Insulin Action on the Internalization of the GLUT4 Glucose Transporter in Isolated Rat Adipocytes*

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A novel method was developed to measure relative amounts of the GLUT4 glucose transporter on the surface of intact fat cells and to monitor the action of insulin on cell surface glucose transporters as they internalize into intracellular membranes. The method takes advantage of two predicted trypsin cleavage sites in the major exofacial loop of this transporter protein. Treatment of cyanide-poisoned rat adipocytes with 1 mg/ml trypsin at 37 °C for 30 min produced an immunoactive GLUT4 protein species in subsequently isolated plasma membranes that migrated with higher mobility (apparent $M_r$ = 35,000) than native GLUT4 (apparent $M_r$ = 46,000) on SDS-polyacrylamide gel electrophoresis. This proteolyzed GLUT4 protein was absent in the intracellular low density microsomes. Insulin treatment of adipocytes for 20 min prior to sequential additions of cyanide and trypsin caused a 16-fold increase in the proteolytically cleaved GLUT4 species. Incubation of fresh fat cells with trypsin caused a rapid and progressive appearance of the proteolyzed GLUT4 species in the intracellular low density membranes as well as plasma membranes. After 5 min of trypsinization, 66% of the total cleaved GLUT4 in these cells had moved into the low density membranes. Insulin treatment markedly decreased the internalized cleaved GLUT4 to 20% of the total. These data indicate the following: 1) trypsinization of the GLUT4 transporter protein on intact fat cells is a convenient means to monitor the extent of transporter recruitment to the plasma membrane by insulin, as well as to estimate GLUT4 internalization rates; and 2) the action of insulin on glucose transporter redistribution to the cell surface is associated with a marked inhibition of the fraction of cell surface GLUT4 transporters internalized per unit time.

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Insulin exerts a rapid action on muscle and fat cells that increases glucose uptake by at least an order of magnitude (for reviews, see Refs. 1-4). These tissues express erythrocyte-type (GLUT1) and muscle/adipocyte type (GLUT4) glucose transporter isoforms, which catalyze facilitative diffusion of hexoses across the plasma membrane. GLUT4 appears to be the major transporter that mediates the insulin response (5-10). Thus, the increased responsiveness of hexose transport to insulin action during differentiation of precursor fibroblast-like cells to mature muscle or fat cells correlates with the de novo expression of GLUT4 protein in the latter. Expression of the GLUT4 protein is restricted to those insulin responsive cells and is present at much higher levels than GLUT1 (5-10). Furthermore, overexpression of the human GLUT1 protein in cultured mouse 3T3-L1 adipocytes elevates basal glucose transport activity but fails to influence the increment in glucose uptake caused by insulin action (11).

A major mechanism involved in the action of insulin on glucose transport is an acute redistribution of transporter proteins from intracellular stores to the plasma membrane where they can catalyze sugar uptake (12-14). This effect has been observed by quantifying GLUT1 and GLUT4 protein content in isolated plasma membranes derived from control versus insulin-treated cells (10, 11, 15), and by immuno-electron microscopy of intact adipocytes using specific anti-GLUT1 and anti-GLUT4 antibodies (16-18). Increases in plasma membrane content of GLUT4 due to insulin action usually range from 2- to 5-fold using the former technique, whereas 13-40-fold increases have been obtained with the latter. This membrane redistribution of GLUT4 in response to insulin could be due to its increased exocytosis, decreased endocytosis, or both. In this study it is shown that trypsinization of intact fat cells causes proteolytic cleavage of cell surface GLUT4 near its N terminus, as predicted by the presence of two basic residues at positions 50 and 63 in a putative major exofacial loop (6-8). It is further demonstrated that during brief trypsinization, a markedly reduced fraction of these readily identified cell surface GLUT4 proteins internalize to intracellular membranes in insulin-treated cells.

MATERIALS AND METHODS

Cell Isolation—Adipocytes were isolated by collagenase (Boehringer Mannheim) digestion of epididymal fat pads from 150-200-g male Sprague-Dawley rats (Taconic Farms, Inc.) using Krebs-Ringer/ Hepes, pH 7.4, supplemented with 2% bovine serum albumin (Inter- gen) and 2 mM pyruvate (19). Cells were resuspended at a dilution of about 2.5 × 10⁶ cells/ml (1 ml of packed cells/3 ml of buffer), and equilibrated for 30 min at 37 °C prior to stimulation with insulin and digestion with trypsin. Insulin was generously provided by Dr. Ronald Chance, Eli Lilly Research Laboratories.

Metabolic Poisoning of Isolated Cells—In order to inhibit membrane recycling events, control or insulin-treated cells were incubated with the above buffer at 37 °C in the presence of 2 mM potassium cyanide for 20 min.

Trypsin Digestion of Isolated Cells—Following insulin treatment and metabolic poisoning where indicated, TPCK-treated trypsin (Sigma) was added to the isolated cells at a final concentration of 1 mg/ml and digestion proceeded for various times. At the end of the digestion period, soybean trypsin inhibitor (Sigma) was added to a final concentration of 2 mg/ml and the cells were quickly washed twice with Krebs-Ringer/Hepes/pyruvate buffer containing trypsin inhibitor and 2% albumin prior to homogenization and membrane preparation.

Preparation of Cellular Membrane Fractions—Plasma membranes

The abbreviations used are: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; ATB-BMPA, 2-N-4-(1-azido-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine.

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and low density microsomes were prepared by modification of previous methods (19). Cells were homogenized for 10 strokes at 22°C with a motor-driven Teflon/glass homogenizer in 24 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, trypsin inhibitor, and phenylmethylsulfonyl fluoride. The homogenate was brought to 0°C and centrifuged for 20 min at 16,000 × g. The resulting pellet containing the plasma membranes was resuspended in 6 ml of 10 mM Tris- HCl, pH 7.4, 1 mM EDTA (Buffer A) using 10 strokes of a Dounce homogenizer and layered onto a sucrose cushion (1.2 M sucrose in Buffer A). This homogenate was separated into pellet and supernatant by centrifugation at 100,000 × g for 1 h. Plasma membranes were removed from the top of the sucrose cushion and washed with 25 ml of Buffer A. The plasma membranes were recovered from the washing step by centrifugation at 29,000 × g for 30 min. The final plasma membrane pellet was resuspended at a final concentration of approximately 0.6–2 mg/ml. The 16,000 × g supernatant was centrifugated at 48,000 × g for 20 min to obtain a pellet of high density microsomes (not used in this study), and the resulting supernatant was centrifuged for 70 min at 200,000 × g to obtain a pellet of low density microsomes. The low density microsomes were resuspended at a final concentration of approximately 1–3 mg/ml.

**Electrophoresis and Immunoblotting**—Plasma membrane and low density microsomal membrane proteins were solubilized in sample buffer for 30 min at room temperature, resolved by SDS-PAGE using 10% polyacrylamide gels as described by Laemmli (20), and transferred to nitrocellulose at 200 mA for 2 h. The nitrocellulose filters were blocked with solution containing 0.5% gelatin, 0.05% bovine serum albumin, 0.05% Tween 20, 250 mM NaCl, and 10 mM Tris-HCl, pH 7.5. The blocked filters were incubated in rabbit anti-GLUT4 C-terminal antiserum (R1288, 1:1000 dilution) overnight at 4°C. The filters were washed extensively with 10 mM Tris, pH 7.5, 0.05% gelatin, 250 mM NaCl, and 0.05% Tween 20, and bound antibody was detected by incubation of the filters in 125I-protein A (Du Pont-New England Nuclear, approximately 8.3 μCi/μg; 1500 dilution) for 1 h at room temperature. Following extensive washes, the filters were subjected to autoradiography using Kodak XAR film and intensifying screens. The relative intensities of the bands on the immunoblots were determined using an LKB laser scanning densitometer.

**RESULTS AND DISCUSSION**

A major technical problem in biochemical determinations of cell surface glucose transporter content has been the suspected contamination of isolated plasma membrane preparations used for immunoblotting. We therefore sought a method independent of membrane fractionation, and tested whether trypsinization of intact cells might selectively cleave surface GLUT4 proteins that could then be identified after SDS-PAGE. Isolated rat adipocytes were incubated with 100 nM insulin for 20 min, followed by 2 mM cyanide for 20 min to inhibit membrane recycling, and then treated with 1 mg/ml trypsin for 15, 30, or 45 min prior to homogenization and preparation of plasma membranes and intracellular low density microsomes. As shown in Fig. 1, appearance of a lower M protein, GLUT4 species in the plasma membrane protein is trypsin-dependent and rises in amount during the time course of cell proteolysis. Importantly, virtually no proteolytically cleaved GLUT4 is detectable in the low density microsomes in spite of their higher content of native GLUT4.

In order to test in greater detail whether tryptic cleavage of GLUT4 in intact fat cells was restricted to cell surface transporter proteins, the consequence of transporter recruitment in response to insulin was compared to trypsinization of untreated cells. Fig. 2 depicts immunoblots of plasma membranes isolated from control or insulin-treated rat adipocytes poisoned with cyanide and then trypsinized for 30 min. Only a faint lower M GLUT4 species was observed in plasma membranes from control cells that had been trypsinized, whereas insulin action caused the expected production of proteolized GLUT4 (about 20% of native GLUT4). It is evident that the proteolysis of cell surface GLUT4 proteins is not complete under these reported conditions because native GLUT4 content is still elevated in plasma membranes from cells treated with insulin plus trypsin versus trypsin alone (Fig. 2, compare lanes 6 and 8). It is not clear whether heterogeneity of glucose transporters on the cell surface contributes to the incomplete digestion or whether the reaction is simply not complete by 45 min, because longer treatment causes significant loss of cell viability. Again, very little signal for cleaved GLUT4 was observed in low density microsomes. Quantitative densitometry of plasma membrane immunoblots, with appropriate subtraction of a minor con-

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**Fig. 1.** Time course of proteolytic cleavage of cell surface GLUT4 in insulin-stimulated rat adipocytes treated with trypsin. Rat adipocytes (approximately 4 × 10⁶ cells in 16 ml) were incubated in the presence of 100 nM insulin for 20 min at 37°C. The cells were poisoned with 2 mM KCN for 20 min at 37°C. TPKC-treated trypsin was added at a final concentration of 1 mg/ml for the indicated times at 37°C. Soybean trypsin inhibitor (2 mg/ml final concentration) was added, and the cells were washed twice with Krebs-Ringer/Hepes/pyruvate containing 2% albumin and homogenized in buffered sucrose. Membrane fractions were prepared by differential centrifugation as described under "Materials and Methods." Membrane proteins (60 μg) were resolved by SDS-PAGE and transferred to nitrocellulose filters. The nitrocellulose was incubated with anti-GLUT4 C-terminal peptide antibody (1:1000), and the bound antibody was detected with 125I-protein A. The relative intensities of the proteolyzed GLUT4 bands were determined by scanning laser densitometry. Lanes 1–4, low density microsome proteins; lanes 5–8, plasma membrane proteins.

**Fig. 2.** Proteolytic cleavage of cell surface GLUT4 in control and insulin-treated rat adipocytes by trypsin in cyanide-poisoned rat adipocytes. Rat adipocytes (3 × 10⁶ cells in 12 ml) were incubated in the presence or absence of 100 nM insulin for 20 min at 37°C. The cells were poisoned with 2 mM KCN for 20 min at 37°C. Where indicated, TPKC-treated trypsin was added at a final concentration of 1 mg/ml for 30 min at 37°C. Soybean trypsin inhibitor (2 mg/ml final concentration) was added, and the cells were washed twice with Krebs-Ringer/Hepes/pyruvate containing 2% albumin and trypsin inhibitor and homogenized in buffered sucrose. Membrane fractions were prepared by differential centrifugation as described under "Materials and Methods." Membrane proteins (45 μg/lane) were resolved by 10% SDS-PAGE and transferred to nitrocellulose filters. The nitrocellulose was incubated with anti-GLUT4 C-terminal peptide antibody (1:1000), and the bound antibody (1:1000) was detected with 125I-protein A. The relative intensities of the proteolyzed GLUT4 bands were determined by scanning laser densitometry.
taminating band in the \( M_r = 35,000 \) region, reveals a 16 \( \pm \) 2.5-fold (\( n = 3 \)) increase in plasma membrane-proteolyzed GLUT4 content mediated by insulin action.

The data in Figs. 1 and 2 provide compelling evidence that trypsinization of intact fat cells modifies only those GLUT4 proteins that are on the cell surface membranes. First, the decrease in apparent

The large effect of insulin to impair the localization of newly cleaved GLUT4 in low density microsomes is not observed upon prolonged (15 min) trypsinization (Table I). This could be due to recycling of the cleaved GLUT4 species back to the plasma membrane during this time period or to deleterious effects of prolonged trypsinization on cellular internalization pathways. It is also possible that trypsin itself is internalized over longer time periods and can act on GLUT4 present in the low density microsomes. Thus, short incubations with trypsin must be used in this assay to obtain values
Glucose Transporter Recycling

TABLE I

Effect of insulin on the movement of cleaved cell surface GLUT4 to intracellular membranes

Rat adipocytes (3 × 10⁶ cells in 12 ml) were incubated in the presence or absence of 100 nM insulin for 20 min at 37 °C. Where indicated, TPCK-treated trypsin was added at a final concentration of 1 mg/ml for 5 or 15 min at 37 °C. Soybean trypsin inhibitor (2 mg/ml final concentration) was added, and the cells were washed once with Krebs-Ringer/Hepes/pyruvate containing 2% albumin and trypsin inhibitor, washed once in buffered sucrose, and homogenized. Membrane fractions were prepared by differential centrifugation as described under "Materials and Methods." Membrane proteins (50 µg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose filters. The nitrocellulose was incubated with anti-GLUT4 C-terminal peptide antibody and the bound antibody was detected with 125I-protein A. The relative intensities of the proteolyzed GLUT4 bands were determined by scanning laser densitometry.

| Cell treatment | Trypsinization period | Relative amounts of cleaved GLUT4 in Low density microsomes | Clear GLUT4 in low density microsomes as percent of total |
|----------------|-----------------------|-----------------------------------------------------------|--------------------------------------------------------|
|                | min                   | arbitrary units                                           | %                                                      |
| None           | 5                     | 0.40                                                      | 20 ± 4.9*                                              |
| Insulin (100 nM) | 5                    | 0.20                                                      | 66 ± 5.0*                                              |
| None           | 15                    | 1.1                                                       | 55 ± 11*                                               |
| Insulin (100 nM) | 15                   | 2.2                                                       | 42 ± 6.4*                                               |

* Mean ± standard error of four experiments.
  ** Mean ± standard error of three experiments.

for GLUT4 internalization that correspond well to those observed with other methods (23, 24).

The data presented here indicate that the fraction of cell surface GLUT4 proteins that internalize per unit time is substantially higher in control cells compared to those exposed to insulin (Table I). However, Table I also shows that the absolute amounts of cleaved GLUT4 internalized in 5 min is similar in control versus insulin-treated cells. These values are probably good estimates of endocytosis rates because cleaved GLUT4 internalization is approximately linear for longer than 5 min (e.g. Fig. 3). Thus, the decreased fractional internalization of cleaved GLUT4 in response to insulin could be due to a direct effect of the hormone to inhibit the cellular process of endocytosis, an indirect consequence of increased GLUT4 proteins on the cell surface, or both. This latter possibility would result if a maximal capacity for GLUT4 endocytosis already existed in non-stimulated cells. Our results cannot as yet distinguish between these two possibilities. Some evidence suggests GLUT4 may be internalized in coated vesicles (17, 25). It is possible that the association of GLUT4 with coated pits is already near saturation under control conditions, such that increasing cell surface GLUT4 would have little additional effect on its rate of internalization. On the other hand, recent evidence suggests insulin actually decreases the amount of GLUT4 present in isolated coated vesicles from rat adipocytes (25), indicating a direct effect of insulin to inhibit GLUT4 endocytosis. Clearly, additional experiments will be required to resolve the underlying mechanism related to the findings presented here. However, the magnitude of the insulin-mediated decrease in the fractional GLUT4 internalization rate relative to cell surface transporters suggests this effect contributes significantly to the redistribution of this transporter to the cell surface membrane.

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