Effect of insulin on the rates of synthesis and degradation of GLUT1 and GLUT4 glucose transporters in 3T3-L1 adipocytes

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The effect of continuous insulin stimulation on the rates of turnover and on the total cellular contents of the glucose-transporter proteins GLUT1 and GLUT4 in 3T3-L1 adipocytes was investigated. Pulse-and-chase studies with [³⁵S]methionine followed by immunoprecipitation of GLUT1 and GLUT4 with isoform-specific antibodies revealed the half-lives of these proteins to be 19 h and 50 h respectively. Inclusion of 100 nM insulin in the chase medium resulted in a decrease in the half-lives of both proteins to about 15.5 h. This effect of insulin was specific for the glucose-transporter proteins, as the average half-life of all proteins was found to be 55 h both with and without insulin stimulation. The effect of insulin on the rate of synthesis of the glucose transporters was determined by the rate of incorporation of [³⁵S]methionine. After 24 h of insulin treatment,

the rate of synthesis of GLUT1 and GLUT4 were elevated over control levels by 3.5-fold and 2-fold respectively. After 72 h of treatment under the same conditions, the rate of synthesis of GLUT1 remained elevated by 2.5-fold, whereas the GLUT4 synthesis rate was not different from control levels. Western-blot analysis of total cellular membranes revealed a 4.5-fold increase in total cellular GLUT1 content and a 50% decrease in total cellular GLUT4 after 72 h of insulin treatment. These observations suggest that the rates of synthesis and degradation of GLUT1 and GLUT4 in 3T3-L1 adipocytes are regulated independently and that these cells respond to prolonged insulin treatment by altering the metabolism of GLUT1 and GLUT4 proteins in a specific manner.

INTRODUCTION

The level of glucose in the serum of mammals is subject to strict hormonal control. Insulin stimulates an increase in the rate of glucose uptake of many cells from between 2-fold and 30-fold depending on the cell type and species [1–3].

Recent studies have established that facilitative diffusion of glucose across the plasma membrane of mammalian cells is mediated by a family of structurally related glucose-transporter glycoproteins [4]. These proteins have distinct tissue distribution, biochemical properties and modes of regulation, which presumably allow the uptake of glucose to be precisely controlled under different physiological conditions and in a tissue-specific manner. One isoform of glucose transporter, GLUT1, is expressed at variable levels in many tissues, including the blood/brain barrier, erythrocytes and fibroblasts. GLUT1 resides primarily in the plasma membrane of the cell types in which it is expressed and may be responsible for basal glucose uptake.

Another isoform of glucose transporter, GLUT4, is expressed only in peripheral tissues that respond to insulin, namely skeletal muscle, cardiac muscle and adipose tissue [5–7]. In these insulin-responsive tissues under normal circumstances, GLUT4 is found almost exclusively in an intracellular compartment [8]. An acute insulin challenge results in an increase in glucose transport through the translocation of GLUT4 proteins to the plasma membrane. Therefore it has been proposed that GLUT4 is responsible for much of the insulin-dependent increase in glucose transport observed in insulin-responsive tissues.

Studies of pathophysiological states associated with chronic changes in insulinaemia have suggested that, in addition to its acute effect on the redistribution of glucose transporters from

internal stores to the plasma membrane, insulin may also play a role in the long-term regulation of glucose-transporter concentration in muscle and adipose cells. For example, depleted [9] or enlarged [10] intracellular pools of glucose transporters have been observed in rats in hypo- or hyper-insulinaemic states respectively. Moreover, GLUT4 levels were recently found to be decreased by as much as 65% in adipose tissue from human non-insulin-dependent diabetes mellitus patients [11], a condition that is characterized by both hyperinsulinaemia and hyperglycaemia. These pathophysiological states are accompanied by many metabolic, hormonal and genetic variations that make it difficult to assign unequivocally the observed changes in glucose-transporter number to changes in insulin concentration alone.

In order to clarify the long-term regulatory role of insulin on glucose-transporter synthesis, stability and total cellular amount in the adipose cell, it was necessary to use a system better controlled for the confounding factors occurring in the intact animal. The murine 3T3-L1 pre-adipose cell line, which after confluence acquires the phenotype of mature adipocytes and can be kept viable for extended periods of time, represents an excellent model in which to assess long-term effects of insulin [12]. This process is accompanied by the acquisition of insulin-responsive glucose transport [13] and the expression of the GLUT4 isoform of the glucose transporters [7]. Mature 3T3-L1 adipocytes express both GLUT1 and GLUT4 at similar abundance, and thus provide a useful model system for comparing these two glucose-transporter isoforms within the same cellular environment [14,15].

Measurements of glucose-transporter mRNA in several diabetic human and animal models have failed to correlate with glucose transport activity [16]. This suggests that perhaps there

Abbreviations used: GLUT1, erythrocyte/HepG2 glucose-transporter isoform; GLUT4, adipocyte/muscle glucose-transporter isoform; DMEM, Dulbecco's modified Eagle's medium; C₁₂E₈, octaethylene glycol dodecyl ether; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride.

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are other levels of regulation, in particular modulation of the glucose-transporter protein rates of biosynthesis and degradation. In the present study, the effect of continuous insulin stimulation on the rates of synthesis and degradation of glucose transporters in 3T3-L1 adipocytes was examined by using a pulse-and-chase protocol. Immunoblotting was also used to assess the effect of insulin treatment on the total cellular content of GLUT1 and GLUT4 in these cells.

We demonstrate that the biosynthesis of each glucose-transporter isoform is differentially regulated, and that 3T3-L1 adipocytes respond to prolonged insulin treatment by altering the metabolism of the GLUT1 and GLUT4 isoforms independently of one another.

MATERIALS AND METHODS

Materials

Tissue-culture medium and serum were obtained from GIBCO. [35 S]Methionine and 125 I-Protein A were purchased from ICN. Pig insulin, phenylmethanesulphonyl fluoride (PMSF), pepstatin A, complete and incomplete Freund's adjuvant and keyhole-limpet haemocyanin were from Sigma. Octaethylene glycol dodecyl ether ($C_{12}E_8$) was obtained from Fluka. Protein A-Sepharose was from Pharmacia LKB Biotechnology Inc. Tissue-culture plates (50 mm diam.) were obtained from Nalgene and 100 mm-diam. plates were from Falcon. Other materials and reagents were of the highest grade.

Cell culture

Murine-derived 3T3-L1 fibroblasts were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 10% (v/v) fetal-bovine serum and 1% (v/v) antibiotic/antimycotic solution (100 000 units/ml penicillin, 100 mg/ml streptomycin) in 75 cm² flasks in an atmosphere of 5% CO₂ at 37 °C. At 3 days before confluence, fibroblasts were treated with trypsin and seeded in 50 mm or 100 mm plates to a concentration of 20 000 cells/ml. Fibroblasts were fed every 72 h until 3 days post-confluence and were differentiated into adipocytes as described by Frost and Lane [17]. Mature adipocytes were used between days 10 and 15 after the initiation of differentiation. Adipocytes prepared in this manner have been shown to respond to an acute insulin challenge with a 5-fold increase in glucose uptake [18]. Furthermore, immunoblotting of subcellular membrane fractions with GLUT1- and GLUT4-specific antibodies (as in ref. [15]) demonstrate that both glucose-transporter isoforms can be induced to translocate from an internal organelle to the plasma membrane in response to an acute insulin challenge (results not shown).

Antibodies

The GLUT1-specific antiserum was generated against a peptide corresponding to amino acids 481–492 of the rat brain/HepG2 GT (sequence G-L-F-H-P-L-G-A-D-S-Q-V) and the GLUT4-specific antiserum was generated against a peptide corresponding to amino acids 498–509 of the rat insulin-responsive glucose transporter (IRGT) (sequence T-E-L-E-Y-L-G-P-D-E-N-D). In addition, C linking moieties were added to the N-terminals of both peptides. The peptides were synthesized by the Biotechnology Centre at the Hospital for Sick Children, Toronto, and were coupled to keyhole-limpet haemocyanin as previously described [7]. Male albino rabbits were immunized with 500 µg of conjugate emulsified in complete Freund's adjuvant and boosted every 4 weeks with 300 µg of conjugate emulsified in incomplete Freund's adjuvant.

Labelling with [35 S]methionine

3T3-L1 adipocytes were grown and differentiated in 50 mm plates. Before labelling, cells were rinsed twice with phosphate-buffered saline (PBS), pH 7.4, and were incubated in methionine-free DMEM supplemented with 10% (v/v) dialysed fetal-bovine serum. After 2 h, this medium was removed and the adipocytes were pulsed for 24 h in methionine-free DMEM supplemented with 10% dialysed fetal-bovine serum and 50 µCi of [35 S]-methionine/plate. The labelling medium was removed and the adipocytes were rinsed with PBS. Complete DMEM with or without insulin was added to the plates of labelled adipocytes. When necessary, the medium was changed and the insulin was replaced every 24 h for the 72 h chase period in order to ensure continuous stimulation by the hormone.

Solubilization and immunoprecipitation

Pulsed-and-chased adipocytes were washed twice with ice-cold PBS and solubilized in 1.2 ml of 2% (w/v) $C_{12}E_8$ /PBS containing 0.5 mM PMSF and 6 µM pepstatin A. The cell lysates were transferred to Eppendorf tubes, vortex-mixed vigorously for 1 min, and centrifuged at 12 000 g for 15 min at 4 °C. The supernatants were used for determination of protein concentration and trichloroacetic acid-precipitable radioactivity and were stored at –80 °C.

A 0.3 ml portion of each thawed supernatant was adjusted to 0.5 ml with fresh 2% (w/v) $C_{12}E_8$ and was pre-cleared with 10 µl of rabbit pre-immune serum for 1 h at 4 °C with gentle mixing. Then 50 µl of a 20% (w/v) suspension of Protein A-Sepharose in PBS was added and incubated for 2 h at 4 °C. Protein A-Sepharose beads were collected by centrifuging the mixture for 1 min at 12 000 g in a microfuge, and the supernatant was used for immunoprecipitation of GLUT1 or GLUT4. Antiserum against either GLUT1 or GLUT4 (20 µl) was added and incubated for 1 h at 4 °C. The immune complex was collected by addition of Protein A-Sepharose beads as described above. The Protein A-Sepharose immune complexes were collected and washed once in 1 ml of 50 mM Hepes/1 mM EDTA/150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS, pH 7.4, once more in the same buffer but instead containing 1.0 M NaCl, twice more in the original buffer containing 150 mM NaCl, and finally once in PBS.

SDS/PAGE and fluorography

After aspiration of the final PBS wash, the glucose-transporter proteins were released from the Protein A-Sepharose beads by incubation in 30 µl of SDS sample buffer for 30 min at 37 °C and were separated by SDS/PAGE as described by Laemmli [19] in Bio-Rad Mini-protein II dual-slab cells, 5% stacking and 10% separating polyacrylamide gels. Gels were stained with Coomassie Blue, destained, and soaked for 1 h in water. Gels were then soaked in 1.0 M sodium salicylate for 1 h before being dried down under heat and suction on to filter paper. Dried gels were exposed directly to Kodak XAR-5 films at –80 °C. The fluorograms were quantified by laser densitometry using the 2190-001 Gelscan Program for the LKB 2202 Ultro Scan instrument.

Rates of synthesis of GLUT1 and GLUT4 in 3T3-L1 adipocytes

3T3-L1 adipocytes were grown in 50 mm plates in the presence of 100 nM insulin for up to 72 h. For the longer time points, the medium was changed every 24 h and fresh insulin was added. At the desired times, a plate of adipocytes was rinsed twice with PBS and preincubated for 2 h in methionine-free DMEM supplemented with 10% dialysed fetal-bovine serum and 100 nM

Figure 1 Characterization of the immunoprecipitation of anti-glucose-transporter antibodies

One 50 mm plate of 3T3-L1 adipocytes was labelled with [35 S]methionine for 24 h and solubilized as described in the Materials and methods section. A sample of the total cell lysate underwent immunoprecipitation (IPP) with pre-immune serum (PIS; lane A), with antibodies against the C-terminal peptide of GLUT1 (lane B), or with antibodies against the C-terminal peptide of GLUT4 (lane E). The supernatants from this first immunoprecipitation were divided into two equal portions and a second immunoprecipitation was performed, using GLUT1-specific antibodies on one portion (lanes C and F), and GLUT4-specific antibodies on the other (lanes D and G). GLUT1 and GLUT4 appear as single bands of about 55 kDa (arrowed).

insulin. This medium was removed and the adipocytes were pulsed for 2 h in the same insulin-containing medium with 200 μ Ci of [35 S]methionine/plate. Labelled cells were solubilized and GLUT1 and GLUT4 were immunoprecipitated and separated by SDS/PAGE as described above.

Isolation of total cellular membranes

3T3-L1 adipocytes were grown in 100 mm plates and incubated for up to 72 h in the presence or absence of insulin as previously described. Adipocytes were rinsed twice with ice-cold homogenization buffer (20 mM Tris/HCl, 1 mM EDTA, 255 mM sucrose, pH 7.4) and homogenized in the same buffer in the presence of 0.5 mM PMSF and 6 μ M pepstatin A. The homogenate was centrifuged at 1000 $g_{\max}$ for 3 min to remove large cell debris and unbroken cells. The supernatant from this first spin was then centrifuged for 90 min at 245000 g to yield a pellet of total cellular membranes. Pellets were resuspended in homogenization buffer to a final concentration of 5 mg/ml of protein and were stored at -80°C .

Immunoblotting

Total membrane protein (50 μ g of protein per lane) was separated on 10% polyacrylamide gels and transferred electrophoretically to nitrocellulose filters as previously described [20]. The nitrocellulose was generally blocked for 1 h with 1% (w/v) BSA in PBS containing 0.2% (v/v) Triton X-100 at room temperature. All subsequent incubations used the same buffer. The nitrocellulose was then incubated overnight at 4°C with a 1:300 dilution of the antisera raised against either GLUT1 or GLUT4, washed three times, incubated with ^{125}I -Protein A (1.5 μ Ci/10 ml of buffer) for 1 h at room temperature, washed three times, dried, and exposed to Kodak XAR-5 films at -80°C . Autoradiograms were quantified by laser densitometry as described above.

2-Deoxyglucose uptake

3T3-L1 adipocytes were grown in 24-well plates and were incubated for up to 72 h in the presence of insulin. Adipo-

Figure 2 Effect of insulin on the accumulation and rate of degradation of total cellular protein in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in the absence (●, control) or presence of 100 nM insulin (▽) for up to 72 h. At the indicated times, the protein content of a 50 mm plate was determined. The results shown are expressed as percentage change in total cellular protein relative to the protein content of the plates at time 0 h and represent means $\pm$ S.E.M. of 7 independent experiments (a). 3T3-L1 adipocytes were pulsed with [35 S]methionine for 24 h and chased for different times as described in the Materials and methods section. A sample of the cell lysate from each of the indicated times was assayed for trichloroacetic acid (TCA)-precipitable radioactivity relative to the cells that were not chased. The results shown represent means $\pm$ S.E.M. of 7 independent experiments (b).

cytes were rinsed twice with PBS and incubated for 2 h in uptake buffer (140 mM NaCl, 1.7 mM KCl, 0.9 mM CaCl_2 , 1.47 mM K_2HPO_4 , 0.8 mM MgSO_4 , 0.5% BSA and 25 mM Tris/HCl, pH 7.5) containing 10 mM 2-deoxyglucose. Adipocytes were then rinsed and incubated for 30 min in uptake buffer with no 2-deoxyglucose. When appropriate, 100 nM insulin was included in all incubations up to this point. The uptake buffer was removed by aspiration and 1.0 ml of uptake buffer containing 10 μ M 2-deoxyglucose and 0.4 μ Ci 2-deoxy[^3H]glucose was added to each well for 10 min at room temperature. The cells were washed immediately in ice-cold PBS containing 10 mM glucose and were solubilized in 0.1% SDS. The 2-deoxy-[^3H]glucose taken up by the cells was quantified by liquid-scintillation counting. Non-carrier-mediated uptake was measured in the presence of 25 μ M cytochalasin B. Specific uptake is expressed in pmol/min per mg of cell protein.

Other methods

The protein concentrations of the cell lysates and total cellular membrane fractions were assayed as described by Peterson [21].

Figure 3 Effect of insulin on the rates of degradation of GLUT1 and GLUT4 in 3T3-L1 adipocytes

3T3-L1 adipocytes were pulsed and chased in the absence (●, control) or presence (▽) of 100 nM insulin. Glucose transporters were then immunoprecipitated and separated by SDS/PAGE as described in the Materials and methods section. The glucose-transporter bands from representative autoradiograms are shown: glucose transporters immunoprecipitated from cells chased under control conditions (A) and from cells chased in the presence of 100 nM insulin (B). Quantification by laser densitometry followed by semi-logarithmic transformation of the data confirms that insulin causes a decrease in the half-life of GLUT1 from approx. 19 h to approx. 15.5 h and a decrease in the half-life of GLUT4 from 50 h to 15.5 h. Results shown are expressed as the percentage of ^{35}S label in glucose transporters immunoprecipitated at time 0 h and represent means $\pm$ S.E.M. of 3 and 7 independent experiments for the insulin and control curves respectively.

Trichloroacetic acid-precipitable radioactivity was determined as previously described [22]. Where applicable, results are expressed as means $\pm$ S.E.M. from n separate experiments.

RESULTS

Specificity of the antibodies against GLUT1 and GLUT4

The C-terminal 13 amino acids of GLUT1 and the C-terminal 12 amino acids of GLUT4 coincide at only one position, and therefore the antibodies against these regions of the two glucose-transporter proteins should not cross-react. In agreement with this expectation, GLUT1 and GLUT4 were sequentially immunoprecipitated from the same lysates of 3T3-L1 adipocytes (Figure 1). The mobilities of both glucose-transporter isoforms corresponded to a molecular mass of approx. 55 kDa. The antibodies against both GLUT1 and GLUT4 were shown to immunoprecipitate virtually all of the respective glucose transporter protein under the conditions used in this work (lanes B and E). A subsequent immunoprecipitation step performed with the same antisera of the supernatant of the first immunoprecipitation did not bring down significant amounts of the respective glucose-transporter isoform (compare lane C with lane B for GLUT1 and lane G with lane E for GLUT4). In contrast, GLUT4 and GLUT1 were successfully immunoprecipitated from the supernatants that were first cleared of GLUT1 and GLUT4 respectively (compare lane D with lane B and lane F with lane E). The other precipitated materials are proteins recognized by the antibodies in the serum or that bind to the Protein A-Sepharose beads. Some cross-reactivity with other proteins is expected, as the antisera used for the immunoprecipitations were raised in rabbits, whereas the 3T3-L1 cell line originates from the mouse. The discrete bands at the 66.2 kDa marker are most likely due to

the large mass of the immunoglobulin heterodimer from the serum (seen as the large clear area between 66.2 kDa and 97.2 kDa) pushing many non-specific proteins ahead of it. A similar band appears in the pre-immune-serum lane.

As a further control, Western blotting was performed on immunoprecipitates from unlabelled cells by using the GLUT4-specific monoclonal antibody 1F8. Under these conditions, a single band was detected in the expected molecular-mass range in the lane containing the GLUT4 immunoprecipitate (result not shown). No bands appeared in the lanes in which GLUT1 antiserum or non-immune serum was used for the immunoprecipitation. The reciprocal of this experiment was not performed, as a monoclonal antibody specific for the GLUT1 isoform is not yet commercially available.

Effect of insulin on the rates of degradation of total cellular protein and the glucose transporters

3T3-L1 adipocytes were labelled with [^{35}S]methionine for 24 h so as to achieve maximum labelling of the total cellular protein, and GLUT1 and GLUT4 in particular (results not shown). The adipocytes were then chased for up to 72 h in regular medium containing unlabelled methionine in the presence or absence of 100 nM insulin. Continuous insulin stimulation for 72 h resulted in a 60% increase in the total cellular protein content of the adipocytes, whereas cells chased in regular medium without insulin for the same period of time showed only a 10% increase in their protein content (Figure 2). These data illustrate the anabolic nature of insulin action, which includes an increase in the rates of protein synthesis in adipocytes.

Despite the change in protein content, the rate of degradation of total protein in 3T3-L1 adipocytes, as determined by com-

Figure 4 Effect of insulin on the rate of synthesis of GLUT1 and GLUT4 in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated for up to 72 h in the presence (+) or absence (—) of 100 nM insulin. At the indicated times, adipocytes were pulsed for 2 h also in the presence or absence of 100 nM insulin with [35 S]methionine and GLUT1 (●) and GLUT4 (▽) were immunoprecipitated and quantified as described in the Materials and methods section. For total protein (□), a sample of the cell lysates was assayed for trichloroacetic acid-precipitable radioactivity as described in the legend of Figure 2. The data shown in the graph are from insulin-stimulated cells and are expressed as 35 S label in GLUT1, GLUT4 or total protein compared with levels at time 0 h and represent means $\pm$ S.E.M. of 4 independent experiments.

paring the trichloroacetic acid-precipitable radioactivity from different times of the chase, was not changed by the 72 h of insulin treatment. The average half-life of total cellular protein was about 55 h both with and without insulin stimulation (Figure 2).

In contrast with the observed half-life of trichloroacetic acid-precipitable material, and half-lives of GLUT1 and GLUT4 were found to be quite different, with values of 19 h and 50 h respectively under basal conditions. Moreover, the inclusion of 100 nM insulin during the chase resulted in a decrease in the half-life of both GLUT1 and GLUT4 to a similar value of 15.5 h (Figure 3). Therefore, the half-life of GLUT4 is analogous to the average half-life of total cellular proteins, whereas the half-life of GLUT1 is much lower. Chronic stimulation with 100 nM insulin decreased the half-lives of GLUT1 and GLUT4 to a similar value, but did not significantly alter the average half-life of total cellular protein. When 10 nM insulin was included in the chase medium, the values for the half-lives of average cellular protein, GLUT1 and GLUT4 were found to be similar to those obtained when 100 nM insulin was used (results not shown).

Effect of insulin on the rate of synthesis of GLUT1 and GLUT4

The rate of synthesis of GLUT1 was increased by more than 3.5-fold after 24 h of insulin stimulation and remained elevated above control levels by almost 3-fold after 72 h of insulin stimulation (Figure 4). The rate of synthesis of GLUT4, on the

Figure 5 Effect of insulin on the total cellular GLUT1 and GLUT4 contents in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated with 100 nM insulin for up to 72 h. At the indicated times, the cellular contents of GLUT1 and GLUT4 were determined by immunoblotting of total crude membranes as described in the Materials and methods section. Quantification of the autoradiograms by laser densitometry indicated a 4.5-fold increase in total cellular GLUT1 content (A) and a 50% decrease in total cellular GLUT4 content (B) after 72 h of insulin stimulation. Results shown are means $\pm$ S.E.M. of four independent experiments.

other hand, increased by 2-fold after 24 h of insulin stimulation and returned to control levels after 72 h of stimulation. The fluorogram pictured in Figure 4 does not clearly show a decrease in the rate of synthesis of GLUT4 at the later time points. However, the average of four independent experiments indicates only a slight increase in the rate of synthesis of this protein after 72 h of insulin treatment. Under the same insulin-stimulatory conditions, the rate of synthesis of trichloroacetic acid-precipitable protein increased by 2.5-fold over control levels after 24 h, increased to almost 3-fold after 48 h, and at 72 h remained elevated by over 2-fold (Figure 4). These data demonstrate the selective and specific effects of insulin stimulation on the regulation of the GLUT1 and GLUT4 glucose-transporter isoforms, both with respect to one another and when compared with the synthesis of total cellular protein.

Effect of insulin on the total cellular contents of GLUT1 and GLUT4

From the previous data, the total cellular contents of GLUT1 and GLUT4 would be expected to change dramatically after 72 h of insulin stimulation. Western-blot analysis of total membranes from 3T3-L1 adipocytes grown for up to 72 h in 100 nM insulin revealed that the hormone caused a 4.5-fold increase in total cellular GLUT1 content and a 50% decrease in total cellular GLUT4 content (Figure 5). The rate of glucose uptake by the insulin-treated cells was measured to assess whether the newly synthesized GLUT1 proteins were targeted to the plasma membrane and were functional. After 72 h of insulin treatment, a 3.5-

Figure 6 Effect of insulin on the rate of 2-deoxyglucose uptake in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in the presence of 100 nM insulin for up to 72 h. At the indicated times, the rate of 2-deoxyglucose (10 μ M; 13.2 Ci/mol) uptake was measured as described in the Materials and methods section. The results shown are specific 2-deoxyglucose uptake and are from one experiment, each time performed in quadruplicates.

fold increase in the rate of 2-deoxyglucose uptake was observed (Figure 6).

DISCUSSION

The objective of the present study was to investigate whether insulin exerts regulation on the GLUT1 and GLUT4 glucose transporters at a post-translational level, specifically, on the half-life of these proteins.

Continuous insulin stimulation of 3T3-L1 adipocytes resulted in an increase in 2-deoxyglucose uptake and in the cellular concentration of the GLUT1 glucose transporter that was selective for this protein. These observations suggest that GLUT1 molecules synthesized by 3T3-L1 adipocytes in response to insulin treatment are likely to be present and functional in the plasma membrane. In a previous study, treatment of 3T3-L1 adipocytes with 100 nM insulin for 72 h was observed to cause a 4-fold increase in the rate of 2-deoxyglucose uptake [23]. This increase was accompanied by a 4.1-fold increase in total GLUT1 protein and a 3.3-fold increase in GLUT1 mRNA. These results are in excellent agreement with our present observations. However, no change in either the total cellular concentration of GLUT4 or its mRNA levels after 72 h of insulin treatment were found in that study, whereas we found the cellular concentration of GLUT4 to be decreased to about 50% of its control levels (Figure 5). The reason for this discrepancy is currently unknown; however, chronic insulin treatment has also been found to lower GLUT4 mRNA levels in L6 muscle cells [24]. Similarly, a GLUT1-specific up-regulation has been detected upon glucose deprivation of 3T3-L1 adipocytes [25]. Hence, it is apparent both from the previous work and from the results presented here that GLUT4 plays a minimal role in the long-term response of 3T3-L1 adipocytes to insulin.

The present study extends these observations by investigating whether the observed changes in cellular glucose-transporter number are the result of alterations to the rate of synthesis, the rate of degradation, or some combination of both. These questions have not been examined so far in any study of glucose-transporter regulation.

The rates of synthesis of GLUT1 and GLUT4 were increased respectively by 3.5-fold and 2-fold above basal levels after 24 h of

insulin treatment (Figure 4). However, after 72 h of insulin treatment, the rate of synthesis of GLUT1 remained elevated by 3-fold, whereas that of GLUT4 had returned to basal levels. These data indicate a two-step response for the expression of glucose transporters that is more pronounced for GLUT4. The initial increase in the rate of GLUT4 synthesis after 24 h may simply reflect a non-specific insulin-induced increase in overall protein synthesis within the cell. Indeed, after 24 h the rate of synthesis of GLUT4 is not significantly different from that of total cellular protein. After 72 h of insulin stimulation, the adipocytes have had time to respond more fully to the insulin challenge by altering both the level of glucose-transporter gene transcription and the stability of the resulting mRNA. The data seem to indicate that, between 24 h and 72 h of insulin treatment, GLUT4 was being specifically down-regulated, whereas GLUT1 synthesis was continuously induced. Therefore GLUT1, but not GLUT4, parallels the glucose-transport response of 3T3-L1 adipocytes to long-term changes in their metabolic needs.

The need for tight control of glucose uptake through the control of expression of GLUT1 would explain why this protein has a half-life of only 19 h, whereas the average half-life of total cellular protein is much longer. A more rapid turnover rate for GLUT1 would allow the cellular concentration of this protein to be altered more readily in response to changes in energy requirements that might occur during development or during changes in the environment.

In a recent study, the effect of insulin on the half-life of the insulin-receptor protein was determined by using a pulse-and-chase protocol similar to that used in the present work [26]. Continuous insulin treatment of rat fibroblasts, in which the insulin receptor was over-expressed, led to a decrease in the half-life of this protein from about 9.9 h to 5.7 h. The half-life of total cellular protein was unaffected by the insulin treatment. This observation, together with the results presented here, further emphasizes the existence of a glucose-transport system that is controlled by insulin treatment with respect to the rate of turnover of a few selected proteins.

The half-life of GLUT4 was found to be 50 h under basal conditions. This value is not significantly different from the average half-life of all proteins in these cells. This suggests that GLUT4 would be maintained within the cell as a constitutive protein, possibly in a slow state of flux to and from the plasma membrane.

The proposed model for insulin-stimulated glucose uptake in 3T3-L1 adipocytes includes the recruitment of glucose transporters to the plasma membrane in response to an acute insulin challenge [14,15]. After the removal of insulin, GLUT4 is endocytosed to one of at least two possible locations within the cell: the internal pool from which it was originally recruited, or the lysosome, where it is degraded. The half-life of GLUT4 fell dramatically from 50 h to 15.5 h with chronic insulin stimulation, whereas the half-life of total cellular protein remained unchanged at 55 h. This decrease in the half-life of GLUT4 can be explained by the proposed model. Chronic insulin stimulation may result in a permanent stimulation of the GLUT4 recruitment pathway that would cause a continual cycling of the protein back and forth between its internal storage compartment and the plasma membrane. With every cycle of translocation, however, a certain percentage of the GLUT4 in the plasma membrane would undergo targeting to and degradation in the lysosomal compartment, thus increasing the rate of GLUT4 degradation and eventually depleting the adipocyte's internal recruitable pool of this isoform. This scenario requires experimental verification.

Finally, it is possible that chronic insulin stimulation results in changes in the levels of expression of GLUT1 and GLUT4 by

independent pathways. The increase in GLUT1 levels may simply be a secondary result of the increased energy requirements of the adipocyte to support insulin-stimulated growth. The decrease in GLUT4 levels, however, is probably a direct consequence of continual insulin stimulation of the recruitment pathway that results, eventually, in the down-regulation of this isoform. A better understanding of the intracellular signalling pathways is required before the relative contributions of these two potentially independent effects can be determined.

We are indebted to Dr. Amira Klip for her excellent advice and to Mr. Rob Romanek and Dr. André Marette for critical reading of this manuscript. This work was supported by a grant from the Medical Research Council of Canada (to M.R.P.). R.J.S. is supported by a University of Toronto Open Scholarship.

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Received 10 August 1992/5 November 1992; accepted 12 November 1992