Biochem. J. (1992) 286, 157–163 (Printed in Great Britain)

Glucose regulates its transport in L_8 myocytes by modulating cellular trafficking of the transporter GLUT-1

Rosamiria GRECO-PEROTTO,* Efrat WERTHEIMER,† Bernard JEANRENAUD,* Erol CERASI† and Shlomo SASSON‡§

Departments of ‡Pharmacology and †Endocrinology & Metabolism, The Hebrew University-Hadassah Medical Center, 91120 Jerusalem, Israel, and *Laboratoires de Recherches Métaboliques, Faculty and Department of Medicine, University of Geneva, 1211 Geneva 4, Switzerland

The effect of culture conditions simulating hypo- and hyper-glycaemia on glucose transport and on the subcellular localization of the glucose transporter GLUT-1 was studied in L_8 myocytes. Incubation of the cells with 20 mM-glucose for 25 h decreased the rate of 2-deoxy-D-[^{3}H]glucose (dGlc) uptake to 0.106 ± 0.016 nmol/min per 10^6 cells compared with 0.212 ± 0.025 in cells maintained at 2 mM-glucose (final glucose concentrations at the end of the incubation period were 16–17 mM and 0.7–1.0 mM respectively). An additional 5 h incubation of these cells with medium containing the opposite glucose concentration (i.e. change from 17 mM to 1 mM and from 1 mM to 17 mM) increased the transport rate to 0.172 ± 0.033 nmol/min per 10^6 cells in cultures initially conditioned at high glucose, and decreased the transport to 0.125 ± 0.029 in those conditioned at low glucose. Plasma-membrane- and microsomal-membrane-enriched fractions were prepared from these cells for [^{3}H]cytochalasin B (CB) binding and Western-blot analysis with antibodies against GLUT-1 and GLUT-4. A decrease in glucose concentration increased the number of D-glucose-displaceable CB-binding sites and GLUT-1 protein in the plasma-membrane fraction to the same extent as the increase in dGlc transport. Under downregulatory conditions, the lower dGlc-transport capacity could be accounted for by a decreased number of transporters in the plasma membrane of the cells. No apparent modification of the intrinsic activity of the glucose transporters was observed in up- or down-regulated cells. Under downregulatory conditions, the CB-binding data indicated a large increase in the number of transporters in the intracellular membranes of the myocytes. Western blots of the same membranes also indicated an increase in GLUT-1 content. However, the interaction of the intracellular GLUT-1 protein with the polyclonal antibodies was much weaker than that of the plasma-membrane-associated GLUT-1. The GLUT-4 concentration was too low to permit quantification in membrane fractions. Our findings suggest that autoregulation of glucose transport in L_8 myocytes is accompanied by parallel changes in the number of GLUT-1 transporters in the plasma membrane, and that the rate of transporter degradation may be augmented in the upregulated myocytes. These glucose-induced changes are fully reversible.

INTRODUCTION

The glucose-transport system in skeletal muscle is regulated by hormones, physical activity and metabolic factors. We have shown previously that glucose itself is a potent regulator of its transport and utilization in rat soleus muscles and L_8 myocytes in culture (Sasson & Cerasi, 1986; Sasson *et al.*, 1987). Low glucose concentrations (1–2 mM) upregulate the hexose-transport rate to a maximal level. At glucose concentrations between 2.5 and 4 mM, this rate is decreased by 25–35%; further elevation of the glucose concentration decreases the rate of transport by approx. 2% for each additional 1 mM-glucose in the incubation medium. Kinetic analysis of the hexose transport under these conditions shows that the $V_{max.}$ of the transport is modulated without a significant change in the K_m value, suggesting that glucose controls the number of glucose transporters in the plasma membrane. These effects of glucose are fully reversible and completed within 3–4 h of the initiation of up- or down-regulation (Sasson & Cerasi, 1986).

Walker *et al.* (1990) reported that glucose deprivation augmented the hexose-transport rate 6.3-fold in L_6 myotubes. This was associated with an increase in the number of transporters in the plasma membrane, but only by 1.75-fold, suggesting that the intrinsic activity of the transporter increases concomitantly with its translocation from an internal pool to the plasma membrane.

Translocation of glucose transporters from the internal membrane pool to the plasma membrane has been found in adipocytes (Cushman & Wardzala, 1980; Kono *et al.*, 1981), brown adipose tissue (Greco-Perotto *et al.*, 1987*a*), rat diaphragm (Wardzala & Jeanrenaud, 1981, 1983), heart (Watanabe *et al.*, 1984; Zaninetti *et al.*, 1988) and skeletal muscle (Klip *et al.*, 1987; Hirshman *et al.*, 1988, 1990; Douen *et al.*, 1989; Vilaro *et al.*, 1989). Some, but not all, studies suggest that the intrinsic activity of the transporter is also subjected to regulation (Hyslop *et al.*, 1985; Kahn & Cushman, 1985; Joost *et al.*, 1986; Sternlicht *et al.*, 1988; Karnieli *et al.*, 1989).

The present experiments were designed to investigate whether the changes in the kinetics of hexose transport result from glucose-induced redistribution of glucose transporters between the plasma membranes and internal membranes of L_8 myocytes, and whether the estimated intrinsic activity of the transporter is also modified by glucose.

MATERIALS AND METHODS

Chemicals

2-[1,2-^{3}H]dGlc (30.2 Ci/mmol) and [4-^{3}H]cytochalasin B (CB;

Abbreviations used: CB, cytochalasin B; CE, cytochalasin E; dGlc, 2-deoxy-D-glucose; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GLUT-1, rat brain/erythrocyte/HepG2 glucose transporter subtype; GLUT-4, insulin-regulated adipocyte/skeletal-muscle glucose transporter subtype; PBS, Dulbecco's phosphate-buffered saline.

§ To whom correspondence should be addressed.

18.5 Ci/mmol) were purchased from DuPont–New England Nuclear (Boston, MA, U.S.A. and Zürich, Switzerland). [U-^{14}C]Sucrose (552 mCi/mmol) and ^{125}I-labelled protein A (30 μCi/μg) were from The Radiochemical Centre (Amersham, Bucks., U.K.). D-Glucose was from Merck (Darmstadt, Germany), and dGlc, L-glucose, CB, cytochalasin E (CE) and luciferin–luciferase kit for ATP determination were from Sigma (St. Louis, MO, U.S.A.). Waymouth and glucose-free Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Grand Island, NY, U.S.A.). Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Beth-Haemek, Israel). Reagents for the 5′-nucleotidase and NADPH–cytochrome *c* reductase enzyme assays were from Boehringer (Mannheim, Germany). All other chemicals were reagent grade.

Cells and culture conditions

Myogenic cells of the L_8 cell line, originally established by Dr. D. Yaffe of the Weizmann Institute (Rehovot, Israel), were obtained courtesy of him. The cells were grown as previously described (Yaffe, 1965). In brief, mononucleated myogenic cells were plated in 100 mm gelatin-coated tissue culture plates (10^6 cells/plate) in Waymouth medium supplemented with 15% (v/v) FCS. When the cultures reached confluency they were washed and received glucose-free DMEM supplemented with 10% (v/v) FCS and 2 or 20 mM-glucose. The plates were incubated for 20 h (at this time the glucose concentrations were 0.7–1.0 mM and 16–17 mM respectively). Then some of the plates were washed again and received DMEM supplemented with 10% (v/v) FCS and the opposite glucose concentration (i.e. cells at 0.7–1.0 mM-glucose received medium with 17 mM-glucose and vice versa). After a further 5 h of incubation, the plates were used for either [^{3}H]dGlc-uptake assay or cell fractionation.

Preparation of membrane fractions

Confluent myocytes were scraped off the plates, collected and washed once (centrifuged at 750 *g*) with phosphate-buffered saline (PBS; 0.5 mM-$MgCl_2$,$6H_2O$, 2.7 mM-KCl, 1.5 mM-KH_2PO_4, 9.6 mM-K_2HPO_4 and 137.0 mM-NaCl, pH 7.4). The washed myocytes (approx. 5×10^8 cells for each incubation condition) were homogenized (20 strokes) in 15 ml of ice-cold buffer (10 mM-$NaHCO_3$/5 mM-NaN_3, pH 7.0) with a glass pestle (B. Braun, Molsungen, Germany) connected to a homogenizer (Heidolph model R2R1). All the following steps were carried out at 4 °C. The homogenate was centrifuged at 700 *g* for 5 min and the supernatant was re-centrifuged at 250000 $g_{max.}$ for 180 min. The resulting pellet was resuspended in ice-cold deionized distilled water. To this fraction an equal volume of 0.8 M-LiBr in 20 mM-Tris/HCl, pH 8.5, was added, the suspension was stirred for 15 h and then centrifuged at 700 *g* for 5 min. The resulting pellet was discarded and the supernatant re-centrifuged at 33000 *g* for 15 min. The supernatant was collected and taken for preparation of the microsomal-membrane fraction. To prepare the plasma-membrane fraction, the pellet was resuspended in a buffer containing 0.6 M-KCl and 10 mM-Tris/HCl, pH 8.0, and centrifuged at 250000 $g_{max.}$ for 90 min. The supernatant was discarded and the pellet rehomogenized in water and centrifuged at 6000 *g* for 15 min. The resulting pellet was discarded and the supernatant was centrifuged at 250000 $g_{max.}$ for 60 min. The pellet was resuspended in water and layered on a discontinuous sucrose-density gradient containing 4 ml portions of 35, 31 and 27% (w/v) sucrose in 20 mM-Tris/HCl/1 mM-EDTA, pH 7.4. The gradient was centrifuged at 150000 $g_{max.}$ for 60 min. The interface between the upper water and the 27% sucrose phases was collected, diluted with water and centrifuged at 250000 $g_{max.}$ for 90 min. The resulting pellet was resuspended to a final protein concentration of approx. 1 mg/ml in a buffer containing 255 mM-sucrose, 10 mM-Tris/HCl and 0.2 mM-EDTA, pH 7.5.

The microsomal-membrane fraction was obtained from the supernatant described above. This supernatant was centrifuged at 250000 $g_{max.}$ for 150 min and the resulting pellet was resuspended in water and layered on a sucrose-density gradient as described above. After centrifugation, the pellet at the bottom of the gradient was collected, resuspended and centrifuged at 250000 $g_{max.}$ for 90 min. The pellet was resuspended in the buffer containing 255 mM-sucrose as above. All fractions were prepared simultaneously from the same starting homogenate.

The enrichment in plasma and microsomal membranes was estimated by measuring the changes in the specific activities of 5′-nucleotidase (Avruch & Wallach, 1971) and NADPH–cytochrome *c* reductase (Sottocasa *et al.*, 1967). Protein was measured by the Coomassie Brilliant Blue method (Bradford, 1976) with BSA as standard.

CB binding to membrane preparations

The number of glucose transporters in the various membrane fractions was measured by the equilibrium binding of [^{3}H]CB by the method of Cushman & Wardzala (1980) with the modifications described previously (Greco-Perotto *et al.*, 1987*a*). All samples were pretreated with 2 μM-CE to decrease non-specific binding. Maximal D-glucose-displaceable [^{3}H]CB binding was measured with 500 nM-labelled ligand in the presence of 500 mM-L- or D-glucose, in triplicate. This concentration of CB was chosen because it was shown in earlier studies using brown adipose tissue (Greco-Perotto *et al.*, 1987*b*) and skeletal muscle (Douen *et al.*, 1989) to be a saturating concentration for both microsomal and plasma membranes.

Western-blot analysis

Membranes (10 μg/lane) were separated on an SDS/polyacrylamide gel and analysed by Western blot (Laemmli, 1970) with specific rabbit antisera against synthetic peptides corresponding to the *C*-terminus of GLUT-1 and GLUT-4 (courtesy of Dr. Hans G. Joost, University of Göttingen, Göttingen, Germany) at a dilution of 1:200. Immunoreactive species were detected with ^{125}I-labelled protein A.

Measurement of dGlc uptake

The plates were rinsed eight times with PBS, pH 7.4, and incubated in the same buffer containing 0.1 mM-dGlc and 0.5 μCi of [^{3}H]dGlc/ml, in triplicate. After 5 min at room temperature the incubation was terminated by rinsing the cells five times with ice-cold PBS. They were then digested with 1 ml of 1 M-NaOH for 30–60 min at 40 °C, neutralized with conc. HCl, and portions taken for liquid-scintillation counting. Extracellular space was determined in parallel with 0.1 μCi of [^{14}C]sucrose. The uptake data were calculated on the basis of cell number with correction for extracellular space. We showed previously that 5 μM-CB effectively inhibited the uptake of dGlc when added before the hexose and that the CB-non-inhibitable uptake of dGlc was similar to the dGlc content in the extracellular space. It was also shown that under both up- and down-regulatory conditions the rate-limiting step of dGlc uptake in L_8 myocytes is the transport of the hexose and not its phosphorylation by hexokinase (Sasson & Cerasi, 1986).

ATP determination

Myocytes were washed with PBS as above, collected in hot (90 °C) distilled water and boiled for 10 min. The resulting lysate was used for ATP determination by the luciferin–luciferase

Table 1. dGlc-uptake rates in up- and down-regulated L_8 myocytes, and D-glucose-displaceable [^{3}H]CB binding in plasma-membrane- and microsomal-membrane-enriched fractions of the same cell groups

The uptake of [^{3}H]dGlc was measured in L_8 myocytes incubated as follows: (A) 2 mM-glucose for 25 h, (B) 20 mM-glucose for 20 h followed by 1 mM-glucose for 5 h, (C) 20 mM-glucose for 25 h and (D) 2 mM-glucose for 20 h followed by 17 mM-glucose for 5 h. Specific [^{3}H]CB binding was measured in the plasma-membrane-enriched fractions (PM) and in the microsomal-membrane-enriched fractions (MM). Results are means ± S.E.M. of three different experiments.

Glucose in medium	[^{3}H]dGlc uptake (nmol/min per 10^6 cells)	[^{3}H]CB binding (pmol/mg of membrane protein)	
		PM	MM
(A) 2 mM	0.212±0.025	9.83±0.99	3.55±0.50
(B) 20 mM changed to 1 mM	0.172±0.033	9.66±2.02	4.12±0.32
(C) 20 mM	0.106±0.016	6.22±0.24	6.93±0.44
(D) 2 mM changed to 17 mM	0.125±0.029	5.80±1.39	7.33±0.36

Table 2. Activity of enzyme markers in plasma- and microsomal-membrane fractions obtained from L_8 myocytes

Plasma and microsomal membranes were obtained from L_8 myocytes incubated with 2 mM-glucose for 25 h (A), 20 mM-glucose for 20 h followed by 1 mM-glucose for 5 h (B), 20 mM-glucose for 25 h (C) and 2 mM-glucose for 20 h followed by 17 mM-glucose for 5 h (D) as described in the Materials and methods section. Enrichment of each membrane fraction was assessed by measuring the specific activity of respective marker enzymes. Percentage recovery relates to the value in the initial homogenate. Results are means ± S.E.M. for three individual preparations for each treatment. PM, plasma-membrane fraction; MM, microsomal-membrane fraction.

Glucose in medium	5′-Nucleotidase		NADPH–cytochrome *c* reductase		Protein	
	Activity (μunits/mg of protein)	Percentage recovery	Activity (munits/mg of protein)	Percentage recovery	Concn. (mg/ml)	Percentage recovery
(A) 2 mM						
Homogenate	2.20±0.08	–	13.89±2.11	–	4.96±0.90	–
PM	33.25±3.40	11.0±1.5	15.78±1.55	0.86±0.27	0.99±0.04	0.74±0.14
MM	8.35±1.25	3.7±0.3	47.80±7.15	3.53±0.75	1.03±0.05	1.00±0.17
(B) 20 mM changed to 1 mM						
Homogenate	2.30±0.13	–	11.52±0.93	–	5.34±0.38	–
PM	31.67±5.05	10.0±1.2	12.77±1.78	0.87±0.27	1.12±0.24	0.77±0.12
MM	8.62±0.96	6.0±1.5	49.43±12.18	4.33±1.08	1.46±0.54	1.17±0.51
(C) 20 mM						
Homogenate	2.10±0.12	–	12.99±2.32	–	5.06±0.85	–
PM	29.57±1.38	11.0±2.5	15.53±3.37	1.03±0.44	1.11±0.15	0.73±0.15
MM	8.53±1.43	4.0±0.6	37.50±7.50	2.93±0.62	1.17±0.20	1.03±0.17
(D) 2 mM changed to 17 mM						
Homogenate	2.17±0.27	–	10.21±0.76	–	5.63±0.27	–
PM	36.05±8.50	9.0±0.6	15.84±2.77	0.87±0.03	0.91±0.16	0.57±0.07
MM	10.10±0.20	4.3±0.3	42.72±6.80	4.13±0.19	1.36±0.31	1.00±0.12

method (Lundin & Baltscheffsky, 1978) by using an LKB–Wallac 1250 luminator (Bromma, Sweden).

Glucose determination

The glucose concentration in culture media was determined by the glucose oxidase method by using a Beckman glucose analyser (Fullerton, CA, U.S.A.).

RESULTS

Table 1 presents the effect of changing the glucose concentration on the hexose-transport capacity of L_8 myocytes. Myocytes exposed for 25 h to 20 mM-glucose (condition C) exhibited a decreased rate of transport compared with cells incubated with 2 mM-glucose (condition A). Decreasing the glucose concentration to 1 mM for 5 h (condition B) increased the transport rate by 62%, whereas cells that were incubated for 5 h at 17 mM-glucose after a 20 h incubation at 2 mM-glucose (condition D) decreased their rate of dGlc transport.

Table 1 also shows the D-glucose-displaceable CB-binding data of the plasma-membrane- and microsomal-membrane-enriched fractions of the same cultures. The correlation between autoregulation of the hexose transport and the distribution of D-glucose-displaceable CB-binding sites between the plasma-membrane and the internal-membrane fractions was excellent: on downregulation, the dGlc-transport rate and CB binding in the plasma-membrane fraction were decreased by 47% (compared with the upregulated state), and a similar number of CB-binding sites disappeared from the plasma-membrane fraction and appeared in the microsomal membrane fraction. In the upregulated state, the dGlc uptake increased by 62% and the number of CB-binding sites in the plasma membrane increased by 55%. Moreover, the majority of the transporters in the upregulated state (conditions A and B) was located in the plasma membrane fraction (73 and 67% of the total number of CB-binding sites respectively). In contrast, when the transport was downregulated, a larger proportion of the CB-binding sites was located intracellularly (56 and 53%, conditions C and D respectively).

The above analysis, although commonly used in studies involving subcellular localization of CB-binding sites, may be

Table 3. Calculated total marker enzyme activities recovered in the plasma- and microsomal-membrane fractions

Glucose in medium	Total protein recovered (mg)	Total 5′-nucleotidase activity recovered		Total NADPH–cytochrome *c* activity recovered	
		Activity (μunits)	Percentage recovery	Activity (munits)	Percentage recovery
(A) 2 mM					
Homogenate	74.4	163.68±5.95	–	1033.42±156.98	
PM	0.550	18.28±1.87	11.17±1.14	8.68±0.85	0.84±0.08
MM	0.744	6.21±0.93	3.79±0.57	35.56±9.61	3.44±0.92
(B) 20 mM changed to 1 mM					
Homogenate	80.1	184.23±10.41	–	922.75±74.49	
PM	0.616	19.51±3.11	10.59±1.69	7.86±1.09	0.85±0.12
MM	0.937	8.07±0.89	4.38±0.48	46.32±11.41	5.02±1.24
(C) 20 mM					
Homogenate	75.9	159.39±9.11	–	985.94±176.09	
PM	0.554	16.38±0.76	10.28±0.48	8.60±1.87	0.87±0.19
MM	0.781	6.89±1.12	4.33±0.70	29.28±5.86	2.97±0.59
(D) 2 mM changed to 17 mM					
Homogenate	84.5	183.26±22.81	–	862.23±64.22	
PM	0.481	17.34±4.09	9.46±2.23	7.62±1.33	0.88±0.15
MM	0.844	8.52±0.17	4.65±0.09	36.05±5.74	4.18±0.67

Table 4. Calculated fold purification of marker enzyme activities associated with plasma- and microsomal-membrane fractions

Glucose in medium	5′-Nucleotidase		NADPH–cytochrome *c* reductase	
	Fold purification	Percentage recovery/mg of protein	Fold purification	Percentage recovery/mg of protein
(A) 2 mM				
PM	15.11	20.31	1.14	1.53
MM	3.79	5.09	3.44	4.62
(B) 20 mM changed to 1 mM				
PM	13.77	17.19	1.11	1.38
MM	3.74	5.00	4.29	5.36
(C) 20 mM				
PM	14.10	18.56	1.19	1.57
MM	4.06	5.54	2.88	3.80
(D) 2 mM changed to 17 mM				
PM	16.61	19.67	1.55	1.83
MM	3.93	5.51	4.18	4.95

misleading since entirely pure plasma- or microsomal-membrane fractions are impossible to obtain, especially from muscle cells. Table 2 presents the marker enzyme and protein contents of the myocyte membrane fractions derived from the various incubation conditions. It is apparent that considerable cross-contamination of the marker enzyme activities occurred in all fractions. These data were used to calculate the marker enzyme activities corresponding to the total plasma-membrane and microsomal-membrane pools of the myocyte homogenates (Table 3). Furthermore, by dividing the specific activity of the enzyme in each fraction with that measured in the initial homogenate (Table 2), the fold purifications of the enzymes were calculated, as well as their recovery per mg of protein [by relating the former to the amount of protein recovered in each fraction (Table 4)]. The latter results were used to calculate the total pools of CB-binding sites in the myocytes, by solving the following equations:

$$\text{CB(PM)} = \left(\frac{\%\ \text{rec 5′NUC (PM)}}{100}X\right) + \left(\frac{\%\ \text{rec CYTC (PM)}}{100}Y\right)$$

$$\text{CB(MM)} = \left(\frac{\%\ \text{rec 5′NUC (MM)}}{100}X\right) + \left(\frac{\%\ \text{rec CYTC (MM)}}{100}Y\right)$$

where CB(PM) and CB(MM) are the CB-binding data in plasma and microsomal membranes respectively shown in Table 1; % rec 5′NUC (PM) and % rec 5′NUC (MM) are the percentage recovery per mg of protein of 5′-nucleotidase in plasma and microsomal membranes respectively, as presented in Table 4; % rec CYTC (PM) and % rec CYTC (MM) are the corresponding values for NADPH–cytochrome *c* reductase in Table 3; *X* stands for total homogenate plasma-membrane-associated CB-binding sites, and *Y* for the corresponding microsomal-membrane-associated CB-binding sites. The *X* and *Y* values thus calculated for all the myocyte incubation conditions are presented in Table 5. It may be seen that the CB binding of the total homogenate plasma-membrane pool gave results consistent with those presented in Table 1, and that a close correlation existed between the modulation of hexose transport by glucose and the change in the number of CB-binding sites in the total plasma membranes of myocytes. The results in total homogenate microsomal-membrane fractions were, however, unexpected. Indeed, Table 5 shows that, whereas the ratio between CB-binding sites of total plasma membranes and microsomal membranes in myocytes with upregulated hexose uptake (conditions A and B) was similar to that given in Table 1 (per mg of membrane protein), in

Table 5. Calculated CB-binding per total plasma and microsomal membranes in the initial homogenate

Total D-glucose-displaceable [^{3}H]CB binding for each subcellular compartment was calculated from the fold purifications of the respective marker enzyme activities and their recoveries in each subcellular membrane fraction. PM, plasma membranes; MM, microsomal membranes.

	CB binding (pmol/10^6 cells)	
Glucose in medium	PM	MM
(A) 2 mM	46.47±4.68	25.64±3.61
(B) 20 mM changed to 1 mM	54.08±11.31	26.34±2.10
(C) 20 mM	20.62±0.79	152.30±9.70
(D) 2 mM changed to 17 mM	17.53±4.20	128.57±6.34

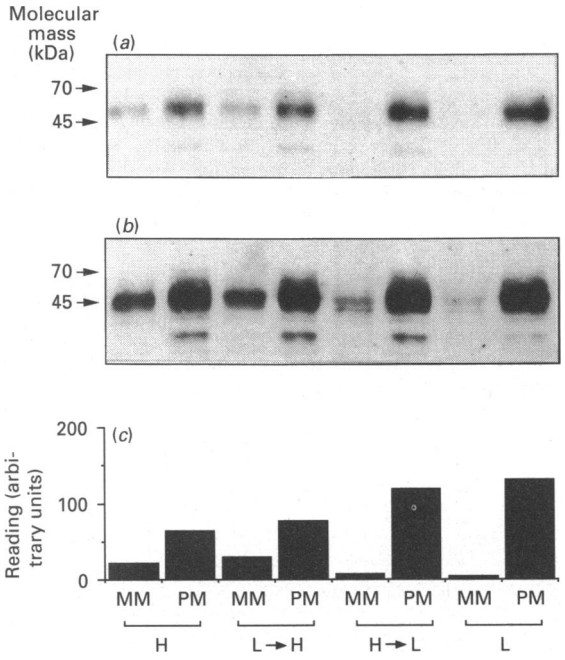

Fig. 1. Western blots of plasma-membrane- and microsomal-membrane-enriched fractions of L_8 myocytes obtained with anti-(GLUT-1) antibodies

L_8 myocytes were incubated for 25 h in 2 mM- (L) or 20 mM- (H) glucose. Other batches were incubated for 20 h in 2 mM-glucose, then transferred to medium containing 17 mM-glucose for 5 h (L → H), and other cultures were incubated for 20 h in 20 mM-glucose and switched to 1 mM-glucose for 5 h (H → L). At the end of the incubation periods, the cells were fractionated to obtain plasma-membrane- (PM) and microsomal-membrane-enriched fractions (MM), which were subjected to PAGE and Western blotted with polyclonal antibodies against the *C*-terminus of GLUT-1. (*a*) Short exposure and (*b*) long exposure of the gels. (*c*) Laser densitometric reading of the blots.

myocytes exposed to high glucose (Table 5, conditions C and D) total CB-binding sites in microsomal membranes were much more abundant. Thus, compared with myocytes exposed to 1–2 mM-glucose, downregulation of transport by 17–20 mM-glucose augmented the total microsomal pool of CB-binding sites 5–6-fold, suggesting that the total cellular CB-binding sites showed a net increase.

Glucose-transporter isoform-specific antibodies were used to characterize the nature of the above CB-binding sites. Under the present myocyte-incubation conditions, Western blots performed with anti-(GLUT-4) antibodies produced very faint signals (results not shown); therefore it could not be assessed whether GLUT-4 distribution is modified by glucose in L_8 myocytes. Fig. 1 presents the Western blots obtained with anti-(GLUT-1) antibodies. The findings in plasma-membrane fractions were quantitatively similar to those obtained with CB binding, densitometric evaluation of the blots showing that downregulation of transport resulted in 41–51 % decrease in the signal, whereas upregulation increased it approx. 2-fold (Fig. 1*a* and *c*). In contrast with the CB-binding data, however, the microsomal membranes reacted poorly with the *C*-terminal GLUT-1 antibodies, distinct signals being obtained only after prolonged exposure (Fig. 1*b*). Nevertheless, this signal was also modulated by the glucose conditioning of the myocytes, 5–10-fold stronger signals being registered in myocytes with glucose transport downregulated by high glucose (Fig. 1*c*). Western blots of total myocyte homogenates did not show significant differences between myocytes conditioned at high or low glucose concentrations (results not shown).

To exclude deleterious metabolic effects of 25 h exposure to a low glucose concentration, myocyte ATP concentrations were measured in all the culture conditions of Table 1. No significant effect of glucose concentration on ATP content was observed under these conditions, the ATP concentration varying between 124±8 and 154±28 pmol/10^6 cells (n = 6).

DISCUSSION

Translocation of glucose transporters from an intracellular compartment to the plasma membrane was first discovered in adipocytes in association with stimulation of hexose transport by insulin (Cushman & Wardzala, 1980; Kono *et al.*, 1981). Translocatable pools of glucose transporters were also identified in rat hindlimb muscles (Klip *et al.*, 1987; Hirshman *et al.*, 1988, 1990; Douen *et al.*, 1989), and in L_6 myotubes in culture (Walker *et al.*, 1990).

In addition to insulin, glucose itself controls the rate of hexose transport in skeletal-muscle cells (Sasson & Cerasi, 1986; Sasson *et al.*, 1987; Cerasi *et al.*, 1989; Wertheimer *et al.*, 1990, 1991). Our present results demonstrate a bicompartmental distribution of glucose transporters in L_8 myocytes. After detailed analysis of the purification and recovery of the marker enzyme activities in the myocyte subcellular fractions, we observed parallelism between the changes in the rate of hexose transport and the number of glucose transporters in the plasma membranes of the cells, determined either by D-glucose-displaceable CB binding or Western blots of GLUT-1 protein. The percentage decreases (on downregulation) were similar to the percentage changes in dGlc uptake in the same cells, indicating that under the experimental conditions used in this study no significant changes in the intrinsic activity of GLUT-1 occurred.

This conclusion does not agree with that of Walker *et al.* (1990) who assessed the distribution of glucose transporters in L_6 myotubes maintained at 25 mM-glucose or 25 mM-xylose for 25 h. Complete glucose withdrawal (in the presence of xylose) resulted in 630 % stimulation of dGlc transport whereas the number of glucose transporters in the plasma-membrane fraction increased by only 75 %. Since the CB-binding data in their study were not corrected for cross-contamination of the subcellular fractions, and because of diverging experimental conditions, including the use of serum-free medium and prolonged complete glucose starvation, it is possible that the two studies describe different mechanisms controlling the distribution and activity of glucose transporters.

The present results agree with previous kinetic studies (Sasson & Cerasi, 1986; Wertheimer *et al.*, 1990) that linked the

autoregulation of hexose transport in L_8 myocytes exclusively to changes in the $V_{max.}$ of the transport, without affecting its K_m. We suggest that the changes observed in the $V_{max.}$ of the transport are a reflection of changes in the number of transporters located in the plasma membrane of the myocytes.

Further analysis of the fractionation data and the results obtained for CB binding and Western blots of the microsomal-membrane fractions attest to the complexity of the autoregulatory mechanism. When CB binding was expressed exclusively per mg of membrane protein, the impression obtained was of a simple translocation/reverse translocation event that distributed the glucose transporters between the plasma and microsomal membranes in the presence of low/high glucose concentrations respectively (Table 1). However, when calculated per total cell membranes (Table 5), a different picture emerged. At the plasma-membrane compartment, glucose did seem to regulate the number of transporters stoichiometrically with the regulation of the transport rate. However, a reverse translocation (or internalization) event is unlikely to be involved in the downregulation of the transport, since total CB-binding sites were not constant (Table 5). Thus in myocytes incubated for 25 h at 20 mM-glucose, CB binding in the total microsomal pool was 126.4 ± 10.53 pmol/10^6 cells higher than in myocytes cultured at 2 mM-glucose, whereas the decrease in CB binding in the total plasma-membrane pool was only 25.9 ± 6.42 pmol/10^6 cells. These results suggest that in myocytes with hexose transport downregulated by high concentrations of extracellular glucose, a decreased rate of transporter translocation from internal pools to the plasma membrane causes intracellular accumulation of transporters and a decrease in their number at the plasma membrane. During upregulation of transport at low glucose, the rate of transporter movement to the plasma membrane is augmented and leads to an appreciable depletion of the intracellular pool. Since the number of CB-binding sites added to the plasma membrane is smaller than expected from the depletion of the intracellular pool, a higher rate of degradation of transporters in the plasma membrane may partially compensate for the increased rate of translocation.

Other experimental findings support this model. We have shown previously (Sasson & Cerasi, 1986) that upregulation starts almost immediately after exposure of myocytes to low glucose concentrations, whereas a lag period of 1–2 h precedes downregulation. Thus the rate of recruitment of transporters to the plasma membrane seems to be fast. On the other hand, in downregulated cells where this process is slow the decrease in the number of glucose transporters in the plasma membrane depends mostly on their turnover rate, which is probably slower under hyperglycaemic conditions. Further support comes from our previous study on the effect of the protein-synthesis inhibitor cycloheximide (Wertheimer *et al.*, 1990). At 1 μM it downregulated the dGlc-transport rate in a manner similar to the effect of high glucose concentrations, suggesting that a short-lived protein is required for translocation of transporters to the plasma membrane. Thus it is reasonable to suggest that downregulation of hexose transport results mainly from decreased trafficking of transporters to the cell surface, and that altered rates of degradation of the transporter also contribute to this process.

In accord with earlier observations, Haney *et al.* (1991) demonstrated, using confocal immunofluorescence imaging, that in 3T3-L1 fibroblasts and HepG2 hepatoma cells overexpressing human GLUT-1, the transporters are mainly localized at the plasma membrane. The Western blots of L_8 myocyte fractions presented here support their observation. It is therefore of great interest that, when GLUT-1 distribution is assessed by the CB-binding technique, a different picture emerges. One possible explanation is that antibodies directed at the *C*-terminal region of GLUT-1 [as in our study and in that of Haney *et al.* (1991)] underestimate the transporters when they are associated with the intracellular vesicles. Such a possibility has been suggested for GLUT-4 in adipocytes (Smith *et al.*, 1991): by using antibodies against the *C*- and *N*-terminal peptides of the transporter, these authors could demonstrate that the *C*-terminus of GLUT-4 is masked in the intracellular compartment, and becomes available after translocation to the plasma membrane. Our findings suggest that a similar situation may exist for GLUT-1.

In conclusion, these studies demonstrate that control of the cellular trafficking of GLUT-1 by glucose (and perhaps other agents) may be an important mechanism by which non-hormonal regulation of glucose uptake occurs.

This study was supported by grants from the Basic Research Foundation administered by the Israel Academy of Sciences and Humanities, The United States–Israel Binational Science Foundation, the Wolfson Foundation, the George Grandis Fund for Medical Research, Grant no. 32.26405.89 of the Swiss National Science Foundation (Berne, Switzerland) and a Grant-in-Aid from Nestlé S.A. (Vevey, Switzerland). Shlomo Sasson is the recipient of the Albert Renold Fellowship Award sponsored by the European Association for the Study of Diabetes. Efrat Wertheimer was a Picciotto Doctoral Fellow. Our sincere thanks go to Dr. S. W. Cushman, National Institutes of Health, Bethesda, MD, U.S.A. for stimulating discussions, and to Rachel Oron for dedicated technical assistance.

REFERENCES

Avruch, J. & Wallach, D. F. H. (1971) Biochim. Biophys. Acta **233**, 334–347

Bradford, M. (1976) Anal. Biochem. **72**, 248–254

Cerasi, E., Glaser, B., Del Rio, G., Sasson, S. & Della Casa, L. (1989) in Frontiers of Diabetes Research: Current Trends in Non-Insulin Dependent Diabetes Mellitus (Alberti, K. G. M. M. & Mazze, R., eds.), pp. 309–320, Elsevier Scientific Publishers, New York

Cushman, S. W. & Wardzala, L. J. (1980) J. Biol. Chem. **255**, 4758–4762

Douen, A. G., Ramlal, T., Klip, A., Young, D. A., Cartee, G. D. & Holoszy, J. O. (1989) Endocrinology **124**, 449–454

Greco-Perotto, R., Assimacopoulos-Jeannet, F. & Jeanrenaud, B. (1987*a*) Biochem. J. **247**, 63–68

Greco-Perotto, R., Zaninetti, D., Assimacopoulos-Jeannet, F., Bobbioni, E. & Jeanrenaud, B. (1987*b*) J. Biol. Chem. **262**, 7732–7736

Haney, P. M., Slot, S. W., Piper, R. C., James, D. E. & Mueckler, M. (1991) J. Cell Biol. **114**, 689–699

Hirshman, M. F., Wallberg-Henriksson, H., Wardzala, L. J., Horton, E. D. & Horton, E. S. (1988) FEBS Lett. **238**, 235–239

Hirshman, M. F., Goodyear, L. J., Wardzala, L. J., Horton, E. D. & Horton, E. S. (1990) J. Biol. Chem. **265**, 987–991

Hyslop, P. A., Kuhn, C. E. & Sauerheber, R. D. (1985) Biochem. J. **232**, 245–254

Joost, H. G., Weber, T. M., Cushman, S. W. & Simpson, I. A. (1986) J. Biol. Chem. **261**, 10033–10036

Kahn, B. B. & Cushman, S. W. (1985) Diabetes/Metab. Rev. **1**, 203–227

Karnieli, E., Moscona, R., Rafaelof, R., Illouz, Y. G. & Armoni, M. (1989) Am. J. Physiol. **256**, E179–E185

Klip, A., Ramlal, T., Young, D. A. & Holoszy, J. O. (1987) FEBS Lett. **224**, 224–230

Kono, T., Suzuki, K., Dansey, L. E., Robinson, F. W. & Belvins, T. L. (1981) J. Biol. Chem. **256**, 6400–6407

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lundin, A. & Baltscheffsky, M. (1978) Methods Enzymol. **57**, 56–72

Sasson, S. & Cerasi, E. (1986) J. Biol. Chem. **261**, 16827–16833

Sasson, S., Edelson, D. E. & Cerasi, E. (1987) Diabetes **36**, 1041–1046

Sasson, S., Kunievsky, B. & Cerasi, E. (1989) Biochem. Pharmacol. **38**, 2655–2661

Smith, R. S., Charron, M. J., Shah, N., Lodish, H. F. & Jarrett, L. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6893–6897

Sottocasa, B. L., Kuylenstierna, B., Ernster, L. & Bergstand, A. (1987) J. Cell Biol. **32**, 415–438

Sternlicht, E., Barnard, R. J. & Grimditch, G. K. (1988) Am. J. Physiol. **254**, E633–E638

Vilaro, S., Palacin, M., Pilch, P. F., Testar, X. & Zorzano, A. (1989) Nature (London) **342**, 798–800

Walker, P. S., Ramlal, T., Sarabina, V., Koivisto, U.-M., Bilan, P. J., Pessin, J. E. & Klip, A. (1990) J. Biol. Chem. **265**, 1516–1523
Wardzala, L. J. & Jeanrenaud, B. (1981) J. Biol. Chem. **256**, 7090–7093
Wardzala, L. J. & Jeanrenaud, B. (1983) Biochim. Biophys. Acta **730**, 49–56
Watanabe, T., Smith, M. M., Robinson, F. W. & Kono, T. (1984) J. Biol. Chem. **259**, 13117–13122
Wertheimer, E., Sasson, S. & Cerasi, E. (1990) J. Cell. Physiol. **143**, 330–336
Wertheimer, E., Sasson, S., Cerasi, E. & Ben-Neriah, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2525–2529
Yaffe, D. (1965) Curr. Top. Dev. Biol. **4**, 37–77
Zaninetti, D., Greco-Perotto, R., Assimacopoulos-Jeannet, F. & Jeanrenaud, B. (1988) Biochem. J. **250**, 277–283

Received 2 December 1991/6 February 1992; accepted 12 February 1992