Cycloheximide decreases glucose transporters in rat adipocyte plasma membranes without affecting insulin-stimulated glucose transport

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This study examines the relationship between insulin-stimulated glucose transport and insulin-induced translocation of glucose transporters in isolated rat adipocytes. Adipose cells were incubated with or without cycloheximide, a potent inhibitor of protein synthesis, for 60 min and then for an additional 30 min with or without insulin. After the incubation we measured 3-*O*-methylglucose transport in the adipose cells, and subcellular membrane fractions were prepared. The numbers of glucose transporters in the various membrane fractions were determined by the cytochalasin B binding assay. Basal and insulin-stimulated 3-*O*-methylglucose uptakes were not affected by cycloheximide. Furthermore, cycloheximide affected neither $V_{\max}$ nor K_m of insulin-stimulated 3-*O*-methylglucose transport. In contrast, the number of glucose transporters in plasma membranes derived from cells preincubated with cycloheximide and insulin was markedly decreased compared with those from cells incubated with insulin alone (10.5 ± 0.8 and 22.2 ± 1.8 pmol/mg of protein respectively; $P < 0.005$). The number of glucose transporters in cells incubated with cycloheximide alone was not significantly different compared with control cells. SDS/polyacrylamide-gel-electrophoretic analysis of [³H]cytochalasin-B-photolabelled plasma-membrane fractions revealed that cycloheximide decreases the amount of labelled glucose transporters in insulin-stimulated membranes. However, the apparent molecular mass of the protein was not changed by cycloheximide treatment. The effect of cycloheximide on the two-dimensional electrophoretic profile of the glucose transporter in insulin-stimulated low-density microsomal membranes revealed a decrease in the pI-6.4 glucose-transporter isoform, whereas the insulin-translocatable isoform (pI 5.6) was decreased. Thus the observed discrepancy between insulin-stimulated glucose transport and insulin-induced translocation of glucose transporters strongly suggests that a still unknown protein-synthesis-dependent mechanism is involved in insulin activation of glucose transport.

INTRODUCTION

One of the major actions of insulin is its ability to stimulate glucose uptake in muscle and adipose tissue [1,2]. The molecular mechanism of insulin-stimulated glucose transport remains unresolved, despite extensive work by different groups. Possible mechanisms to account for this insulin effect include changes in the subcellular distribution of glucose transporters and/or in the intrinsic activity of glucose transporters induced by insulin. Two groups have independently accumulated evidence in support of the hypothesis that insulin stimulates glucose transport by inducing a rapid and reversible translocation of glucose transporters from an intracellular membranous pool to the plasma membrane [3–5]. This insulin-induced translocation of glucose transporters occurs via an energy-dependent process [1,6], probably involving bivalent metal cations [7] and thiol groups [8,9]. However, during the last 2 years, evidence has been accumulated showing that translocation does not fully explain insulin's effects to stimulate glucose transport. For example, Whitesell & Abumrad [10] suggested that insulin stimulates glucose

transport by inducing changes in the affinity rather than in the number of glucose transporters. Another piece of evidence has been published by Joost *et al.* [11], who found that catecholamines inhibit glucose-transport activity through changes in the intrinsic activity of the glucose transporter rather than by altering its subcellular distribution.

The purpose of the present study was to investigate the relationship between insulin-stimulated glucose transport and insulin-induced translocation of glucose transporters in isolated rat adipocytes treated with or without cycloheximide. Furthermore, using a modified two-dimensional electrophoresis technique we studied the effect of cycloheximide on the glucose-transporter isoforms that we have recently identified in rat adipocytes [12]. The data indicate that insulin is still able to stimulate glucose transport fully in cells incubated with cycloheximide, although cycloheximide pretreatment decreases the number of glucose transporters by more than 50%. The observed discrepancy between insulin-stimulated glucose transport and insulin-induced translocation of glucose transporters strongly suggests that a still unknown mechanism, i.e. increased intrinsic activity

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of transporters, is involved in insulin activation of glucose transport.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (160–180 g) were obtained from Charles River Laboratories. Animals were fed on laboratory chow *ad libitum*. Pig monocomponent insulin was generously supplied by Dr. Ronald Chance of Eli Lilly Co. (Indianapolis, IN, U.S.A.). [³H]Cytochalasin B, D-3-O-methyl[U-¹⁴C]glucose and 2-deoxy-D-[³H]glucose were purchased from New England Nuclear, bovine serum albumin (fraction V) was from Armour Pharmaceuticals, collagenase was from Worthington, phloretin was from Spectrum Chemical Corp., and carrier ampholytes were from Pharmacia; other reagents for isoelectric focusing and electrophoresis were from Bio-Rad. All other chemicals were reagent grade and from standard sources.

Adipocyte isolation and incubation

Isolated adipocytes were prepared by a modification [13] of the method of Rodbell [14], in a Krebs-Ringer phosphate buffer containing 128 mM-NaCl, 5.2 mM-KCl, 1.2 mM-CaCl₂, 1.2 mM-MgSO₄, 1.29 mM-KH₂PO₄, 10 mM-Na₂HPO₄, pH 7.4, supplemented with collagenase (2 mg/ml), 4% (w/v) albumin and glucose (3 mM). After digestion, cells were filtered through a 202 µm nylon mesh and washed four times in the above Krebs-Ringer phosphate buffer with 4% albumin (pH 7.4). Isolated and washed rat adipocytes were then incubated under the following conditions: (1) basal, cells were incubated with no additions, in Krebs-Ringer phosphate for 90 min in polypropylene jars at 37 °C in a water bath with mild shaking; (2) insulin, cells were incubated for 60 min as under basal conditions, insulin (7 nM) was added and the cells were incubated for further 30 min; (3) basal + cycloheximide, cells were incubated with cycloheximide (10 µg/ml) for 90 min; and (4) insulin + cycloheximide, cells were incubated with cycloheximide for 90 min and insulin (7 nM) was added after 60 min.

Preparation of subcellular fractions from rat adipocytes

Plasma membrane and low-density microsomal membrane fractions ('microsomes') were prepared by the differential-centrifugation method described by McKeel & Jarett [15] and modified by Karnieli *et al.* [4]. All membrane pellets were resuspended in 'TES' buffer, pH 7.4, containing 20 mM-Tris/HCl, 1 mM-EDTA and 255 mM-sucrose. Leupeptin (2.5 µg/ml), pepstatin (2.5 µg/ml) and Aprotinin (2.5 µg/ml) were added to avoid proteolysis. To prepare the total membrane fraction, cell homogenates were centrifuged at 1000 *g*_{max} for 10 min at 0–1 °C. The post-nuclear supernatant was separated from the pellet and fat cake, and then centrifuged at 430 000 *g*_{max} for 90 min at 4 °C. The resulting pellet was resuspended in 'TES' buffer and comprised the total cellular membrane fraction.

Protein determination and marker-enzyme assays

In each membrane fraction protein was determined by the Coomassie Brilliant Blue method described by Bradford [16], with crystalline bovine serum as the standard (Bio-Rad protein assay). NaF-stimulated

adenylate cyclase activity was measured by the method of Salomon *et al.* [17]; rotenone-insensitive NADH-cytochrome *c* reductase was determined by the method of Dallner *et al.* [18].

Glucose transport

Glucose transport was assayed by measuring initial rates of 3-O-methylglucose influx. All data presented are measurements of zero-*trans* influx, which was assayed in fat-cells by a modification [13] of the method of Whitesell & Gliemann [19]. The portion of cellular uptake owing to diffusion and trapping of the label in the extracellular space was measured by performing parallel reactions with L-[1-¹⁴C]glucose as substrate. All results for transport are corrected for the L-glucose value.

Determination of glucose transporter number

The number of D-glucose-inhibitable cytochalasin B binding sites in the subcellular membrane fractions was determined as previously described [4]. In the presence of cytochalasin E (5 µM), cytochalasin B binding at each ligand concentration (0.025, 0.05, 0.1, 0.2, 0.4 µM) was measured with ~80 µg of membrane protein in the absence and the presence of 500 mM-D-glucose. The difference in binding was taken as the D-glucose-inhibitable component. The number of binding sites (*R*₀) and the affinity constant (*K*_d) were determined by Scatchard [20] analysis.

Photochemical cross-linking of membranes, and SDS/polyacrylamide-gel electrophoresis

Subcellular membrane fractions prepared from rat adipocytes were resuspended in 50 mM-sodium phosphate buffer (pH 7.4), to a final concentration of 1–5 mg of membrane protein/ml. Membranes were incubated with or without D-glucose (500 mM) for 30 min at 4 °C and then for an additional 10 min with [³H]cytochalasin B (10 µM). The membranes were then photochemically cross-linked by using a high-intensity u.v. lamp at 253 nm for 5 min.

Membranes covalently cross-linked to [³H]cytochalasin B were solubilized by boiling for 5 min in Laemmli [21] sample buffer and then analysed by SDS/polyacrylamide-gel electrophoresis as previously described [22]. Prestained molecular-mass marker proteins (Bethesda Research Laboratories) were used to estimate the molecular mass of the ³H-labelled membrane proteins. Proteins corresponding to a molecular mass of 40–55 kDa were excised from the gels and electrophoretically eluted as described by Hunkapiller *et al.* [23].

Isoelectric focusing

The extracted and concentrated SDS-solubilized glucose transporters were subjected to isoelectric focusing on cylindrical polyacrylamide gels. The gel composition was as follows: 4% acrylamide, 0.25% *NN'*-methylene-bisacrylamide, 2% Nonidet P-40, 8 M-urea, 2% carrier ampholytes pH 4–6.5 and 5–8 (Pharmacia), 0.11% *NNN'*-tetramethylethylenediamine and 0.02% ammonium persulphate. Isoelectric focusing was performed in 13 cm-long glass tubes (internal diam. 5 mm). The catholyte was 30 mM-NaOH and the anolyte was 10 mM-H₃PO₄. The samples were electrofocused at 1 mA constant current for 1 h and at 400 V for the remainder of the run.

Fig. 1. 3-*O*-Methylglucose uptake by basal, insulin-treated and cycloheximide-plus-insulin-pretreated rat adipocytes

Isolated rat adipocytes were incubated at 37 °C for a total time of 90 min; basal cells without any additions, 'insulin' cells with 7 nM-insulin for the last 30 min, and 'cycloheximide+insulin' cells with 10 µg of cycloheximide/ml for 60 min, followed by 30 min with 7 nM-insulin. After incubation, glucose transport was measured in the three groups with 3-*O*-[¹⁴C]methylglucose at a tracer concentration. Experiments were performed four times; data represent means ± S.E.M.

RESULTS

Effect of cycloheximide on 3-*O*-methylglucose transport

Fig. 1 illustrates the effects of cycloheximide treatment on insulin-stimulated glucose transport activity in isolated rat adipose cells. Insulin (7 nM) stimulates glucose transport 7.1 ± 0.4-fold over basal values, and cycloheximide pretreatment (10 µg/ml) does not affect insulin-stimulated glucose uptake (6.6 ± 0.5-fold over basal). Similar results were obtained with 2-deoxy-D-[³H]glucose as the glucose analogue (results not shown). In order to investigate whether cycloheximide affects the affinity of the glucose uptake system, we measured zero-*trans* rates of 3-*O*-methylglucose transport at various 3-*O*-methylglucose concentrations. Fig. 2 shows a Line-

Fig. 2. Lineweaver-Burk plot of initial rates of 3-*O*-methylglucose transport

Incubation conditions were as described in legend to Fig. 1. Zero-*trans* rates of influx were measured at various 3-*O*-methylglucose concentrations. Reaction times were varied, so that only initial rates were measured (30 % of equilibrium space). Results are the averages of three experiments, each done in quadruplicate. The unit of *v* is fmol/s per 40 µl of 40 % adipocyte cells.

weaver-Burk plot of initial rates of 3-*O*-methylglucose transport. Cycloheximide affected neither the maximal velocity (V_{max}) nor the affinity (K_m) of the 3-*O*-methylglucose uptake system.

Effect of cycloheximide on the subcellular distribution of glucose transporters

Subcellular membrane fractions from basal, insulin- and cycloheximide-treated insulin-stimulated rat adipose

Table 1. Effect of cycloheximide on membrane protein recovery and specific enzyme activities in subcellular membrane fractions from rat adipocytes

Results are averages of at least four individual experiments, ± S.E.M.

Membrane fraction	Treatment	Protein recovery (mg/preparation)	NaF-stimulated adenylate cyclase (pmol/min per mg)	Cytochrome <i>c</i> reductase (µM/min per mg)
Plasma membrane	Basal	1.96 ± 0.51	178 ± 9	0.45 ± 0.02
	Insulin	2.42 ± 0.50	213 ± 17	0.44 ± 0.03
	Cycloheximide	2.93 ± 0.68	201 ± 9	0.40 ± 0.03
Low-density microsomes	Basal	1.71 ± 0.71	4.1 ± 0.2	1.04 ± 0.04
	Insulin	1.70 ± 0.63	4.1 ± 0.3	1.06 ± 0.05
	Cycloheximide	2.20 ± 0.65	3.7 ± 0.3	1.12 ± 0.04

Fig. 3. Effect of cycloheximide on the subcellular distribution of glucose transporters in basal and insulin-stimulated rat adipocytes

Incubation conditions were as described in the legend to Fig. 1, except for an additional group of basal cells that were incubated with cycloheximide for 60 min. Transporter numbers were determined by D-glucose-inhibitable cytochalasin B binding as described in the Materials and methods section. Results are the averages $\pm$ S.E.M. of eight to ten experiments, each done in duplicate. The total numbers of glucose transporters (pmol/mg of protein) for all experimental conditions, with inter-group statistical analysis, are: BPM, 8.5 ± 1.5 ; BCPM, 8.2 ± 7 ; IPM, 22.8 ± 1.6 ; ICPM, 10.8 ± 1.7 ; BLDM, 39.5 ± 3.5 ; BCLDM, 35.2 ± 3.4 ; ILDM, 23.0 ± 2.6 ; ICLDM, 14.9 ± 2.5 ; BPM versus BCPM versus ICPM, not significant; IPM versus BPM, BCPM or ICPM, $P < 0.01$; ILDM versus ICLDM, $P < 0.05$. Abbreviations: B, basal; I, insulin; PM, plasma membrane; LDM, low-density microsomes; C, cycloheximide.

cells were prepared by differential ultracentrifugation [4]. Cycloheximide pretreatment (10 µg/ml) of adipocytes before subcellular-membrane fractionation did not affect membrane protein recovery or specific marker-enzyme activities in the subcellular-membrane fractions, as shown in Table 1. The number of glucose transporters in each of the membrane fractions was then determined by using the cytochalasin B binding assay. The results presented in Fig. 3 indicate that the number of glucose transporters is markedly decreased in plasma membranes from cells treated with cycloheximide followed by incubation with insulin for 30 min (10.5 ± 0.8 pmol/mg of protein), compared with the number of glucose transporters in plasma membranes from insulin-treated cells (22.2 ± 1.8 pmol/mg of protein, $P < 0.005$). Furthermore, cycloheximide did not change the concentration of glucose transporters in the absence of insulin in either plasma membranes (8.3 ± 1.5 pmol/mg of membrane protein under basal conditions versus 7.8 ± 0.6 pmol/mg of protein in cycloheximide-treated plasma membranes) or low-density microsomes, where we detected 39.2 ± 3.8 pmol of glucose transporter/mg of membrane protein in basal membranes, compared with 35.1 ± 3.7 pmol of glucose transporter/mg in cyclo-

Fig. 4. Effect of cycloheximide on glucose transporter numbers in total membrane fractions prepared from basal, insulin-treated and cycloheximide-plus-insulin-treated cells

Incubation conditions were as described in the legend to Fig. 1. After the incubation, adipocytes were homogenized and the post-nuclear supernatant was centrifuged at $430\,000\ g_{max}$ for 90 min as described in the Materials and methods section. Binding sites were assessed by the D-glucose-inhibitable cytochalasin-B-binding assay. Results are averages of 3 experiments, $\pm$ S.E.M.

heximide-pretreated low-density microsomes. However, in the insulin-stimulated low-density microsomes cycloheximide treatment caused a significant decrease in glucose transporters compared with control insulin-stimulated low-density membranes (22.4 ± 3.4 versus 14.6 ± 3.3 pmol/mg of protein, $P < 0.05$). The K_d for cytochalasin B binding in plasma membranes was not significantly different in cells incubated with or without cycloheximide: $K_d = 119 \pm 5.8$, 125 ± 8.9 , 128 ± 5.1 nM in basal, insulin-treated and cycloheximide-plus-insulin-treated membranes respectively.

Table 1 indicates that acute cycloheximide treatment did not influence membrane recovery or yield. However, to assess further the possibility that the effect of cycloheximide to decrease glucose transporter number in insulin-stimulated membranes was due to different recoveries of transporters during the subcellular fractionation scheme, we measured glucose transporter numbers in total membrane fractions derived from basal, insulin-stimulated and cycloheximide-plus-insulin-stimulated cells as shown in Fig. 4. Preincubation of cells with 10 µg of cycloheximide/ml before homogenization induces a 50% decrease in glucose transporter number in total membrane fractions (post-nuclear-supernatant membranes pelleted at $430\,000\ g_{max}$) compared with both basal and insulin-stimulated cells (14 ± 1.0 , 13 ± 1.8 and 7 ± 0.8 pmol/mg of membrane protein in basal, insulin-treated and cycloheximide-plus-insulin-treated cells respectively).

Effect of cycloheximide on glucose transporter structure

Next, we investigated the effect of cycloheximide on the electrophoretic profile of [3 H]cytochalasin-B-labelled plasma membranes. Plasma membranes were irradiated with [3 H]cytochalasin, a potent inhibitor of glucose transport, which can be photochemically cross-linked to the glucose transporter by high-intensity u.v. light as

Fig. 5. Effect of cycloheximide on the electrophoretic profile of [^3H]cytochalasin-B-labelled plasma-membrane proteins

Plasma membranes from insulin-treated (a) and cycloheximide + insulin-treated cells (b) were incubated with 500 mM-D-glucose (●) or -L-glucose (○) before incubation with [^3H]cytochalasin B for 10 min at 4 °C. The membranes were irradiated with u.v. light at 254 nm for 5 min. Membrane protein (250 μg) was applied to 9%-acrylamide gels and analysed by SDS/polyacrylamide-gel electrophoresis. Arrow indicates the position of a 43 kDa marker protein.

described in the Materials and methods section. The results shown in Fig. 5 indicate that cycloheximide did not change the molecular mass of the glucose transporter as estimated by SDS/polyacrylamide-gel electrophoresis; in both membrane fractions we detected a D-glucose-inhibitable protein of molecular mass 45 kDa. On the other hand, cycloheximide treatment clearly led to a marked 66% decrease of the D-glucose-inhibitable portion of glucose transporters in insulin-treated plasma membranes (Fig. 5b), in confirmation of the data obtained by using the cytochalasin B binding assay (Fig. 3).

To characterize further the effect of cycloheximide on the rat adipocyte glucose transporter at the biochemical level, we used a modified two-dimensional-electrophoresis protocol [12]. The first step in this protocol involves SDS/polyacrylamide-gel electrophoresis, as described above (Fig. 5). The proteins of molecular mass 40–50 kDa were then excised from the gel, electrophoretically extracted, concentrated, and subjected to isoelectric focusing as described in the Materials and methods section. Fig. 6 shows the results when basal, insulin-treated and cycloheximide-plus-insulin-treated low-density microsomes were used as the starting material. There is heterogeneity of glucose transporters in the low-density microsomes, one glucose transporter isoform focusing at pH 6.4, and the other at pH 5.6 (Fig. 6a). Insulin caused a decrease only of the pI-5.6 glucose-transporter isoform, whereas the pI-6.4 isoform is not sensitive to insulin (Fig. 6b). As previously described, only the pI-5.6 isoform exists in plasma membranes, and its concentration is markedly increased by insulin [12]. Cycloheximide did not alter the pI values of either isoform, but did lead to the disappearance of the pI-5.6 glucose-transporter isoform and caused a 47% decrease

in the pI-6.4 transporter isoform in the low-density microsomes (Fig. 6c). In plasma membranes from insulin-plus-cycloheximide-treated cells, the pI of the pI-5.6 isoform was unchanged, but the concentration of this transport species was decreased by ~50% (results not shown).

DISCUSSION

Our major goal in initiating these studies was to gain further insight into the biochemical mechanism of insulin-stimulated glucose transport. Thus we have investigated the effect of cycloheximide, a potent inhibitor of protein synthesis, on insulin-stimulated glucose transport and simultaneously on the subcellular distribution of glucose transporters in isolated adipocytes. The findings indicate that cycloheximide markedly decreases the number of glucose transporters in plasma membranes derived from insulin-stimulated cells (Fig. 3). Of particular importance is the finding that this 50% decrease in plasma-membrane glucose carriers is not associated with a concomitant decrease in insulin-stimulated glucose uptake as assessed by measuring 3-O-methylglucose transport (Fig. 1).

Thus cycloheximide led to a marked dissociation between insulin's ability to stimulate glucose transport and its effect to recruit transporters to the plasma membrane, suggesting that some other process, such as an increase in transporter intrinsic activity, must underlie insulin stimulation of transport in these cells. One might argue that the effect of cycloheximide treatment to decrease glucose transporters in insulin-stimulated plasma membranes is due to differences in recovery of the transport protein in the various membrane fractions. The fact that cycloheximide did not affect the number of glucose transporters in plasma membranes and low-

Fig. 6. Isoelectric focusing of SDS/polyacrylamide-gel-electrophoresis-purified photoaffinity-labelled rat adipocyte glucose transporters from (a) basal, (b) insulin-treated and (c) cycloheximide-plus-insulin-treated low-density microsomes

The [^3H]cytochalasin-B-labelled low-density microsomes migrating at 40–50 kDa were excised from an SDS/polyacrylamide gel and, after elution from the gel, were subjected to isoelectric focusing. Arrows indicate the isoelectric points of the focused protein peaks. $\blacktriangle$, pH.

density microsomes prepared from basal cells weighs against this assumption. Furthermore, as shown in Table 1, the recovery of specific marker-enzyme activities and membrane protein was similar in the various membrane fractions. Finally, we measured the number of glucose transporters in total membrane fractions and found that cycloheximide causes a $\sim 50\%$ decrease in the total number of cellular transporters, which is in good

agreement with the data obtained after subcellular fractionation.

Our findings that cycloheximide causes decreased transporter number in insulin-stimulated plasma membranes without affecting whole-cell transport might be explained by an increase in transport K_m . However, the results shown in Fig. 2 indicate that cycloheximide affects neither the maximum velocity (V_{max}) nor the affinity (K_m) of 3-*O*-methylglucose transport. Cycloheximide might also cause a structural alteration in the transporter, such that the binding of cytochalasin B is impaired. However, analysis of [^3H]cytochalasin-B-labelled membranes revealed that cycloheximide treatment has no effect on the apparent molecular mass nor on the pI value of the glucose-transport protein. Furthermore, the ability of $20\ \mu\text{M}$ -cytochalasin B to inhibit glucose transport in cycloheximide-treated cells was not altered (results not shown).

Baly & Horuk [24] have presented data showing that cycloheximide causes a decrease in transporter number without affecting insulin-stimulated glucose transport. They used an antiserum raised against the human erythrocyte glucose transporter, and could detect no discernible differences in molecular mass (analysed by SDS/polyacrylamide-gel electrophoresis) induced by cycloheximide pretreatment, but they were able to detect the effect of cycloheximide to decrease glucose transporter numbers in insulin-treated membranes, using Western-blotting techniques. Thus several lines of evidence support to notion that cycloheximide pretreatment induces a decrease in the number of glucose transporters only in insulin-stimulated cells. Kono and colleagues [6] reported that cycloheximide has no effect on glucose transporter numbers. However, they used a different methodology, i.e. reconstitution of glucose transporters into artificial liposomes, and their conclusion was based on the assumption that no change in the intrinsic activity of the glucose transporter occurs upon insulin treatment. Our data presented above, in combination with the results of Kono *et al.* [6], therefore suggest that the intrinsic activity is higher in glucose transporters from insulin-plus-cycloheximide-treated cells compared with controls. The mechanism by which cycloheximide decreases the number of glucose transporters in plasma membranes of insulin-stimulated cells remains speculative. In this regard, however, we have recently shown that glucose transporters in the low-density microsomal fraction exist in at least two different isoforms with different charge properties, whereas those in the plasma membrane are more homogeneous in structure [12,25]. In the present study we assessed the effects of cycloheximide treatment on the glucose transporter isoforms. Basal, insulin-treated and cycloheximide-plus-insulin-treated low-density microsomes were subjected to a modified two-dimensional-electrophoresis protocol. The results confirm our recently published data [12,25] that there is heterogeneity of glucose transporters in the intracellular pool, one isoform focusing at pI 6.4 and the other at pI 5.6. Insulin decreases the pI-5.6 glucose-transporter isoform in the low-density microsomes and induces the translocation of only this isoform to the plasma membrane, whereas the pI-6.4 glucose-transporter isoform is not sensitive to the hormone [12,25] and is a precursor to the mature pI-5.6 species. Cycloheximide treatment of insulin-stimulated low-density microsomes did not alter the charge pro-

perties of the transporter species, but did markedly decrease the amount of both the pI-5.6 and pI-6.4 glucose-transporter isoforms. Two-dimensional electrophoresis of plasma membranes derived from insulin-plus-cycloheximide-treated cells revealed no effect of cycloheximide on the pI of the transporter, which still focused at pH 5.6. However, a 50% decrease in the amount of [³H]cytochalasin-B-labelled pI-5.6 glucose transporter was detectable (results not shown). From these results we hypothesize that cycloheximide treatment leads to decreased synthesis or enhanced turnover of the transporter, as is indicated by the diminished pI-6.4 glucose-transporter isoform. Furthermore, one might speculate that cycloheximide inhibits the conversion of the pI-6.4 isoform into the translocatable pI-5.6 isoform, since we could not detect any pI-5.6 isoform in the cycloheximide-treated low-density-microsomes. In consequence, there are very few, if any, glucose transporters available for translocation, which leads to the near-basal numbers of transporters in cycloheximide-treated insulin-stimulated plasma membranes.

The finding that insulin is still able to stimulate glucose transport fully in cycloheximide-pretreated cells, despite the decrease in glucose transporters, supports the idea that insulin-induced translocation of glucose transporters does not adequately explain the mechanism by which insulin stimulates glucose transport in intact cells. Other workers [11,26] have published evidence showing that changes in the intrinsic activity of the glucose transporter might represent an additional physiological mechanism by which insulin stimulates cellular glucose uptake. Clearly, an increase in intrinsic activity of glucose transporters in cycloheximide-pretreated insulin-stimulated cells is consistent with our results. Further studies will be necessary to investigate whether this apparent change in intrinsic activity is accompanied by molecular modifications of the glucose-transport protein.

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