Translocation of the brain-type glucose transporter largely accounts for insulin stimulation of glucose transport in BC3H-1 myocytes

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Insulin-stimulated glucose transport was examined in BC3H-1 myocytes. Insulin treatment lead to a 2.7 ± 0.3 -fold increase in the rate of deoxyglucose transport and, under the same conditions, a 2.1 ± 0.1 -fold increase in the amount of the brain-type glucose transporter (GLUT 1) at the cell surface. It has been shown that some insulin-responsive tissues express a second, immunologically distinct, transporter, namely GLUT 4. We report here that BC3H-1 myocytes and C2 and G8 myotubes express only GLUT 1; in contrast, rat soleus muscle and heart express 3–4 times higher levels of GLUT 4 than GLUT 1. Thus translocation of GLUT 1 can account for most, if not all, of the insulin stimulation of glucose transport in BC3H-1 myocytes. On the other, hand, neither BC3H-1 myocytes nor the other muscle-cell lines are adequate as models for the study of insulin regulation of glucose transport in muscle tissue.

INTRODUCTION

Both fat and muscle have long been known to respond to insulin by exhibiting an increased rate of glucose transport [1]. In the early 1980s, two laboratories independently described a molecular basis for this stimulation [2,3]. Glucose transporters are recruited from an intracellular pool to the plasma membrane in response to insulin. This process is termed translocation, and the increase in plasma membrane transporters is responsible, at least in part, for the increased rate of glucose transport into the cell. A large body of evidence has established this mechanism in adipocytes [4]. In addition, translocation has been demonstrated in skeletal muscle [5] and heart [6].

Debate, however, has surrounded the question of whether translocation is the sole mechanism involved in the stimulation of transport by insulin. In an effort to answer this question we developed a method to assay glucose transporter at the cell surface [7]. This method consists of labelling surface glycoproteins, including the glucose transporter, with galactose oxidase and [^3H]borohydride and then isolating the transporter by immunoadsorption. The label in the transporter provides a measure of the amount of transporter in the plasma membrane. We originally applied this method to 3T3-L1 adipocytes and reported that there was only a 2.6-fold increase in the plasma-membrane glucose transporter, whereas the increase in the rate of glucose transport was found to be 11-fold [7]. Since then it has become evident that the basis for this discrepancy may lie in the fact that 3T3-L1 adipocytes contain two types of glucose transporter [11,12], rather than one, as assumed at the time. One type of transporter present in 3T3-L1 adipocytes is the type found in many tissues; its cDNA has been cloned from human hepatoma cells [9], rat brain [10] and 3T3-L1 adipocytes [11]. We refer to it as 'GLUT 1' in the present paper. The second, more recently discovered, transporter, termed 'GLUT 4', is found only in tissues (fat and muscle) with insulin-responsive transport [11–15]. GLUT 4 undergoes marked translocation to the plasma membrane in response to insulin. In our study of 3T3-L1 adipocytes, only the translocation of GLUT 1 was measured.

Here we describe the application of this method for measuring translocation to BC3H-1 myocytes. BC3H-1 myoblasts differentiate into myocytes that have characteristics of both smooth and skeletal muscle [16,17]. Upon differentiation the number of insulin receptors increases and glucose uptake is acutely stimulated by insulin [18]. We demonstrate that, in contrast with 3T3-L1 adipocytes, insulin stimulation of glucose transport in BC3H-1 cells is almost entirely accounted for by translocation of GLUT 1. Using a newly developed immunological method to determine the contents of GLUT 1 and GLUT 4, we show that this result is explained by the absence of detectable GLUT 4 in BC3H-1 myocytes. Unlike heart and skeletal muscle, two other muscle cell lines (C2 and G8) also lack detectable levels of GLUT 4.

MATERIALS AND METHODS

Materials

NaB^3H_4 (5–10 Ci/mmol) was from Amersham International. The solid was dissolved at 50 mCi/ml in 50 mM-NaOH, frozen in liquid N_2 and stored at -70°C . Galactose oxidase (from *Dactylium dendroides*; product no. G3385) was from Sigma Chemical Co. The enzyme was dissolved in 150 mM-NaCl/10 mM-sodium phosphate, pH 7.4 (PBS) at 1000 units/ml (units as defined by Sigma), held at 50°C for 30 min in order to inactivate any inherent proteinase activity [19] and stored in aliquots at -70°C . Rabbit antiserum against the C-terminal peptide (residues 477–492) of GLUT 1 was a gift from Dr. S. A. Baldwin (Royal Free Hospital, Hampstead, London N.W.3, U.K.) [20]. Antibodies were affinity-purified from this serum as described in [20]. Rabbit antiserum against the C-terminal peptide (residues 491–509) of GLUT 4 was prepared by the method described in [21], and antibodies were then purified by chromatography on the immobilized peptide [21]. All other reagents were the same as those in [7].

Cell culture

The BC3H-1 murine cell line was obtained from Dr. Stanley

Abbreviations used: PBS, phosphate-buffered saline (for composition and pH, see the text); KRP, Krebs–Ringer/phosphate buffer (for composition and pH, see the text); $\text{iPr}_2\text{P-F}$, di-isopropyl fluorophosphate; NEM, *N*-ethylmaleimide; Ep 475, *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane; C_{12}E_8 , octa(ethylene glycol) ether.

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Froehner of this Institution and is the one described in [28]. BC3H-1 myoblasts were cultured and differentiated on 35 mm-diameter plates as described in [18], with the exception that the plates were not collagen-coated. Myoblasts were plated at 2000/cm², and the myocytes used on day 11 after plating. The cells were incubated in 2 ml of serum-free Dulbecco's modified Eagle's medium for 3 h before use; this medium was replaced every hour.

Plates of confluent differentiated C2 and G8 cells were, respectively, kindly provided by Dr. Paul Gardner and Dr. Richard Comi of this institution. The C2 line was originally obtained from Dr. Zach Hall [29] and was cultured as described in [22]. The G8 line was from the American Type Culture Collection and was cultured as described in [23].

Hexose transport

Hexose transport by BC3H-1 myocytes was measured by the uptake of 2-deoxyglucose [7]. Plates (35 mm) were incubated at 37 °C in 1 ml of Krebs-Ringer/phosphate buffer (KRP) (136 mM-NaCl/4.7 mM-KCl/1.25 mM-CaCl₂/1.25 mM-MgSO₄/5 mM-sodium phosphate, pH 7.4), containing 300 nM-insulin or vehicle. Uptake was initiated by the addition of 50 µl of 2-deoxy[1,2-³H]-glucose, such that the final concentration was 5 µM with 0.5 µCi/ml. After 1 or 2 min, uptake was terminated by three rapid washes with ice-cold PBS; the monolayer was solubilized in 1 ml of 1% Triton X-100, and the radioactivity was measured. To correct for non-specifically associated radioactivity, uptake was measured in the presence of 25 µM-cytochalasin B, a potent inhibitor of transport, and these values subtracted from the values obtained in the absence of cytochalasin B. In the presence of cytochalasin B, the transport rates were typically less than 10% of those measured in the absence of the inhibitor. Under these conditions, the uptake of deoxyglucose was found to be linear over a 5 min period (results not shown). In a previous study it was shown that there are equivalent stimulations of uptake in response to insulin with either 2-deoxy-D-glucose, which is phosphorylated by hexokinase, or 3-O-methyl-D-glucose, which is not; this finding indicates that transport is the rate-limiting step in the uptake of 2-deoxyglucose under these conditions [18].

Cell-surface labelling of GLUT 1

This method is a slight modification of that previously described by us [7]. Plates (35 mm) of BC3H-1 myocytes were incubated in KRP at 37 °C in the presence of 300 nM-insulin or vehicle for 15 min. The cells were then quickly washed with ice-cold KRP. All subsequent operations were at 0–4 °C. The cells were incubated with 40 units of galactose oxidase in 1 ml of KRP for 1 h. After the galactose oxidase treatment, the cells were washed in KRP, and the oxidized carbohydrate reduced by the addition of an aliquot of the stock NaB³H₄ (2 mCi) to 1 ml of KRP buffer. An equimolar amount of HCl (2 µmol) was added immediately before NaB³H₄ to maintain the pH at 7.4. After a final wash in KRP, the cells on each plate were lysed in 1.5 ml of 2% octa(ethylene glycol)ether (C₁₂E₈) in PBS containing the proteinase inhibitors di-isopropyl fluorophosphate (iPr₂P-F, 0.2 mM) *N*-ethylmaleimide (NEM, 5 mM), pepstatin A (1 µM) and *L*-trans-epoxysuccinyl-leucylamido-(3-methyl)butane (Ep 475, 10 µM). The lysate was centrifuged at 190 000 *g*_{max} for 1 h and the supernatant removed. The GLUT 1 in the supernatant was immunoprecipitated with 3 µg of affinity-purified antibodies against the C-terminal peptide.

The proteins in the immunoprecipitate were separated on 10%-(w/v)-polyacrylamide slab gels, and the ³H content of the gel slices was measured. The total radioactivity in the GLUT 1 was determined by summing the radioactivity in the slices

corresponding to the molecular-mass range 45–55 kDa (generally slices 6, 7 and 8), and subtracting from this sum a background value. The latter was obtained by assuming the background of each slice in the peak to have a value between those of the slices immediately adjacent to the transporter peak (generally slices 5 and 9; see Fig. 1 below). The relative amount of GLUT 1 was determined by quantitative immunoblotting as described in [24]. About 35% of the total GLUT 1 in a 35 mm dish of BC3H-1 cells was recovered in the SDS samples of the immunoprecipitates, as determined by comparison of the GLUT 1 content of these samples with that in an SDS sample of the cells on a 35 mm plate.

Determination of GLUT 1 and GLUT 4 content

The amounts of each glucose-transporter species in the different cell types were determined by quantitative immunoblotting using appropriate standards for both GLUT 1 and GLUT 4. Standards for GLUT 1 were prepared from human erythrocyte membranes in which the transporter makes up 5.2% of the total protein [25]. Membranes were solubilized in SDS/PAGE sample buffer and diluted in this buffer to give the amounts of GLUT 1 indicated in the Figure legends. Standards of GLUT 4 were similarly made from a preparation of rat adipocyte low-density microsomes [32]. The total transporter content of this preparation was 40 pmol of glucose transporters/mg of protein as measured by cytochalasin B binding [31]. Of this amount, over 90% is GLUT 4 [26, 30; S. Brown & G. E. Lienhard, unpublished work].

Samples of the different muscle-cell lines were prepared by washing the plate three times with ice-cold PBS and solubilizing them in either 0.5 ml (for BC3H-1) or 1 ml (for G8) per 35 mm plate or 1.5 ml per 60 mm plate (for C2) of SDS/PAGE sample buffer containing 20 mM-dithiothreitol, iPr₂P-F (0.5 mM), pepstatin A (1 µM) and Ep 475 (10 µM). The DNA in the samples was sheared by passing them through a 25-gauge needle. For analysis of rat tissues, the heart and both soleus muscles were removed from a 170 g Sprague-Dawley male rat and frozen at once in liquid N₂. The frozen tissue was powdered and then homogenized in 5 ml of buffer [20 mM-Hepes (pH 7.2)/120 mM-NaCl/0.2 mM-iPr₂P-F/5 mM-NEM/10 µM-Ep475/pepstatin A (1 µg/ml)]/g of tissue with a VirTis VirTishear for 30 s at setting 80. Membranes were solubilized by adding a 1/20 vol. of 20% Triton X-100 and vortex-mixing the solution intermittently over the next 1 h. Insoluble material was pelleted by centrifugation at 1000 *g*_{max} for 20 min. The supernatant was removed, and SDS samples were prepared. The amounts of protein in the supernatant/g of tissue were 88 and 38 mg for heart and soleus muscle respectively. The SDS samples of the muscle-cell lines and tissues, together with the standards for GLUT 1 and GLUT 4, were electrophoresed on 10%-polyacrylamide slab gels and transferred to nitrocellulose as described in [24]. For detection of GLUT 1, the nitrocellulose was generally blocked for 1 h with 3% BSA in buffer A (PBS/1 mM-EDTA/0.1% Triton X-100) and treated overnight with antibodies (1 µg/ml) against the GLUT 1 C-terminal peptide in the blocking medium. The nitrocellulose was then washed five times for 10 min each with buffer A and then exposed to ¹²⁵I-labelled goat antibodies against rabbit IgG (0.07 µCi/ml; du Pont-NEN) in 3% BSA/buffer A for 3 h. Finally the nitrocellulose was washed five times with buffer A. For GLUT 4, the nitrocellulose was blocked with 5% (w/v) non-fat dry milk in PBS and then treated with antibodies (8 µg/ml) against the C-terminal peptide of GLUT 4 in 1% dry milk/PBS. After five washes with buffer A, the blot was exposed to 0.07 µCi of ¹²⁵I-labelled second antibody/ml in 1% dry milk/PBS for 3 h and then washed five times with buffer A. The procedure used for GLUT 4 was also used for GLUT 1 in the single case of the rat tissue samples (see Fig. 3), because these samples gave a higher background with some non-specific bands

when blocked with albumin. In this case the antibodies against the C-terminal peptide of GLUT 1 were present at 3 µg/ml. The nitrocellulose blots were subjected to autoradiography, and radioactivity in the GLUT bands was measured by cutting and counting as detailed in [24]. The amounts of GLUT 1 and GLUT 4 were calculated by comparison with the standards run on the same blots (see Figs. 2 and 3 below). Protein content in the various samples was measured by means of a modification of the Lowry method [27].

RESULTS

Insulin stimulation of GLUT 1 translocation in BC3H-1 myocytes

We have previously shown that the GLUT 1 in the plasma membrane of 3T3-L1 adipocytes can be labelled by oxidation of residues in the carbohydrates moiety with galactose oxidase, followed by reduction with NaB³H₄ [7]. This labelling method

has been shown to provide an assay with which to measure the appearance of additional GLUT 1 at the plasma membrane in response to insulin [7]. Fig. 1 presents the results from a representative experiment of this type performed with BC3H-1 cells. The gels show a peak of label at about *M_r* 50000, characteristic of GLUT 1. There is also a small peak at *M_r* 100000, which probably represents incomplete removal of contaminant proteins from the immunoprecipitate, since its appearance was variable. In response to insulin there was increased incorporation of ³H into GLUT 1. The inset to Fig. 1 shows the immunoblots of the immunoprecipitates; it illustrates that there were similar amounts of GLUT 1 in the samples from basal and insulin-treated BC3H-1 cells. Thus the increase in ³H label in response to insulin is due to an increased amount of GLUT 1 at the cell surface, rather than a change in the recovery of GLUT 1 upon immunoprecipitation.

The relationship between the insulin-dependent increases in plasma-membrane GLUT 1 content and hexose transport was examined by performing both the labelling of GLUT 1 and the assay of transport on the same day with groups of 35 mm plates that had been plated together from the same stock of cells. Cells were treated with 300 nM-insulin for 15 min at 37 °C, a condition which leads to the full stimulation of transport (results not shown). The representative experiment in Fig. 1 demonstrates that, in response to insulin, there was about a 2-fold increase in the label associated with GLUT 1. Table 1 summarizes the data from five independent cell platings. The average increase in the label associated with GLUT 1 was 2.1-fold, whereas 2-deoxyglucose transport was stimulated 2.7-fold. A previous study reported that insulin stimulated 2-deoxyglucose transport in BC3H-1 myocytes from 1.8- to 3.0-fold in five different platings of cells [18]. The insulin effect was maximal at 200 nM-insulin or greater, and required 15 min to develop fully [18].

GLUT 1 and 4 contents of BC3H-1 myocytes

The discovery of a second glucose transporter found exclusively in insulin-sensitive tissues led us to determine the amounts of both GLUT 1 and GLUT 4 in BC3H-1 cells. To do this, we employed quantitative immunoblotting with antibodies specific for the C-terminal peptides of GLUT 1 and GLUT 4. As standards we used human erythrocyte membranes for GLUT 1 and rat adipocyte low-density microsomes for GLUT 4 (see the

Fig. 1. Labelling of the cell surface GLUT 1 in basal (○) and insulin-treated (●) BC3H-1 myocytes

The cells were labelled and GLUT 1 was isolated as described in the Materials and methods section. The values are means ± S.D. for two (basal) and three (insulin-treated) samples, each from separate plates. Markers for molecular mass (*M*) in kDa are indicated at the top. The inset (lanes a-e) is an immunoblot for GLUT 1 in the two basal (a,b) and the three insulin (c-e) samples. The GLUT 1 was immunoprecipitated from a whole-cell lysate and so represents both cell-surface and intracellular transporter. The specific radioactivity in the GLUT 1 band of the immunoblot for the five samples is (in c.p.m.): a, 2298; b, 1845; c, 1662; d, 1527; e, 1593. Although in this experiment the average amount of GLUT 1 in the immunoprecipitates from the basal cells was slightly larger (1.3-fold) than the amount from insulin-treated cells, in the four other experiments summarized in Table 1 there was no significant difference (values for the amount of GLUT 1 (basal) to amount of GLUT 1 (insulin-treated): 0.93, 1.09, 0.99 and 1.03). The sharp upper band in the immunoblot is the heavy chain-light chain dimer of the antibodies in the immunoprecipitate (about 75 kDa). Abbreviations: T. D., tracking dye; GT, glucose transporter.

Table 1. Effects of insulin on the label in cell-surface GLUT 1 and the rate of 2-deoxyglucose uptake in BC3H-1 myocytes

GLUT 1 at the cell surface in basal and insulin-treated BC3H-1 myocytes was assessed by labelling, and the rates of 2-deoxyglucose uptake were measured as described in the Materials and methods section. In each experiment, the values are derived from duplicate or more determinations of ³H incorporation and 2-deoxyglucose uptake for both basal and insulin-treated 35 mm plates from the same plating of cells used on the same day for both procedures. Individual values were within ± 10% of the average. Typical values for 2-deoxyglucose uptake in these experiments were 25 and 57 pmol/min per 10⁶ cells for basal and insulin-treated cells respectively.

Expt.	Ratio (insulin-treated/basal)	
	³ H label in GLUT 1	dGlc uptake
1	2.36	3.22
2	2.23	2.26
3	1.71	3.65
4	2.03	1.95
5	1.95	2.39
Mean ± S.E.M....	2.06 ± 0.12	2.69 ± 0.32

Fig. 2. Quantification of GLUT 1 and GLUT 4 in BC3H-1 myocytes by immunoblotting

(a) GLUT 1: human erythrocyte GLUT 1 standards are in lanes a–e with the amount in ng of GLUT/lane given below each lane. Two amounts of basal (lanes f and g) and insulin-treated (lanes h and i) BC3H-1 myocytes are shown. SDS samples of cells were prepared as described in the Materials and methods section and 1/25 (lanes f and h) or 1/50 (lanes g and i) of a 35 mm plate (about 0.9 mg of protein/plate) were run. (b) GLUT 4: rat adipocyte GLUT 4 standards are shown (lanes a–e) with the amounts in ng given below each lane. The same samples of BC3H-1 cells as in lanes f–i of (a) were analysed (lanes f–i). The autoradiograms were developed for 18 h.

Materials and methods section). It is known that the C-terminal peptide of GLUT 1 in the human, rat and mouse are identical [9–11]; the same is true for GLUT 4 [11–15]. On the other hand, the two peptides differ markedly in sequence. In agreement with the latter, neither preparation of the antibodies against the C-terminal peptides cross-reacted with the other GLUT on immunoblots. This specificity was demonstrated by immunoprecipitating GLUT 1 and GLUT 4 from 3T3-L1 adipocytes with their respective antibodies. When the immunoprecipitates were blotted for both transporters, only GLUT 1 was detected in the sample immunoprecipitated with antibodies to GLUT 1 and, conversely, only GLUT 4 was seen in the immunoprecipitates using the GLUT 4 antibodies (K. Kitagawa, D. M. Calderhead and G. E. Lienhard, unpublished work).

Fig. 2(a) presents the autoradiogram samples of basal and insulin-treated BC3H-1 cells. Quantification of these results indicates that there is 230 ng of GLUT 1/35 mm plate of BC3H-1 cells (equivalent to 260 ng/mg of protein). Fig. 2(b) shows an autoradiogram of the same samples of BC3H-1 cells, but this time immunoblotted for GLUT 4. It is evident that no GLUT 4 is detectable in BC3H-1 cells. We estimate that if there had been 0.5 ng of GLUT 4 in the sample (corresponding to 12.5 ng per 35 mm plate), this transporter would have been detected; thus the amounts of GLUT 1 in these cells is at least 18 times the amount of GLUT 4.

GLUT 1 and 4 contents of other muscle-cell lines and tissues

In an effort to determine whether other muscle-cell lines were similar to BC3H-1 cells, we examined the murine muscle-cell lines C2 and G8, both of which have been used as muscle models [22,23]. Figs. 3(a) and 3(b) demonstrate that these lines showed a pattern of expression similar to the BC3H-1 line; they both contained significant amounts of GLUT 1 (C2, 310 ng/mg of protein; G8, 585 ng/mg of protein) and no detectable GLUT 4. Triton X-100 extracts of rat heart and soleus muscle were also analysed for their content of the two GLUT species by this method (Figs. 3c and 3d). Unlike the cell-culture lines, both these tissues contain considerably more GLUT 4 than GLUT 1 (heart, 5.0 μ g of GLUT 4/g of tissue and 1.3 μ g of GLUT 1/g of tissue;

Fig. 3. Quantification of GLUT 1 and GLUT 4 in G8 cells, C2 cells and rat tissues by immunoblotting

(a) GLUT 1 in C2 and G8 cells. Erythrocyte GLUT 1 standards (ng) (lanes a–e) and two amounts of SDS samples of cells are shown. For G8 cells 1/50 (f) or 1/100 (g) of a 35 mm plate (about 0.7 mg protein per plate) was run. For C2 cells 1/75 (h) or 1/150 (i) of a 60 mm plate (about 3.6 mg of protein/plate) were run. (a) GLUT 4 in G8 and C2 cells. Same as in (a), except rat adipocyte GLUT 4 standards (in ng) are shown in lanes a–e. (c) GLUT 1 in rat soleus muscle and heart. Lanes a–d, human erythrocyte GLUT 1 standards (ng). Lanes e–h, Triton X-100 extracts of soleus muscle (70 and 35 μ g of protein in lanes e and f respectively) and heart (70 and 35 μ g of protein in lanes g and h respectively). (d) GLUT 4 in soleus muscle and heart. As in (c), except lanes a–d contain rat adipocyte GLUT 4 standards (ng).

soleus, 2.2 μg of GLUT 4/g of tissue and 0.7 μg of GLUT 1/g of tissue).

DISCUSSION

In response to insulin, the rate of glucose transport into BC3H-1 cells was increased 2.7-fold. Under identical conditions, the GLUT 1 content of the plasma membrane increased 2.1-fold. Thus, in BC3H-1 myocytes, the translocation of GLUT 1 can account for 80 % of the increase in transport rate. This finding is in marked contrast with the observation with 3T3-L1 adipocytes, where the translocation of GLUT 1 can account for only 25 % of the increase in transport rate (see the Introduction and see [7,8]). Quantification of the amounts of GLUT 4 in these two cell types provided an explanation for the contrast. BC3H-1 myocytes do not contain detectable amounts of GLUT 4 (GLUT 1/GLUT 4 > 18). On the other hand, both GLUT 1 and GLUT 4 are present in 3T3-L1 adipocytes (GLUT 1/GLUT 4 ~4; D. M. Calderhead & G. E. Lienhard, unpublished work). It remains possible that BC3H-1 myocytes contain a very low level of GLUT 4, not detectable by immunoblotting, which contributes to the insulin stimulation of glucose transport, and that this explains the finding that the stimulation of transport is slightly higher than the increase in cell-surface GLUT 1.

A previous study has shown the presence of both GLUT 1 and GLUT 4 in skeletal muscle and heart [12], but the actual amounts of these was not determined. The results presented here shows that both tissues contain much more GLUT 4 than GLUT 1. In contrast, besides BC3H-1 myocytes, the other two muscle-cell lines, C2 and G8, also lack detectable GLUT 4. Skeletal muscle is the peripheral tissue in the which most insulin-stimulated glucose utilization occurs [33,34], and it would be useful to have a muscle-cell line in which to study this process. Clearly none of these lines is appropriate.

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