Unique mechanism of GLUT3 glucose transporter regulation by prolonged energy demand: increased protein half-life

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L6 muscle cells survive long-term (18 h) disruption of oxidative phosphorylation by the mitochondrial uncoupler 2,4-dinitrophenol (DNP) because, in response to this metabolic stress, they increase their rate of glucose transport. This response is associated with an elevation of the protein content of glucose transporter isoforms GLUT3 and GLUT1, but not GLUT4. Previously we have reported that the rise in GLUT1 expression is likely to be a result of *de novo* biosynthesis of the transporter, since the uncoupler increases GLUT1 mRNA levels. Unlike GLUT1, very little is known about how interfering with mitochondrial ATP production regulates GLUT3 protein expression. Here we examine the mechanisms employed by DNP to increase GLUT3 protein content and glucose uptake in L6 muscle cells. We report that, in contrast with GLUT1, continuous exposure to DNP had no effect on GLUT3 mRNA levels. DNP-stimulated glucose transport was unaffected by the protein-synthesis inhibitor cycloheximide. The increase in GLUT3 protein mediated by DNP was also insensitive to cycloheximide, paralleling the response of glucose uptake, whereas the rise in GLUT1 protein levels was blocked by the inhibitor. The GLUT3 glucose transporter may therefore provide the majority of the glucose transport stimulation by DNP, despite elevated levels of GLUT1 protein. The half-lives of GLUT3 and GLUT1 proteins in L6 myotubes were determined to be about 15 h and 6 h respectively. DNP prolonged the half-life of both proteins. After 24 h of DNP treatment, 88 % of GLUT3 protein and 57 % of GLUT1 protein had not turned over, compared with 25 % in untreated cells. We conclude that the long-term stimulation of glucose transport by DNP arises from an elevation of GLUT3 protein content associated with an increase in GLUT3 protein half-life. These findings suggest that disruption of the oxidative chain of L6 muscle cells leads to an adaptive response of glucose transport that is distinct from the insulin response, involving specific glucose transporter isoforms that are regulated by different mechanisms.

INTRODUCTION

Facilitative glucose transport across the plasma membrane is fundamental to mammalian energy metabolism and is mediated by tissue-specific glucose transporter (GLUT) proteins. In cells such as muscle, the transport of glucose across the plasma membrane is rate-limiting for its utilization [1,2]. To date, seven glucose transporter genes have been identified, designated *GLUT1* to *GLUT7* [3]. Of these, three isotypes (GLUT1, GLUT3 and GLUT4) are expressed in rat L6 skeletal muscle cells [4,5], a cell line that retains many properties of skeletal muscle [6,7]. In mammalian tissues, GLUT1 is ubiquitously expressed and is thought to provide basal cellular glucose uptake because of its significant localization at the plasma membrane of many cell types [8]. GLUT4 is expressed in insulin-responsive tissues such as fat, and skeletal and cardiac muscle, where it responds to an acute insulin challenge by translocating rapidly from an intracellular-membrane storage site to the plasma membrane [9,10]. GLUT3 is principally important in neurons and in developing muscle [11,12]. In mammals, GLUT3 expression is greatest in the brain, placenta and platelets [11,13,14]. In humans, GLUT3 may play a role in fetal skeletal muscle glucose transport, since GLUT3 mRNA is present at this stage of muscle development [11]. In culture, GLUT3 is expressed only in the L6 muscle and UMR 106-01 osteosarcoma cell lines [15]. L6 cells allow for the identification of the particular role of this transporter in comparison with those of the well characterized GLUT1 and GLUT4 transporters.

Many mammalian cells have the capacity to adapt to situations of high energy demand by increasing their rate of glucose uptake. Such situations include environmental stress and metabolic challenges to the oxidative chain, such as exercise, hypoxia and mitochondrial inhibition. 2,4-Dinitrophenol (DNP) is an uncoupler of oxidative phosphorylation which rapidly decreases cellular ATP levels [16]. However, cells adapt to this and other stressors by elevating glucose transport in order to increase the anaerobic production of ATP [17]. The anabolic hormone insulin is a physiological stimulator of glucose transport. The increase in glucose uptake induced by energy stressors and insulin in L6 muscle cells occurs in two stages. The initial phase is associated with a redistribution of GLUT3, GLUT1 and GLUT4 to the cell surface [18], which reaches a maximum within 15–20 min of stimulation and is maintained for up to 60 min. In the prolonged presence of these activators, renewed effects can be observed as early as 90–120 min, with a further increase in glucose uptake beyond the acute phase. This increase continues for up to 24 h, reaching a maximum of 4-fold, and is known to result from an elevated cell content of GLUT3 and GLUT1 proteins, but not of GLUT4 protein [19,20].

The regulation of glucose transport activity in the adaptive biosynthetic response to environmental signals and hormones is complex, and evidence has accumulated for regulation of glucose transporters at the transcriptional, post-transcriptional and post-translational levels in several tissue culture cell lines [21–24]. For the most part, this phenomenon has been studied extensively for the GLUT1 and GLUT4 isoforms [21,22,25,26]. However, con-

Abbreviations used: GLUT1, erythrocyte glucose transporter; GLUT3, neuronal glucose transporter; GLUT4, insulin-responsive glucose transporter; DNP, 2,4-dinitrophenol; α-MEM, α-minimum essential medium; FBS, fetal bovine serum; $C_{12}E_8$, octaethylene glycol dodecyl ether.

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siderably less is known about the mechanisms that underlie the regulation of GLUT3 biosynthesis [15,23,24,27–29]. Given the importance of GLUT3 in neurons [30] and muscle development [12], it is of interest to characterize mechanisms that regulate its expression.

The present study examined the effects on GLUT3 protein and mRNA levels of prolonged exposure of L6 myotubes to mitochondrial uncoupling in comparison with the effects on GLUT1 expression. The results indicate a unique mechanism of control elicited by metabolic stress that is isoform-specific and, at least for GLUT3, largely post-translational.

EXPERIMENTAL

Materials

Tissue culture medium, methionine-free α-minimum essential medium (α-MEM), serum and other tissue culture reagents were obtained from Life Technologies (Burlington, ON, Canada). Human insulin was kindly donated by Eli Lilly Canada (Toronto, ON, Canada). DNP, cytochalasin B and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Octaethylene glycol dodecyl ether ($C_{12}E_8$) was purchased from Fluka (Ronkonkoma, NY, U.S.A.). Protein A–Sepharose was from Pharmacia Biotechnology Inc. (Uppsala, Sweden). [^{35}S]-Methionine, [γ-^{32}P]dCTP and Amplify were purchased from Amersham Inc. (Oakville, ON, Canada). 2-[^{3}H]Deoxyglucose was obtained from DuPont (Boston, MA, U.S.A.). ^{125}I-Protein A and ^{125}I-labelled sheep anti-mouse IgG were purchased from ICN (Irvine, CA, U.S.A.). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL, U.S.A.). Bradford protein assay reagent was from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Polyclonal antibody against GLUT1 used for immunoprecipitation was generated as previously described [26]. Polyclonal anti-GLUT1 antibody used for immunoblotting was a gift from Dr. R. Reithmeier (Department of Biochemistry, University of Toronto). Polyclonal anti-GLUT3 antibody was generated as described previously [31]. Monoclonal antibody McK1 to the α_1-subunit of the Na^+/K^+-ATPase was a gift from Dr. K. Sweadner (Massachusetts General Hospital, Boston, MA, U.S.A.). Plasmids containing full-length cDNAs for GLUT1 (prGT4-12), GLUT3 (pmGLUT3-6) and glyceraldehyde-3-phosphate dehydrogenase were kindly provided by Dr. M. Birnbaum (Department of Cell Biology, Harvard Medical School, Cambridge, MA, U.S.A.), Dr. C. F. Burant (Department of Medicine, University of Chicago, Chicago, IL, U.S.A.) and Dr. H. Elsholtz (Department of Clinical Biochemistry, University of Toronto) respectively.

Cell culture

L6 muscle cells were maintained in myoblast monolayer culture in α-MEM containing 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) antibiotic/anti-mycotic solution (10000 units/ml penicillin G, 10 mg/ml streptomycin and 25 mg/ml amphotericin B) in an atmosphere of 5 % CO_2 at 37 °C as described previously [32]. Cells were maintained in continuous passage by trypsinization of subconfluent cultures using 0.25 % (w/v) trypsin. Myoblasts were seeded in medium containing 2 % (v/v) FBS at $\approx 4 \times 10^4$ cells/ml in 10 cm-diameter dishes and used at 6–8 days post-seeding for total membrane preparations, RNA isolation and [^{35}S]methionine labelling. L6 cells seeded in 12-well plates were used for glucose uptake experiments. Cells were fed fresh medium every 48 h and used at the stage of myotubes.

2-[^{3}H]Deoxyglucose uptake

After various treatments, myotube monolayers were rinsed twice with glucose-free Hepes-buffered saline solution (140 mM NaCl, 20 mM Hepes/Na, pH 7.4, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$). Glucose uptake was measured as described previously [33] using 10 μM 2-[^{3}H]deoxyglucose (1 μCi/ml) for 5 min at room temperature. Non-specific uptake was determined in the presence of 10 μM cytochalasin B and was subtracted from total uptake. After the uptake period, the radioactive solution was removed and the cells were rinsed three times with cold stop solution (0.9 % NaCl). Cells were lysed in 0.05 M NaOH and cellular radioactivity was quantified by liquid scintillation counting using an LKB 1217 β-radiation counter. Protein content was determined using the Bradford assay [34]. Each condition was assayed in triplicate.

Total membrane preparation and immunoblotting

After the appropriate treatments, total membranes were isolated as described previously [32] and protein content was determined using the bicinchoninic acid assay. Membrane samples (40 μg) were subjected to SDS/PAGE on 10 % gels. Glucose transporter isoforms were detected by immunoblotting as previously described [32], using polyclonal anti-GLUT3 antibody or polyclonal anti-GLUT1 antibody at a dilution of 1:1000, or monoclonal anti-(Na^+/K^+-ATPase α_1-subunit) at 1:500 dilution, in 150 mM NaCl, 50 mM Tris/HCl, 1 % (w/v) BSA, 0.04 % Nonidet P-40 and 0.02 % NaN_3 (pH 7.5) [5]. Glucose transporter proteins were visualized with ^{125}I-Protein A for polyclonal antibodies and with ^{125}I-labelled sheep anti-mouse antibodies followed by Phosphorimager analysis for monoclonal antibody detection.

RNA isolation and Northern blot hybridization

Total RNA was isolated and Northern blots were performed exactly as described previously [19,35].

[^{35}S]Methionine labelling and immunoprecipitation of glucose transporters

Labelling of L6 cells with [^{35}S]methionine and glucose transporter immunoprecipitation was performed essentially according to a previously established procedure [26]. Briefly, L6 myotubes, grown and differentiated in 10 cm-diameter plates, were rinsed twice with PBS, pH 7.4, containing 1 % (v/v) antibiotic/anti-mycotic solution prior to incubation for 2 h in methionine-free α-MEM supplemented with 2 % (v/v) dialysed FBS and 1 % (v/v) antibiotic/anti-mycotic. The medium was removed and myotubes were pulsed for 4 h in fresh methionine-free α-MEM containing 100 μCi of [^{35}S]methionine/plate. The labelling medium was removed and the cells were rinsed twice with PBS. For chasing, complete α-MEM with or without 0.5 mM DNP or 100 nM insulin was added to the labelled cells for 2–48 h, with medium replaced every 12 h to ensure continuous stimulation.

Pulsed-and-chased myotubes were washed twice with cold PBS and lysed in 1.0 ml of 2 % (w/v) $C_{12}E_8$/PBS containing 0.4 mM PMSF, 1 μM leupeptin and 1 μM pepstatin A. The cell lysates were transferred to Eppendorf tubes, vortex-mixed vigorously for 1 min and centrifuged at 12000 ***g*** for 15 min at 4 °C. A portion of the supernatants was used for determination of protein concentration and trichloroacetic acid-precipitable radioactivity, and was stored at −80 °C. A 0.9 ml portion of each supernatant was pre-cleared with 10 μl of rabbit pre-immune

serum for 1 h at 4 °C with gentle mixing, followed by a 2 h incubation with 25 μl of a 50% (v/v) suspension of Protein A–Sepharose in PBS. Immunocomplexes were collected by centrifuging the mixture for 1 min at 12000 ***g*** in a Microfuge, and the supernatant was used for immunoprecipitation of GLUT3 or GLUT1. Antiserum against either GLUT3 or GLUT1 was added, followed by incubation for 4 h at 4 °C. The immune complexes were collected with Protein A–Sepharose beads as described above and were washed once with 1 ml of Wash I (50 mM Hepes, 1 mM EDTA, 150 mM NaCl and 1% Nonidet P-40), once with Wash II (Wash I containing 1.0 M NaCl), twice more with Wash I, and finally once with PBS. The immune complexes were incubated in 30 μl of 2 × Laemmli sample buffer for 30 min at 37 °C and were separated by SDS/PAGE (10% gels). Gels were stained with Coomassie Blue, destained and soaked for 30 min in Amplify®. The polyacrylamide gels were then vacuum dried under heat and suction on to filter paper, and exposed to Phosphorimager analysis.

Statistical analysis

Detection and quantification of ^{125}I and ^{35}S-labelled GLUT isoforms was performed with a Molecular Dynamics Phosphorimager System (Sunnyvale, CA, U.S.A.). Statistical analysis was performed using the ANOVA test (Fisher; multiple comparisons).

RESULTS

Time courses of GLUT3 and GLUT1 induction by DNP

For all experiments described herein, cells were stimulated with a concentration of 0.5 mM DNP, shown previously to maximally stimulate glucose uptake in L6 myotubes [36]. Cell viability was unaffected at this concentration for up to 30 h. The exposure of L6 myotubes to 0.5 mM DNP caused a rapid and progressive increase in glucose uptake that peaked at 8 h and was sustained for up to 24 h (Figure 1A). We have reported previously [20] that incubation with DNP for 18 h elevated the content of GLUT3 and GLUT1 glucose transporters in L6 myotubes. In contrast, no effect on GLUT4 expression was observed [20]. To determine the time course of the induction of GLUT3 and GLUT1, Western blot analysis of the glucose transporter proteins was performed on total membrane fractions from L6 cells incubated for up to 18 h in the presence or absence of 0.5 mM DNP (Figure 1B). Representative immunoblots of GLUT3 and GLUT1 are shown in Figure 1(B), and the results from four experiments are summarized in Figure 1(C). The DNP-mediated increases in GLUT3 and GLUT1 followed similar time courses. There was a slow phase of increase in GLUT3 and GLUT1, followed by steep increases in both isoforms after 6–8 h of treatment with DNP. However, the effect of DNP on GLUT1 at this time was more pronounced than its effect on GLUT3. After 18 h of exposure to DNP, GLUT3 and GLUT1 proteins were elevated by 3- and 5-fold respectively.

GLUT3 and GLUT1 mRNA responses to DNP

It is known that chronic insulin treatment causes an elevation in the levels of GLUT3 and GLUT1 mRNAs in L6 muscle cells, leading to increased expression of the corresponding proteins [16,20,37]. To test whether the same phenomenon occurs with prolonged DNP treatment, Northern blot analysis was performed using total RNA extracted from L6 muscle cells treated for 18 h with or without 0.5 mM DNP. Interestingly, in contrast with the effects of insulin (positive control; Figure 2A), GLUT3 mRNA

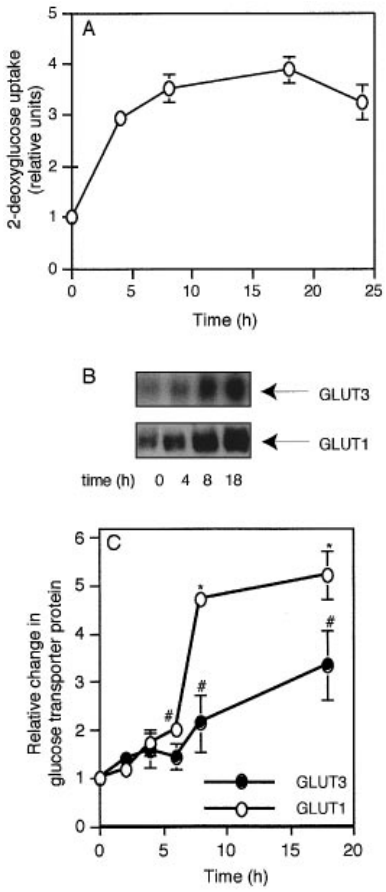


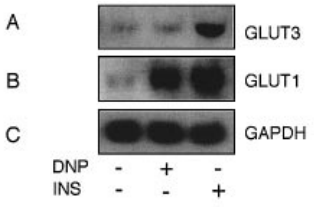
Figure 1 Time courses of DNP-stimulated glucose transport and of DNP-dependent increases in GLUT3 and GLUT1 steady-state protein levels

L6 myotubes were treated with 0.5 mM DNP in α-MEM containing 2% (v/v) FBS for the indicated time period, with all incubations initiated so that each experimental group was incubated for the entire 18 h time period before further processing. (A) 2-[^{3}H]Deoxyglucose uptake was determined as described in the Experimental section. Glucose transport in untreated cells (time = 0 h) was assigned a value of 1.0, and all other values are expressed in relative units. Each data point represents the mean ± S.E.M. for three independently performed experiments. (B) Total membrane fractions from each experimental group were immunoblotted for GLUT3 and GLUT1 as described in the Experimental section. To ensure equality of protein loading, all membranes were also probed for the α_1-subunit of the Na^+/K^+-ATPase, a membrane protein whose expression is not altered on continuous exposure to DNP [4,20] (results not shown). Representative immunoblots for time-dependent changes in GLUT3 and GLUT1 in response to DNP are shown. (C) Immunoblots were quantified and the results are expressed as the means ± S.E.M. of four independent experiments. The content of GLUT3 (●) or GLUT1 (○) protein from untreated cells was assigned a value of 1.0, and all other values are expressed in relative units. Statistically significant compared with untreated control: $^*P < 0.001$; $\#P < 0.05$.

levels were unaltered by treatment of L6 myotubes with DNP for 18 h (Figure 2A). However, continuous exposure to DNP led to an increase in the steady-state levels of GLUT1 mRNA (Figure 2B), similar to the effects of insulin (Figure 2B). Therefore, although DNP increased GLUT3 protein levels, it did not increase the steady-state levels of GLUT3 mRNA.

Effect of cycloheximide on glucose transporters and glucose transport

To assess the requirement for ongoing protein synthesis in the responses of increased glucose transport and transporter content, L6 muscle cells were treated with the protein-synthesis inhibitor



	Steady-State GLUT mRNA Level		
	Control	DNP	Insulin
GLUT3	1.00±0.0	0.86±0.14	3.61±0.47
GLUT1	1.00±0.0	4.11±0.26	4.42±0.23

Figure 2 Steady-state GLUT3 mRNA is not elevated by DNP treatment

Total RNA was isolated from L6 myotubes incubated with 0.5 mM DNP or 100 nM insulin (INS) for 18 h, as described in the Experimental section. Samples of 20 μg of RNA were analysed by Northern blotting using a GLUT3 (A) or GLUT1 (B) riboprobe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was probed as a loading control (C). The Northern blots of four independent experiments were quantified, and their values are expressed relative to the respective untreated control, which was given the value of 1.

cycloheximide for the duration of DNP challenge. On DNP treatment of L6 myotubes, GLUT3 protein levels remained elevated during co-incubation with 2 μg/ml cycloheximide (Figures 3A and 3B), while the increase in GLUT1 protein was completely blocked by the inhibitor (Figures 3A and 3D). For comparison, the effects of cycloheximide on insulin-mediated GLUT3 and GLUT1 induction were also examined. In contrast with that induced by the uncoupler, the insulin-stimulated elevation in GLUT3 protein was fully prevented by cycloheximide (Figures 3C and 3B), as was that in GLUT1 protein (Figures 3C and 3D). The low concentration of cycloheximide used in these experiments (2 μg/ml) did not directly inhibit or activate glucose transport, since basal levels of transporters (Figures 3B and 3D) and glucose uptake (Figure 3E) were not affected by the drug.

As shown in Figure 3E, the majority of glucose transport in response to chronic DNP treatment remained elevated in the presence of 2 μg/ml cycloheximide, yet the insulin-stimulated glucose transport was completely blocked. Thus, in the face of cycloheximide inhibition of protein synthesis, the DNP-stimulated increase of GLUT3 protein was paralleled by the continued elevation of glucose transport.

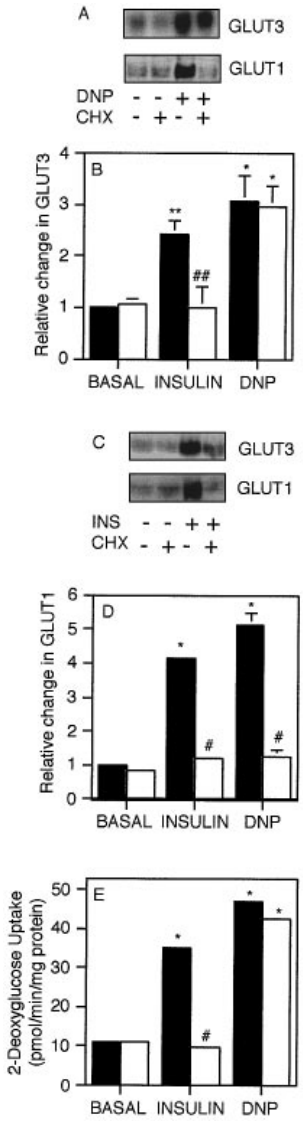


Figure 3 Induction of GLUT3 protein levels by DNP treatment is insensitive to cycloheximide

Differentiated L6 cells were preincubated for 60 min in the presence (open bars) or absence (filled bars) of 2 μg/ml cycloheximide (CHX), followed by subsequent incubation with 0.5 mM DNP or 100 nM insulin for 18 h with or without the continuous presence of CHX, as required. Total membrane fractions were prepared and subjected to immunoblot analysis as described in the Experimental section. To assess equality of protein loading, all Western blot membranes were also probed for the α_1-subunit of the Na^+/K^+-ATPase, expression of which is not altered by continuous exposure to DNP or insulin [4,20] (results not shown). Representative immunoblots are shown for GLUT3 and GLUT1 from DNP-treated (A) and insulin-treated (C) cells, and the results are quantified in (B) (GLUT3) and (D) (GLUT1) as the means ± S.E.M. of four independently performed experiments. (E) Uptake of 2-[^{3}H]deoxyglucose was determined under the same conditions as described for (A) and (C). Each data bar represents the mean ± S.E.M. of triplicate determinations within a representative experiment, independently performed three times. Where error bars are absent, errors are too small to be seen. Significant differences: $^*P < 0.0001$, $^{**}P < 0.05$ compared with basal; $\#P < 0.0001$, $\#\#P < 0.05$ compared with respective control in the absence of cycloheximide.

Effect of DNP on the half-lives of glucose transporters

To assess whether post-translational mechanisms are responsible for the sustained elevation of GLUT3 protein mediated by DNP, we examined the effects of the uncoupler on the rate of degradation of glucose transporter proteins. L6 myotubes were metabolically labelled with [^{35}S]methionine for 4 h, a time sufficient to substantially label most cellular proteins (results not shown). After chasing for the indicated times with media containing unlabelled methionine in the presence or absence of 0.5 mM DNP, the amount of label associated with glucose transporter immunoprecipitates was determined. Under basal conditions, the profiles of the decay of radiolabel for the two GLUT isoforms were clearly different. In untreated cells the GLUT3 label decreased by 25% by 8 h of chasing and had dropped to 75% at the end of the 24 h chase period (Figure 4B). In contrast, labelled GLUT1 had fallen by 75% within 8 h of chase time and remained at this level up to 24 h (Figure 4D). The half-lives of GLUT3 and GLUT1 in L6 myotubes were calculated to be 15 h and 6 h respectively. The half-life of GLUT3 was markedly prolonged by DNP. More than 80% of the radioactive label remained associated with GLUT3 immunoprecipitates after 24 h of exposure to DNP (Figure 4B). After 24 h, the half-life of GLUT1 had also risen with DNP treatment (Figure 4D), albeit

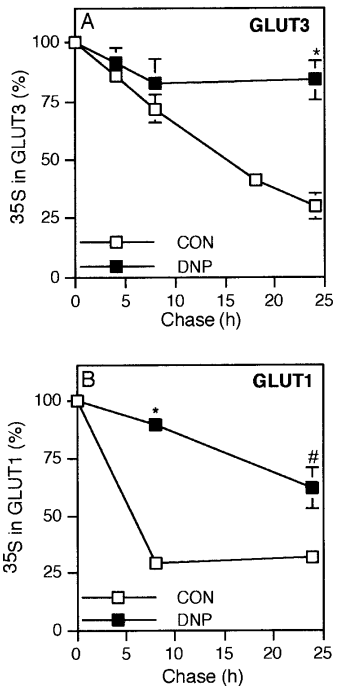


Figure 4 Long-term DNP treatment leads to an increase in the half-life of GLUT3 protein

L6 myotubes were pulsed and chased in the absence (□; CON) or presence (■) of 0.5 mM DNP. Glucose transporters were then immunoprecipitated and separated by SDS/PAGE (10% gels) as described in the Experimental section. The labelled GLUT3 (A) and GLUT1 (B) immunoprecipitates were quantified by Phosphorimager analysis. Results are expressed as the percentage of ^{35}S label remaining in glucose transporters compared with that at 0 h of chase time (100%), and represent means ± S.E.M. of six independent experiments. Pre-immune complexes and immune supernatants contained no immunologically detectable glucose transporters (results not shown). Significantly greater than untreated control: $*P < 0.0001$; $\#P < 0.01$.

to a lesser extent than that of GLUT3. The actual changes in half-lives could not be calculated directly, since this would require more than 24 h of chasing in the presence of DNP, which would compromise the viability of L6 muscle cells. Nevertheless, the results in Figure 4 show clearly that the degradation of GLUT3 was significantly inhibited by DNP.

DISCUSSION

In this study we have analysed the effects of chronic (18 h) treatment with DNP on GLUT3 and GLUT1 regulation in L6 myotubes, and compared them with the known effects of prolonged insulin treatment. Prior studies have shown that, unlike that mediated by insulin, the increase in glucose uptake mediated by DNP is insensitive to the protein-synthesis inhibitor cycloheximide [16,38]; however, the transporter(s) responsible for sustaining glucose transport was not investigated. Here we demonstrate the chronic DNP-mediated elevation of GLUT1 mRNA and protein, suggesting that *de novo* biosynthesis of this isoform occurred; hence cycloheximide was able to block the DNP-stimulated elevation of GLUT1. On the other hand, chronic DNP treatment elevated GLUT3 protein in L6 myotubes without changing the steady-state GLUT3 mRNA levels. Moreover, the uncoupler attained this increase independently of transcription or translation of new GLUT3 protein, but rather by a protein stabilization mechanism. Since DNP-stimulated glucose uptake and GLUT3 protein levels remained elevated during co-incubation with cycloheximide, we suggest that GLUT3 makes a significant contribution to the DNP-stimulated component of glucose transport.

GLUT1 may be functionally silent

Interestingly, although chronic DNP treatment elevated both GLUT3 and GLUT1 protein levels, the elimination of the GLUT1 component by cycloheximide had a minimal effect on DNP-stimulated glucose transport (Figures 3D and 3E). Lack of correlation between GLUT1 levels and glucose transport has been noted in earlier reports. For example, glucose deprivation of L6 muscle cells augmented GLUT1 mRNA, GLUT1 protein and glucose transport; the GLUT1 induction was cycloheximide-sensitive, but the glucose transport stimulation was not [38]. The inhibitors of oxidative phosphorylation rotenone and azide also have discrepant effects on GLUT1 protein content and glucose uptake in L6 muscle cells [16]. A reasonable explanation of our results could be that the GLUT3 glucose transporter is responsible for the majority of the glucose transport that is stimulated by DNP, irrespective of the elevated GLUT1 protein levels, i.e. GLUT1 is silent during metabolic stress. This conclusion is not without precedent. Nishimura et al. [39] observed that the catalytic efficiency of GLUT4 was far greater than that of GLUT1 when both transporters were co-expressed in *Xenopus* oocytes. Moreover, Xia and Lo [40] have proposed that GLUT1 protein may normally exist in an inactive form in L6 muscle cells, and that the GLUT3 and GLUT4 isoforms suffice to provide the cell with essential glucose transport activity. It is likely, then, that GLUT3 serves a specific purpose in the metabolic adaptation of L6 muscle cells to stimuli that compromise energy supply, such as disruption of the oxidative chain and glucose deprivation.

Unique regulation of GLUT3

Here we report for the first time that the half-lives of GLUT3 and GLUT1 proteins are approx. 15 h and 6 h respectively in L6 myotubes. Prolonged exposure to DNP reduced the turnover rate of both proteins. The more rapid turnover of the GLUT1 protein compared with the GLUT3 protein in untreated cells accounts for the more evident stabilization of GLUT1 during early hours of chasing in the presence of DNP. However, up until the 8 h time point, the turnover rates of both GLUT1 and GLUT3 proteins in the presence of DNP are similar. By 24 h of DNP treatment, which coincides more closely with the time point examined for all other steady-state GLUT mRNA and protein analyses (e.g. 18 h) in this study, GLUT3 has a much lower turnover rate than GLUT1. This difference could be a result of the much greater intrinsic rate of degradation of GLUT1 than of GLUT3 seen in the untreated cells. The experimental design of the [^{35}S]methionine pulse–chase labelling eliminates a protein synthesis component. Thus DNP induces only a moderate stabilization of the GLUT1 protein, while inducing a marked stabilization of the GLUT3 glucose transporter in L6 myotubes.

Previous reports examining the effects of energy demand (glucose deprivation) and insulin on glucose transporter turnover have focused on GLUT4 and GLUT1 in 3T3-L1 cells [21,26,41]. In those studies, insulin was shown to increase the rate of degradation of GLUT4 and GLUT1, while nutrient depletion led to increased stability of GLUT1, in 3T3-L1 adipocytes. We have observed that insulin increases GLUT3 and GLUT1 half-lives only slightly in L6 myotubes (results not shown). Moreover, in adipocytes from fasting rats, the GLUT4 degradation rate was also increased [42]. Therefore the regulation of glucose trans-

porter half-life is different in muscle and fat cells, and is isoform-specific.

Elevations in GLUT1 mRNA and protein have been reported in response to chronic stimulation with energy stressors, such as hypoxia and ischaemia, and with insulin in the brain [22,23] and glucose deprivation, hypoxia and mitochondrial uncouplers/inhibitors in muscle [16,36,38], fat [16,43] and other tissues and cell lines [44,45]. For the most part, GLUT1 expression is controlled pre-translationally, and the effects of insulin or other stimuli result from increased mRNA stability [25,37,46] or enhanced rates of transcription [38,47], as well as from enhanced protein synthesis [26], and do not involve mechanisms that stabilize glucose transporter proteins. Our present observations of the effects of DNP on GLUT1 expression are in good agreement with these previous studies. Compared with that of GLUT1, relatively little is known about how the expression of GLUT3 is regulated. Physiological changes such as hypoxia/ischaemia, chronic hypoglycaemia and starvation have been shown to induce GLUT3 protein in the immature rat brain [23] and rat brain neurons [22], and to increase GLUT3 mRNA in mouse brain [29]. It has also been reported that chronic insulin increased GLUT3 protein and mRNA in L6 muscle cells [4,20] and UMR 106-01 osteosarcoma cells [15]. However, the cellular mechanisms responsible for mediating these changes in GLUT3 expression are unknown. We propose a unique mechanism of regulation whereby chronic metabolic challenge elicits GLUT3 protein stabilization.

In conclusion, the increases in the levels of GLUT3 and GLUT1 proteins mediated by mitochondrial uncoupling occur by distinct mechanisms; the expression of the latter is governed largely by pre-translational processes, whereas the elevation of GLUT3 is determined by increasing the half-life of the protein. In contrast with DNP, the hormone insulin regulates both transporters by similar protein-synthesis-dependent processes. These studies extend our understanding of the need for the expression of multiple glucose transport proteins in the same tissue for the regulation of glucose and energy homoeostasis.

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