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Retention of Insulin-Stimulated D-Glucose Transport Activity by Adipocyte Plasma Membranes Following Extraction of Extrinsic Proteins

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Plasma membrane vesicles prepared from adipocytes incubated with insulin exhibited accelerated D-glucose transport activity characteristic of insulin action on intact fat cells. Both control and insulin-stimulated D-glucose transport activities were inhibited by cytochalasin B and thiol reagents. Extraction of plasma membranes with dimethylmaleic anhydride eluted 80% of the protein from plasma membrane vesicles. The two major glycoprotein bands (94,000 and 78,000 daltons) and small amounts of a 56,000-dalton band were retained in dodecyl sulfate gels of the extracted membranes. Both control and insulin-activated D-glucose transport activities were retained by plasma membrane vesicles extracted with dimethylmaleic anhydride. Cytochalasin B binding activity was also retained by extracted membrane vesicles and D-glucose uptake into extracted vesicles derived from untreated or insulin-treated fat cells was inhibited by cytochalasin B. These results suggest that the modification of the adipocyte hexose transport system elicited by insulin action is not altered by a major purification step which involves quantitative extraction of extrinsic membrane proteins.

Key words: dimethylmaleic anhydride, cytochalasin B

One well-known effect of insulin is the rapid stimulation of hexose transport in adipose and muscle tissue. Neither the effect of insulin nor the mechanism of sugar transport have been well defined at the molecular level. Numerous investigators have attempted to identify a discrete biochemical modification of the adipocyte plasma membrane in response to insulin [1–12]. The complexity of the adipocyte membrane and the presence of many substances which are involved in processes other than hexose transport make it

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extremely difficult to quantitate biochemical alterations which might occur as a consequence of insulin action. It has been well documented that plasma membrane vesicles derived from fat cells retain the stereospecific hexose transport activity found in whole cells [13, 14]. It has also been found that membrane preparations derived from insulin-treated fat cells have an accelerated rate of D-glucose uptake compared to vesicles derived from untreated adipocytes [15, 16]. It was the aim of this study to isolate plasma membrane vesicles from insulin-treated or untreated fat cells and to partially purify the D-glucose transport system of these preparations by extracting components not involved in transport.

Extensive research with red cell ghosts has revealed that NaI and dimethylmaleic anhydride can be used to remove extrinsic proteins from the membrane surface [17–19]. Brenner et al [20] have recently published a report which shows that dimethylmaleic anhydride and NaI can similarly be used to extract extrinsic proteins from the surface of human adipocyte plasma membrane vesicles and to partially purify the D-glucose transport system of that preparation. In addition, Shanahan and Czech [21] have recently reported that dimethylmaleic anhydride can be used to extract 80% of the protein from rat adipocyte plasma membrane vesicles without removing D-glucose transport activity. This communication will document that the stimulatory effect of insulin on D-glucose uptake in fat cells is retained by membrane vesicle preparations which undergo this rather extensive extraction procedure, which results in partial purification of the glucose transport system.

MATERIALS AND METHODS

Isolation of Fat Cells

White fat cells were obtained by enzymatic digestion of the parametrial adipose tissue of female rats (Charles River CD strain), weighing 180–230 g, fed laboratory chow ad libitum. Adipose tissue was pooled and minced, and 9 g was added to small plastic bottles containing 9 ml of 3% albumin solution in Krebs-Ringer phosphate buffer, pH 7.4, which also contained 9 mg of crude collagenase (Clostridium histolyticum, Worthington). The albumin buffer was prepared fresh daily and the pH was adjusted to 7.4 with NaOH after the addition of bovine serum albumin fraction V (Armour lot no. N 10101). After a 60 min incubation at 37°C, cells were filtered through one layer of nylon chiffon, washed twice with a warm buffer containing 1.5% albumin in Krebs-Ringer phosphate buffer, pH 7.4, and pooled.

Preparation of Plasma Membrane Vesicles

Cells were resuspended in a solution containing 3% albumin in Krebs-Ringer phosphate buffer, pH 7.4, and divided into two aliquots. Porcine insulin (10 m units/ml) was added to one aliquot and the cells were then placed in plastic bottles (10 ml per bottle) and incubated for 15 min at 37°C. When large quantities of adipocytes were used, the cells were divided into several small batches during the incubation with insulin and the subsequent homogenization. Preliminary experiments revealed that larger samples were less sensitive to insulin-activation of D-glucose transport. After low-speed centrifugation, the cells were resuspended in Krebs-Ringer phosphate buffer, pH 7.4, and homogenized with five strokes of a Teflon pestle in a tight-fitting glass-homogenizer at room temperature. The insulin-treated cells were homogenized in buffer which contained 10 m units/ml insulin. The homogenate was centrifuged at 8,500g for 10 min; the supernatant and the

fluffy white material on the surface of the pellet were then centrifuged for 35 min at 32,000g. The pellet was resuspended in 1–3 ml of Krebs-Ringer phosphate buffer and dispersed for 5 sec using a Brinkman Polytron apparatus. This relatively simple procedure yielded a preparation rich in plasma membrane vesicles, although electron microscopy revealed contamination with microsomes and mitochondria. Studies with purified plasma membrane vesicles, prepared on sucrose density gradients gave similar results. Since the rate of sugar diffusion into any contaminating intracellular compartments was accounted for with studies utilizing L-glucose, facilitated D-glucose uptake into membrane vesicles could be determined in these relatively crude preparations.

Extraction With Dimethylmaleic Anhydride

Extraction of plasma membranes was similar to the method of Steck and Yu [17]. Membranes (4–8 mg protein) from insulin-treated or untreated fat cells (40–60 g) were resuspended in 4 ml of buffer (10 mM Tris, 1 mM ethylenediaminetetracetic acid [EDTA], pH 7.4). An aliquot of 0.5 ml was saved and the remainder was added to 52.5 ml H_2O. Dimethylmaleic anhydride (112 mg) was added with constant stirring and the pH was maintained between 8.0 and 8.5 with the addition of 2 N NaOH. After acid evolution was ended, the solution was centrifuged for 35 min at 32,000g. The pellet was resuspended in an appropriate volume of Krebs-Ringer phosphate buffer, pH 7.4 (usually 1–2 ml) for uptake or binding studies.

Glucose Transport Assay

Plastic test tubes containing 100 μl of a suspension of plasma membrane vesicles (0.5–1.5 mg protein per milliliter) dispersed in Krebs-Ringer phosphate buffer, pH 7.4, were incubated at room temperature for at least 5 min with or without appropriate additions. Transport was initiated by the addition of 5 μl of D-(^{3}H)-glucose (2 μCi per tube, 0.2–1.0 mM). The tubes were shaken vigorously by hand at the start of incubation and at various times the uptake was stopped by the addition of 3 ml of ice-cold Krebs-Ringer phosphate buffer, pH 7.4. The samples were quickly poured onto Millipore HA filters (pore size 0.45 μ, diameter 25 mm) attached to a Doerr vacuum pump and washed twice with 3 ml ice-cold buffer. The filters were soaked in distilled water with three changes before use. The total time taken to filter and wash the membranes was less than 10 sec. After drying, the filters were immersed in 4 ml of liquid scintillation fluid containing 33% (v/v) Triton X-100 in toluene and 4 g/liter Omnifluor (New England Nuclear).

Net uptake of D-glucose is the total amount of D-glucose uptake minus the amount of glucose uptake caused by diffusion. This component could be determined either by using L-(^{3}H)glucose or by adding cytochalasin B (10 μg per tube) to the sample before addition of D-(^{3}H)glucose. All uptake determinations were performed in triplicate and the values reported are the means of several experiments.

(^{3}H)Cytochalasin B Binding

Binding studies were performed in a manner similar to that described for the D-glucose transport assays above. Routinely, 50 or 100 μl of membranes were preincubated at 4°C with or without 1 or 2 μl of cytochalasin B (10 mg/ml) for 5 min, and then 0.5 or 1 μl respectively of (^{3}H)cytochalsin B (0.1–0.65 μM) was added. After various times at 4°C, the assay was stopped by the addition of 3 ml ice-cold buffer, poured onto Millipore HA filters (pore size 0.45 μ, diameter 25 mm) and washed twice with 3 ml of cold buffer. The filters were dried and prepared for liquid scintillation spectrometry as described above.

High-affinity binding was taken as the amount of label bound which could be inhibited by the presence of 100 μm unlabeled cytochalasin B. Blanks were run containing buffer and (^{3}H)- cytochalasin B without membranes.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Gel electrophoresis of plasma membranes was performed as previously described [21]. Membranes were solubilized with 2% sodium dodecyl sulfate (SDS) and 4 M urea, briefly heated at 100°, and run on 5% polyacrylamide gels in the presence of mercaptoethanol. The reservoir buffer contained 0.1% sodium dodecyl sulfate, and electrophoresis was performed over night at a current of 4 mA per gel. The gels were stained for protein with Coomassie Blue stain and electrically destained in a Savant destainer.

Reagents

Cytochalasin B was obtained from Aldrich Chemical Co and radiolabeled chemicals from New England Nuclear. All other chemicals were obtained from Sigma Chemical Co.

RESULTS

The uptake of D- vs L-glucose by adipocyte plasma membrane vesicles is depicted in Figure 1. D-glucose uptake is more rapid than L-glucose uptake, which confirms that these vesicles retain stereospecific hexose transport activity [13, 14]. Cytochalasin B, a mold metabolite which inhibits D-glucose uptake into fat cells [22], also inhibits D-glucose uptake by these membrane vesicles. The entry of D-glucose in the presence of cytochalasin B is similar to the simple diffusion of L-glucose into these vesicles (Fig 1).

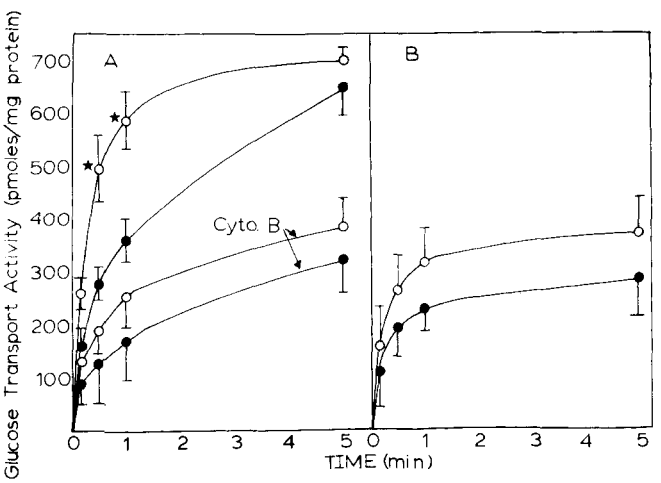

Fig 1. Uptake of D- vs L-glucose by membrane vesicles prepared from untreated or insulin-treated fat cells. Adipocytes were incubated with (○—○) or without (●—●) insulin (10 m units/ml) for 15 min at 37° C and membrane vesicles were prepared. Uptake of D-glucose (A) or L-glucose (B) was assayed at various times in Krebs-Ringer phosphate buffer, pH 7.4, at room temperature. Cytochalasin B (100 μM) when present was added to samples at least 15 min before uptake was measured. Data represent the mean values for three experiments ± standard error.
*) $p < 0.05$.

Vesicles prepared from insulin-treated adipocytes had a significantly accelerated initial rate of D-glucose uptake when compared to vesicles prepared from untreated adipose cells. The effects of insulin on D-glucose transport were most apparent during the first minute of hexose uptake. In the presence of cytochalasin B, D-glucose entry into insulin-activated vesicles was markedly reduced to a level comparable to L-glucose uptake; hexose diffusion into insulin-activated vesicles was slightly greater than entry into control vesicles but the difference was not significant. These results confirm the observations of Carter, Avruch, and Martin [15, 16] that enhanced D-glucose transport activity is found in membrane vesicles prepared from insulin-treated adipocytes. In addition, these findings demonstrate that cytochalasin B sensitivity is retained in both control and insulin-activated membrane vesicles.

Membrane vesicles prepared from both control and insulin-activated adipocytes were extracted with dimethylmaleic anhydride, and the densitometric scans of the electrophoretic patterns developed on dodecyl sulfate gels depicted in Figure 2 reveal that extraction causes the release of considerable amounts of membrane proteins. Two major

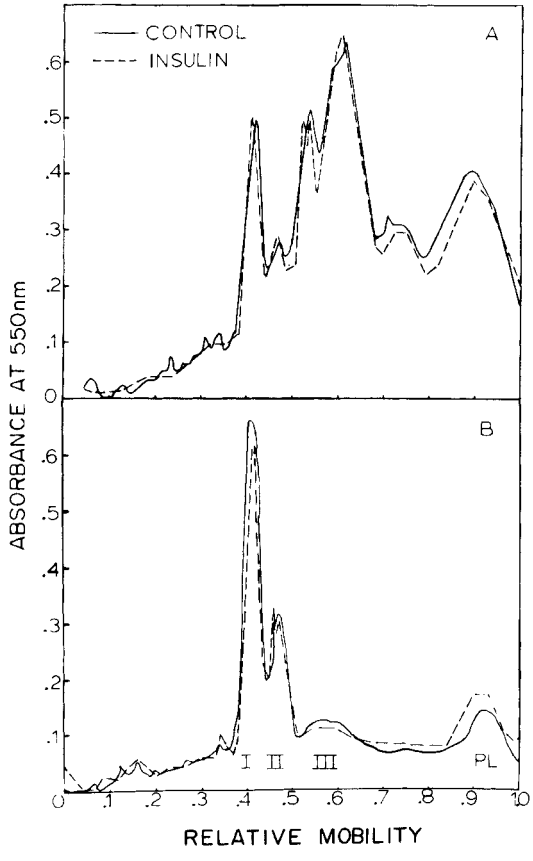

Fig 2. Densitometric scans of SDS polyacrylamide gels of plasma membrane vesicles derived from control and insulin-treated adipocytes before and after extraction with dimethylmaleic anhydride. Vesicles were prepared from fat cells as described in Figure 1 and gels were stained with Coomassie Blue. A) Whole plasma membrane vesicles; B) vesicles after extraction with dimethylmaleic anhydride. Bands I, II, and III refer to molecular weight species of 94,000, 78,000, and 56,000, respectively [21]. PL) Phospholipid region of the gels.

glycoprotein bands, corresponding to proteins with apparent molecular weights of 94,000 and 78,000 daltons, respectively, as well as a minor band of lower-molecular-weight material (56,000 daltons), are the only Coomassie Blue-staining components of the adipocyte membrane which are retained after treatment with dimethylmaleic anhydride. Extraction of 70–80% of the total membrane protein was routinely accomplished and numerous experiments have yielded similar electrophoretic patterns [21]. There was no difference between control and insulin-activated membrane vesicles in terms of their electrophoretic profile either before or after extraction (Fig 2). This observation suggests that the effect of insulin on the adipocyte D-glucose transport system does not involve a major change in the structure of membrane proteins and agrees with the result of Avruch, Carter, and Martin [16], using intact fat cell plasma membranes.

Vesicles from insulin-activated or untreated adipose cells were then tested for glucose uptake and (^{3}H)cytochalasin B binding activity before and after extraction with dimethylmaleic anhydride. The uptake of D-glucose was measured for 15 sec and results were compiled as pmoles D-glucose accumulated per milligram protein per 15 sec. These data were then converted to percentages with respect to transport activity in control plasma membranes to allow us to normalize the data from individual experiments. The data in Table I again demonstrate that before extraction of membranes, D-glucose uptake was accelerated in vesicles derived from insulin-activated adipocytes. Cytochalasin B inhibited D-glucose uptake to the level found for simple diffusion, while net uptake (the difference between D-glucose uptake in the presence and absence of cytochalasin B) was considerably greater in insulin-activated vesicles. After extraction with dimethylmaleic anhydride, the uptake of D-glucose per milligram protein was somewhat decreased in both the presence and absence of cytochalasin B, while the level of net transport did not change appreciably. The net uptake of D-glucose by protein-depleted vesicles prepared from insulin-activated fat cells was more rapid than uptake by vesicles from untreated cells, demonstrating that the effect

TABLE I. Effect of Insulin on D-Glucose Transport and (^{3}H)Cytochalasin B Binding Before and After Extraction of Rat Adipocyte Plasma Membranes with DMMA

Pretreatment	Extraction	% of control D-glucose transport rate[a]			% of control (^{3}H)cytochalasin B binding[b]
		Control	+Cytochalasin B	Net	
None	None	100 ± 5	65 ± 3	35	100 ± 5
Insulin	None	145 ± 12[c]	72 ± 5	73	100 ± 3
None	DMMA	65 ± 8	28 ± 4	37	114 ± 9
Insulin	DMMA	99 ± 5[c]	32 ± 3	67	96 ± 12

Plasma membrane vesicles were prepared from adipocytes treated with or without insulin before cellular disruption. An aliquot of each sample was then extracted with dimethylmaleic anhydride (DMMA) as described in the text. Vesicles were resuspended in Krebs-Ringer phosphate buffer and briefly sonicated before each assay. Cytochalasin B (100 μM) was added where indicated for 15 min before the assay. Data represent the mean of three experiments ± standard error, and the values for 100% transport activity ranged from 180 to 880 pmoles glucose accumulated per milligram protein per min for transport and from 3.8 to 9.7 pmoles (^{3}H)cytochalasin B bound per milligram protein for 100% binding activity.

[a]Transport was measured for 15 sec at room temperature.

[b]Binding was measured for 25 min at 4°C.

[c]$p < 0.01$ compared to sample without insulin treatment.

of insulin on hexose transport activity had not been reversed by treatment with dimethylmaleic anhydride. It is interesting to note that the specific activity of (^{3}H)cytochalasin B binding did not change after extraction of membranes with dimethylmaleic anhydride. A recent report by Wardzala, Cushman, and Lienhard [23] suggested that ghosts prepared from insulin-treated fat cells had a lower K_d for high-affinity cytochalasin B binding; we have not observed a consistent effect of insulin on cytochalasin B binding in these membrane vesicles, either before or after extraction with dimethylmaleic anhydride.

The observation that the specific activity of glucose transport and cytochalsin B binding did not increase during extraction of extrinsic proteins with dimethylmaleic anhydride in these experiments might be due to one of the following reasons: Dimethylmaleic anhydride may have selectively removed some carrier proteins from the vesicles or inactivated a portion of those carriers remaining in the membrane or both; alternatively, 20–30% of the vesicles in the dimethylmaleic anhydride-extracted preparation may be native membranes which have escaped extractions of membrane proteins and therefore retained glucose transport activity. The gel patterns presented in Figure 2 would argue against this latter possibility, since many other protein bands would be visible in the gels if the extraction were not complete. In this regard, we have elected to use rather high concentrations of dimethylmaleic anhydride to elicit this complete extraction of extrinsic proteins at the expense of partially inhibiting transport activity. It should also be noted that values for specific activity varied considerably among experiments and we have observed significant increases in specific activity for glucose transport and cytochalasin B binding in some experiments.

DISCUSSION

The mechanism by which insulin rapidly stimulates hexose transport activity is unknown at present (for recent review, see Czech [24]). One approach to this problem is the purification of hexose transport proteins from an insulin-sensitive cell type and the biochemical characterization of such proteins derived from untreated and insulin-treated cells. A key requisite in this strategy is that during the purification procedure the transport proteins must retain the biochemical modification which is elicited by insulin. The present study clearly demonstrates that this requisite is fulfilled for at least one major purification step, which involves elution of extrinsic membrane proteins. The retention of the insulin effect subsequent to extensive protein extraction with dimethylmaleic anhydride indicates a remarkable stability of whatever modification is caused by the hormone.

Several proposed mechanisms for insulin action [1–5, 8–11] are consistent with our findings, and covalent modification of membrane proteins may be responsible for the stability of the insulin effect on hexose transport. Czech and associates [9–11] have shown that sulfhydryl oxidizing agents can mimic the action of insulin on fat cell hexose transport and N-ethylmaleimide can stabilize the glucose transport activity of fat cells in either the basal or the activated state; these observations led to the hypothesis that the hexose transport protein(s) contain sensitive sulfhydryl residues which exist in a reduced, low-V_{max} conformation and an oxidized, high-V_{max} conformation.

Another form of covalent modification which has been implicated in insulin action on the sugar transport system is a phosphorylation-dephosphorylation cycle. Chang and Cuatrecasas [8] have found that adenosine triphosphate (ATP) inhibits insulin-activated hexose transport without interfering with basal transport activity in fat cells, while Randle

and Smith [25] suggested long ago that phosphorylation of the transport system may be associated with low activity and that dephosphorylation may trigger its activation. Benjamin and Singer [1] found that phosphorylation of a fat cell protein (140,000 daltons) was stimulated by addition of insulin, while phosphorylation of another protein (60,000 daltons) was stimulated by epinephrine. More recently, Benjamin and Singer [2] reported that dibutyryl cyclic AMP (adenosinemonophosphate) also increased phosphorylation of the 60,000-dalton protein, while insulin increased phosphorylation of a 50,000-dalton protein as well. Avruch and his colleagues [3–5] have also performed detailed analyses of the incorporation of ^{32}Pi into membrane proteins, and these investigatiors have implicated cyclic AMP-independent phosphorylation in insulin action, while confirming the role of cyclic AMP-dependent phosphorylation in the action of epinephrine.

Interestingly, the addition of insulin directly to plasma membrane vesicles [14], or to vesicles extracted with dimethylmaleic anhydride, does not accelerate D-glucose uptake (unpublished observations). It is clear that the intracellular components lost upon cellular disruption are not necessary to maintain basal or insulin-activated D-glucose uptake; if these components were directly involved in hexose transport, it is unlikely that the stereospecific sugar transport depicted in Figure 1 for membrane vesicles, or shown in Table I for extracted vesicles, would be present. Recent advances have been made toward the identification of both insulin receptors and hexose transport components on the fat cell plasma membrane, yet virtually nothing is known about the process which links these two systems. Thus, purification of the D-glucose transport system of the adipocyte may represent the most feasible method for identifying the molecular basis of insulin action, particularly if covalent modification of the protein is involved.

Steck and Yu [17] have shown that erythrocyte membrane glycoproteins with strong hydrophobic associations with the membrane were most resistant to extraction by dimethylmaleic anhydride. High-affinity cytochalasin B binding sites [18] and D-glucose transport activity [19] are known to be resistant to extraction with dimethylmaleic anhydride in red cell membranes. These findings confirm that the hexose transport system of both adipocytes and erythrocytes are associated with hydrophobic intrinsic proteins which can be partially purified by extraction of extrinsic proteins from the membrane surface. The material remaining after extraction of adipocyte membrane vesicles with dimethylmaleic anhydride does not necessarily consist solely of three homogeneous proteins. Isoelectric focusing of extracted membrane vesicles reveals considerable heterogeneity of Schiff reagent-staining material, while enzymatic analysis has revealed the presence of Na^+-K^+ and Mg^{++}-ATPase activities (unpublished observations). Nevertheless, the dimethylmaleic anhydride extraction procedure described herein does represent a dramatic purification step. The retention of insulin-activated transport activity provides substantial confidence that the insulin effect might survive the further steps necessary to complete the purification of this transport system.

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