO rats was significantly higher than in group C. However, in female OLETF rats, this was not observed, even in the presence of higher blood glucose levels in group D. Judging from these data, it appears that the plasma insulin level in group D female OLETF rats was not sufficiently high to compensate for the

higher demand for insulin due to insulin resistance caused by the high-energy diet.

In positive control OLETF rats, HGO was higher and glucose uptake in muscle and fat tissues was lower compared with those in the normal control, group C male LETO rats, indicating obvious insulin resistance in both the peripheral tissues and the liver. For group D female OLETF rats, glucose uptake in muscle was about half of that in group C but HGO values were similar. According to Villar-Palasi and Farese,[25] D-glucose uptake by isolated gastrocnemius in the GK rat is only about 50% of that observed in Wistar rats, and this defect correlates with a defect in the activation of glycogen synthase. The decrease in glucose uptake in the muscle of OLETF rats might also be due to such an effect.

The issue remains as to what was responsible for the decreased glucose uptake in these insulin-resistant rats. Glucose transport into a cell by the insulin-responsive glucose transporter, GLUT4, constitutes the rate-limiting step for glucose uptake in adipose tissue and muscle.[26] GLUT4 expression in retroperitoneal fat tissue and skeletal muscle was not significantly different between group D and C female OLETF rats, although insulin resistance in muscle was obvious in the former group. Muscle tissue is responsible for most of the insulin-stimulated glucose disposal in vivo.[27] There are several reports of a modest decrease in GLUT4 mRNA and protein levels in muscle tissue of streptozotocin-induced diabetic rats.[28-30] However, there are several lines of evidence against the altered expression of GLUT4 in skeletal muscle of patients with insulin resistance,[31,32] which are consistent with our findings. Insulin resistance in skeletal muscle may involve impaired GLUT4 function or translocation and not transporter depletion. In this study, only the amounts of total membrane GLUT4 protein were measured, and the translocation between plasma membrane and internal membrane and the function of GLUT4 after insulin stimulation clearly require further examination.

The other possible cause of insulin resistance, TNF-α expression,[33] was also examined (Fig 7). There was no significant difference in TNF-α expression in adipose tissue between group D and C female OLETF and male LETO rats. Hotamisligil et al[33] reported that expression of TNF-α mRNA was increased in adipose tissue of genetically obese rodents, but was not elevated in chemically (monosodium glutamate) induced obese rats. Obesity induced by a cafeteria diet may not be sufficiently severe to induce enhanced TNF-α expression. Furthermore, the expression of TNF-α in male OLETF rats was not elevated compared with that in normal male LETO rats. Although OLETF rats became obese, insulin-resistant, and diabetic, they were different from genetically obese mice, ob/ob, db/db, fa/fa, and tub/tub, in terms of TNF-α expression.[33]

In conclusion, female OLETF rats became insulin-resistant and diabetic when they became obese as a result of feeding a high-energy diet, and a major site of the insulin resistance was skeletal muscle. However, these changes were mild in male LETO rats that similarly became obese. Based on these findings, it is reasonable to presume that both environmental and genetic factors play important roles in the development of diabetes mellitus in OLETF rats.

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