ORIGINAL PAPER

Nagoya J. Med. Sci. **68**. 155 ~ 163, 2006

IMPAIRED INSULIN-REGULATED MEMBRANE AMINOPEPTIDASE TRANSLOCATION TO THE PLASMA MEMBRANE IN ADIPOCYTES OF OTSUKA LONG EVANS TOKUSHIMA FATTY RATS

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ABSTRACT

Insulin-regulated membrane aminopeptidase (IRAP) translocates to the plasma membrane with glucose transporter-4 (GLUT4) on insulin stimulation. However, this may be impaired in patients at risk of diabetes. Recently a novel technique has been developed to assess cell surface IRAP activity dynamically using a fluorogenic membrane impermeable substrate. In this study we measured the cell surface IRAP activity and 3-*0*-[*methyl*-^{14}C]-D-Glucose uptake in adipocytes isolated from Otsuka Long Evans Tokushima Fatty rats (OLETF), developed as a model of type 2 diabetes mellitus, to evaluate whether the translocation of GLUT4/IRAP vesicles is affected. On the addition of insulin, the cell surface IRAP activity promptly increased to reach equilibrium in a hormone dose-dependent manner. OLETF rats showed significantly lower equilibrium activity than control rats ($P<0.01$). Time to reach the equilibrium was also significantly longer in the OLETF case, and adipocytes isolated from OLETF rats demonstrated both a delay and a reduction in 3-*0*-[*methyl*-^{14}C]-D-Glucose uptake. This impairment in all parameters was alleviated by treatment with pioglitazone. Continuous measurement of cell surface IRAP activity allowed accurate evaluations of GLUT4/IRAP vesicle translocation and of the establishment of defects in OLETF rats.

Key Words*:* Insulin-regulated membrane aminopeptidase, Translocation, Otsuka Long Evans Tokushima Fatty rats, Type 2 diabetes mellitus, Glucose transporter 4

INTRODUCTION

Peripheral insulin resistance plays an important role in the pathogenesis of type 2 diabetes mellitus. Insulin normally increases glucose uptake in sensitive tissues such as muscle and adipose tissue by stimulating the translocation of the insulin-sensitive glucose transporter 4 (GLUT4) from intracellular storage sites to the plasma membrane. The impairment of glucose uptake in these tissues appears to be a major cause of insulin resistance. Garvey *et al*. (1998) have described an

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abnormal accumulation of GLUT4 under basal conditions and impaired translocation in type 2 diabetes skeletal muscles. Therefore, a detailed estimation of the insulin-stimulated translocation of GLUT4 might be critical for defining the pathology of peripheral insulin resistance. Since experimental system is required for the elucidation of mechanisms, we selected the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a well established model for obese type 2 diabetes mellitus (Kawano *et al*. 1992, 1994). Indeed, GLUT4 protein and mRNA levels in adipose tissue from OLETF rats were significantly reduced compared with those in control rats (Furuta *et al*. 2002).

Pioglitazone, an analogue of thiazolidinedione, has been reported to improve peripheral insulin resistance in type 2 diabetic patients (Florkowski *et al*. 2002; Henry *et al*. 2003) and in diabetic animal models (Ikeda *et al.* 1997; Furuta *et al.* 2002). It has also been suggested to facilitate translocation of GLUT4 by increasing phosphorylation of the insulin receptors. Previous estimations of the movement of GLUT4 from intracellular vesicular storage sites to plasma membranes have been dependent on exogenous protein expression or immunological procedures. However, it is difficult to dynamically evaluate translocation in impaired adipocytes from insulin resistant individuals and to determine the effects of therapeutic agents in vivo.

Insulin-regulated membrane aminopeptidase (IRAP) was discovered to be a major protein in GLUT4 vesicles (Mastick *et al.* 1994; Kandror *et al.* 1994, 1995) and shown to be identical to oxytocinase/placental leucine aminopeptidase (Rogi *et al.* 1996; Ross *et al.* 1996). IRAP, a type II membrane protein with extracellular catalytic sites, has interesting and unique characteristics among vesicle proteins that behave like GLUT4; it is not only sequestered in the same intracellular vesicles as GLUT4 under basal conditions, but also redistributes to the cell surface after stimulation with insulin. The translocation of GLUT4/IRAP vesicles is complete once their trafficking, docking, and fusion to the plasma membranes have been accomplished. Proteolytic analysis revealed that IRAP is present in sealed GLUT4/IRAP-containing vesicles with its active site in the lumen, and that it displays enzyme activity after fusion of the plasma membranes. Recently Ross *et al*. (1996) have developed a novel technique to measure cell surface IRAP activity, and we have demonstrated its applicability to the dynamic assessment of IRAP translocation in intact cells (Nakamura *et al.* 2000).

In the present study, we evaluated the time required for GLUT4/IRAP vesicle translocation to plasma membranes by examining the time-lapse cell surface IRAP catalytic activity in diabetic OLETF rats, and investigated the effects of pioglitazone on the translocation.

MATERIALS AND METHODS

Animals and reagents

Four-week-old male OLETF rats and non-diabetic control male Long-Evans Tokushima Otsuka (LETO) rats were provided by the Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Sprague-Dawley rats (27 to 30 weeks) were purchased from Chubu Kagaku Shizai (Nagoya, Japan). Wortmannin was purchased from Sigma (St. Louis, MO). Pioglitazone, provided by Takeda Chemical Industries Co., Ltd. (Osaka, Japan), was suspended in 0.5% methylcellulose solution for administration orally at 3 mg/kg per day to 30-week-old OLETF and to LETO rats by stomach tube for 10 days (Kawano *et al*. 1992). The methylcellulose (0.5%) vehicle was administered to controls (n=4, each). The animal protocol used was approved by the Nagoya University Committee for Animal Investigations.

Cell preparation and treatment

Isolated brown adipocytes were prepared by collagenase digestion (Rodbell *et al*. 1964) from

the adipose tissue of pioglitazone-treated and pioglitazone-untreated OLETF/LETO rats. After overnight fasting, adipocytes were isolated from the brown adipose tissues by digestion with 2.5 mg ml^{-1} collagenase, as described previously (Furuta *et al.* 2002). Digested fat pads were filtered through a 100 μm Nylon Cell Strainer (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the cells were washed three times with Krebs-bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin, 45% Percol, 5 mM glucose, and 200 nM adenosine. Aliquots were used to determine the cell concentrations, as described previously (Furuta *et al.* 2002).

Measurement of cell surface IRAP activity

Cell surface IRAP activity was assayed by the method detailed previously (Martz *et al.* 1986) using 7-amino-4-(*S*-glutathionyl) methylcoumarin, L-lysine amide (Molecular Probes, Inc., Eugene, OR) with aminoacyl derivatives as fluorogenic substrates. Time course changes in adipocyte cell surface IRAP activity were assayed at 37˚C in a recording fluorescence spectrometer (model RF5000, Shimadzu, Kyoto, Japan) with excitation at 365 nm and emission at 455 nm in a 1.0 ml cuvette (type FM20-UV-3, GL Sciences Inc., Tokyo, Japan) with a 45 mm path length. Fluorescence measurement of 0.5 ml of cell suspensions with 1μM substrate in the cuvette was carried out every 2 sec. During this process, various concentrations of insulin were added and measurements were continued.

Assay of 3-0-[methyl-^{14}C]-D-Glucose uptake

Assays of 3-*0*-[*methyl*-^{14}C]-D-Glucose uptake were performed using a modification of the method described earlier (Martz *et al*. 1986; Jhum *et al*. 1991; Wheitesel *et al*. 1985). Following preparation, cells were added to KRPB buffer with 5% albumin and incubated at 37˚C. Suspensions (30 μl) were then mixed with 6 μl of labelled 3-*0*-[*methyl*-^{14}C]-D-Glucose (22.2kBq) (ICN Pharmaceuticals, Inc., Irvine, CA) and 3 μl of insulin (final 10^{-7}M) at time *t*=0. Uptake at 37˚C was stopped after a given time by the addition of 200 μl of ice-cold KRPB buffer containing 200 μM phloretin. After addition of 80 μl silicon oil topped with 20 μl corn oil, they were centrifuged for 15 sec (12,500 rpm), after which oils were removed and the supernatant was immediately decanted. The cell pellets were suspended in 50 μl of KRPB buffer and counted by liquid scintillation to give intracellular counts. In the same way, the supernatant was counted to yield extracellular counts. The uptake rate of 3*OMG* was calculated as the total intracellular $count^{-1}$.

Statistical analysis

All results are expressed as mean ± SD values. Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS) for Windows. Differences between OLETF and LETO rats in cell surface IRAP activity and 3*OMG* uptake were analyzed by two-way ANOVA with Scheffe's test, where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of experimental animals

Compared with LETO rats, OLETF rats showed significantly higher levels of fasting plasma glucose, insulin, and triglyceride (Table 1). Pioglitazone treatment reduced the elevation in plasma insulin in OLETF rats, but no effects were observed in LETO rats.

Time-lapse measurement of cell surface IRAP activity

Figure 1-A illustrates the results of time course measurements of cell surface IRAP activity of adipocytes from SD rats. Without insulin the RFU value over time showed a constant gradient, but once insulin was added, the cell surface IRAP activity promptly rose after an initial spike downwards, and reached a plateau level that continued for 15 min with no remarkable change

Table 1 Characteristics of the experimental animals.

	LETO/Pg(–)	LETO/Pg(+)	OLETF/Pg(–)	OLETF/Pg(+)
Body weight (g)	462 ± 23	475 ± 24	618 ± 28**	606 ± 25
Fasting plasma glucose (mg dl^{-1})	234 ± 16	254 ± 17	379 ± 61**	335 ± 76
Fasting plasma insulin (pg ml^{-1})	1640 ± 170	1753 ± 59	9715 ±125**	7891 ± 55††
Fasting plasma triglyceride (mg dl^{-1})	32 ± 12	29 ± 16	115 ± 17**	76 ± 27

Data are expressed as mean ± S.D. from four LETO and OLETF rats.
** $P<0.01$ vs. LETO/Pg (-) rats †† $P<0.01$ vs. pioglitazone-untreated OLETF rats

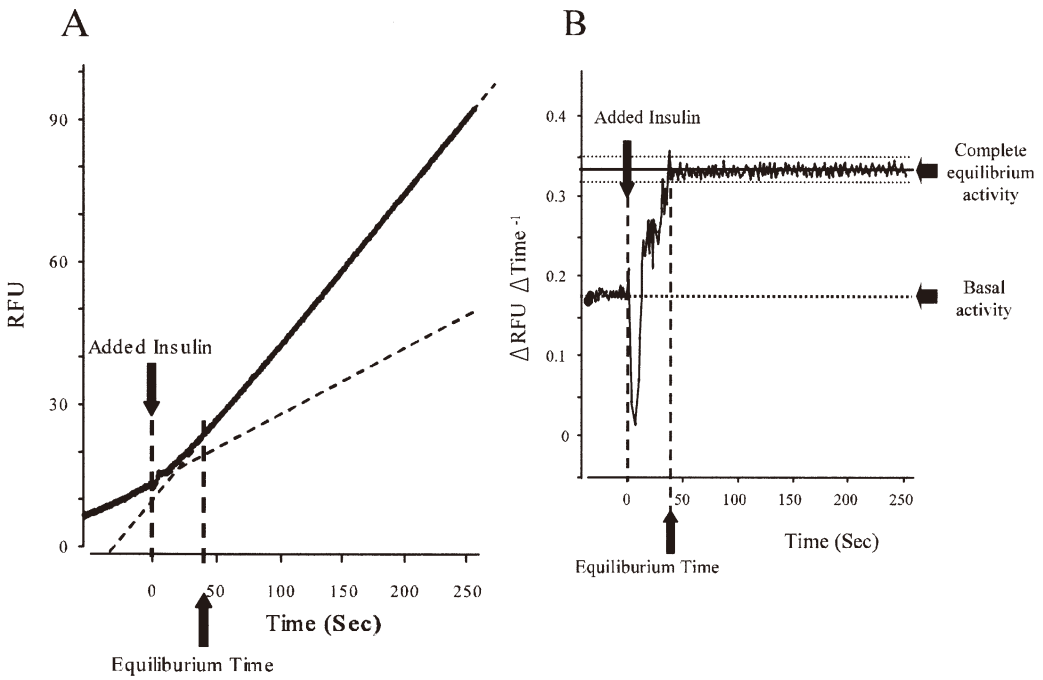

Fig. 1 Time course change in cell surface IRAP activity in adipocytes from SD rats.
Cell surface IRAP activity was assayed using a membrane impermeable fluorogenic substrate as described in Materials and Methods. Fluorescence measurement of 0.5 ml isolated rat adipocyte suspensions with 1 μM substrate in cuvette was carried out every 2 sec in a recording fluorescence spectrometer. A: RFU value over time B: Changes in cell surface IRAP activity (ΔRFU $\Delta Time^{-1}$) with time. Value without insulin was defined as "Basal value". "Complete equilibrium activity" was determined as based on mean values of ΔRFU $\Delta Time^{-1}$ at 200–250 sec. We also defined time required to reach complete equilibrium (range within complete equilibrium value ± 2%) as "Equilibrium time".

(data not shown).

Data for ΔRFU ΔTime^{-1} (IRAP activity) are given in Fig. 1-B. After reaching a stable value, ΔRFU ΔTime^{-1} remained steady. The value after equilibrium (completed equilibrium activity) was determined as the mean value of ΔRFU ΔTime^{-1} for 200–250 sec. We also defined the time required for achieving equilibrium activity (the range within the equilibrium activity ± 2%) as "equilibrium time".

On the addition of wortmannin (known to inhibit GLUT4/IRAP translocation to the plasma membrane), the effects of insulin were significantly inhibited (data not shown).

Complete equilibrium activity and time of OLETF and LETO rats

As shown in Fig. 2, the ratio of cell surface IRAP activity rose on stimulation with insulin in a dose-dependent manner. OLETF rats (*n=4*) exhibited a significantly lower equilibrium activity than LETO rats (*n=4*) at each insulin concentration (P<0.01). Pioglitazone improved the equilibrium activity of OLETF rats so that it was close to the level of LETO rats (P<0.01), while no significant effects were noted in the latter.

Figure 3 demonstrates the time to reach equilibrium. A high concentration of insulin appreciably reduced activities in both LETO and OLETF rats. The time for OLETF rats was significantly longer than for LETO rats at each insulin concentration (P<0.01). Similar to equilibrium activity, pioglitazone improved the time to equilibrium of OLETF rats (P<0.01), with no influence on LETO rats.

3OMG uptake

To elucidate the association of initial IRAP translocation and glucose transport, we compared the time course of insulin stimulation of glucose transport activity in LETO and OLETF rats. As shown in Fig. 4, glucose transport was significantly reduced and delayed in OLETF rats (P<0.01)

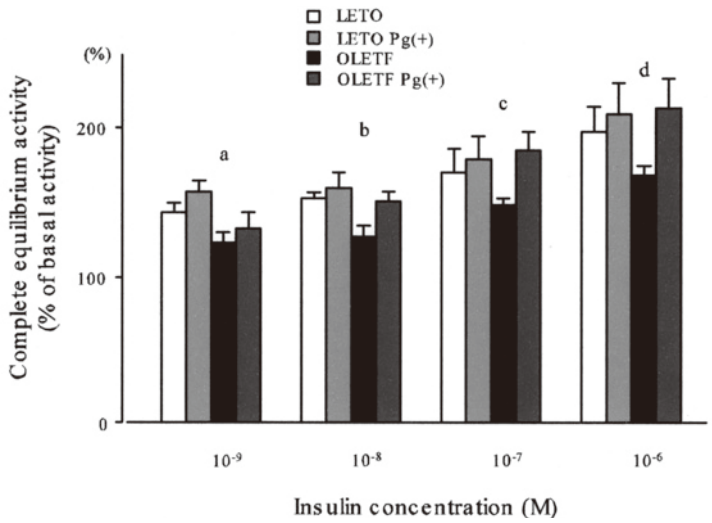

Fig. 2 Effects of insulin and pioglitazone on complete equilibrium activity of adipocytes from OLETF and LETO rats.
Complete equilibrium activity for cell surface IRAP activity stimulated with various insulin concentrations was determined. Pioglitazone (Pg. (+)) was administrated to OLETF and LETO rats orally for 10 days (3 mg kg^{-1}). Results are mean ± S.D. values (n=4). Different characters represent statistically significant differences (P<0.05).

but improved on the administration of pioglitazone, whereas in LETO rats the drug exerted no remarkable effects on either maximum activity or $t_{1/2}$.

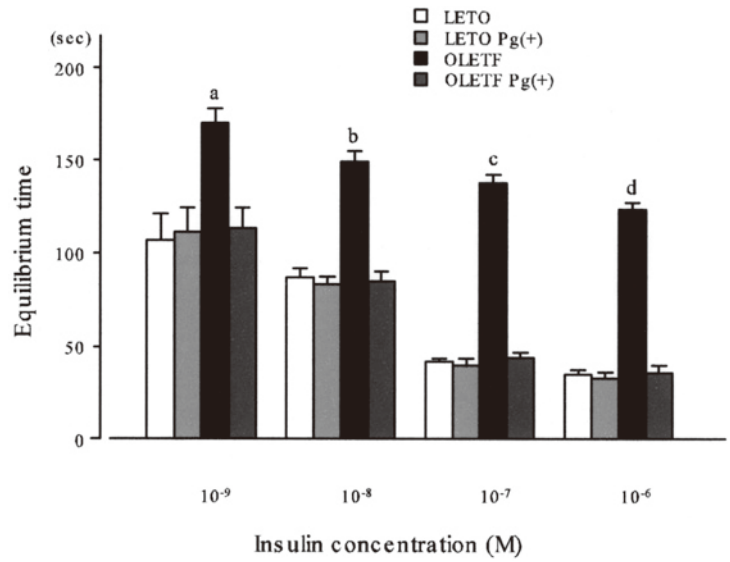

Fig. 3 Effects of insulin and pioglitazone on time to equilibrium for adipocytes from OLETF and LETO rats.
Equilibrium time for cell surface IRAP activity stimulated with various insulin concentrations was analyzed. Pioglitazone reduced time for OLETF rats close to LETO values, while no effects were observed in the latter. Results are mean ± S.D. values (n=4). Different character denotes statistically significant difference ($P<0.05$).

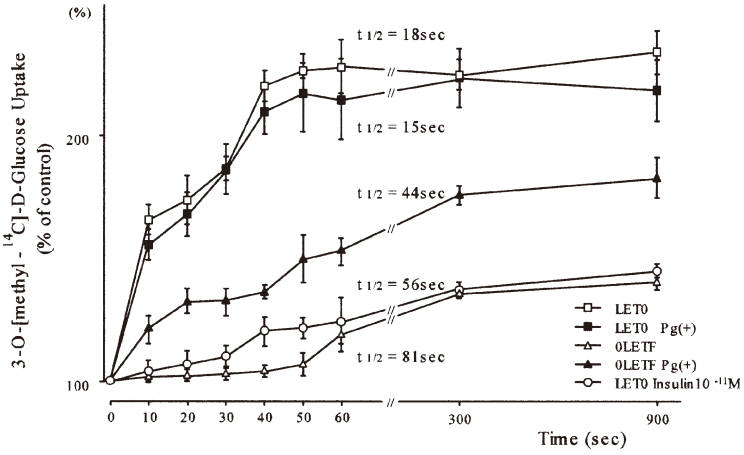

Fig. 4 Time course of insulin stimulation of glucose transport activity.
Isolated adipocytes in KRPB buffer with 5% albumin were incubated at 37˚C. Labelled 3*OMG* (22.2kBq) and 3 µl of insulin (final 10^{-7}M) were added to suspensions at time *t*=0. White circles are for insulin 10^{-11}M-treated LETO rats. Results are mean ± S.D. (n=3).

DISCUSSION

The present measurements of the time-course of changes in cell surface IRAP activity of native adipocytes using membrane impermeable substrates revealed that the translocation of GLUT4/IRAP was reduced in the OLETF strain, but improved on the application of pioglitazone. Our findings are in agreement with defects in the trafficking of GLUT4/IRAP-containing vesicles of adipocytes isolated from human type 2 diabetes using the immunological technique described by Maianu *et al*. (2001).

The kinetics of GLUT4/IRAP vesicle translocation in living cells have been determined using the plasma membrane sheet assay, a fusion protein of the cytoplasmic domain of IRAP as well as the transmembrane and extracellular domains of the transferring receptor, or the IRAP or GLUT4-GFP fusion protein. Cell surface biotinylation methods have also been employed for this purpose after arresting the internalization of GLUT/IRAP. However, using these techniques, the time required to evaluate translocation is relatively long (>1 min). In contrast, our novel method of measuring the cell surface IRAP activity of adipocytes allowed a continuous determination of the amount of IRAP on the plasma membrane, and established that insulin (10^{-7}M) stimulated translocation to rapidly reach an equilibrium in less than 50 sec in uncomplicated adipocytes, which then persisted for 15 min in the presence of insulin. In addition, the time-course of cell surface IRAP activity indicated that the time to achieve both equilibrium and the final activity relative to the basal activity depended on the insulin dose.

The equilibrium activity and time in our diabetic model rats (OLETF) were significantly shortened and prolonged, respectively, compared with the control rats (LETO). Actually, 3*OMG* uptake was impaired in terms of both the initial uptake rate and final transport activity. This was not simply due to a decline in the maximal transport activity, since a reduction in insulin concentration to 10^{-11} M for LETO rats resulted in levels similar to those in OLETF rats receiving 10^{-7} M. Pioglitazone treatment in the diabetic rats completely restored equilibrium activity and time in the time-course measurements of IRAP activity as well as $t_{1/2}$ for 3*OMG* uptake. As the total amount of GLUT4 protein in adipose tissue is known to be reduced in these rats, the rate of glucose transport can be altered by a change in the amount of this protein present on the plasma membrane in the presence of insulin. On the other hand, diabetes does not affect IRAP protein expression, but rather alters its subcellular distribution; more IRAP is observed on the plasma membrane under basal conditions in control rats. Since we described equilibrium IRAP activity as a percentage of the basal condition, a distribution abnormality could explain the reduced value of IRAP in diabetic OLETF rats.

Some researchers have reported not only a decrease in GLUT4 protein expression, but also defects in GLUT4 translocation under obese or diabetic conditions (Toide *et al.* 1997; Garvey *et al.* 1998). A reduction in insulin receptor autophosphorylation may be one cause of impaired GLUT4 translocation (Slieker *et al*. 1990). Pioglitazone is reported to increase GLUT4 protein and mRNA in the adipose tissue of animals with diminished GLUT4 and to simultaneously increase insulin receptor autophosphorylation. In this study, both the equilibrium value and time were restored after pioglitazone treatment in OLETF rats, whereas the glucose uptake was not restored completely.

In conclusion, the present approach to a continuous measurement of cell surface IRAP activity was able to determine the time required to reach equilibrium for GLUT4/IRAP vesicle translocation after insulin stimulation in living cells without protein modification. We thereby established defects in this translocation in diabetic OLETF rats, which are presumably associated with their insulin resistance. To clarify the pathogenesis of insulin resistance, further detailed studies for determining the mechanism of the translocation of GLUT4/ IRAP employing this novel technique

would appear warranted.

ACKNOWLEDGMENTS

This work was funded in part by a Grant-in-Aid (15591740) to A.I. from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the JAOG Ogyaa Donation Foundation.

REFERENCES

1) Florkowski C.M.: Management of co-existing diabetes mellitus and dyslipidemia: defining the role of thiazolidinediones. *Am. J. Cardiovasc. Drugs*, 2, 15–21 (2002).
2) Furuta M., Yano Y, Gabazza E.C., *et al.*: Troglitazone improves GLUT4 expression in adipose tissue in an animal model of obese type 2 diabetes mellitus. *Diabetes Res. Clin. Pr.*, 56, 159–171 (2002).
3) Garvey W.T., Maianu L., Zhu J.H., Brechtel-Hook G., Wallace P. and Baron A.D.: Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J. Clin. Invest.*, 101, 2377–2386 (1998).
4) Henry R.R.: Insulin resistance: from predisposing factor to therapeutic target in type 2 diabetes. *Clin. Ther.*, 25, B47–63 (2003).
5) Ikeda H., Taketomi S., Sugiyama Y., Souda T. and Meguro H.: Effect of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. *Jpn. J. Pharmacol.*, 25, 337–343 (1997).
6) Jhun B.H., Hah J.S. and Jung C.Y.: Phenylarsine oxide causes an insulin-dependent, GLUT4-specific degradation in rat adipocytes. *J. Biol. Chem.*, 266, 22260–22265 (1991).
7) Wheitesel R.R. and Nakada A.A.: Increased affinity predominates in insulin stimulation of glucose transport in the adipocyte. *J. Biol. Chem.*, 260, 2894–2899 (1985).
8) Kawano K., Hirashima T., Mori S., Saitoh Y., Kurosumi M. and Natori T.: Spontaneous long-term hyperglycemic rat with diabetic complications: Otsuka Long–Evans Tokushima Fatty (OLETF) strain. *Diabetes*, 41, 1422–1428 (1992).
9) Kawano K., Hirashima T., Mori S., and Natori T.: OLETF (Otsuka Long–Evans Tokushima Fatty) rat: a new NIDDM rat strain. *Diabetes Res. Clin. Pr.*, 24, S317–320 (1994).
10) Kandror K.V. and Pilch P.F.: Identification and isolation of glycoproteins that translocate to the cell surface from GLUT4-enriched vesicles in an insulin-dependent fashion. *J. Biol. Chem.*, 269, 138–142 (1994).
11) Kandror K.V., Coderre L., Pushkin A.V. and Pilch P.F.: Comparison of glucose-transporter-containing vesicles from rat fat and muscle tissues: evidence for a unique endosomal compartment. *Biochem. J.*, 307, 383–390 (1995).
12) Maianu L. Keller S.R. and Garvey W.T.: Adipocytes exhibit abnormal subcellular distribution and translocation of vesicles containing glucose transporter 4 and insulin-regulated aminopeptidase in type 2 diabetes mellitus: implications regarding defects in vesicle trafficking. *J. Clin. Endocrinol. Metabol.*, 86, 5450–5456 (2001).
13) Martz A., Mookerjee B.K. and Jung C.Y.: Insulin and phorbol esters affect the maximum velocity rather than the half-saturation constant of 3-*O*-methylglucose transport in rat adipocytes. *J. Biol. Chem.*, 261, 13606–13609 (1986).
14) Mastick C.C., Aebersold R. and Lienhard G.E.: Characterization of major protein in GLUT4 vesicles: concentration in the vesicles and insulin-stimulated translocation to the plasma membrane. *J. Biol. Chem.*, 269, 6089–6092 (1994).
15) Nakamura H., Itakura A., Okamura M. *et al.*: Oxytocin stimulates the translocation of oxytocinase of human vascular endothelial cells via activation of oxytocin receptors. *Endocrinology*, 141, 4481–4485 (2000).
16) Rodbell M.: Metabolism of isolated fat cells: effect of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.*, 239, 375–380 (1964).
17) Rogi T., Tsujimoto M., Nakazato H., Mizutani S. and Tomoda Y.: Human placental leucine aminopeptidase/oxytocinase. *J. Biol. Chem*., 271, 56–61 (1996).
18) Ross S.A., Scott H.M., Morris N.J., *et al.*: Characterization of the insulin-regulated membrane aminopeptidase in 3T3-L1 adipocytes. *J. Biol. Chem.*, 271, 3328–3332 (1996).
19) Slieker L.J., Roberts E.F., Shaw W.N. and Johnson W.T.: Effect of streptozocin-induced diabetes on insulin-

receptor tyrosine kinase activity in obese Zucker rats. *Diabetes*, 39, 619–625 (1990).
20) Toide K., Man Z.W., Asahi Y., *et al.*: Glucose transporter levels in a male spontaneous non-insulin-dependent diabetes mellitus rat of Otsuka Long-Evans Tokushima Fatty strain. *Diabetes Res. Clin. Pr.*, 38, 151–160 (1997).