Rapid Preparation of a Plasma Membrane Fraction from Adipocytes and Muscle Cells: Application to Detection of Translocated Glucose Transporter 4 on the Plasma Membrane

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The aim of this study was to establish a rapid preparation of plasma membrane from adipocytes and muscle cells to detect translocated glucose transporter (GLUT) 4. A plasma membrane fraction was prepared by sequential centrifugation with buffer containing detergents, and its purity was estimated by detecting insulin receptor β-subunit (IR/β). After insulin stimulus, GLUT4 translocation was observed in 3T3-L1 adipocytes and L6 myotubes. It was found that IR/β and GLUT4 levels on the plasma membrane decreased in adipose and muscle with intake of a 29% lard diet for 14 weeks. Hence, this method should be useful for rapid preparation of the plasma membrane fraction.

Key words: glucose transporter 4 (GLUT4); insulin receptor β-subunit; plasma membrane; high-fat diet

Glucose transporter 4 (GLUT4), an essential protein in the incorporation of glucose into adipocytes and muscle cells, plays an important role in glucose homeostasis. GLUT4 translocates from the endoplasmic reticulum to the plasma membrane under insulin stimulus. Therefore, determination of GLUT4 translocation due to various stimuli is important to make clear the molecular mechanisms of the insulin-stimulated glucose transport system. Several methods have been performed to evaluate GLUT4 translocation. For example, the plasma membrane fraction is obtained by a sucrose density-gradient centrifugation method using an ultracentrifuge, and the translocated GLUT4 on the plasma membrane is detected by Western blotting analysis. In another method, the immunostaining technique is employed to detect tagged GLUT4 on the cell surface, after expression of c-myc- or green fluorescence protein-tagged GLUT4 in the cells, but these methods require their complexity of handling with considerable time. Moreover, the latter method is difficult to apply to animal experiments. Hence, we attempted to establish a rapid and simple method for preparation of the plasma membrane fraction using a microtube-centrifuge without the sucrose density-gradient and ultracentrifuge. We applied to this method to the detection of GLUT4 translocation on the plasma membrane of mouse 3T3-L1 adipocytes and rat L6 myotubes, which have been used to study glucose metabolism in response to acute insulin stimulus. We also applied it in preparing the plasma membrane from adipose tissue and muscle of mice.

The culture and differentiation of 3T3-L1 fibroblasts and L6 myoblasts were performed as described previously. To prepare the plasma membrane and post-plasma membrane fractions, these cells were harvested with buffer A (50 mM Tris, pH 8.0, and 0.5 mM dithiothreitol, DTT) containing 0.1% (v/v) Nonidet P-40 (NP-40), protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 1 mM Na₃VO₄), and homogenated with a hand microtube-homogenizer. The homogenate was transferred to a 1-ml syringe and sheared by passing it three times through a 25-gauge needle. Each homogenate was centrifuged at 1,000 × g for 10 min at 4 °C, and the precipitate was suspended in NP-40-free buffer A containing the same protease and phosphatase inhibitors, stood on ice for 1 h with occasional mixing, and recentrifuged at 1,000 × g for 10 min at 4 °C. The precipitate obtained was suspended again in buffer A containing 1.0% (v/v) NP-40 and the same protease and phosphatase inhibitors, stood on ice for 1 h with occasional mixing, and centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was collected and stored as the plasma membrane fraction at −80 °C until use. The supernatant from the first and second 1,000 × g centrifugation was gathered and centrifuged again at 16,000 × g for 20 min at 4 °C.

Abbreviations: DTT, dithiothreitol; GLUT, glucose transporter; IR/β, insulin receptor β-subunit; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate
The supernatant obtained was collected and used as a post-plasma membrane fraction. To obtain cell lysate, other cultured cells were harvested with a lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1.0% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] sodium dodecyl sulfate [SDS], and 0.5 mM DTT) containing the same protease and phosphatase inhibitors, stood on ice for 1 h with occasional mixing, and centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was referred as a cell lysate.

Animal treatment in this study conformed to the “Guidelines for the care and use of experimental animals, in Rokkodai Campus, Kobe University.” Male C57BL/6J mice (4 weeks old) were housed in a temperature-controlled (20–25°C) room at 60 ± 5% humidity under a 12-h light-dark cycle. The mice were acclimatized for 7 d with distilled water and commercial chow. They were divided at random into two groups of three each, and fed a standard diet (AIN-93M) or high-fat diet containing 29% lard6) with ad libitum intake of tap water. After 14 weeks of feeding, diet was withheld for 12 h and the mice were killed. Epididymal white adipose tissue and muscle were taken, washed with 1.15% (w/v) KCl, and weighed. After each tissue was chopped into small pieces, it was homogenated with 3 volumes of buffer A or lysis buffer, followed by preparation of the plasma membrane and post-plasma membrane fractions or the cell lysate as described above.

Proteins in each fraction or cell lysate were separated by SDS–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto a polyvinylidenefluoride membrane (GE Healthcare Bio-Science, Piscataway, NJ), followed by blocking of the non-specific binding sites with a commercial blocking solution (Nacalai Tesque, Kyoto, Japan). The membrane was washed out with Tris buffered saline containing 0.05% (v/v) Tween20 (TBST) six times for 5 min each, and incubated with the corresponding primary antibody, as described in the figure legends, for 1 h at room temperature. After it was washed out with TBST under the same conditions, the membrane was incubated with the appropriate secondary antibody conjugated with horseradish peroxidase, as described in the figure legends, for 1 h at room temperature. Specific immune complexes were detected by the ECL plus Western blotting system (GE Healthcare Bio-Science).

First we investigated whether our fractionation procedure was applicable in obtaining a pure plasma membrane fraction. As shown in Fig. 1, insulin receptor β-subunit (IRβ), which are located on the plasma membrane of the cells, were detected in the prepared plasma membrane fraction and cell lysate from 3T3-L1 adipocytes and L6 myotubes, while they were not detected in the post-plasma membrane fraction. β-Actin and cytochrome c, which are distributed in the cytosol and mitochondria respectively, were detected in the post-plasma membrane fraction and cell lysate from both types of cells, while they were not detected in the plasma membrane fraction. These results indicate that the purity of the plasma membrane fraction was relatively high and that, at least, the proteins distributed in the cytosol and mitochondria were not contained in the obtained plasma membrane fraction under our experimental conditions. Therefore, our devising fractionation is applicable in preparing plasma membrane fractions from cultured cells.

To apply this method for the detection of translocated GLUT4 on plasma membrane from the endoplasmic reticulum, 100 or 1,000 nm of insulin was treated to 3T3-L1 adipocytes and L6 myotubes for 15 min and the plasma membrane fraction was prepared, separately (Fig. 2). As the results, the GLUT4 level in the plasma membrane fraction was increased by insulin in a dose-dependent manner, while that in the post-plasma membrane fraction was decreased inversely. The GLUT4 level in the cell lysate remained unchanged. Since these results are consistent with the action of insulin on the glucose transport system,7) it was
confirmed that the fractionation method used here is appropriate to detect GLUT4 translocation from intracellular sites to the plasma membrane.

To apply this method in animal experiments, detection of GLUT4 in the subcellular fractions was performed in mice fed a 29% high-fat diet for 14 weeks (Fig. 3). The body weights of the mice in the high-fat diet group and the control group fed a standard diet were 44.3 ± 2.3 g and 29.7 ± 0.9 g, respectively, at the end of the feeding period. The weights of mesenteric adipose tissue were 1.58 ± 0.31 g and 0.29 ± 0.06 g, respectively, and those of epididymal adipose tissue were 1.53 ± 0.10 g and 0.51 ± 0.02 g, respectively. Moreover, plasma glucose levels were 292 ± 57 mg/dl and 99 ± 12 mg/dl, respectively, and plasma insulin levels were 3,140 ± 810 pg/ml and 872 ± 263 pg/ml, respectively. These results indicate that the high-fat diet caused obesity accompanied by hyperglycemia and hyperinsulinism. In the adipose tissue and muscle of mice, the high-fat diet decreased GLUT4 levels in all the fractions as compared with the standard diet. These results indicate that the constitutive expression levels of GLUT4 in adipose tissue and muscle were downregulated by feeding of the high-fat diet, resulting in a reduction in the GLUT4 levels in both the plasma membrane and the post-plasma membrane fractions. In previous studies, it was reported that long-term intake of a high-fat diet led to decrease in the GLUT4 of adipose tissue and muscle.8–10) Our results are in agreement with these previous reports, indicating that this method is also applicable in animal experiments. It is noteworthy that

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**Fig. 2.** Insulin-Stimulated GLUT4 Translocation to the Plasma Membrane in 3T3-L1 Adipocytes and L6 Myotubes.

Differentiated 3T3-L1 adipocytes and L6 myotubes on 35-mm dish were serum-starved in appropriate media containing 0.25% (w/v) bovine serum albumin for 18 h. The cells were treated with 100 or 1,000 nM insulin, or with 1 μl/ml 10% (v/v) acetic acid alone as a vehicle control for 15 min. The plasma membrane and post-plasma membrane fractions and cell lysate were prepared from these cells. GLUT4 was detected by Western blotting using anti-GLUT4 antibody goat IgG at 1:1,000 (Santa Cruz Biotechnology) and anti-goat IgG antibody at 1:30,000 (Santa Cruz Biotechnology). Typical results are shown from experiments at least in triplicate.

**Fig. 3.** Downregulation of GLUT4 and IRβ in Adipose Tissue and Muscle of Mice Fed the High-Fat Diet.

Mice were fed a standard or high-fat diet for 14 weeks, and levels of GLUT4 and IRβ were detected in the plasma membrane and post-plasma membrane fractions and cell lysate from adipose tissue and muscle of mice by Western blotting using the same antibodies as described in Figs. 1 and 2. Each panel shows the results for three different animals.
intake of the high-fat diet also decreased the IRβ levels in the plasma membrane fraction and cell lysate from adipose tissue and muscle. IRβ plays an important role in the insulin-signalling pathway, and its downregulation leads to insulin resistance, but there is no report demonstrating downregulation of IRβ by a high-fat diet, and this is, to our knowledge, the first report elucidating this issue. In conclusion, our method is a convenient and rapid preparation method for the plasma membrane fraction, and it is applicable in the detection of GLUT4 translocation in cultured cells and experimental animals.

References