

Plasma Membrane Content of Glucose Transporter 4 in Skeletal Muscle and Visceral Fat in OLETF Rats Treated with Troglitazone

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The exact mechanism by which troglitazone improves insulin sensitivity is not well understood. Eight 35-week-old male diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats were treated with troglitazone (30 mg/kg body weight/d) for 20 d (OLETF-T). Body composition, glucose tolerance, serum lipid profile and expression of glucose transporter 4 (Glut 4) in OLETF-T were compared with those in 8 male control OLETF rats and in 18 normal Long Evans Tokushima Otsuka (LETO) rats. Body weight, visceral fat weight, and pancreas weight in OLETF-T rats were significantly lower than those in OLETF rats ($p < 0.05$). Furthermore, troglitazone treatment attenuated atrophy and fibrosis of the pancreas. Serum concentrations of glucose, triglyceride, total cholesterol and immunoreactive insulin (IRI) were also significantly lower in OLETF-T rats. Expression of Glut 4 in plasma membrane fractions of skeletal muscle and visceral fat was detected by Western blot. The amount of Glut 4 protein in skeletal muscle in OLETF rats was 52% of that in LETO rats, and 75% for OLETF-T rats. In visceral fat, Glut 4 expressions in OLETF and OLETF-T rats were 38% and 83%, respectively, of that in LETO rats. Thus, treatment with troglitazone prevented the decrease of Glut 4 expression seen in OLETF rats. Glucose tolerance was improved significantly by the treatment, and the amount of secreted IRI in response to oral glucose tolerance test was 1798 pM, 702.2 pM, and 1103.5 pM, in OLETF, OLETF-T and LETO rats, respectively. The data presented suggest that treatment with troglitazone increased the Glut 4 expression in both skeletal and visceral fat tissues of OLETF rats, which may result in the improvement of insulin sensitivity and preservation of pancreas function.

Key words — OLETF rat, glucose transporter 4, troglitazone

INTRODUCTION

Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a strain of spontaneous non-insulin dependent diabetes mellitus (NIDDM) model with characteristics of innate polyphagia, rapid weight gain and accumulation of visceral fat.¹⁻³ OLETF rats exhibit hypertriglyceridemia and hyperinsulinemia in the early phase of their life, and develop impaired glucose tolerance or NIDDM after about 18 weeks of age. It has been reported that deficiency of cholecystokinin-A receptor gene, and recessive gene for diabetes may be involved in the development of NIDDM in this strain.⁴⁻⁶ Earlier studies have shown that the decreased expression of glucose transporter 4 (Glut 4) in the skeletal muscle and abdominal fat

contributes, at least in part, to the development of insulin resistance in this strain.^{7,8}

Troglitazone is an anti-diabetic agent of the thiazolidinedione family, and exerts insulin-sensitizing activity through activation of peroxisome proliferator-activated receptor-gamma (PPAR- γ), a member of the steroid nuclear receptor superfamily. A number of studies have shown that treatment with troglitazone improves insulin sensitivity and serum lipid profile.^{9,10} Though previous studies indicated that troglitazone increased insulin receptor in insulin receptor decreased animal models¹¹ and improved insulin receptor tyrosine kinase activity *in vivo*,¹² the mechanism by which this agent improves insulin sensitivity of post-receptor has not been clarified yet. Since Glut 4 plays a pivotal role in regulating glucose metabolism, we attempted to determine whether treatment with troglitazone alters the expression of Glut 4 in skeletal muscle and visceral fat tissues in OLETF rats, and to observe the effect

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of troglitazone on Glut 4 expression and the correlation between changes in the function and morphology of pancreas islet.

MATERIALS AND METHODS

Reagents — Bovine serum albumin (BSA) and phenylmethylsulfonyl fluoride (PMS) were purchased from Sigma (St. Louis, MO, U.S.A.). An enhanced chemiluminescence (ECL) Western blotting detection system kit, peroxidase-labeled antibody, and autoradiography film (Hyperfilm-ECL) were obtained from Amersham Inc. (Amersham, Bucks, U.K.). Anti-Glut 4 antibody was a product of Eas Costa Mesa (CA, U.S.A.). Other chemicals were purchased from Beijing Chemicals (Beijing, China). Troglitazone was provided by Sankyo Pharmaceuticals (Tokyo).

Animals — Tokushima Research Institut (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) provided OLETF and LETO rats. They were raised at the Animal Centre in the Academy of Military Medical Sciences, Beijing, China, and fed *ad libitum*. Oral glucose tolerance test (OGTT, 2 g/kg-body weight) was performed in OLETF rats at the age of 35 weeks. Blood samples were obtained from tail veins at 30 min intervals for up to 2 h. Animals were judged diabetes when peak levels of serum glucose were higher than 16.8 mmol/l or the levels at 120 h were higher than 11.2 mmol/l. Sixteen diabetic rats were randomly divided into two groups of 8 animals each. Rats in one group was treated daily with troglitazone (30 mg/kg) for 20 d (called OLETF-T). The other 8 rats without any treatment served as controls (OLETF). Long-Evans Tokushima Otsuka (LETO) rats, a diabetes-resistant counterpart of OLETF rats, was also used as a control strain. On day 20, OGTT was administered to all three groups. All the rats were then fasted overnight and anesthetized with sodium pentobarbital. Blood was drawn from the vena cava for measurement of serum concentrations of total cholesterol (TC), triglyceride (TG) and immunoreactive insulin (IRI). Adipose tissue were collected and weighed, and part of the tissues was immediately frozen at -80°C for subsequent analysis of Glut 4 expression. Muscle tissues were obtained from the right hind limb and also stored at -80°C . The pancreas was fixed in neutralized 10% formalin for histological examination.

Glut 4 Expression — Adipose and muscle tis-

sues were powered in liquid nitrogen and homogenized at 4°C in the buffer containing 10 mmol/l NaHCO_3 , 250 mmol/l sucrose, 5 mmol/l NaN_3 , and 0.1 mmol/l PMSF as described by Klip *et al.*¹³⁾ with slight modifications. All the subsequent procedures were carried out at 4°C . The obtained homogenates were centrifuged at $1200 \times g$ for 10 min. The supernatant was retained and the pellet was resuspended, homogenized, and again centrifuged for 10 min at $1200 \times g$. The first and second supernatants were combined and centrifuged at $9000 \times g$ and the resultant supernatant was centrifuged at $227000 \times g$ for 75 min to produce crude membrane fractions. These fractions were resuspended in the buffer, and 100 mg of the protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by the method of Lowry *et al.* using BSA as a standard.¹⁴⁾ The separated proteins were electroblotted to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) and the membranes were reacted with rabbit anti-Glut 4 antibody (1 : 500 diluted). The antibody-bound Glut 4 protein was visualized by autoradiography using the ECL method. The relative amount of Glut 4 on the film was measured with a densitometer.

Blood Analysis — Serum glucose concentrations were measured using α -glucose monitor (Boeringer Mannheim) with an oxidative method. Serum immunoreactive insulin was determined by radioimmunoassay kit (North Isotop Research). Serum total cholesterol, triglyceride and HDL-cholesterol levels were measured with a Cobas Mira auto-chemical analyzer.

Histological Examination — Paraffin-embedded tissues were sectioned and stained with hematoxylin-eosin according to the standard method.

Statistical Analysis — All the values were expressed as the mean \pm S.D. Statistical significance was tested by two-tailed *t*-test. *p*-Values less than 0.05 were considered significant.

RESULTS

The effect of troglitazone on glucose tolerance is shown in Table 1a. Although fasting serum glucose levels did not differ among the three groups, glucose tolerance and insulin secretion in OLETF were markedly impaired compared to those in control LETO rats (Table 1b). Treatment with troglitazone

Table 1a. Serum Glucose Levels in Rats of Different Groups

Time after treatment	LETO <i>n</i> = 18	OLETF <i>n</i> = 8	OLETF-T <i>n</i> = 8
0	6.4 ± 0.5	7.3 ± 1.1	6.9 ± 0.7
30 min	8.6 ± 4.9	14.6 ± 1.6***	11.8 ± 2.1***,##
60 min	8.4 ± 1.2	17.9 ± 2.3***	11.6 ± 2.6***,###
120 min	6.7 ± 1.3	18.2 ± 7.5***	10.5 ± 1.5***,###

Each value represents the mean ± S.D. (mmol/l)

Table 1b. Summary of Immunoreactive Insulin Levels in Rats of Different Groups

<i>n</i>	LETO 18	OLETF 8	OLETF-T 8
	1798.4 ± 452.2	702.2 ± 364.1**	1103.5 ± 407.3*.#

Each value represents the mean ± S.D. (pmmol/l); OGTT = oral glucose tolerance test; LETO = Long-Evans Tokushima Otsuka; OLETF = Otsuka Long-Evans Tokushima fatty rats without treatment; OLETF-T = Otsuka Long-Evans Tokushima fatty rats treated with troglitazone; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (significantly different from LETO); #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 (significantly different from LETO).

Table 2. Serum Lipid Profile in LETO, OLETF and OLETF-T Rats

<i>n</i>	LETO 18	OLETF 8	OLETF-T 8
TC	2.1 ± 0.3	2.4 ± 0.3***	2.1 ± 0.3
TG	0.4 ± 0.2	1.8 ± 1.0***	1.2 ± 0.7**.,##
HDL-C	1.6 ± 0.3	1.4 ± 0.2	1.5 ± 0.4

Each value represents the means ± S.D. (mmol/l); LETO = Long-Evans Tokushima Otsuka; OLETF = Otsuka Long-Evans Tokushima Fatty rats without treatment; OLETF-T = Otsuka Long-Evans Tokushima Fatty rats treated with troglitazone; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (significantly different from LETO); ##*p* < 0.01 (significantly different from LETO).

in OLETF-T rats resulted in a significant improvement in these two factors although the responses were still different from those in LETO rats.

Serum TC and TG levels in OLETF rats were significantly higher than those LETO rats. Troglitazone treatment resulted in a significant reduction of both serum TG and TC (Table 2). There was no difference in HDL-C levels between OLETF and OLETF-T rats.

The effect of troglitazone on body weight (BW), visceral fat weight (VFW) and pancreas weight (PW) is summarized in Table 3. Troglitazone significantly reduced both BW and VFW and increased PW. Histological changes showed that there was hypertrophy of visceral fat cells, and infiltration of fat cells

Table 3. Body Weight (BW), Visceral Fat Weight (VFW) and Pancreas Weight (PW) in LETO, OLETF and OLETF-T Rats

	LETO <i>n</i> 18	OLETF <i>n</i> 8	OLETF-T <i>n</i> 8
BW	446.8 ± 15.2	596.8 ± 11.2***	550.8 ± 32.3***,###
VFW	13.9 ± 2.1	53.4 ± 9***	39.5 ± 10.1***,###
PW	1.6 ± 0.4	0.9 ± 0.1***	1.2 ± 0.3**.#

LETO = Long-Evans Tokushima Otsuka; OLETF = Otsuka Long-Evans Tokushima Fatty rats without treatment; OLETF-T = Otsuka Long-Evans Tokushima Fatty rats treated with troglitazone; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (significantly different from LETO); #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 (significantly different from OLETF).

into skeletal muscle tissues in OLETF rats, in which marked fat deposition, fibrosis, mononuclear cell infiltrations and fatty degeneration in β -cells of the islet appeared in the pancreas. But in OLETF-T rats, troglitazone treatment significantly attenuated these abnormalities (shown in Fig. 1–3).

Western blot analysis revealed that expression of Glut 4 in membrane fractions per gram of protein were 32% in visceral fat and 52% in skeletal muscle in OLETF rats compared to those in LETO rats, whereas the expression in OLETF-T rats was 71% for visceral fat and 75% for skeletal muscle tissues. Thus, expression of Glut 4 was significantly higher in both visceral fat (*P* < 0.01) and skeletal muscle (*P* < 0.05) in OLETF-T rats than those in OLETF rats (shown in Fig. 4–5).

DISCUSSION

Diabetic OLETF rats were found to have increased body weight, increased fat tissue in the body, hypertriglyceridemia, and decreased expression of Glut 4 in skeletal muscle and adipose tissues, as shown in previous studies.^{1–3,7,8} It is very likely that these abnormalities are responsible for insulin resistance in this NIDDM animal model. The present histological study also demonstrated atrophy of pancreas associated with fibrosis, lymphocytic infiltration and deposition of fat droplets in β -cells, which may account for impaired insulin secretion in response to glucose in OLETF rats. The functional and histological abnormalities of the pancreas may be consequences of insulin resistance. A recent study by Man *et al.*¹⁵ has shown that triglyceride inhibits insulin secretion, at least in part, by negatively regulating glucokinase activity in pancreas islet cells.

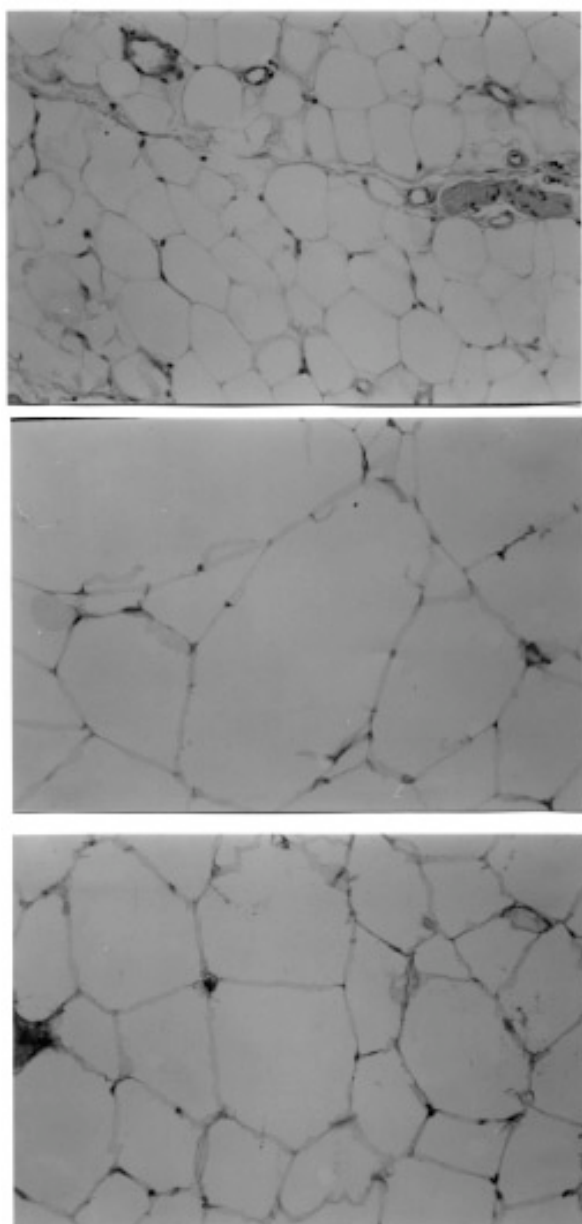


Fig. 1. Histological Changes of Visceral Fatty Cells in Different Groups

Typical light-microscopic photographs of visceral fat tissue from LETO (a), OLETF (b), and OLETF-T (c) rats are shown. The visceral fat cell from OLETF rats were hypertrophic and some of them became fusion blebs. Visceral fat cells from OLETF-T rats were slightly enlarged and partly normal ($\times 200$, hematoxylin-eosin stain).

The data presented in this study showed that treatment with troglitazone restores to normal the majority of abnormalities described above. Troglitazone reduced body weight, body fat weight, serum triglyceride and cholesterol levels. Infiltration of fat in skeletal muscle tissues and in the pancreas was also significantly attenuated. These effects were associated with a marked improvement

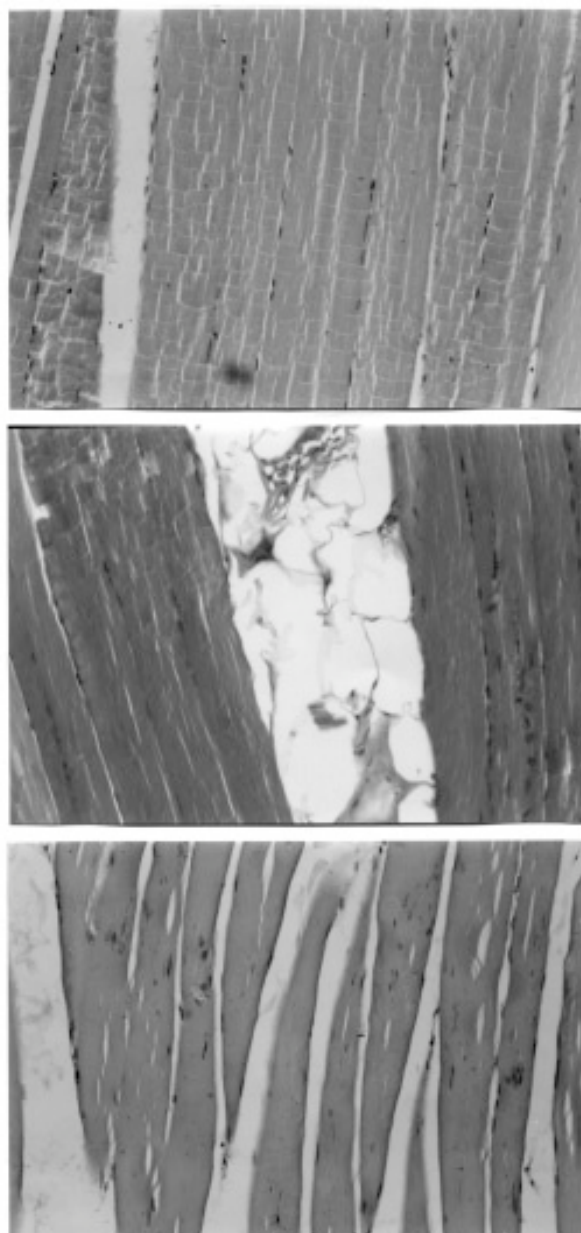


Fig. 2. Histological Appearances of Skeletal Muscles From LETO (a), OLETF (b) and OLETF-T (c)

Fatty infiltration can be seen in the skeletal muscles from OLETF rats, but this condition is improved in those from OLETF-T rats ($\times 200$, hematoxylin-eosin stain).

of insulin resistance. Importantly, troglitazone prevented the decrease of expression of Glut 4 observed in non-treated OLETF rats. Glut 4 plays a pivotal role in regulating glucose uptake and utilization. Therefore, over expression of Glut 4 in troglitazone-treated OLETF rats may be one the factor responsible for the improved insulin resistance. The mechanism by which troglitazone increases Glut 4 expression is not clear at present. It has been shown that this antidiabetic reagent binds and activates PPAR-

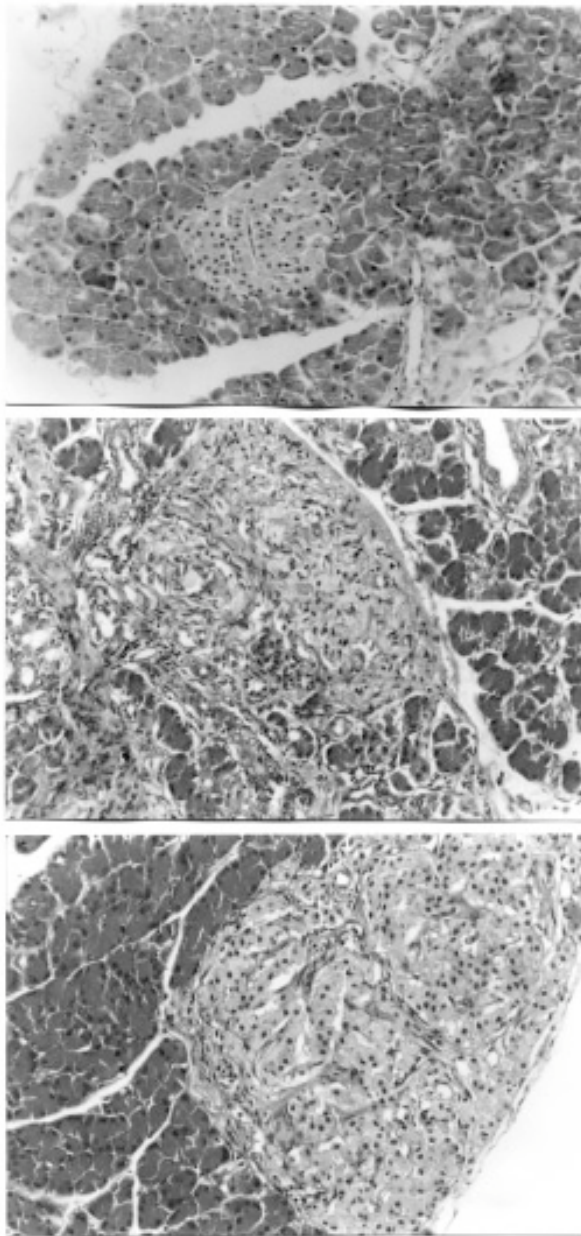


Fig. 3. Histological Changes of the Pancreas Islets From LETO (a), OLETF (b) and OLETF-T (c) Rats

The pancreatic islet became larger with mononuclear cell infiltration, fibrosis and fatty deposition in OLETF rats. The lesion of the islet were significantly improved in OLETF-T rats, and normal pancreas islets can be seen in this group ($\times 200$, Chematoxylin-eosin stain).

γ receptor. Activation of PPAR- γ may lead to stimulation of Glut 4 synthesis at transcriptional or translational levels. Further studies are required to clarify this point.

In summary, the data presented here demonstrate that troglitazone improves glucose tolerance in association with reduction of serum triglyceride levels and attenuation of morphological abnormalities in the pancreas of OLETF rats. Troglitazone signifi-

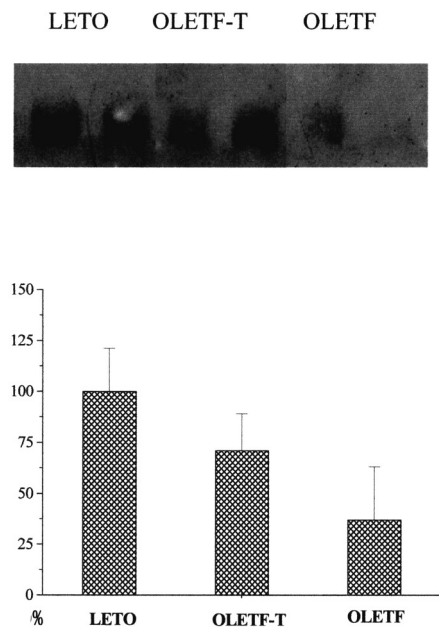


Fig. 4. Quantity of Glucose Transporter 4 (Glut4) Expression in the Plasma Membrane of the Adipocytes in Different Strains of the Rats

a) Glut 4 protein in the plasma membrane from adipocytes of different rat strains was analyzed by Western blot and the results are shown here. b) Scanning data of the Western blot analysis are expressed in arbitrary units. Statistically significant differences ($p < 0.01$) existed between LETO and OLETF-T, OLETF-T and OLETF, and LETO and OLETF.

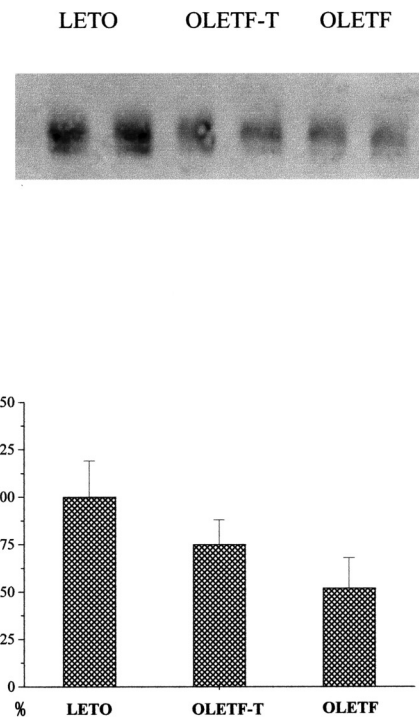


Fig.5. Quantity of Glut 4 Expression in the Plasma Membrane of the Skeletal Muscle Cells in Different Rat Strains

a) Glut 4 protein in the plasma membrane from skeletal muscle cells of different groups was analyzed by Western blot. b) Scanning data of the results are expressed in arbitrary units. Statistically significant differences existed between LETO and OLETF-T, OLETF-T and OLETF, and LETO and OLETF.

cantly increased Glut 4 expression in membrane fractions of both skeletal and adipose tissues. These findings suggest that the increased expression of Glut 4 might be one of the mechanisms by which insulin sensitivity is improved in this rat model of NIDDM.

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