

Plasma membrane content of insulin-regulated glucose transporter in skeletal muscle of the male Otsuka Long-Evans Tokushima Fatty rat, a model of non-insulin-dependent diabetes mellitus

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Received 17 February 1997; revised version received 24 March 1997

Abstract The male Otsuka Long-Evans Tokushima Fatty (OLETF) rat shows insulin resistance in skeletal muscle and visceral obesity. To obtain information on the mechanism of the insulin resistance in the diabetic rats, we examined the content of insulin-regulated glucose transporter (GLUT4) in skeletal muscles. The results indicate that the total content of the transporter is significantly decreased ($P < 0.05$) in muscles of the diabetic rats. Plasma membrane content of the GLUT4 protein in muscles of the diabetic rats was increased in the basal state as compared to control rats. Hyperinsulinemic clamps increased GLUT4 levels in the plasma membrane of control rats but failed to do so in the diabetic rats. The distribution of GLUT4 in OLETF rat is reminiscent of the characteristics of human non-insulin-dependent diabetes mellitus.

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Key words: Otsuka Long-Evans Tokushima Fatty rat; Insulin-regulated glucose transporter; Skeletal muscle; Non-insulin-dependent diabetes mellitus model

1. Introduction

Glucose uptake in muscle cells is considered to be mediated by glucose transporter proteins. Skeletal muscle expresses at least two glucose transporters. One is GLUT1, a glucose transporter which is mainly responsible for basal glucose uptake, and the other is GLUT4, an insulin-regulated glucose transporter which is considered to be translocated from intracellular membranes to the plasma membrane by insulin, resulting in an increased muscle glucose uptake [4,5]. In human non-insulin-dependent diabetes mellitus (NIDDM) patients, impaired expression of GLUT4 in skeletal muscle has not been observed [6–8]. Recently, it was reported that plasma membrane content of GLUT4 in NIDDM patients was increased in the basal state and failed to increase after euglycemic hyperinsulinemic clamps [3].

The male Otsuka Long-Evans Tokushima Fatty (OLETF) rat, which is a model of NIDDM, is an inbred strain with innate polyphagia, rapid body weight gain, and accumulation of intra-abdominal fats [1,2]. We previously examined the existence of insulin resistance in OLETF rats by euglycemic hyperinsulinemic clamp experiments in vivo. In these experiments, the glucose infusion rate in OLETF rats is found to be

about 25–60% of that in the control strain, Long-Evans Tokushima Otsuka (LETO) rats. Hindlimb perfusion experiments showed that insulin-stimulated glucose uptake in OLETF rats is 55% of that of LETO rats, confirming skeletal muscle insulin resistance. However, no major defects in the insulin receptor functions in vitro, including insulin receptor binding, receptor autophosphorylation, and receptor tyrosine kinase activity, in skeletal muscle of this diabetic model were observed [2]. To examine whether an abnormal GLUT4 expression and/or membrane distribution may be responsible for insulin resistance in skeletal muscle of OLETF rats, we examined GLUT4 protein levels in total and isolated plasma membrane in the basal state and after a hyperinsulinemic clamp. GLUT1 contents in the muscle plasma membrane of these rats were also examined.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, fraction V) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). An enhanced chemiluminescence (ECL) Western blotting detection system, peroxidase-labeled antibody, and autoradiography film (Hyperfilm-ECL) were obtained from Amersham Int. (Amersham, Bucks., UK). Anti-GLUT4 and GLUT1 antibodies were obtained from Eastacres (Costa Mesa, CA, USA). Antibody against Na^+/K^+ -ATPase $\alpha 1$ subunit was obtained from UBI (Lake Placid, NY, USA). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). LETO and OLETF rats were raised at the Tokushima Research Institute (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan).

2.2. Membrane preparation and Western blot analysis

Male OLETF rats and age-matched LETO rats were used in all experiments. Euglycemic hyperinsulinemic clamp experiments in 30-week-old rats were performed as described previously [2]. After overnight starvation, the animals were anesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.). Hindlimb skeletal muscles were excised and immediately frozen at -80°C . The sample was powdered in liquid nitrogen and was homogenized in buffer containing 10 mmol/l NaHCO_3 , 250 mmol/l sucrose, 5 mmol/l NaN_3 , and 0.1 mmol/l PMSF at 4°C as described by Klip et al. [9], with slight modifications. All subsequent steps were carried out at 4°C . Homogenates were centrifuged at $1200 \times g$ for 10 min. The supernatant was retained and the pellet was resuspended, homogenized, and centrifuged at $1200 \times g$ for 10 min. The first and second supernatants were combined and centrifuged at $9000 \times g$ for 10 min. The resulting supernatant was again centrifuged at $227\,000 \times g$ for 75 min to produce total crude membranes, and the pellet was resuspended in the buffer. The plasma membrane fraction was isolated by the method of Galante et al. [10]. Briefly, approximately 3 g of tissue was powdered and homogenized in 0.2 mol/l Tris-HCl, 1 mmol/l EDTA, 255 mmol/l sucrose, 0.5 mg/ml bacitracin, 1 mmol/l PMSF, 0.2 mg/ml benzamidine, 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, and 1 mmol/l orthovanadate (pH 7.4). Homogenate was centrifuged at $8700 \times g$ for 20 min. The pellet was resuspended in buffer as described above, and centrifuged at $750 \times g$ for 10 min. The resulting supernatant was cen-

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Abbreviations: OLETF, Otsuka Long-Evans Tokushima Fatty; GLUT4, insulin-regulated glucose transporter; NIDDM, non-insulin-dependent diabetes mellitus; LETO, Long-Evans Tokushima Otsuka

trifuged again at $12000 \times g$ for 30 min. The pellet was resuspended in the buffer and layered on a 38.3% sucrose cushion. After ultracentrifugation at $58800 \times g$ for 30 min, the pellet was collected at the sucrose interface. Finally, the plasma membrane- and t-tubule-containing fraction was ultracentrifuged at $183000 \times g$ for 40 min. The entire preparation was performed on ice. Na^+, K^+ -ATPase $\alpha 1$ subunit was used as a marker of the plasma membrane fraction by immunoblotting with its specific antibody. The total crude muscle membrane (100 μg) and the plasma membrane (10 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) followed by treatment with rabbit anti-GLUT4 or anti-GLUT1 antibody (1:500) and a secondary peroxidase-labeled antibody (1:1000). Protein concentration was determined by the Lowry method using BSA as a standard [11]. The glucose transporter protein was detected using a non-radioisotopic ECL method as a fluorogram on autoradiography film, and the density of the band at the glucose transporter position was measured using an Ultrascan XL, Enhanced Laser Densitometer (LKB, Bromma, Sweden). Membrane to membrane normalization was performed by the application of same sample.

2.3. Blood analysis

Serum glucose, serum cholesterol, serum NEFA (Wako Pure Chemicals, Osaka, Japan) and serum triglyceride (Ono, Osaka, Japan) were analyzed using commercial kits. Serum insulin was determined by radioimmunoassay (Amersham).

2.4. Statistical analysis

All values are expressed as the mean \pm S.E.M. Statistical significance was examined by two way analysis of variance followed by two-tailed *t*-test at each week of age. *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Characteristics of the animals

The body weight, fasting serum glucose, serum triglyceride, serum cholesterol, serum NEFA, and serum insulin of the LETO and OLETF rats used for these experiments are shown in Table 1. Body weights of OLETF rats were significantly higher than those of LETO rats at 6 ($P < 0.001$) and 30

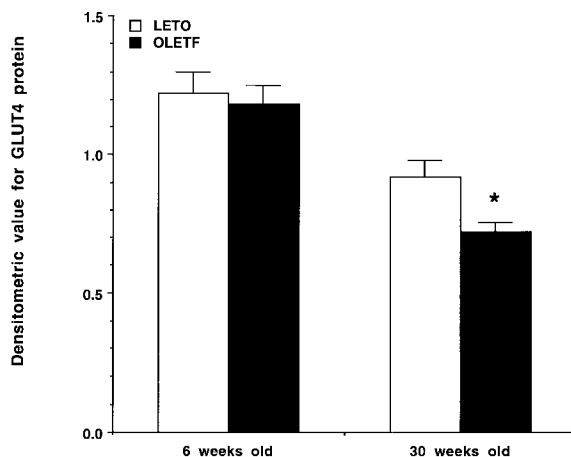


Fig. 1. Western blot analysis of GLUT4 in skeletal muscle of LETO and OLETF rats. The muscle membranes (100 μg of protein) from 6- and 30-week-old LETO and OLETF rats were applied to SDS-PAGE and electroblotted to polyvinylidene difluoride membrane followed by treatment with anti-GLUT4 antibody and a second peroxidase-labeled antibody as described in Section 2. The GLUT4 protein was detected using an ECL method as a fluorogram on autoradiography film. Densitometric values for protein level of GLUT4 from skeletal muscle of 6- and 30-week-old LETO and OLETF rats are shown. Results are means \pm S.E.M., * $P < 0.05$ vs. age-matched LETO rats ($n = 5$).

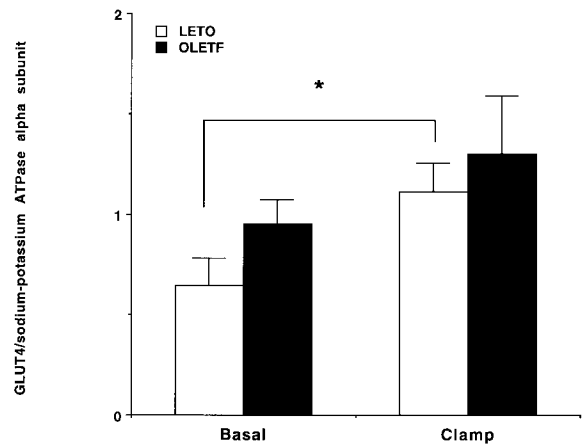


Fig. 2. Plasma membrane content of GLUT4 proteins in hindlimb muscle of LETO and OLETF rats before (Basal) and after the euglycemic hyperinsulinemic clamp (Clamp) experiments. Na^+, K^+ -ATPase $\alpha 1$ subunit was used as marker protein in plasma membrane of skeletal muscle in these rats. The Na^+, K^+ -ATPase $\alpha 1$ subunit and the GLUT4 proteins were detected using an ECL method as a fluorogram on autoradiography film before and after the clamp experiment. Densitometric values for protein level of GLUT4 from skeletal muscle of 30-week-old LETO and OLETF rats are shown. Results are means \pm S.E.M., * $P < 0.05$ ($n = 5$).

($P < 0.01$) weeks of age. Serum triglyceride of OLETF rats significantly increased 3.7- and 8.4-fold at 6 ($P < 0.001$) and 30 ($P < 0.01$) weeks of age, respectively. Serum cholesterol was higher in OLETF rats than in LETO rats at 30 weeks of age ($P < 0.05$). Serum NEFA was significantly increased in OLETF rats at 30 ($P < 0.05$) weeks of age. Serum insulin was higher in OLETF rats at 30 weeks of age ($P < 0.05$).

3.2. Euglycemic hyperinsulinemic clamp experiments

Blood glucose concentrations during the clamp experiments were 4.92 ± 0.02 and 5.47 ± 0.02 mmol/l (not significantly different), and the glucose infusion rate values were 12.2 ± 1.6

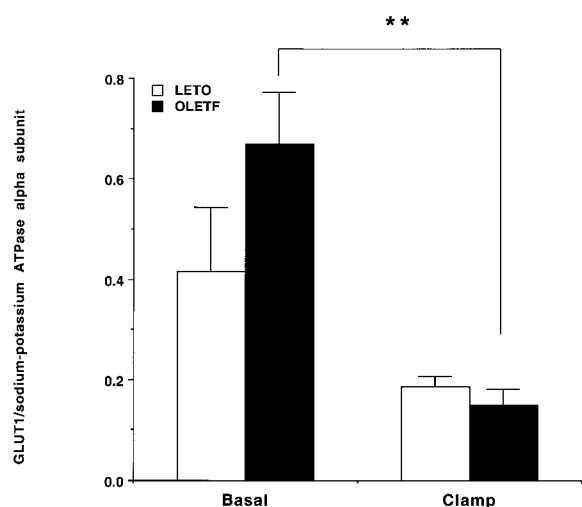


Fig. 3. Plasma membrane content of GLUT1 proteins in hindlimb muscle of LETO and OLETF rats before (Basal) and after the euglycemic hyperinsulinemic clamp (Clamp) experiments. Densitometric values for protein level of GLUT1 from skeletal muscle of 30-week-old LETO and OLETF rats are shown as described in Fig. 2 legend. Results are means \pm S.E.M., ** $P < 0.01$ ($n = 5$).

Table 1

Characteristics of the Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats

Age (weeks)	6 weeks		30 weeks	
	LETO	OLETF	LETO	OLETF
Body weight (g)	145 ± 4	196 ± 8***	507 ± 4	638 ± 18**
Fasting serum glucose (mmol/l)	4.71 ± 0.33	4.55 ± 0.16	6.71 ± 0.44	7.99 ± 0.55
Fasting serum triglyceride (mmol/l)	0.466 ± 0.060	1.72 ± 0.09***	0.376 ± 0.053	3.15 ± 0.40**
Fasting serum cholesterol (mmol/l)	2.40 ± 0.04	2.28 ± 0.10	2.47 ± 0.04	3.98 ± 0.39*
Fasting serum NEFA (μmol/l)	614 ± 50	821 ± 116	602 ± 56	873 ± 70*
Fasting serum insulin (μg/l)	0.539 ± 0.047	0.562 ± 0.034	1.84 ± 0.33	4.54 ± 0.78*

Means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. age-matched LETO rats ($n = 5$).

and $3.08 \pm 1.33 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ ($P < 0.01$) in LETO and OLETF rats, respectively. Insulin concentrations after the clamp experiments were 10.8 ± 1.9 in control and $16.3 \pm 1.7 \text{ μg/l}$ in the diabetic rats (not significant).

3.3. Western blot analysis of GLUT4

To study the mechanism of insulin resistance of OLETF rats, Western blot analysis of GLUT4 protein level in skeletal muscle was carried out at 6 and 30 weeks of age (the prediabetic and diabetic stages, respectively). When compared with the control strain, GLUT4 protein level in OLETF rats showed no significant differences at 6 weeks and was significantly lower ($P < 0.05$) at 30 weeks of age (Fig. 1). The Na^+, K^+ -ATPase $\alpha 1$ contents were not different in these rats (data not shown). We next investigated the plasma membrane content of GLUT4 protein in these rats. The experiments showed that the muscle GLUT4 protein content was increased by 49% in the diabetic rats in the basal state. Furthermore, hyperinsulinemia increased plasma membrane GLUT4 content significantly in the control rats but not in diabetic muscle (Fig. 2).

3.4. Western blot analysis of GLUT1

GLUT1 protein content in plasma membrane of skeletal muscle was not different in these two types of rats in either the basal or the insulin-stimulated state (Fig. 3). Interestingly, GLUT1 protein levels in plasma membranes of skeletal muscle of these rats were decreased after the clamp states.

4. Discussion

GLUT4 protein level in total membrane content in 30-week-old OLETF rat was decreased significantly compared to LETO rats (Fig. 1). The GLUT4 levels in skeletal muscle of other animal models were the same as or lower than in controls. In diabetic Zucker *fa/fa* and viable yellow mice, the GLUT4 levels in skeletal muscle were decreased [12]. In *db/db* mice, no differences were observed compared to lean mice [13]. From these studies and the results in humans [6–8], it has not been confirmed that the total contents of GLUT4 protein reflect the decrease of insulin-stimulated glucose uptake in skeletal muscle, indicating that investigations of the translocation and activity of the GLUT4 protein will be important. However, there were only few studies where translocation of the GLUT4 protein was examined possibly mainly because of the difficulty of fractionating skeletal muscle membranes. King et al. [14] reported a defect in GLUT4 translocation in muscle of Zucker rats. However, Galante et al. [10] found that there were no differences in GLUT4 translocation

in muscles of lean and obese rats. The discrepancy may have resulted from differences in the methods of fractionation. Recently, it was shown that in NIDDM patients, the plasma membrane content of GLUT4 was higher than in controls and no increase was observed after insulin infusion [3]. These findings are similar to our results in muscles of OLETF rats (Fig. 2). Na^+, K^+ -ATPase $\alpha 1$ subunit protein is a housekeeping protein of plasma membranes, and therefore we used this protein as internal standard to quantify GLUT4 protein content in this fraction. After euglycemic hyperinsulinemic clamps, GLUT4 content in the plasma membrane fraction was increased significantly in the LETO rats, but not significantly in the diabetic rats. STZ-induced hyperglycemic rats [15] and the hyperglycemic hyperinsulinemic obese model [16] showed GLUT1 contents in plasma membranes were higher than those of controls. The GLUT1 content was also increased in OLETF rats. Interestingly, after insulin infusion, the plasma membrane GLUT1 contents were decreased in both rats. This novel observation deserves further investigation.

In conclusion, the redistribution of plasma membrane GLUT4 content in the diabetic rat is reminiscent of that of human NIDDM. The OLETF rat is a very useful model for the elucidation of insulin resistance in NIDDM patients.

Acknowledgements: We thank Drs. Amira Klip (The Hospital for Sick Children, Toronto) and Andre Marette (Laval University, Quebec) for useful suggestions. Also, we appreciate Dr. Yasuhide Mitumoto (Otsuka Pharmaceuticals) for technical advice and useful suggestions.

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