Substrate autoregulation of glucose transport: hexose 6-phosphate mediates the cellular distribution of glucose transporters

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Summary Exposure of rat skeletal muscle and skeletal muscle cell lines to high glucose levels results in a time- and dose-dependent reduction of the rate of hexose uptake, paralleled by a reduction in the plasma membrane density of glucose transporters. The mechanism of this process was investigated in cultured L8 myocytes. Low concentrations (0.5–2.0 mmol/l) of deoxyglucose mimicked the downregulatory action of 20 mmol/l glucose both regarding the time-course and magnitude of the effect, but in an irreversible manner. A dose-dependent relationship between intracellular accumulation of deoxyglucose 6-phosphate and the magnitude of the downregulatory response was observed. Depletion of intracellular deoxyglucose 6-phosphate restored the rate of hexose transport to the control level. The reduction of hexose transport activity by deoxyglucose occurred independently of ATP depletion which by itself produced the opposite effect. The effects of

deoxyglucose and high glucose on hexose transport were associated with reduced transport maximal velocity and GLUT1 transporter abundance in the plasma membranes of myocytes, as assessed by cell surface biotinylation. The reduction of myocyte GLUT1 mRNA content, observed after exposure to high glucose, did not accompany the transport downregulatory action of deoxyglucose. We suggest that hexose 6-phosphate is the mediator of the downregulatory signal for subcellular redistribution of GLUT1 in L8 myocytes. The signal responsible for reducing the GLUT1 mRNA level may be related to glucose metabolites downstream of the hexokinase reaction. [Diabetologia (1997) 40: 30–39]

Keywords Glucose transporters, GLUT1, glucose, deoxyglucose, deoxyglucose 6-phosphate, glucose 6-phosphate, hexose 6-phosphate, skeletal muscle, myocyte, diabetes mellitus.

The hexose transport system in skeletal muscle is subject to complex regulation. We have previously shown that hexose uptake and utilisation in cultured rat myocytes and myotubes and in isolated rat soleus

muscles in vitro are dependent on the D-glucose concentration to which the cells and muscles are pre-exposed [1–5]. Similar effects of glucose withdrawal and refeeding have been reported in cultured L6 [6] and BC3H1 skeletal muscle cells [7] as well as in human skeletal muscle [8]. The maximal velocity of hexose transport ($V_{\max}$) was reduced in L8 skeletal muscle cells exposed to increasing glucose concentrations (0.5–20.0 mmol/l) in a concentration-dependent manner, while the affinity (K_m) was unaffected [2]. Cytochalasin B (CB) binding and GLUT1 Western blotting of enriched plasma- and microsomal-membrane fractions revealed that high glucose concentrations modulated the subcellular distribution of GLUT1, reducing their number at the plasma membrane of the cell [1]. Similar autoregulatory effects of glucose

Received: 1 August 1996 and in revised form: 17 October 1996

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Abbreviations: AIB, α -aminoisobutyric acid; BSA, bovine serum albumin; CB, cytochalasin B; dGlc, 2-deoxy-D-glucose; dGlc-6-P, 2-deoxy-D-glucose 6-phosphate; DNP, dinitrophenol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MeGlc, 3-O-methylglucose; NHS-LC biotin, sulphosuccinimidyl 6-(biotinamido) hexanoate; PMSE, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline.

were found in a variety of cells (for a review see [9]). In addition, high glucose levels induced a time-dependent reduction in the GLUT1 mRNA abundance in L8 myocytes [5] and other cells studied [9]. It has been proposed that the downregulation of glucose transport and utilisation in skeletal muscle via this autoregulatory mechanism may be important for the induction of reduced peripheral glucose utilisation and insulin resistance in diabetes mellitus [10–12].

The nature of the intracellular signal that operates the downregulation of glucose transport is unknown. Glucose is rapidly metabolised in muscle cells; any of its metabolites responsible for initiating the downregulation of transport may be expected to be short-lived and therefore difficult to identify. We therefore chose to use the glucose analogue 2-deoxy-D-glucose (dGlc) to study the modulation of the hexose transport system of L8 muscle cells. This analogue is transported by glucose transporters and phosphorylated by hexokinase, but it is not metabolised further. The findings of the present report suggest that hexose 6-phosphate interacts with the intracellular sensing mechanisms for the downregulation of glucose transport.

Materials and methods

Materials. 2-[1, 2-³H]dGlc (40 Ci/mmol) was purchased from American Radiolabelled Chemicals (St. Louis, Mo., USA). [Methyl-³H]aminoisobutyric acid (23.5 Ci/mmol), 3-O-[methyl-³H]glucose (79 Ci/mmol), [U-¹⁴C]sucrose (442 mCi/mmol), and [³²P]-deoxycytidine 5'-triphosphate (~3000 Ci/mmol) were from Dupont-New England Nuclear (Boston, Mass., USA). Rediprime DNA labelling system and Rapid-hyb hybridisation buffer were obtained from the Radiochemical Centre (Amersham, UK). Antipain, aprotinin, bovine serum albumin (BSA), CB, dGlc, glycine, luciferine-luciferase kit for ATP determination, 3-O-methylglucose (MeGlc), phenylmethylsulphonyl fluoride (PMSF), saponin and streptavidin-agarose beads were obtained from Sigma Chemicals (St. Louis, Mo., USA). D-glucose was from Merck (Darmstadt, Germany). Sulphosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC biotin) was from Pierce (Rockford, Ill., USA). Leupeptin, α -macroglobulin, and positively charged nylon membranes were obtained from Boehringer Mannheim (Mannheim, Germany). Dowex-1-chloride (4% cross-link) was from Dowex Chemical Co., obtained through Sigma Chemicals. MicroSpin columns for purification of labelled probes were from Pharmacia Biotechnology (Uppsala, Sweden). Triton X-100 was obtained from BDH Chemicals (Poole, UK). Waymouth medium and fetal calf serum (FCS) were from Biological Industries (Kibbutz Beth-Haemek, Israel). Glucose-free Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories (Grand Island, N.Y., USA). All other chemicals and reagents were reagent or molecular biology grade.

Cells and culture conditions. Myocytes of the L8 cell line were kindly donated by Dr. D. Yaffe (Weizmann Institute of Science, Rehovot, Israel). The cells were grown as previously described [2]. The glucose concentration of FCS was ~5 mmol/l, consequently glucose-free incubates in reality contained ~1.0 mmol glucose/l.

Measurement of dGlc and α -aminoisobutyric acid (AIB) uptake. The L8 culture plates were rinsed five times with 2 ml PBS (in mmol/l: 137 NaCl, 2.7 KCl, 9.6 Na₂HPO₄, 1.5 KH₂PO₄, 0.5 MgCl₂, pH 7.4) at room temperature; thereafter, 1 ml PBS containing 0.1 mmol/l dGlc, 0.5 μ Ci [³H]dGlc and 0.25 μ Ci [¹⁴C]sucrose was added to triplicate plates, and incubated for 5 min at room temperature. The uptake of [³H]dGlc into L8 myocytes was linear up to 15 min [2]. CB-noninhibitable dGlc uptake was less than 5% of total uptake. The uptake of dGlc was calculated after correction for the extracellular dGlc as determined with [¹⁴C]sucrose.

The uptake of AIB was measured similarly, using 0.1 mmol/l AIB, 1.0 μ Ci [³H]AIB and 0.25 μ Ci [¹⁴C]sucrose. The incubation was carried out for 5 min at room temperature.

Measurement of MeGlc uptake. The cultures were rinsed as described above and incubated at room temperature for 10 s with 1 ml PBS containing 0.1 mmol/l MeGlc, 2.0 μ Ci [³H]MeGlc and 0.25 μ Ci [¹⁴C]sucrose. The uptake was terminated by the addition of 10 μ mol/l CB for an additional 10 s, followed by five washes with 2 ml ice-cold PBS. One set of control plates received PBS containing 10 μ mol/l CB one min before the addition of MeGlc, and served to determine the CB-noninhibitable MeGlc transport (10–20% of total uptake). CB-inhibitable MeGlc uptake was calculated after correction for the CB-noninhibitable uptake and extracellular [³H]MeGlc, as determined with [¹⁴C]sucrose.

Measurement of intracellular free and phosphorylated dGlc. A modification of the method of Tsuboi and Petricciani was used [13]. Myocytes grown to confluency were incubated with 5 ml PBS containing dGlc at the desired concentrations and 10 μ Ci [³H]dGlc. The incubation was terminated by five rapid washes with 10 ml ice-cold PBS containing 10 μ mol/l CB. The cells were then treated with 4 ml of 0.2% (v/v) Triton X-100 in water at room temperature, collected and treated further with 3 ml chloroform. After centrifugation 1-ml aliquots of the water phase were passed through ion-exchange columns (Dowex-1-chloride). Free dGlc was eluted with 10 ml water, phosphorylated dGlc with 10 ml 4N HCl. Recovery was above 90%. Under all incubation conditions free dGlc accounted for only 5–10% of the total radioactivity eluted.

Cell surface biotinylation. Surface biotinylation of L8 myocytes was adapted from Shetty et al. [14] and Levy-Toledano et al. [15]. L8 myocytes in 100 mm culture plates were washed with ice-cold PBS and incubated with 0.5 mg/ml NHS-LC biotin in PBS for 30 min at 4°C. The reaction was stopped by rinsing the plates three times with 15 mmol/l glycine in ice-cold PBS. The cells were then collected and solubilised for 30 min on ice in 1 ml solubilisation buffer (150 mmol/l NaCl, 50 mmol/l Hepes, pH 7.4, 1% (v/v) Triton X-100, 2 mmol/l PMSF, 2 μ g/ml aprotinin, 0.1 mmol/l antipain, 0.2 mmol/l leupeptin and 0.5 U/ml α -macroglobulin). The supernatant was separated by a 2-min centrifugation in an Eppendorf centrifuge and mixed with 50 μ l streptavidin-agarose beads (1 mg streptavidin/1 ml gel in solubilisation buffer containing 0.1 mmol/l PMSF). The suspension was gently mixed for 30 min at 4°C and the beads sedimented by centrifugation. The bead pellet was washed with 150 mmol/l NaCl, 10 mmol/l Tris, pH 7.0, containing 2 mmol/l PMSF and 0.2 mmol/l leupeptin. The final pellet was resuspended in 120 μ l Laemmli buffer (1.2-fold concentrated, without bromophenol blue [16]) and incubated for 30 min at 65°C. The supernatant was separated from the beads by centrifugation, collected and kept at –70°C until used.

Western blot analysis. Protein content in the streptavidin-purified membrane preparations was determined by the Bio-Rad

DC Protein assay (Bio-Rad Laboratories, Hercules, Calif., USA), using BSA standard dissolved in the same buffer of the membrane preparation. Western blots were performed as described earlier [5], with specific rabbit antiserum against synthetic rat GLUT1 N-terminus (1:500 dilution).

Cell permeabilisation. Permeabilisation of L8 myocytes was performed according to Johnson et al. [17]. In short, cultured cells were chilled to 4°C and treated with permeabilisation buffer (20 mmol/l HEPES, pH 7.4, 10 mmol/l EGTA, 150 mmol/l KCl, 5 mmol/l oxalic acid-dipotassium salt, 6 mmol/l ATP and 50 µg/ml saponin) for 10 min in an ice bath. The cells were then washed gently and incubated with ice-cold PBS for a 20 min recovery (resealing) period followed by 2 min incubation with PBS at room temperature. This step was repeated with PBS at 37°C after which the cells were incubated with DMEM with the specified additions.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from 2–3 confluent 100-mm plates by the RNAzol method (Cinna/Biotex, Friendswood, Texas, USA) according to the manufacturer's instructions. Twenty microgram aliquots were separated by electrophoresis on 1% agarose-formaldehyde gels containing 0.04% ethidium bromide, RNA was transferred to nylon membranes and prehybridised for 3 h at 65°C in Rapid-hyb hybridisation buffer, followed by hybridisation to random-primed ³²P-labelled cDNA probes of rat GLUT1 [5] and mouse α_1 tubulin (1.1 kb fragment obtained from Dr. P. Dobner, University of Massachusetts Medical School, Boston, Mass., USA), purified by MicroSpin columns.

ATP determination. The myocytes were washed eight times with PBS, collected in hot (90°C) double-distilled water and boiled for 10 min. The resulting lysate was taken for ATP determination by the luciferine-luciferase method [18] using an LKB-Wallac luminator (Bromma, Sweden).

Glucose determination. Glucose in aliquots of incubation media was determined by the glucose oxidase method using a Beckman Glucose Analyzer (Fullerton, Calif., USA).

Statistical analyses were performed by Student's *t*-test. Results are expressed as mean $\pm$ SEM. Absence of bars in figures indicates that the SEM is smaller than the symbol.

Results

Effect of dGlc on hexose transport in L8 myocytes. The effect of dGlc was investigated in upregulated L8 myocytes (preincubated for 20 h with DMEM containing 2.0 mmol/l glucose). The cells were washed and received glucose-free DMEM supplemented with 2.0 mmol/l dGlc or 20.0 mmol/l glucose, and the rate of [³H]dGlc uptake was determined at the indicated times. Shortly after its introduction dGlc initiated a downregulatory process of hexose transport, with a half maximal effect at 2.2 h (Fig. 1). This dGlc-induced downregulation was similar to the effect induced by 20.0 mmol/l glucose. The effect of dGlc was specific: while both 20.0 mmol/l glucose and 2.0 mmol/l dGlc elicited downregulation of hexose uptake, neither affected the uptake of AIB in L8 myocytes (Table 1).

Fig. 1. Effect of dGlc on hexose transport. L8 myocytes were preincubated in DMEM containing 2.0 mmol/l glucose for 20 h, cells were then washed and received fresh DMEM containing 2.0 mmol/l glucose (□), 20.0 mmol/l glucose (○), or 2.0 mmol/l dGlc (●). At the indicated times the cells were washed extensively and the uptake of [³H]dGlc was determined as described under Experimental Procedures. Mean $\pm$ SEM, *n* = 3

Table 1. Effect of dGlc on AIB uptake

Treatment	dGlc uptake pmol · 10 ⁶ cells ⁻¹ · min ⁻¹	AIB uptake pmol · 10 ⁶ cells ⁻¹ · min ⁻¹
Control	126.0 $\pm$ 6.7	34.6 $\pm$ 4.2
→ 20.0 mmol/l glucose	77.8 $\pm$ 2.3 ^a	35.8 $\pm$ 4.2
→ 2.0 mmol/l dGlc	68.6 $\pm$ 3.1 ^a	30.2 $\pm$ 3.8

L8 myocytes were incubated for 20 h at 2.0 mmol/l glucose (control), then washed and switched to 20.0 mmol/l glucose or 2.0 mmol/l dGlc for additional 5 h. [³H]dGlc and [³H]AIB uptake assays were performed as described in Methods. Mean $\pm$ SEM, *n* = 3. ^a *p* < 0.05 compared to control

To determine whether the downregulatory effect of dGlc was restricted to the transport mechanism or combined with changes in the hexokinase reaction we compared the uptake rates of [³H]dGlc and [³H]MeGlc in myocytes treated with dGlc or high glucose (Table 2). The uptake rates of both glucose analogues were reduced in a comparable manner, indicating that both agents modulated the transport step rather than the hexokinase reaction.

The effect of dGlc was dose-dependent (Fig. 2): cells maintained for 20 h at 2.0 or 20.0 mmol/l glucose received fresh medium containing increasing concentrations of dGlc for 5 h; upregulated myocytes (pretreated with 2.0 mmol/l glucose) reduced the rate of hexose transport dose-dependently up to 7.0 mmol/l dGlc, while at higher dGlc concentrations an escape from the downregulatory response was observed. In downregulated myocytes (pretreated with 20.0 mmol/l glucose) the rate of hexose transport remained low with dGlc up to 7 mmol/l, while higher dGlc concentrations increased the rate of hexose transport.

In contrast to the reversible effect of glucose, the dGlc-induced downregulation of hexose transport

Table 2. Rates of [3 H]dGlc and [3 H]MeGlc uptake

Treatment	MeGlc uptake		dGlc uptake	
	pmol $\cdot$ 10 ⁶ cells ⁻¹ $\cdot$ 10 s ⁻¹	(%)	pmol $\cdot$ 10 ⁶ cells ⁻¹ $\cdot$ min ⁻¹	(%)
Control	15.1 $\pm$ 1.4	100	202.6 $\pm$ 2.7	100
→ 2.0 mmol/l glucose	14.1 $\pm$ 1.0	93.4 $\pm$ 6.6	194.4 $\pm$ 34.6	96.0 $\pm$ 17.1
→ 20.0 mmol/l glucose	4.7 $\pm$ 0.4 ^a	31.1 $\pm$ 2.6	76.5 $\pm$ 20.1 ^a	37.8 $\pm$ 9.9
→ 2.0 mmol/l dGlc	4.4 $\pm$ 0.5 ^a	29.1 $\pm$ 3.3	97.3 $\pm$ 8.6 ^a	48.0 $\pm$ 4.2

L8 myocytes were incubated with DMEM supplemented with 2.0 mmol/l glucose for 20 h (control). The cells were then washed and received fresh medium with the indicated hexose

additions and incubated for 5 h, washed again and taken for the standard [3 H]dGlc or [3 H]MeGlc uptake assays as described in Methods. Mean $\pm$ SEM, $n = 3$. ^a $p < 0.05$ compared to control

Fig. 2. dGlc dose-response curve. L8 myocytes were preincubated for 20 h with DMEM containing 2.0 (●) or 20.0 mmol/l glucose (○), then washed and incubated with fresh DMEM containing increasing concentrations of dGlc (0.5–20.0 mmol/l) for additional 5 h, after which the cells were washed extensively and the rate of [3 H]dGlc was measured. Mean $\pm$ SEM, $n = 3$

was long-lasting. Figure 3A shows that exposure of the cells to 20.0 mmol/l glucose for 2 h resulted in 21% reduction in the rate of hexose transport; change of the incubate to 2.0 mmol/l glucose at this time restored the rate of uptake to the initial level in a typical upregulatory response. However, washing the cells 30 min or 2 h after exposure to 2.0 mmol/l dGlc, and maintaining them at 2.0 mmol/l glucose, did not prevent the progression of the downregulatory response, which reached a similar low level of uptake as cultures exposed continuously to 2.0 mmol/l dGlc (Fig. 3B). These data suggest that the downregulatory response initiated by dGlc is dependent upon the accumulation of a long-lasting intracellular signal.

Relationship between intracellular content of 2-deoxy-D-glucose 6-phosphate (dGlc-6-P) and downregulation of hexose transport. Since incubation of myocytes with dGlc results in intracellular accumulation of dGlc-6-P, we measured its content and correlated it to the degree of downregulation of transport. Myocytes were exposed continuously to 2.0 mmol/l dGlc, and the rate of hexose uptake and the cellular dGlc-6-P content were determined at various times up to

Fig. 3A, B. dGlc-induced prolonged downregulation of hexose transport. (A) L8 myocytes were preincubated for 20 h with DMEM supplemented with 2.0 mmol/l glucose, then washed and incubated for 5 h with fresh DMEM containing 20.0 mmol/l glucose (●). At 2 h, some of the cultures were transferred to 2.0 mmol/l glucose (○). (B) After preincubation with 2.0 mmol/l glucose the cells received 2.0 mmol/l dGlc (■). In some of the culture plates, the medium was changed to 2.0 mmol/l glucose after 30 min (□) or 2 h (△). The uptake of [3 H]dGlc was measured at the indicated times. Arrows indicate the time the medium was changed to 2.0 mmol/l glucose. Mean $\pm$ SEM, $n = 3$

5 h. Figure 4 shows a curvilinear relationship between the cellular content of dGlc-6-P and the magnitude of the reduction in hexose uptake. Accumulation of ~ 5 and ~ 20 nmol dGlc-6-P/10⁶ cells was sufficient to induce half-maximal and maximal downregulatory effects, respectively.

Further evidence for the role of intracellular dGlc-6-P in initiating and maintaining downregulation of

Fig 4. Relationship between intracellular dGlc-6-P and downregulation of hexose transport. L8 myocytes were preincubated for 20 h with DMEM containing 2.0 mmol/l glucose. The cells were washed and received fresh medium with 2.0 mmol/l dGlc. Intracellular dGlc-6-P and $[^3\text{H}]\text{dGlc}$ uptake were measured at 20 and 40 min and at 1.0, 1.5, 2.0, 2.5 and 5.0 h. Uptake results are expressed as percentage of the downregulation from time zero ($93.9 \pm 4.7 \text{ pmol dGlc} \cdot 10^6 \text{ cells}^{-1} \cdot \text{min}^{-1}$). Mean $\pm$ SEM, $n = 3$. Horizontal and vertical bars give the SEM of dGlc-6-P accumulation and of downregulation of transport, respectively

Fig 5. Effect of cell permeabilisation on up- and downregulation of hexose transport. L8 myocytes were preincubated for 20 h with DMEM containing 2.0 or 20.0 mmol/l glucose. Some of the plates were then washed and permeabilised (open symbols), while the rest were washed in a saponin-free permeabilization buffer (closed symbols). Cells that were preincubated with 2.0 mmol/l glucose received 20.0 mmol/l glucose (●, ○); those preincubated with 20.0 mmol/l glucose received 2.0 mmol/l glucose (▲, △). The uptake of $[^3\text{H}]\text{dGlc}$ was measured at the indicated times. Mean $\pm$ SEM, $n = 3$

hexose transport comes from premeabilisation experiments. Figure 5 shows control experiments in which cell permeabilisation did not interfere with the course of up- or downregulation of hexose transport. Therefore, this method was suitable to study the effect of dGlc-6-P efflux from dGlc-treated cells. Figure 6A shows a rapid 80% depletion of cell-associated dGlc-6-P following permeabilisation. Control cells that were treated in parallel with saponin-free

Fig 6A, B. Effect of cell permeabilisation on intracellular content of dGlc-6-P and dGlc-induced downregulation of hexose transport. (A) L8 myocytes preincubated in DMEM containing 2.0 mmol/l glucose for 20 h were washed and incubated with 2.0 mmol/l dGlc and $2.5 \mu\text{Ci } [^3\text{H}]\text{dGlc}$ for 1 h. The plates were then treated with permeabilisation buffer without (□) or with (■) saponin. The intracellular content of labelled dGlc was determined at the indicated times, following washes and solubilisation of the cells. (B) L8 myocytes were preincubated in DMEM containing 2.0 mmol/l glucose for 20 h. Some cells were then washed and received DMEM containing 2.0 mmol/l glucose (○). Other cells were washed and incubated with 2.0 mmol/l dGlc (●). After 1 h some of these cells were treated with permeabilisation buffer without (□) or with (■) saponin, and incubated further with DMEM containing 2.0 mmol/l glucose. The uptake of $[^3\text{H}]\text{dGlc}$ was measured at the indicated times. Mean $\pm$ SEM, $n = 3$

buffer lost the phosphorylated metabolite at a slower rate, probably due to the activity of intracellular phosphatase(s). The data depicted in Figure 6B show that the depletion of dGlc-6-P following treatment with 2.0 mmol/l dGlc and permeabilisation reversed the downregulatory response to dGlc, and returned the rate of hexose transport to the level typical for incubation with low glucose. Moreover, control cells, pretreated with 2.0 mmol/l dGlc and then treated with saponin-free buffer, continued the downregulation process.

Effect of dGlc on myocyte ATP content. Phosphorylation of dGlc consumes ATP which by itself could affect cellular functions as hexose transport. Figure 7 shows that myocyte ATP was indeed reduced

Fig. 7. Effect of dGlc on ATP content. Myocytes were preincubated for 20 h with DMEM containing 2.0 mmol/l glucose, washed and incubated for additional 5 h with fresh medium containing increasing dGlc concentrations. At the end of the incubation the cells were taken for [3 H]dGlc uptake assay (●) and total cellular ATP determination (○) as described under Experimental Procedures. Mean $\pm$ SEM, $n = 3$

under the experimental conditions described for Figure 2; however, there was no clear correlation between reduction in cell ATP and the degree of transport downregulation. Thus, a significant downregulatory effect was observed with 0.5 mmol/l dGlc without significant changes in ATP. Severe ATP depletion induced by 20.0 mmol/l dGlc was not accompanied by a downregulatory response; on the contrary, the cells responded by increasing the transport. To investigate the role of ATP depletion alone we studied the effect of the uncoupler DNP. Table 3 shows that 0.25 mmol/l DNP abolished the downregulation of the uptake induced by 20.0 mmol/l glucose, and augmented the upregulation induced by 2.0 mmol/l glucose. In parallel, the ATP content was reduced by 24.9 and 28.1%. Thus, reduction in cell ATP content is associated with upregulation rather than downregulation of hexose transport. These results therefore negate a role for ATP depletion in dGlc-induced downregulation of hexose uptake. Furthermore, pronounced ATP depletion ($> 70\%$)

overrode the downregulatory effect of dGlc, as observed in myocytes incubated with 20.0 mmol/l dGlc for 5 h (Figs. 2 and 5). Therefore, the observed dGlc-induced reduction of hexose transport may be an underestimate of the true downregulatory effect of the analogue, partially opposed by the upregulatory effect of ATP depletion.

Kinetic analysis of dGlc-induced downregulation of hexose transport. Table 4 summarises the kinetic parameters derived from experiments in which uptake data of up- and downregulated myocytes following treatments with 2.0 mmol/l dGlc were analysed according to Lineweaver and Burk [19]. dGlc treatment reduced the maximal velocity ($V_{\max}$) of the transport system without significant effect on the affinity (K_m) for dGlc.

Effect of dGlc on plasma membrane GLUT1 in L8 myocytes. To investigate further the nature of the dGlc effect we determined the plasma membrane content of GLUT1, the major glucose transporter of these cells, using cell surface biotinylation in intact cells. Figure 8 shows the results of a representative experiment: surface GLUT1 content in cells incubated for 20 h at 20.0 mmol/l glucose was 45% of that observed in cells incubated with 2.0 mmol/l glucose; also hexose uptake was reduced by $\sim 50\%$. When myocytes preincubated at 2.0 mmol/l glucose were treated with 2.0 mmol/l dGlc or 20.0 mmol/l glucose for additional 5 h, cell surface GLUT1 was decreased by 72 and 70%, respectively, while the rate of hexose transport was reduced by 73 and 67%. The total cell content of GLUT1, determined by Western blotting of crude membranes (containing both plasma- and internal membranes), was not affected by the above treatments (data not shown).

Effect of dGlc on GLUT1 mRNA in L8 myocytes. High glucose concentrations not only downregulate hexose transport, but also reduce the content of GLUT1 mRNA in L8 myocytes [5]. Figure 9 shows

Table 3. Effect of DNP on hexose uptake and ATP content

Treatment	dGlc uptake		ATP content	
	pmol $\cdot 10^6$ cells $^{-1} \cdot \text{min}^{-1}$	(%)	pmol/10 6 cells	(%)
2.0 mmol/l glucose (20 h)	150.1 $\pm$ 4.7	100	475.0 $\pm$ 24.0	100
→ 20.0 mmol/l glucose	99.4 $\pm$ 6.5 ^a	66.2 $\pm$ 4.3	439.9 $\pm$ 29.3	92.6 $\pm$ 6.2
→ 20.0 mmol/l glucose + DNP	139.3 $\pm$ 10.5 ^b	92.8 $\pm$ 7.0	356.6 $\pm$ 5.8 ^{a,b}	75.1 $\pm$ 1.2
20.0 mmol/l glucose (20 h)	80.2 $\pm$ 6.2	100	440.2 $\pm$ 30.2	100
→ 2.0 mmol/l glucose	136.6 $\pm$ 1.9 ^a	170.3 $\pm$ 2.4	473.6 $\pm$ 24.4	107.6 $\pm$ 5.5
→ 2.0 mmol/l glucose + DNP	160.6 $\pm$ 1.0 ^{a,b}	200.2 $\pm$ 1.2	316.6 $\pm$ 3.6 ^{a,b}	71.9 $\pm$ 0.2

L8 myocytes were incubated for 20 h with 2.0 or 20.0 mmol/l glucose, then washed and incubated for additional 5 h with DMEM containing the opposite glucose concentration with or without 0.25 mmol/l DNP, and taken for [3 H]dGlc uptake

assay and determination of ATP content as described in Methods. Mean $\pm$ SEM ($n = 3$). ^a $p < 0.05$ compared to control; ^b significantly different from the value obtained for the incubation with 20.0 mmol/l glucose

Table 4. Kinetic analysis of the effect of dGlc

Treatment	K_m (mmol/l)	V_{max} (nmol dGlc · 10 ⁶ cells ⁻¹ · min ⁻¹)
2.0 mmol/l glucose	1.68 ± 0.12	4.16 ± 0.43
2.0 mmol/l glucose → dGlc	1.88 ± 0.09	2.26 ± 0.19 ^a
20.0 mmol/l glucose	1.50 ± 0.21	2.31 ± 0.47 ^a
20.0 mmol/l glucose → dGlc	1.71 ± 0.59	1.95 ± 0.85 ^a

L8 myocytes were incubated with DMEM supplemented with 2.0 or 20.0 mmol/l glucose for 20 h. The cells were then washed and received fresh DMEM containing the same glucose concentration or 2.0 mmol/l dGlc, and incubated for additional 5 h. The kinetic parameters of dGlc uptake were calculated from dose-response experiments according to Lineweaver and Burk [19]. Mean ± SEM, $n = 3$ to 6 experiments. ^a $p < 0.05$, significantly different from the 2.0 mmol/l glucose control

Fig. 8. Effect of dGlc on myocyte plasma membrane GLUT1. L8 myocytes were incubated for 20 h in 2.0 mmol/l (L) or 20 mmol/l glucose (H). The cultures then received fresh medium containing the same glucose concentrations, and some cultures preincubated with 2 mmol/l glucose received fresh medium containing either 20 mmol/l glucose (L > H) or 2.0 mmol/l dGlc (L > dG). After additional 5 h incubation the cells were biotinylated and processed as described under Experimental Procedures. Samples (5 µg/lane) of biotinylated proteins were resolved electrophoretically and GLUT1 protein was visualized with anti-GLUT1 (N-terminus) polyclonal antibody. B denotes control of crude membranes prepared from rat brain

Fig. 9. Effect of dGlc on myocyte GLUT-1 mRNA. L8 myocytes were preincubated with 2.0 mmol/l glucose for 20 h (L). The cells were then washed and incubated for additional 5 h with fresh medium containing 20.0 mmol/l glucose (L > H) or 2.0 mmol/l dGlc (L > dG). Total RNA was extracted from the cells, separated electrophoretically and hybridised with rat GLUT1 and α_1 tubulin probes as described under Experimental Procedures

that pre-exposure of the cells to 2.0 mmol/l glucose for 20 h and incubation at 20.0 mmol/l glucose for additional 5 h reduced the GLUT1 mRNA content by 54%. In contrast, the mRNA level following 5 h exposure of the cells to 2.0 mmol/l dGlc was 137% of that observed in control cells.

Discussion

Results from our [1–5, 20, 21] and other laboratories [9] over recent years convincingly demonstrated that extracellular glucose is an important feed-back regulator of its transport through the cell membrane. Glucose transport in skeletal muscle is the limiting step for hexose metabolism in toto, therefore regulation of this step by glucose can be expected to result in the modulation of all aspects of cellular glucose metabolism. Glucose concentrations of the magnitude often seen in diabetic patients are capable of substantially reducing the muscle glucose transport and utilization [3, 8]. Indeed, hyperglycaemia per se has been proposed to be one of the causes of reduced peripheral glucose disposal in diabetic patients [10–12, 22]. However, the mechanism by which glucose effects this action is not clear.

Extracellular glucose produces a signal which affects the subcellular distribution of glucose transporters, thus influencing their content in the plasma membrane of the myocyte [1, 6, 23]. The intracellular signal(s) involved in this autoregulatory response is not known. L-glucose was ineffective [2]; evidently interaction with the glucose transporter is necessary. It was therefore deemed suitable to investigate whether transport alone, transport and phosphorylation at position 6, or further metabolism are necessary to elicit the downregulatory signal of glucose. The glucose analogue dGlc is transported into the cells by the hexose transport system, interacts with hexokinase, but is not metabolized beyond the dGlc-6-P step. This study shows that low dGlc concentrations can mimic the downregulatory action of high glucose levels.

Deoxyglucose-6-P, like glucose-6-P, can inhibit the hexokinase activity. However, the fact that the rate of hexose uptake, measured with both [³H]dGlc and [³H]MeGlc, was similarly reduced by high glucose or dGlc treatments indicates that the transport mechanism and not the hexokinase reaction in L8 myocytes is the target of the autoregulatory signal. GLUT1 is the main glucose transporter of L8 myocytes [1, 5]. Direct measurement of GLUT1 content in the plasma membrane of the myocytes demonstrated that dGlc, like high glucose concentrations, reduced the density of transporters. The cell surface biotinylation technique used to measure the cell surface content of glucose transporters was chosen because it is simple and not complicated with low yields and cross-contamination of enriched-membrane preparations obtained by the alternative method of subcellular fractionation. The data obtained by this procedure showed a good correlation between the content of GLUT1 in the plasma membrane of the myocytes and the reduction in hexose transport capacity, induced by 2.0 mmol/l dGlc or 20.0 mmol/l glucose. The total content of GLUT1 transporters was not changed during relatively short exposures to dGlc or

high glucose concentrations (data not shown), indicating that the steady state level of the transporter was not affected.

Deoxyglucose reduces the cell ATP content. However, we could not relate this action to the effect of the analogue on hexose transport. Indeed, other procedures that reduce ATP (hypoxia, anoxia, uncouplers of oxidative phosphorylation) were shown to augment hexose transport and induce translocation of glucose transporters to the plasma membrane in a variety of cells and tissues [24]. Also in the present study the uncoupler DNP, while lowering the cell ATP level, stimulated the hexose transport in L8 myocytes. Therefore, the effect of low concentrations of dGlc on the hexose transporter is independent of its ATP consuming effect.

The effect of dGlc seems to be mediated by its major metabolite, dGlc-6-P. A short exposure to low dGlc concentrations was sufficient to initiate and drive to completion the downregulation of hexose transport in myocytes. The time required for half maximal downregulatory effect of dGlc was inversely related to the time of exposure (data not shown). A curvilinear relationship existed between intracellular dGlc-6-P concentration and the degree of downregulation of hexose transport. Furthermore, permeabilisation of the dGlc-treated cells, resulting in loss of more than 80 % of cell dGlc-6-P, reversed the downregulatory process. In control cells dGlc-6-P disappeared at a slow rate, probably as a result of phosphatase activity; however, the intracellular dGlc-6-P concentration remained significantly high to sustain the downregulatory mechanism. Control experiments indicated that the mild saponin treatment had no significant effect on either up- or downregulation of hexose transport. This confirms a previous study showing that this method did not compromise cell integrity and function [17]. Our findings verify the study by Naftalin and Rist [25] in which preloading rat thymocytes with dGlc (1–10 mmol/l) decreased the rate of MeGlc uptake. Similar results observed by Foley and Huecksteadt [26] in adipocytes were ascribed to the effect of increased intracellular hexose-6-P. Wheeler [27], on the other hand, found no similar effects of glucose-6-P on hexose transport into liposomes from reconstituted adipocyte membrane proteins. Glucose-6-P had no effect on hexose transport activity also in plasma membrane vesicles prepared from skeletal muscles [28]. Taken together, we conclude that the effect of hexose-6-P on glucose transporters requires intact cells, indicating that a mechanism of transporter redistribution in the cells is the target of this glucose metabolite.

The proposed effect of hexose-6-P in initiating downregulation of glucose transport is strongly supported by studies in which the content of glucose-6-P was determined in skeletal muscle biopsies of diabetic patients. Yki-Järvinen et al. [29] reported that

the content of glucose-6-P was 2.3 times higher in biopsies of vastus lateralis muscles of diabetic compared to non-diabetic subjects. Young et al. [30] found an inverse correlation between muscle glucose-6-P and glucose disposal, and suggested that the main control over carbohydrate disposal in muscle occurred after glucose-6-P, as increased insulin-stimulated glucose disposal was associated with a reduction in muscle glucose-6-P. The pool of glucose-6-P is determined by the sum of the rates of glycogen synthesis and glycolysis. Reduced glycogen synthesis in skeletal muscles of diabetic patients may augment the glucose-6-P levels [31, 32]. Rothman et al. [33], on the other hand, using ^{31}P nuclear magnetic resonance spectroscopy, found lower glucose-6-P levels in muscles of diabetic patients compared to control subjects under hyperglycaemic-hyperinsulinaemic clamps. In this context it is important to note the early studies of Sims and Landau [34] and Dully et al. [35] which suggested distinctive insulin-responsive and insulin non-responsive pools of glucose-6-P in skeletal muscles. Our results also indicate that ~ 20 % of cell-associated dGlc-6-P could not be mobilised following saponin treatment. Considering these early studies and the present observations it seems important to determine the steady state levels and compartmentalisation of glucose-6-P pools and their relation to glucose transport and utilization in skeletal muscles of normal and diabetic patients. In our study, relatively high intracellular levels of dGlc-6-P were required to induce maximal downregulation of hexose transport and reduction of cell surface GLUT1. This may be due to a lower affinity of the signalling system to dGlc-6-P. This correlates well with a three-orders of magnitude lower affinity of this compound to glucose-6-P dehydrogenase compared to that of the natural substrate, glucose-6-P [36, 37]. Thus, dGlc-6-P may mimic the effects of glucose-6-P, albeit at higher concentrations.

The role ascribed here to phosphorylated glucose or dGlc in cellular signalling and transduction, coupled to subcellular localization of GLUT1, is not unusual. For instance, mannose-6-P is a high affinity ligand for the insulin-like growth factor II/mannose-6-P receptor and the cation-dependent mannose-6-P receptor. Both receptors regulate the transport of lysosomal enzymes from the trans-Golgi to a prelysosomal compartment and the cell surface [38–40]. Further work will have the challenge of identifying the structures that interact with hexose-6-P and control the cellular location of glucose transporters.

Glucose has been shown to regulate the expression and steady-state level of GLUT1 mRNA in several types of cells [9]. We have shown before that glucose reduces the content of GLUT1 mRNA in L8 myocytes in a dose- and time-dependent manner [5]. The observation that, unlike glucose, dGlc had no downregulatory effect on GLUT1 mRNA level in

myocytes suggests that glucose operates two distinct regulatory systems in muscle. One, shared by dGlc, affects the subcellular distribution of GLUT1 and consequently the rate of hexose uptake, while the second reduces the content of GLUT1 mRNA, probably at the transcription level [5]. The latter is apparently induced by a signal generated downstream of the hexokinase reaction during glucose metabolism.

Acknowledgements. We thank Ms. A. Davarashvili for excellent technical help. This study was supported by grants from The Israel Ministry of Health, and a donation by Mr. and Mrs. Jack Sasson. Efrat Wertheimer was a Picciotto Doctoral Fellow.

References

- Greco-Perotto R, Wertheimer E, Jeanrenaud B, Cerasi E, Sasson S (1992) Glucose regulates its transport in L8 myocytes by modulating cellular trafficking of the transporter GLUT-1. *Biochem J* 286: 157–163
- Sasson S, Cerasi E (1986) Substrate regulation of glucose transport system in rat skeletal muscle: characterization and kinetic analysis in isolated soleus muscle and skeletal muscle cells in culture. *J Biol Chem* 261: 16827–16833
- Sasson S, Edelson D, Cerasi E (1987) In vitro autoregulation of glucose utilization in rat soleus muscle. *Diabetes* 36: 1041–1046
- Wertheimer E, Sasson S, Cerasi E (1990) Regulation of hexose transport in L8 myocytes by glucose: possible sites of interaction. *J Cell Physiol* 143: 330–336
- Wertheimer E, Sasson S, Cerasi E, Ben-Neriah Y (1991) The ubiquitous glucose transporter GLUT-1 belongs to the glucose-regulated family of stress-inducible proteins. *Proc Natl Acad Sci (USA)* 88: 2525–2529
- Walker PS, Ramlal T, Sarabia V, et al. (1990) Glucose transport activity in L6 muscle cells is regulated by the coordinate control of subcellular glucose transporter distribution, biosynthesis, and mRNA transcription. *J Biol Chem* 265: 1516–1523
- El-Kabbi IM, Roser S, Pollet RJ, Cushman SW, Wilson CM (1994) Regulation of GLUT1 glucose transporter in cultured myocytes: total number and subcellular distribution as determined by photoaffinity labeling. *Biochem J* 30: 35–40
- Zierath JR, Galuska D, Nolte LA, Thorne A, Smedegaard-Kristensen J, Wallberg-Henriksson H (1994) Effects of glycaemia on glucose transport in isolated skeletal muscle from patients with NIDDM: in vitro reversal of muscular insulin resistance. *Diabetologia* 37: 270–277
- Klip A, Tsakiridis T, Marette A, Ortiz PA (1994) Regulation of expression of glucose transporters by glucose: a review of studies in vivo and in cell cultures. *FASEB J* 8: 43–53
- Cerasi E, Glaser B, Del-Rio G, Sasson S, Della-Casa L (1989) The clinical significance of insulin resistance in NIDDM: studies with continuous subcutaneous insulin infusion. In: Alberti KGMM, Mazze R (eds) *Frontiers of diabetes research: current trends in non-insulin-dependent diabetes mellitus*. Elsevier Science Publishers, New York, pp 309–320
- Rossetti L, Giaccari A, DeFronzo RA (1990) Glucose toxicity. *Diabetes Care* 13: 610–630
- Yki-Järvinen H (1990) Acute and chronic effects of hyperglycaemia on glucose metabolism. *Diabetologia* 33: 579–585
- Tsuboi KK, Petricciani JC (1975) Concentrative accumulation (active transport) of 2-deoxy-D-glucose in primate fibroblasts. *Biochem Biophys Res Commun* 62: 587–593
- Shetty M, Loeb JN, Vikstrom K, Beigi FI (1993) Rapid activation of Glut-1 glucose transporter following inhibition of oxidative phosphorylation in Clone 9 cells. *J Biol Chem* 268: 17225–17232
- Levy-Toledano R, Caro R, Hindman LH, Taylor SI (1993) Streptavidin blotting: a sensitive technique to study cell surface proteins: application to investigate autophosphorylation and endocytosis of biotin-labeled insulin receptors. *Endocrinology* 133: 1803–1808
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685
- Johnson JA, Gray MO, Karliner JS, Chen C-H, Mochly-Rosen D (1996) An improved permeabilisation protocol for the introduction of peptides into cardiac myocytes: application to protein kinase C research. *Circulation Res* (in press)
- Ludin A, Baltscheffsky M (1978) Determination of creatine kinase isoenzymes in human serum by an immunological method using purified firefly luciferase. In: DeLuca M (ed) *Methods in enzymology*, Vol. 57. Academic Press, New York, pp 56–72
- Segal HS (1975) *Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems*. John Wiley & Sons, New York
- Kaiser N, Sasson S, Peener EP, et al. (1993) Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes* 42: 80–89
- Sasson S, Gorowitz N, Joost JH, King GL, Cerasi E, Kaiser N (1996) Regulation by metformin of hexose transport system in vascular endothelial and smooth muscle cells. *Br J Pharmacol* 117: 1318–1324
- Efendic S, Luft R, Wajngot A (1984) Aspects of the pathogenesis of type 2 diabetes. *Endocrine Rev* 5: 395–410
- Koivisto U-M, Martinez-Valdez H, Bilan PJ, Burdett E, Ramlal T, Klip A (1991) Differential regulation of GLUT-1 and GLUT-4 glucose transport systems by glucose and insulin in L6 muscle cells in culture. *J Biol Chem* 266: 2615–2621
- Wheeler TJ (1988) Translocation of glucose transporters in response to anoxia in heart. *J Biol Chem* 263: 19447–19454
- Naftalin RJ, Rist RJ (1990) Effects of phorbol, dexamethasone and starvation on 3-O-methyl-D-glucose by rat thymocytes. *Biochem J* 265: 251–259
- Foley JE, Huecksteadt TP (1984) Glucose-6-phosphate effects on deoxyglucose, glucose and methylglucose transport in rat adipocytes. *Biochim Biophys Acta* 805: 313–316
- Wheeler TJ (1989) Effects of three proposed inhibitors of adipocyte glucose transporter on the reconstituted transporter. *Biochim Biophys Acta* 979: 331–340
- Kristiansen S, Wojtaszewski JF, Juel C, Richter EA (1994) Effect of glucose-6-phosphate and pH on glucose transport in skeletal muscle plasma membrane giant vesicles. *Acta Physiol Scand* 150: 227–233
- Yki-Järvinen H, Sahlin K, Ren JM, Koivisto VA (1990) Localization of rate-limiting defect for glucose disposal in skeletal muscle of insulin-resistant type I diabetic patients. *Diabetes* 39: 157–167
- Young AA, Bogardus C, Stone K, Mott DM (1988) Insulin response of components of whole-body and muscle carbohydrate metabolism in humans. *Am J Physiol* 254: E231–E236

31. Thorburn AW, Gumbiner B, Bulacan F, Brechtel G, Henry RR (1991) Multiple defects in muscle glycogen synthase activity contribute to reduced glycogen synthesis in non-insulin dependent diabetes mellitus. *J Clin Invest* 87: 489–495
32. Shulman GI, Rothman DL, Jue T, Stein RA, DeFronzo RA, Shulman RG (1990) Quantitation of muscle glycogen synthesis in normal subjects and subjects with noninsulin-dependent diabetes by ^{13}C nuclear magnetic resonance spectroscopy. *N Engl J Med* 322: 223–228
33. Rothmann DL, Shulman RG, Shulman GI (1992) ^{31}P nuclear magnetic resonance measurements of muscle glucose-6-phosphate. Evidence for reduced insulin-dependent muscle glucose transport or phosphorylation activity in non-insulin-dependent diabetes mellitus. *J Clin Invest* 89: 1069–1075
34. Sims EA, Landau BR (1966) Insulin responsive and insulin nonresponsive pools of glucose-6-phosphate in diaphragmatic muscle. *Fed Proc* 25: 835–839
35. Dully CC, Bocek RM, Beaty CH (1969) Presence of two or more glucose-6-phosphate pools in voluntary skeletal muscle and their sensitivity to insulin. *Endocrinology* 84: 855–860
36. Sasson S, Oron R, Cerasi E (1993) Enzymatic assay of 2-deoxyglucose 6-phosphate for assessing hexose uptake rates in cultured cells. *Anal Biochem* 215: 309–311
37. Manchester JK, Chi M, Carter M-Y, Pusateri ME, McDougal DB, Lowry OH (1990) Measurement of 2-deoxyglucose and 2-deoxyglucose-6-phosphate in tissues. *Anal Biochem* 185: 118–124
38. Kornfeld S, Mellman I (1989) The pathogenesis of lysosomes. *Annu Rev Cell Biol* 5: 483–525
39. Stein M, Zijderhand-Bleekemolen JE, Geuze H, Hasilik A, von Figura K (1987) Mr 46,000 mannose 6-phosphate specific receptor: its role in targeting of lysosomal enzymes. *EMBO J* 6: 2677–2681
40. Szebenyi G, Rotwein P (1991) Differential regulation of mannose-6-phosphate receptors and their ligands during the myogenic development of C2 cells. *J Biol Chem* 266: 5534–5539