Decrease in Triglyceride Accumulation in Tissues by Restricted Diet and Improvement of Diabetes in Otsuka Long-Evans Tokushima Fatty Rats, a Non–Insulin-Dependent Diabetes Model

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With respect to the connection between triglyceride (TG) and non–insulin-dependent diabetes mellitus (NIDDM), previous reports have shown that TG accumulation in the liver and muscle is one of the causes of insulin resistance, and TG accumulation in pancreatic islets induces impairment of pancreatic β-cell function. This experiment examined the relationship between an amelioration of hypertriglyceridemia (HTG), a decrease in TG accumulation in tissues, and an improvement of NIDDM by food restriction. In this experiment using Otsuka Long-Evans Tokushima fatty (OLETF) rats developing NIDDM and Long-Evans Tokushima Otsuka (LETO) rats as controls, sequential changes in body weight and TG content in tissue were measured and biochemical blood tests, an insulin euglycemic clamp test, and histopathologic examination of the pancreas and liver were performed. OLETF rats were allocated to a food-satiated group (satiated) or 30% food-restricted group (restricted). As a result, several findings were more evident in the restricted group than in the satiated group: (1) reductions in body weight and intraabdominal fat weight, decreases in plasma TG, insulin, and glucose levels, a decrease in the TG secretion rate, and an increase in plasma lipoprotein lipase (LPL) activity, (2) decreases in the TG content in the liver, pancreas, and muscle, (3) improvement of the glucose infusion rate (GIR), and (4) a marked reduction of TG accumulation in the liver and pancreatic islets on histopathologic examination. These results indicate that the improved HTG caused a reduction in TG accumulation in the liver and muscle, thereby improving insulin resistance. Moreover, the decrease in TG accumulation in pancreatic islets suggests an improvement of pancreatic β-cell function.

DIET THERAPY is well known among the effective therapies for non–insulin-dependent diabetes mellitus (NIDDM). Diet therapy is considered to ameliorate abnormalities in glucose and lipid metabolism by improving insulin resistance and abnormal insulin secretion. A loss of body weight and reduction in intraabdominal fat by diet therapy are reported to greatly contribute to the improvement of abnormalities in glucose metabolism.

The mechanism of the amelioration of abnormal glucose metabolism was reported to be facilitation of glucose uptake in muscle and adipose tissue and improvement of glucose metabolism in the liver, which causes a reduction in blood glucose, thereby improving pancreatic β-cell dysfunction.[1-6] Unger[7] reported that high blood levels of free fatty acid (FFA) induced triglyceride (TG) accumulation in muscle and pancreatic islets, resulting in insulin resistance and impaired pancreatic β-cell function, and advocated a concept of lipotoxicity as a cause of NIDDM. In Otsuka Long-Evans Tokushima fatty (OLETF) rats, a model of spontaneous NIDDM,[8] mild obesity and intraabdominal fat accumulation develop, accompanied by hypertriglyceridemia (HTG) after 6 weeks of age and hyperglycemia and insulin resistance after 12 weeks of age. Focusing on early-onset HTG in OLETF rats, we demonstrated that HTG induced TG accumulation in pancreatic islets, causing a decrease in glucokinase activity, and led to an impairment of pancreatic β-cell function, and reported that this was 1 of the factors behind the occurrence of NIDDM in OLETF rats.[9]

In the present study, the following experiments were performed using OLETF rats after NIDDM onset to examine the connection between an amelioration of HTG, a decrease in TG accumulation in tissues, and an improvement of NIDDM by a restricted diet: (1) sequential changes in plasma TG, glucose, and insulin levels during food restriction, (2) changes in the TG secretion rate in the liver, plasma lipoprotein lipase (LPL) activity, and TG content in the hepatic, muscular, and pancreatic tissues after food restriction, (3) changes in insulin euglycemic clamp test results, and (4) histopathologic changes associated with TG accumulation in the liver and pancreas.

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Submitted March 15, 1999; accepted July 15, 1999.

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0026-0495/00/4901-0023$10.00/0

MATERIALS AND METHODS

Animals and Composition of Experimental Groups

Using 60 male OLETF rats in which an oral glucose tolerance test (OGTT) at 25 weeks of age confirmed the development of NIDDM and 30 male diabetes-resistant counterparts, Long-Evans Tokushima Otsuka (LETO) rats, as normal controls, experiments were performed from 25 to 40 weeks of age. OLETF rats were allocated to 2 groups: the first group maintained on food satiation (satiated group, n = 30) or the second group in which 30% of the food intake in the satiated group was restricted (restricted group, n = 30). These 2 groups and LETO rats maintained on food satiation (control group, n = 30) were subjected to experiments. Animals were raised on the conditions of Specific Pathogens Free (SPF) and food satiation until the experiment was started. CRF-1 (protein 23.1%, carbohydrate 53.5%, and fat 5.9%; Oriental Yeast, Tokyo, Japan) solid food for general breeding of rats was used, and water was given ad libitum.

Measurement of Body Weight and Blood Tests

Body weight was measured once per week. Blood tests were performed every 5 weeks from 25 to 40 weeks of age. Using blood collected from the caudal artery of rats fasted for 16 hours, plasma levels of TG, glucose, insulin, FFA, and cholesterol were measured. Plasma TG were determined using Lipidos Liquid (Ono Pharmaceutical, Osaka, Japan). Plasma glucose levels were measured using the Glucose B-Test Wako kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin was determined using an enzyme-linked immunosorbent assay (ELISA) Insulin kit (Morinaga Biochemical Industries,

Tokyo, Japan). Plasma FFA levels were measured using the NEFA C-Test Wako kit (Wako Pure Chemical Industries). Plasma cholesterol was determined using the Cholesterol E-Test Wako kit (Wako Pure Chemical Industries).

OGTT

An OGTT was performed every 5 weeks. Glucose (2 g/Kg) was orally administered to rats fasted for 16 hours, and then blood was collected from the caudal artery at 0, 30, 60, 90, and 120 minutes. Plasma glucose levels were measured by the method already described. The diagnosis of diabetes was made based on plasma glucose levels during the OGTT, and animals showing a peak level of 300 mg/dL or higher and 200 mg/dL or higher at 120 minutes were regarded as the diabetic type. Plasma insulin levels were determined by ELISA at 0, 60, and 120 minutes during the OGTT.

Measurement of TG Secretion Rate From the Liver and Plasma LPL Activity

At the end of the experiment (40 weeks of age), the TG secretion rate from the liver and plasma LPL activity were measured in 5 rats from each experimental group. For measurement of the TG secretion rate, 60 mg/100 g body weight Tyloxapol (Triton WR-1339; Nacalai Tesque, Kyoto, Japan), which has an inhibitory effect on LPL, was administered to rats fasted for 16 hours by intravenous injection. The difference in the plasma TG level 60 minutes after administration versus before administration was regarded as the TG secretion rate.[10] For measurement of LPL activity, 100 U/kg body weight heparin (Novo Nordisk, Copenhagen, Denmark) was intravenously injected into rats fasted for 16 hours. Then, the serum collected 10 minutes later was mixed with active substrate buffer and rat serum, bovine serum albumin, Intralipid (Pharmacia, Stockholm, Sweden). Then, 1.0 mol NaCl was added to inhibit LPL. After incubation at 37°C for 60 minutes, FFA was measured and the resulting value was regarded as LPL activity.[11]

Anatomy and Determination of TG Content in the Hepatic, Muscular, and Pancreatic Tissues

At the end of the experiment, animals in each group (n = 10) were euthanized under anesthesia, and the intraabdominal fat (fat in the epididymis, mesentery, and retroperitoneum), liver, soleus, and pancreas were removed and weighed. Portions of the liver, soleus, and pancreas were pulverized in liquid nitrogen, and then TG was extracted by the Folch method (other portions were used for pathologic examination).[12] The extracted TG was measured by the method already described.

Insulin Euglycemic Clamp Test

At the end of the experiment, rats in each group (n = 10) were anesthetized with pentobarbital (50 mg/kg), and 10% glucose and insulin each were infused from the right and left femoral vein. The insulin infusion rate was 30 mU/min/kg until 3 minutes after the experiment started, 20 mU/min/kg from 3 to 6 minutes, 14 mU/min/kg from 6 to 10 minutes, and 10 mU/min/kg from 10 to 60 minutes. Plasma glucose levels were measured every 4 minutes until 40 minutes after the experiment started, and then every 2 minutes from 40 to 60 minutes. The glucose infusion rate (GIR) was calculated so that the plasma glucose level would reach 110 mg/dL using a pocket-sized computer, and the GIR value at each time point was recorded. The sum of GIRs from 40 to 60 minutes was compared with each experimental group.

Histopathologic Examination of the Liver and Pancreas

The liver and pancreas were fixed in 10% Formalin overnight, frozen using Tissue-Tec OCT (Miles, Elkhart, IN), and then cut into 7- to 10-μm tissue sections. These sections were stained with Oil Red O,[13] and the stained sections of the hepatic tissue were examined under a microscope. The Oil Red O–stained sections of pancreatic tissue were subjected to primary reaction with anti-insulin antibody (guinea pig anti-insulin polyclonal antibody 1:400; Biomedia, Foster City, CA) followed by secondary reaction with fluorescein isothiocyanate (FITC)-labeled anti–guinea pig IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) to obtain sections double-stained for insulin and fat. The stained sections of pancreatic tissue were observed under a fluorescent microscope (Olympus AX 80; Olympus Provis, Tokyo, Japan) at 490 nm for FITC and 564 nm for Oil Red O.

Statistical Analysis

Sequential changes in body weight and plasma glucose, TG, cholesterol, FFA, and insulin levels in the fasting state were evaluated by ANOVA with repeated measurements. At each time point, the comparison between the satiated group and restricted group was assessed by *t* test. For the TG secretion rate from the liver, LPL activity, intraabdominal fat weight, and TG content in the hepatic, muscular, and pancreatic tissues, actual values were compared by *t* test between the satiated group and restricted group and between the control group and satiated group. In these analyses, a *P* level less than .05 was regarded as significant.

RESULTS

General Characteristics of OLETF Rats

Table 1 shows changes in body weight, intraabdominal fat weight, and plasma glucose, insulin, TG, FFA, and cholesterol levels in the fasting state with aging in OLETF and LETO rats. Body weight and intraabdominal fat weight markedly increased with aging in OLETF. Plasma glucose and insulin showed the same tendency as the body weight and intraabdominal fat weight in OLETF, and were significantly higher than the values in LETO at 12 and 30 weeks of age. FFA levels were higher in OLETF versus LETO at every week of age, but there were no

Table 1. General Characteristics of LETO and OLETF Rats

Characteristic	LETO			OLETF		
	6 wk	12 wk	30 wk	6 wk	12 wk	30 wk
Body weight (g)	152.1 ± 3.0	353.3 ± 8.0	518.2 ± 6.0	175.6 ± 4.0	420.1 ± 14.0†	660.4 ± 15.0‡
Intraabdominal fat (g)	2.25 ± 3.0	9.02 ± 0.17	24.21 ± 0.5	2.84 ± 0.2	20.39 ± 1.16†	87.96 ± 2.36‡
Plasma glucose (mg/dL)	83.3 ± 3.4	111.9 ± 5.4	115.9 ± 5.8	83.0 ± 3.1	133.7 ± 7.9*	163.6 ± 8.5†
Plasma insulin (pg/mL)	791.0 ± 130.0	957.2 ± 140.0	1,534 ± 140.0	841.2 ± 40.0	1,230.3 ± 160.0*	4,799.5 ± 342.0‡
Plasma TG (mg/dL)	45.4 ± 3.5	59.9 ± 1.8	60.5 ± 4.4	121.4 ± 11.5‡	185.5 ± 18.9‡	322.1 ± 33.9‡
Plasma FFA (mmol/L)	0.62 ± 0.04	0.46 ± 0.03	0.62 ± 0.03	0.77 ± 0.07	0.61 ± 0.1	0.71 ± 0.07*
Plasma cholesterol (mg/dL)	87.4 ± 2.3	77.3 ± 3.5	91.3 ± 1.9	72.7 ± 3.5	70.4 ± 7.0	145.8 ± 4.6†

NOTE. Data are the mean ± SE. After overnight fasting, blood was withdrawn from the vena cava (n = 10).
*$P < .05$, †$P < .01$, ‡$P < .001$ *v* LETO.

significant differences between 6 and 30 weeks of age. Cholesterol was significantly increased in OLETF compared with LETO at 30 weeks of age. The plasma TG level in OLETF was 2.6 times that in LETO at 6 weeks of age, 3.1 times at 12 weeks, and 5.3 times at 30 weeks, showing significantly higher values.

Alterations in Body Weight and Biochemical Parameters

Figure 1 illustrates sequential changes in body weight and biochemical parameters in the satiated group, restricted group, and control group from 25 to 40 weeks of age. Body weight and plasma TG in the fasting state were significantly decreased in the restricted group compared with satiated group after 30 weeks of age, and decreased to the same level as in the control group at 40 weeks of age (Fig 1A and B). Plasma insulin and glucose levels in the fasting state were significantly diminished in the restricted group after 35 weeks of age (Fig 1C and D). Plasma FFA and cholesterol in the fasting state were significantly decreased in the restricted group compared with the satiated group after 35 weeks of age, but the difference in plasma cholesterol between the 2 groups was not due to a decrease in the restricted group but to an increase in the satiated group (Fig 1E and F).

TG Secretion Rate From the Liver and Plasma LPL Activity

The TG secretion rate from the liver in each experimental group at 40 weeks of age is shown in Fig 2A. The TG secretion rate in the restricted group decreased to 73.1% of that in the satiated group, which was almost the same value as in the control group. Plasma LPL activity in the restricted group was 1.2 times higher than that in the satiated group (Fig 2B).

Weight and TG Content of Intraabdominal Fat, Liver, Soleus, and Pancreas

The weights for intraabdominal fat, liver, and soleus in each experimental group at 40 weeks of age are shown in Table 2. Intraabdominal fat weight and liver weight in the restricted group significantly decreased to 59.7% and 69.9%, respectively, of the weights in the satiated group, but there was no significant difference in the weight of the soleus. Figure 3 illustrates TG content in the hepatic, muscular, and pancreatic tissues in each experimental group at the end of the experiment (40 weeks of age). The TG content in the restricted group markedly decreased to 21.2% of the level in the satiated group for the liver, 56.5% for the soleus, and 45.3% for the pancreas.

Insulin Euglycemic Clamp Test

Insulin euglycemic clamp test results in each experimental group at the end of the experiment (40 weeks of age) are shown in Fig 4. The sum of GIRs from 40 to 60 minutes in the restricted group increased to about 5 times that in the satiated group (109.87 ± 10.31 *v* 20.81 ± 2.38 mg/kg/min; $P < .001$), showing almost the same level as the control group (122.55 ± 7.91 mg/kg/min).

Changes in Plasma Glucose and Insulin Levels During the OGTT

Changes in plasma glucose during OGTT in each experimental group at 40 weeks of age are illustrated in Fig 5A. Plasma glucose at each time point of 30, 60, 90, and 120 minutes was

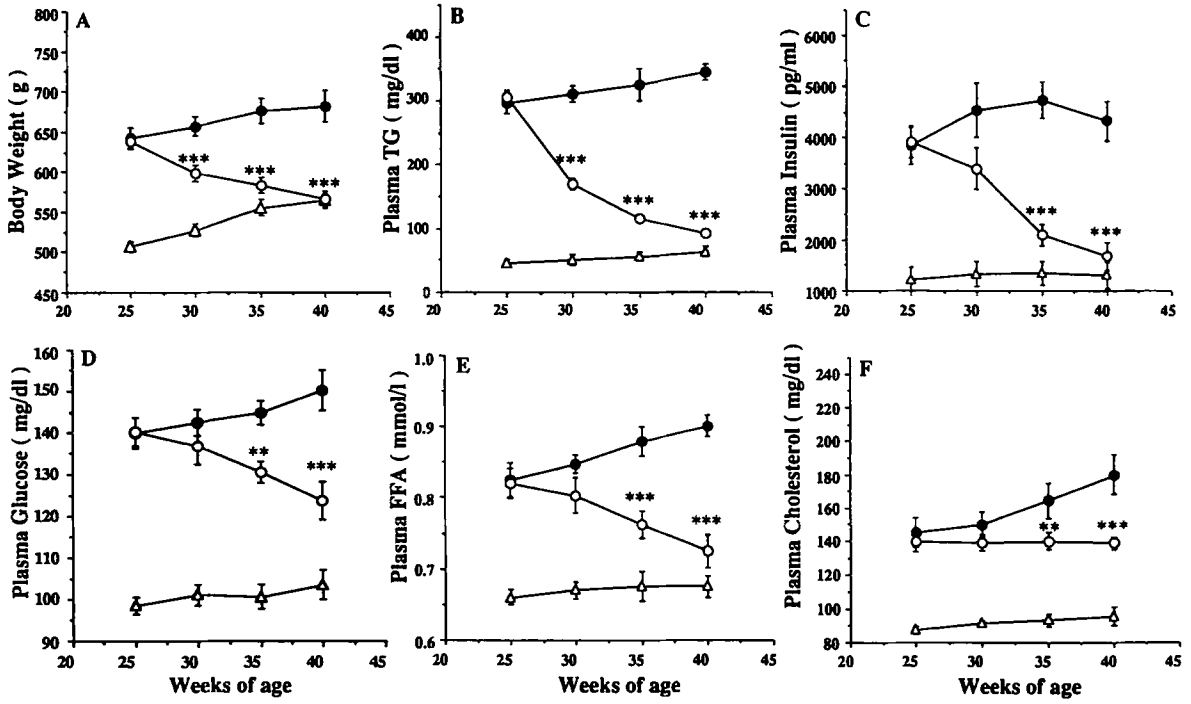

Fig 1. Changes in body weight and blood chemical parameters in the satiated group (●, n = 30) and restricted group (○, n = 30) of OLETF rats and LETO rats (△, n = 30) during the experiment. After overnight fasting, blood was collected from the tail vein. Values are the mean ± SE. *$P < .05$, **$P < .01$, ***$P < .001$ *t* test) *v* OLETF (satiated group). The overall mean and interaction were significantly different between the satiated group and restricted group in every parameter ($P < .001$ by repeated measurement).

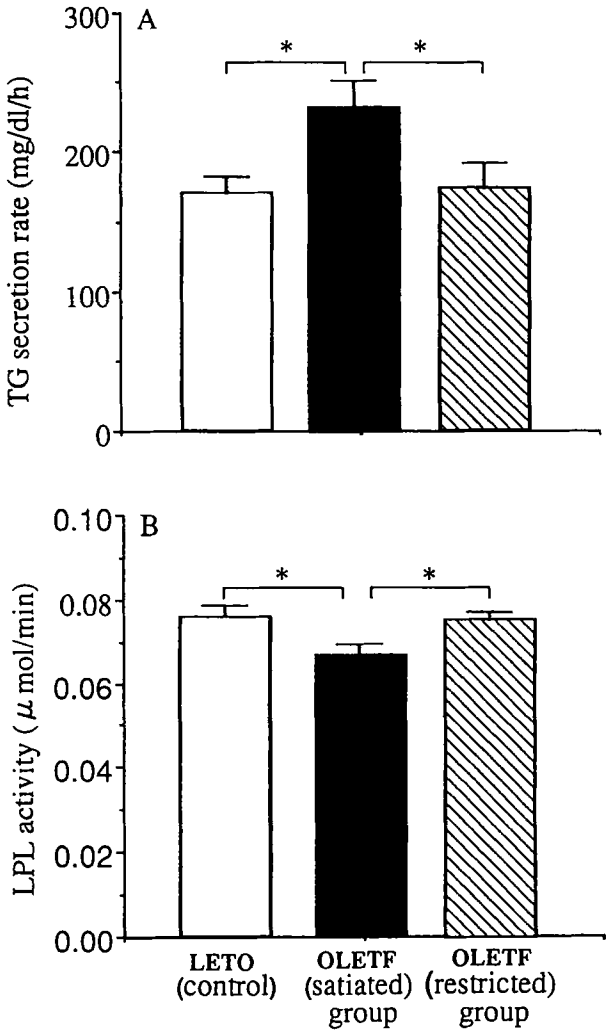

Fig 2. Changes in TG secretion rate (A) and LPL activity (B) in the satiated group (■, n = 5) and restricted group (▨, n = 5) of OLETF rats and LETO rats (□, n = 5) at 40 weeks of age (end of the experiment). Values are the mean ± SE. *$P < .05$ (*t* test) *v* OLETF (satiated group).

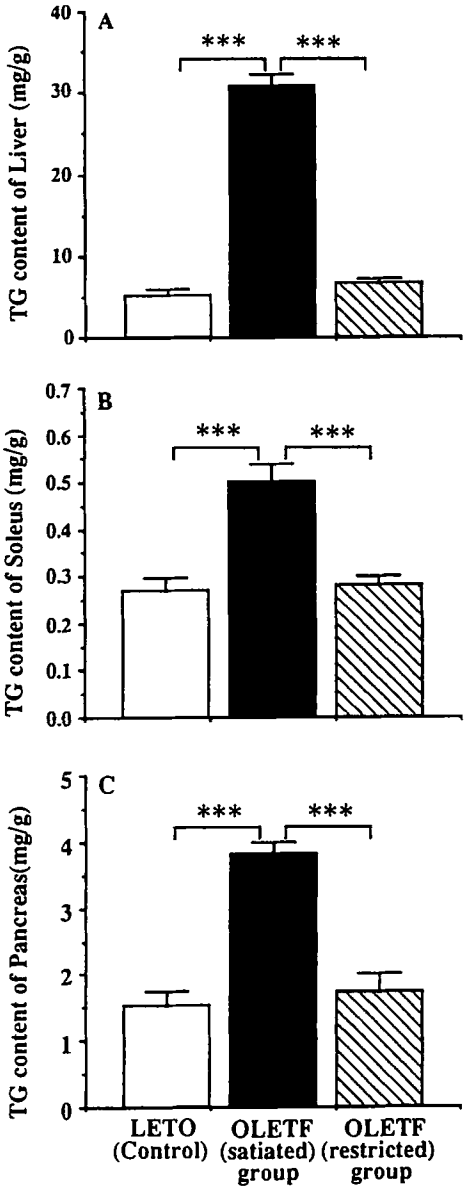

Fig 3. Changes in TG content in liver (A), soleus (B), and pancreas (C) in the satiated group (■, n = 10) and restricted group (▨, n = 10) of OLETF rats and LETO rats (□, n = 10) at 40 weeks of age (end of the experiment). Values are the mean ± SE. ***$P < .001$ *t* test) *v* OLETF (satiated group).

significantly decreased in the restricted group compared with the satiated group. The sum of plasma glucose during the OGTT significantly decreased to about 77.8% (1,154.9 ± 32.6 mg/dL) of the value in the control group (1,633.8 ± 43.1 mg/dL). The plasma insulin level during the OGTT in each experimental group is shown in Fig 5B. Plasma insulin was significantly decreased at each time point of 0, 60, and 120 minutes in the restricted group compared with the satiated group. The sum of plasma insulin during the OGTT significantly decreased to approximately 58.5% (7.79 ng/mL) of the value in the satiated group (13.31 ng/mL).

Table 2. Organ and Tissue Weight at the End of the Experiment

Organ/Tissue (g)	LETO (control)	OLETF	
		Satiated Group	Restricted Group
Liver	13.2 ± 0.22*	21.97 ± 0.94	15.37 ± 0.23*
Kidney	2.85 ± 0.12*	3.61 ± 0.11	2.91 ± 0.11*
Soleus	0.37 ± 0.02*	0.40 ± 0.01	0.39 ± 0.01
Intraabdominal fat	35.88 ± 4.53*	82.04 ± 6.46	48.99 ± 2.93*

NOTE. Data are the mean ± SE (n = 10).
*$P < .001$ *v* OLETF satiated group.

Histopathologic Examination of the Liver and Pancreas

In the liver of the satiated group at 40 weeks of age, fat infiltration, vacuolation, and destruction of the hepatic cords were evident (Fig 6B), while in the restricted group and control group, such changes were not noted (Fig 6A and C). Fat droplets

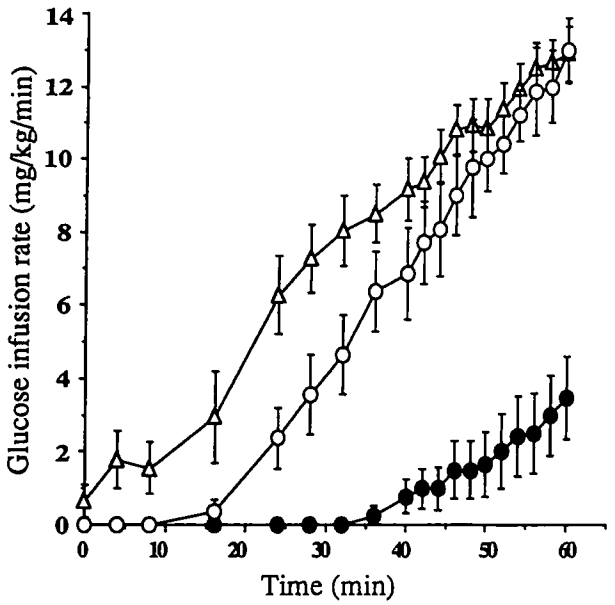

Fig 4. Comparison of glucose infusion rate in the satiated group (●, n = 10) and restricted group (○, n = 10) of OLETF rats and LETO rats (△, n = 10) at 40 weeks of age (end of the experiment). Values are the mean ± SE. The overall mean and interaction were significantly different between the satiated group and restricted group ($P < .001$ by repeated measurement).

were markedly accumulated and insulin granules were decreased in pancreatic β cells in the satiated group. Moreover, the pancreatic islets were divided into island-like portions due to salient fibrosis accompanied by mild atrophy (Fig 6E). However, in the restricted group, pancreatic β cells demonstrated little accumulation of fat droplets, sufficient concentrations of insulin granules, and mild fibrosis (Fig 6F).

DISCUSSION

With regard to the relationship between the amelioration of abnormal glucose metabolism and improvement of NIDDM by a restricted diet, it has been clarified that facilitated glucose uptake in muscle and normalized glucose metabolism in the liver improve insulin resistance and reduced carbohydrate influx improves hyperglycemia, preventing pancreatic β-cell exhaustion.[5,6] However, concerning the relationship between the amelioration of abnormal lipid metabolism and improvement of NIDDM, in particular, the causal relationship between a reduction of HTG and an improvement of NIDDM has not been sufficiently elucidated. Previously, HTG has been regarded as a consequence of NIDDM, but recently, HTG was found before NIDDM onset in patients and animal models of NIDDM.[14-18] HTG induced by loading a high-fat diet was reported to cause TG accumulation in the liver and skeletal muscle, leading to insulin resistance.[19-23] Furthermore, in Zucker diabetic fatty and OLETF rats, which are animal models of NIDDM, TG accumulated in the pancreatic islets, findings suggesting that HTG may not be a consequence of NIDDM, but instead 1 of the factors involved in the pathogenesis of NIDDM.[24,25]

In OLETF, since HTG developed at 6 weeks of age and abnormal insulin secretion and hyperglycemia at 12 weeks, it was considered that HTG recognized in the early phase may have some influence on the development of NIDDM (Table 1).[9] Recently, it was reported that FFA inhibited glucose phosphorylation in vitro[26] and induced insulin resistance in vivo.[27] Plasma FFA was significantly higher in OLETF versus LETO, and this could cause the decrease in glucose transporter 4[28,29] and the reduced activity of hexokinase 2 and concentration of glucose-6-phosphate in skeletal muscle.[30] In OLETF, it was also reported that the intraabdominal fat caused a high portal FFA concentration, influenced lipid and glucose metabolism, respectively, and led to insulin resistance in the liver.[31,32] These results proved that abnormal lipid metabolism may be an important factor involved in insulin resistance in OLETF. In our experiments, the plasma TG level in the restricted OLETF group was decreased preceding reductions in plasma FFA, insulin, and glucose levels (Fig 1). This was considered due to suppressed TG secretion from the liver and elevated plasma LPL (Fig 2). In the restricted group, intraabdominal fat weight and TG content in skeletal muscle and hepatic tissue decreased to the same levels as those in the control group (Table 2 and Fig 3A and B), and almost no fat infiltration was observed in the liver (Fig 6C). Moreover, the GIR was improved on the insulin euglycemic clamp test in the restricted group (Fig 4). These results suggest that a marked reduction in TG accumulation in the liver and skeletal muscle ascribed to a reduction of HTG and plasma FFA may play a key role in the improvement of insulin resistance by food restriction.

In this study, we could not perform experiments on insulin

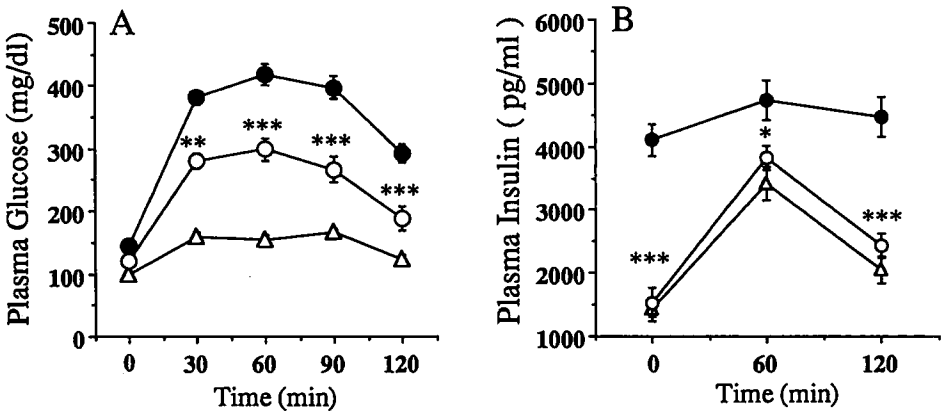

Fig 5. Plasma glucose (A) and insulin (B) responses in the OGTT in the satiated group (●, n = 30) and restricted group (○, n = 30) of OLETF rats and LETO rats (△, n = 30) at 40 weeks of age (end of the experiment). Rats were fasted for 16 hours before the test. Values are the mean ± SE. *$P < .05$, **$P < .01$, ***$P < .001$ (t test) v OLETF (satiated group). The overall mean and interaction were significantly different between the satiated group and restricted group in A and B ($P < .001$ by repeated measurement).

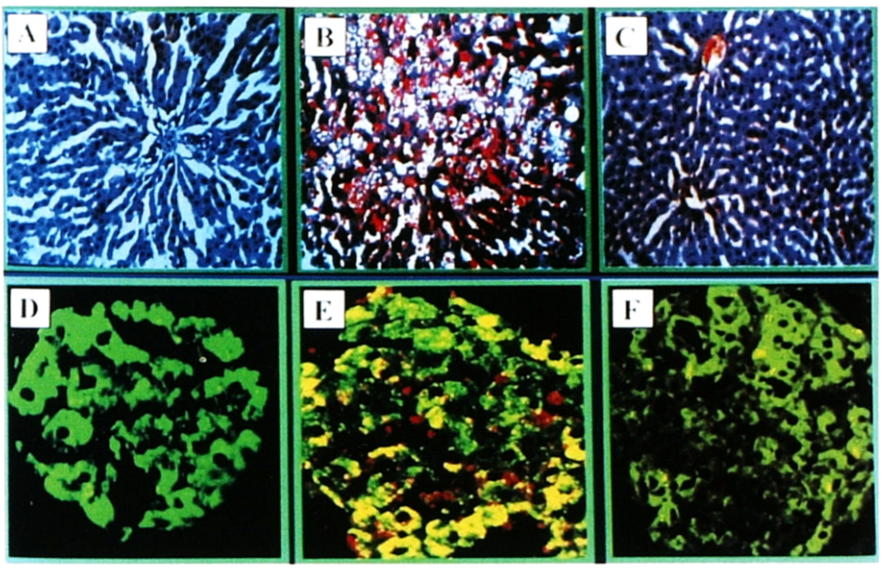

Fig 6. Optical microscopic features of the liver of OLETF rats in (B) satiated and (C) restricted groups, and in (A) LETO rats at 40 weeks of age (end of the experiment). By staining with Oil Red O, fat droplets (TG) were accumulated largely in the hepatic cells (red in B) and were not found in C and A. Fat-insulin double-staining optical microscopic features of the islets of OLETF rats in (E) satiated and (F) restricted groups, and in (D) LETO rats. Staining with Oil Red O and anti-insulin. Deposition of fat droplets (TG) was found in the islets (red), even in enlarged β cells (yellow), in E. Insulin was shown clearly (green) and deposition of fat droplets (TG) was not found in F and D. Original magnification ×300.

secretion in OLETF rats of advanced age because of the severe fibrosis in the pancreatic islets. However, in the restricted group, at least pancreatic β-cell function seemed to be restored, considering the decreased TG accumulation in the pancreatic tissue (Fig 3C), improved fibrosis on histopathologic examination, as well as a marked reduction in fat deposits and a sufficient concentration of insulin granules in pancreatic β cells (Fig 6F). Moreover, since the sum of insulin levels during the OGTT diminished, showing almost the same pattern of plasma insulin secretion as LETO, the response to glucose loading in pancreatic β cells was thought to be recovered in the restricted group (Fig 5B). The improved insulin tolerance and preserved β cell function were considered among the causes of the improvement of glucose tolerance in OLETF (Fig 5A).

Although the TG uptake pathway in the muscle and pancreatic islets has not been fully defined, in general, TG is degraded into FFA by LPL and reconstructed into TG after entering cells, and these reactions are reversible in blood and tissues. In addition, in many tissues and cells, the presence of the very–low-density lipoprotein (VLDL) receptor has been confirmed, and the possibility that TG may be taken up by cells via this receptor has also been reported.[33-35] A recent study demonstrated that the low-density lipoprotein (LDL) receptor was present in pancreatic β cells of humans and rats, and LDL and VLDL were taken up by the cells via this receptor.[36] It is unlikely that the pathway via VLDL receptor is the main pathway of TG uptake by cells in a normal state, but the role of this pathway in HTG needs to be investigated further.

In the present experiments, it was considered that food restriction suppressed TG synthesis in the liver, facilitated peripheral TG degradation, and decreased exogenous TG inflow, ameliorating HTG. The reduced intraabdominal fat and decreased TG accumulation in skeletal muscle and hepatic tissue were thought to result in an improvement of insulin resistance. In this experiment, although we could not directly confirm the improvement of β-cell dysfunction, some amelioration in morphology, a decrease of fat droplets, a sufficient concentrations of insulin granules, and mild fibrosis were found in pancreatic islets. In conclusion, the amelioration of abnormal lipid metabolism by food restriction is considered to play an important role in improving hyperglycemia and hyperinsulinemia in OLETF rats.

ACKNOWLEDGMENT

We are grateful to Dr Kenji Shima, Professor, Department of Laboratory Medicine, School of Medicine, University of Tokushima, and Dr Takashi Natori, Palm Research Institutes, for providing valuable advice and guidance during these experiments.

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