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Insulin-induced translocation of glucose transporters in rat hindlimb muscles

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Insulin causes a translocation of glucose transporters from intracellular microsomes to the plasma membrane in adipocytes. To determine whether insulin has a similar effect in rat hindlimb muscles, we used glucose-inhibitable cytochalasin B binding to estimate the number of glucose transporters in membrane fractions from insulinized and control muscles. Insulin treatment caused an approx. 2-fold increase in cytochalasin B-binding sites in a plasma membrane fraction and an approx. 70% decrease in cytochalasin B-binding sites in an intracellular membrane fraction. In order to detect this effect of insulin, it was necessary to develop a procedure for isolating a plasma membrane fraction and an intracellular membrane fraction that were not contaminated with sarcoplasmic reticulum. Our results show that, as in adipocytes, insulin stimulates translocation of glucose transporters from an intracellular membrane pool to the plasma membrane in hindlimb skeletal muscles.

Glucose transport; Insulin action; (Skeletal muscle)

1. INTRODUCTION

Studies on adipocytes have shown that insulin causes translocation of glucose transporters from an intracellular pool into the plasma membrane [1–5]. This process is thought to play a major role in the stimulation of glucose transport by insulin [1–5]. Skeletal muscle is the major site of insulin-mediated glucose transport accounting for ≈90% of insulin-stimulated glucose removal from the blood [6], while adipose tissue probably accounts for less than 5% of insulin-mediated glucose uptake [7,8]. The question of whether or not insulin also causes a translocation of glucose transporters from an intracellular pool into the plasma membrane in skeletal muscle is therefore of great interest. However, in contrast to the wealth of information regarding the effect of insulin on the distribution of glucose transporters in adipocytes, there is a paucity of similar data on skeletal muscle.

This lack of information is due to the technical difficulty in obtaining adequate separation of a plasma membrane fraction containing glucose transporters from an intracellular pool of 'microsome' bound glucose transporters, largely because of heavy contamination with other membrane fractions, particularly the sarcoplasmic reticulum. To our knowledge, Wardzala and Jeanrenaud [9,10], who studied rat diaphragms incubated with insulin, are the only investigators to report that insulin causes translocation of glucose transporters from an intracellular pool to the plasma membrane in a skeletal muscle. That no report of a similar finding by other investigators has been published in the 6 years since their first paper [9] attests to the technical difficulties involved.

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We tried to apply the methods of Wardzala and Jeanrenaud to rat hindlimb muscles, but despite much effort were unable to separate adequately the glucose transporter containing plasma membrane and microsomal fractions. We have, therefore, developed another procedure for isolating these fractions from sufficiently large quantities of skeletal muscle to allow cytochalasin B-binding studies over a range of concentrations. Using this procedure, we were able to show that perfusing rat hindlimb muscle with a high concentration of insulin results in an increase in D-glucose-protectable cytochalasin B-binding sites in the plasma membrane with a concomitant decrease in cytochalasin B-binding sites in an intracellular membrane fraction.

2. MATERIALS AND METHODS

2.1. *Reagents*

Cytochalasin B, cytochalasin E, D- and L-glucose, and gradient-grade sucrose were obtained from Sigma. Porcine insulin (Regular Iletin) was obtained from Eli Lilly. [^{3}H]Cytochalasin B was from Amersham.

2.2. *Animals and perfused hindlimb preparation*

Male specific pathogen-free Sprague-Dawley rats weighing 250–350 g were obtained from Sasco (Omaha, NE) and maintained on a diet of Purina chow and water. After an overnight fast, the rats were anesthetized with sodium pentobarbital (50 mg/kg body wt). Surgical preparation of the hindquarter, with placement of catheters in the abdominal aorta and inferior vena cava, was performed as described by Ruderman et al. [11]. The perfusion apparatus and procedure have been described previously [12]. The perfusion medium consisted of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 4 g/100 ml of bovine serum albumin, 8 mM glucose, and 0 or 1000 μU/ml insulin. The perfusion medium was oxygenated [12], and both the perfusion medium and the rat hindquarter preparation were maintained at 37°C. The hindlimbs were initially washed out with 50 ml of perfusion medium. A 20 min long flow-through perfusion at a flow rate of 20 ml/min was then begun; arterial and venous samples of perfusate were taken after 10 and 20 min. The arteriovenous glucose concentration differences and perfusate flow rate were used to calculate the rate of glucose uptake. Glucose concentration was measured using a YSI model 23A glucose analyzer (Yellow Springs Instrument). Immediately after perfusion, the muscles of both hindlimbs were dissected out, trimmed of fat and connective tissue while kept on ice, and then frozen in liquid N_2. Muscles were kept at −70°C until used for preparation of membranes.

2.3. *Membrane preparation*

Membrane isolation was carried out by a modification of three previously described procedures for the isolation of skeletal [13] and smooth [14] muscle membranes. The final protocol is illustrated in detail in fig.1. Essential characteristics of this purification procedure are the use of a low setting during the Polytron homogenization, a large dilution factor and the inclusion of NaN_3 in the buffer solution (to prevent both bacterial growth and redistribution of glucose transporters, in a manner akin to KCN [15]). The final step in the purification consisted of a 16 h centrifugation on discontinuous sucrose gradients (25, 30 and 35% sucrose, w/w). Membranes were collected from each sucrose layer, washed by 10-fold dilution in 10 mM $NaHCO_3$/0.25 M sucrose, 5 mM NaN_3 and recovered by high-speed centrifugation. Samples were immediately assayed for cytochalasin B binding. They were then stored at −20°C and used within a week for subsequent enzyme marker analysis. 5′-Nucleotidase activity was measured as in [16]. Sarcoplasmic reticulum Ca^{2+}-dependent ATPase and sarcolemmal Mg^{2+}-dependent ATPase activities were assayed according to Hidalgo et al. [17]. Phosphodiesterase I activity was measured as described [13].

D-Glucose-protectable binding of [^{3}H]cytochalasin B was measured at equilibrium with the indicated concentrations of cytochalasin B in the presence of 5 μM cytochalasin E and 200 mM of either D- or L-glucose as described previously [13,18,19]. The membranes were separated from the incubation medium by rapid (2 s) filtration through GF/B Whatman filters as reported in [13,18,19] or by ultracentrifugation [1,19] as stated. Binding at each concentration of [^{3}H]cytochalasin B was tested in triplicate in the presence of D- or L-glucose. D-Glucose-protectable binding of cytochalasin B is recognized as a confident

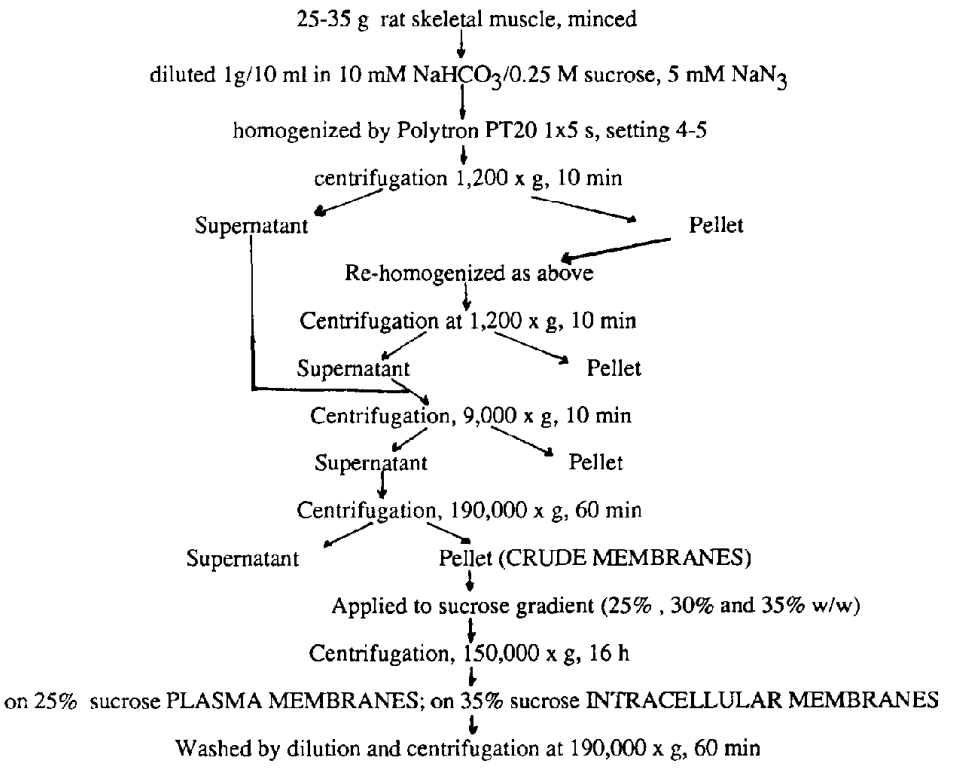

Fig.1. Schematic representation of the isolation procedure for plasma membranes and intracellular membranes from the rat hindquarter muscles.

measurement for the estimation of glucose transporters [20].

2.4. *Statistics*

Student's *t*-test for unpaired observations was used to evaluate the significance of differences between muscles treated with insulin and control muscles not treated with insulin.

3. RESULTS AND DISCUSSION

3.1. *Effect of insulin on glucose uptake by rat hindlimb*

Perfusion of rat hindquarter preparations with medium containing 1000 μU/ml of insulin resulted in an approx. 5-fold increase in the rate of glucose uptake. The rate of glucose uptake with no insulin in the perfusion medium averaged 33.3 ± 3.4 μmol glucose/10 min per hindquarter, compared to 175 ± 16 μmol/10 min per hindquarter when the perfusate contained insulin ($P < 0.001$; values are means ± SE for 15 rat hindquarter preparations per group). This approx. 5-fold increase in glucose uptake is close to the maximal insulin response attainable in the perfused rat hindquarter preparation [21]. The control and insulinized muscles from the perfused hindlimbs were used for isolation of membranes.

3.2. *Membrane isolation and characterization*

A crude membrane preparation was isolated from 25–35-g aliquots of control and insulinized rat hindlimb muscles according to the scheme outlined in fig.1. Separation of the muscle membrane fractions was performed using a discontinuous sucrose density gradient. On the basis of preliminary findings, the fractions floating on 25 and 35% sucrose were used in the present study. As shown in table 1, the 25% sucrose fraction was enriched in the plasma membrane marker enzymes 5′-nucleotidase and Mg^{2+}-ATPase relative to the crude membrane fraction. In contrast, the 35% sucrose fraction was relatively poor in 5′-nucleotidase and Mg^{2+}-ATPase activities. In

Table 1

Enzyme activities of membranes from muscles of rat hindquarter muscles

Fraction	Protein, mg/fraction (mg/g tissue)		5′-Nucleotidase (nmol/min per mg protein)		Mg^{2+}-ATPase (μmol/min per mg protein)		Ca^{2+}-ATPase (μmol/min per mg protein)	
	Control	Insulin	Control	Insulin	Control	Insulin	Control	Insulin
Crude membranes	54 ± 6 (1.69)	63 ± 7 (1.88)	81 ± 9	64 ± 7	1.0 ± 0.2	0.9 ± 0.1	0.35 ± 0.11	0.45 ± 0.19
25% sucrose	1.9 ± 0.3 (0.06)	2.1 ± 0.2 (0.06)	305 ± 25	245 ± 19	3.2 ± 0.4	2.7 ± 0.5	non-detectable	non-detectable
35% sucrose	10.8 ± 2 (0.34)	11.1 ± 2 (0.33)	74 ± 10	76 ± 11	1.2 ± 0.2	1.2 ± 0.2	non-detectable	non-detectable

Results are means ± SE of 5 independent membrane preparations, each performed with 25–35 g of tissue isolated from control and insulin-treated rats. The separate contributions of the Mg^{2+}-ATPase and Ca^{2+}-ATPase were assessed in parallel determinations containing Mg^{2+}, in the presence and absence of Ca^{2+} (the latter in the presence of EGTA). Under the conditions of this assay, the Mg^{2+}-ATPase corresponds to a surface membrane ATPase [17,18], and the Ca^{2+}-ATPase corresponds to the sarcoplasmic reticulum ATPase, as shown in [17]

some preparations the activity of a third plasma membrane marker enzyme, phosphodiesterase I, was also tested. The results indicated a 6-fold enrichment in this activity in the 25% sucrose fraction relative to the crude membranes, and a relative depletion in the 35% sucrose fraction (not shown).

Sarcoplasmic reticulum Ca^{2+}-ATPase activity was not detectable in either fraction. (The sarcoplasmic Ca^{2+}-ATPase activity was largely recovered in the pellet of the gradient.) This separation of sarcoplasmic reticulum from the membrane fractions under study is important, because in preliminary studies in which muscle was homogenized more vigorously (15 s at a Polytron setting of 6) there was abundant Ca^{2+}-ATPase activity in the 35% sucrose fraction, and the effect of insulin on D-glucose-inhibitable cytochalasin B-binding sites (see below) was obscured. The key to prevention of contamination by sarcoplasmic reticulum appears to be the short duration and relatively low intensity of the Polytron homogenization in a large volume.

The data in table 1 suggest that the 25% sucrose fraction is enriched in plasma membranes, while the 35% fraction is depleted of plasma membranes. Furthermore, the 35% sucrose fraction is not sarcoplasmic reticulum; instead, this fraction may represent an intracellular organelle in skeletal muscle that, as in adipocytes, contains glucose transporters (see below). At present, there does not appear to be a specific marker, other than an insulin-induced decrease in cytochalasin B binding, for the intracellular membranes endowed with glucose transporters [5].

Interestingly, insulin treatment caused a small but consistent decrease in 5′-nucleotidase activity in the 25% sucrose membrane fraction, with no change in the 35% sucrose fraction (table 1). The biological significance of this finding remains to be determined. Neither the Mg^{2+}-ATPase activity nor the protein yield was influenced by the insulin treatment. Freezing the muscles did not alter the distribution of marker enzymes or cytochalasin B-binding sites, as judged by parallel assays in membranes prepared simultaneously from fresh and frozen muscles.

3.3. *Cytochalasin B binding*

Membrane fractions for cytochalasin B-binding studies were prepared from insulinized and control rat hindlimb muscles in five separate experiments. D-Glucose-protectable cytochalasin B binding to the 25 and 35% sucrose fractions was measured at three cytochalasin B concentrations. Fig.2A shows the effect of perfusion of the muscles with a high concentration of insulin on the distribution of D-glucose-inhibitable cytochalasin B-binding sites in

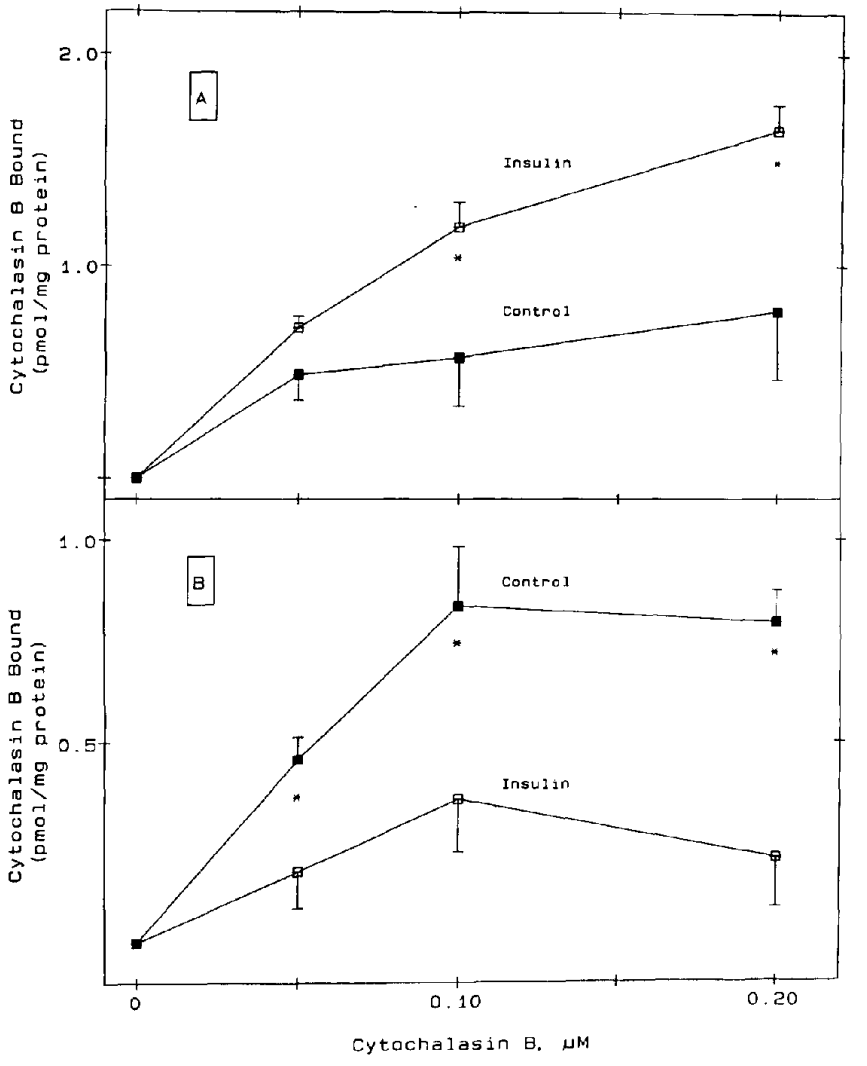

Fig.2. Effect of treatment of rat skeletal muscle with insulin on cytochalasin B binding to a plasma membrane fraction (A) and to an intracellular membrane fraction (B). The hindlimbs were perfused with (□) or without (■) insulin for 30 min, and the hindlimb muscles were used for preparation of the two membrane fractions (see sections 2,3 for details). Points are the means ± SE for 5 muscle preparations. * Insulin treated vs control, $P < 0.05$.

the membrane fractions. The 25% sucrose fraction from the insulin-treated muscles showed a significant increase in D-glucose-inhibitable cytochalasin B-binding sites per mg protein compared to the same fraction from control muscles. Since the 25% sucrose fraction is enriched in plasma membranes, as reflected in the activity of the marker enzymes 5'-nucleotidase, phosphodiesterase I and Mg^{2+}-ATPase, this finding suggests that, as in adipocytes [5], insulin causes an increase in the number of glucose transporters in the plasma membrane in skeletal muscle. In contrast, treatment of skeletal muscle with insulin resulted in a significant decrease in D-glucose-inhibitable cytochalasin B binding to the membranes in the 35% sucrose fraction (presumably an intracellular membrane fraction) (fig.2B).

Cytochalasin B binding was low but measurable in the crude membrane fraction. In contrast to the change in cytochalasin B binding in the 25 and

Table 2

Specific activity and recovery of cytochalasin B binding to muscle membranes

Fraction	Specific activity (pmol bound/mg protein)		Recovery (pmol bound/fraction)	
	Control	Insulin	Control	Insulin
25% sucrose	0.79 ± 0.36	1.63 ± 0.16	1.63 ± 0.81	3.34 ± 0.34
35% sucrose	0.80 ± 0.09	0.22 ± 0.15	8.38 ± 1.38	2.47 ± 1.67

Data from cytochalasin B binding at 0.2 μM [^{3}H]cytochalasin B. Results are means ± SE of 5 independent preparations

35% sucrose fractions caused by the insulin treatment, the crude membranes did not display a change in cytochalasin B binding (not shown).

In two membrane preparations, cytochalasin B binding was also measured by the equilibrium centrifugation method. The 25% sucrose fractions from control muscles gave a binding of 1.26 pmol/mg protein, compared to 3.11 pmol/mg in the corresponding fractions from insulin-treated muscles. The 35% sucrose fraction from control muscles gave a binding of 1.72 pmol/mg, but only 0.89 pmol/mg protein in the corresponding fraction from insulin-treated muscles.

Table 2 shows the D-glucose-inhibitable cytochalasin B-binding data obtained at 0.2 μM cytochalasin B (using the ultrafiltration method). The results are expressed both per mg protein and as the total amount of D-glucose-protectable cytochalasin B binding recovered in each fraction. When expressed as binding per mg membrane protein, there was a 2-fold increase ($P < 0.05$) in cytochalasin B binding in the plasma membrane enriched, 25% sucrose fraction from the muscles treated with insulin relative to controls. In the 35% sucrose fraction, presumably containing an intracellular membrane fraction from muscles perfused with insulin, there was an approx. 70% decrease ($P < 0.01$) in cytochalasin B binding (per mg protein) relative to the corresponding control fraction. When expressed as the total cytochalasin B binding recovered in each fraction, there was also a 2-fold increase ($P < 0.05$) in binding in the 25% sucrose fraction from insulin-treated muscles vs that from control muscles. Conversely, there was a 70% decrease ($P < 0.01$) in total cytochalasin B binding in the 35% sucrose fraction from insulin-treated muscles relative to control muscles.

The abundance and variety of membranous organelles in skeletal muscles make it very difficult to isolate a plasma membrane fraction and an intracellular glucose transporter-containing microsomal fraction that are not heavily contaminated with other membrane fragments, particularly sarcoplasmic reticulum. As a consequence, the present results depend markedly on the procedure followed to isolate the membrane fractions. Insulinized and control muscles showed comparable distributions and recoveries of marker enzymes for plasma membrane; indeed, if anything, in the plasma membrane fraction from insulin-treated muscle there was a decrease of 5′-nucleotidase activity, in contrast to the increase in cytochalasin B binding (tables 1,2). Hence, when viewed in the context of the information available on adipocytes [1–5], our results show that insulin stimulates the translocation of glucose transporters from an intracellular membranous organelle to the plasma membrane in skeletal muscle. This may underlie at least in part the concomitant stimulation by insulin of glucose uptake into rat hindquarter muscles.

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