RESEARCH COMMUNICATION

Phosphatidylinositol 3-kinase and the actin network are not required for the stimulation of glucose transport caused by mitochondrial uncoupling: comparison with insulin action

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In L6 myotubes insulin stimulates glucose transport through the translocation of glucose transporters GLUT1, GLUT3 and GLUT4 from intracellular stores to the plasma membrane. An intact actin network and phosphatidylinositol 3-kinase activity are required for this process. Glucose transport is also stimulated by the mitochondrial ATP-production uncoupler dinitrophenol. We show here that, in serum-depleted myotubes, dinitrophenol induced translocation of GLUT1 and GLUT4, but not GLUT3.

This response was not affected by inhibiting phosphatidylinositol 3-kinase or disassembling the actin network. Insulin, but not dinitrophenol, caused tyrosine phosphorylation of several polypeptides, including the insulin-receptor substrate-1 and mitogen-activated protein kinase. Similarly, insulin, but not dinitrophenol, caused actin reorganization, which was inhibited by wortmannin. We conclude that insulin and dinitrophenol stimulate glucose transport by different mechanisms.

INTRODUCTION

The insulin signalling cascade begins with tyrosine phosphorylation of the insulin receptor and activation of its tyrosine kinase. The activated receptor phosphorylates insulin-receptor substrate-1 (IRS-1) at multiple tyrosine residues, creating docking sites for a number of signalling molecules containing Src homology 2 (SH2) domains, such as growth-factor-receptor binding protein 2 (Grb2) and phosphatidylinositol 3-kinase (PI3k) [1]. Binding of the SH2 domain of Grb2 to tyrosine-phosphorylated IRS-1 allows its associated guanine-nucleotide exchanger Sos ('son of sevenless') to activate the Ras/mitogen-activated protein kinase (MAPK) cascade and stimulate cell proliferation [1]. PI3k consists of a regulatory 85 kDa subunit (p85) and a catalytic 110 kDa subunit (p110) which phosphorylates inositol lipids on the D-3 position. Binding of p85 to tyrosine-phosphorylated IRS-1 stimulates the p110 catalytic activity [2].

In muscle and fat-cells, insulin stimulates glucose transport by the translocation of intracellularly stored glucose transporters to the plasma membrane. The mechanism of glucose transporter traffic and the signals leading to its initiation are largely unknown. Recent studies in adipocytes and fibroblasts [3–8] show that inhibition of PI3k activity abolishes the ability of insulin to stimulate glucose transport and to induce recruitment of glucose transporters to the plasma membrane. We have observed that wortmannin, a potent and relatively specific inhibitor of PI3k [9], inhibits both basal glucose transport and its stimulation by insulin in L6 skeletal-muscle cells [10]. In a recent study we also showed that the stimulation of glucose transport by insulin and the translocation of glucose transporters from internal stores to plasma membrane require an intact actin network. Disassembly of the actin network by cytochalasin D abolished insulin-induced stimulation of glucose transport without affecting the basal rate of uptake [11].

Insulin, similar to other growth factors, causes a dynamic ruffling of the plasma membrane in both fibroblasts and differentiated cells [2,12–14]. This phenomenon, caused by reorganization of the actin network, involves localized actin polymerization under the plasma membrane [14]. Recent work in epidermoid carcinoma cells (KB) has shown that insulin mediates this effect on the actin network by the activation of PI3k [13]. It is not known whether a relationship exists between the activation of actin reorganization causing membrane ruffling and the stimulation of glucose transport in response to insulin.

In differentiated L6 myotubes, glucose transport is also elevated by stimuli that increase energy demand. Hypoxia, dinitrophenol (DNP) and rotenone stimulate glucose transport by the recruitment of glucose transporters to the cell surface [15,16]. It is not known whether PI3k activity or the actin network is required for glucose uptake and transporter traffic in response to these stimuli. The objective of this study was to explore whether conditions that increase energy demand utilize elements of the insulin signalling pathway in order to elevate glucose transport. In particular, the involvement of protein tyrosine phosphorylation, PI3k activity and the actin network in the stimulation of glucose transport by DNP were examined. We observed that DNP stimulates glucose transport by recruitment of the GLUT1 and GLUT4 glucose transporters to the plasma membrane without involvement of PI3k or of organized actin filaments.

EXPERIMENTAL

Materials

α -Minimum Essential Medium (α -MEM), fetal-bovine serum (FBS) and antibiotic/antimycotic solution were obtained from GIBCO/BRL (Burlington, Ont., Canada). Wortmannin, cytochalasin B, cytochalasin D, DNP and 2-deoxy-D-glucose (2DG)

Abbreviations used: GLUT1, erythrocyte glucose transporter; GLUT3, neuronal glucose transporter; GLUT4, insulin-responsive glucose transporter; IRS-1, insulin-receptor substrate-1; SH2, Src homology 2; Grb2, growth-factor-receptor binding protein 2; PI3k, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; p85, PI3k regulatory 85 kDa subunit; p110, PI3k catalytic 110 kDa subunit; DNP, dinitrophenol; α -MEM, α -Minimum Essential Medium; 2DG, 2-deoxy-D-glucose; ECL, Enhanced Chemiluminescence; FBS, fetal-bovine serum.

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and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Deoxy-D-[^3H]glucose (^3H -2DG) was purchased from DuPont (Boston, MA, U.S.A.). Human insulin was obtained from Eli Lilly Canada Inc., Toronto, Ont., Canada. Polyclonal anti-GLUT1 and anti-GLUT4 glucose-transporter antibodies were purchased from East Acres Laboratories (Southbridge, MA, U.S.A.). The polyclonal anti-GLUT3 antibody was kindly given by Dr. I. A. Simpson (Bethesda, MD, U.S.A.). The Enhanced Chemiluminescence (ECL) reagents were purchased from Amersham (Oakville, Ont., Canada). All electrophoresis and immunoblotting reagents were obtained from Bio-Rad (Mississauga, Ont., Canada).

Cell culture and incubations

Monolayers of L6 muscle cells were grown to the stage of myotubes as previously described [17] in α -MEM containing 2% (v/v) FBS, 100 units/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin and 250 ng/ml amphotericin B at 37 °C in an atmosphere of air/ CO_2 (19:1). The cells were grown in 2.5 cm-diameter wells for transport and tyrosine-phosphorylation experiments, or in 15 cm-diameter dishes for membrane preparations. L6 myotubes were incubated in serum-depleted medium (α -MEM containing 0.1% FBS) for 5 h before experimental manipulations. Cells were routinely incubated without or with 100 nM wortmannin serum-depleted medium for 40 min or 1 μM cytochalasin D for 2 h before assays, unless otherwise stated in the Figure legends. Cultures were stimulated with 100 nM insulin or 0.5 mM DNP for the last 30 min before the transport assay unless otherwise stated.

Detection of tyrosine-phosphorylated proteins

Tyrosine phosphorylation of proteins *in vivo* was detected as described by Lamphere and Lienhard [18]. Briefly, myotubes grown in 12-well plates (with identical amounts of protein in each well) were serum-depleted and treated with or without 100 nM insulin or 0.5 mM DNP for 5 min. The cells were immediately washed twice with ice-cold PBS and lysed in a solution containing 4% SDS, 10 mM dithiothreitol, 115 mM Tris/HCl (pH 6.8), 10% glycerol, 0.25 mg/ml Bromophenol Blue, protease inhibitors (100 μM phenylmethanesulphonyl fluoride, 10 μM E-64, 1 μM pepstatin and 1 μM leupeptin) and phosphatase inhibitors (40 mM NaF, 7.5 mM sodium pyrophosphate and 1.5 mM Na_3VO_4). The lysates were passed five times through a 27-gauge needle to shear DNA and boiled for 3 min. A 50 μg sample of total protein from each condition was separated by SDS/PAGE (7.5% gels), transferred on to poly(vinylidene difluoride) membranes and probed by immunoblotting with an anti-phosphotyrosine antibody.

Fluorescence microscopy

L6 muscle cells were grown to myotube stage on glass coverslips. Myotubes were serum-depleted and treated with or without 100 nM wortmannin for 40 min. During the last 30 min of this period the cells were stimulated with or without 100 nM insulin or 0.5 mM DNP. After these incubations, myotubes were washed twice with serum-free medium, fixed with 4% paraformaldehyde for 20 min, washed with 100 mM glycine in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min and washed (3 $\times$ 15 min) with PBS. Fixed and permeabilized cells were incubated for 1 h at room temperature with rhodamine-labelled phalloidin (1 unit/coverslip). To assess sample autofluorescence, cells were treated for 1 h with PBS without rhodamine-labelled phalloidin. The fluorescently labelled actin

network of myotubes was viewed with an inverted Nikon fluorescence microscope (at $\times 360$ magnification and photographed with Kodak ISO 400 slide film).

Other assays

Cytochalasin B-sensitive 2-DG uptake was measured for 10 min as described previously [11]. Plasma membranes and internal membranes were prepared as reported previously [11,19,20]. SDS/PAGE and immunoblotting were carried out essentially as described previously [17]. Primary antibodies were used as described in the Figure legends and were detected with ^{125}I -Protein A (0.1 $\mu\text{Ci}/\text{ml}$). Where indicated, horseradish-peroxidase-conjugated anti-rabbit IgG and the ECL method were used. The fluorographs were densitometrically scanned with a Discovery Series DNA 35 gel scanner or a Molecular Dynamics PhosphorImager system. Statistical analysis was performed by the Anova test (Fisher, multiple comparisons).

RESULTS AND DISCUSSION

Insulin, hypoxia and contraction stimulate glucose transport into skeletal muscle through the recruitment of glucose transporters to the cell surface [21–23]. The mechanism of stimulation of glucose transport by hypoxia has been postulated to be the same as that triggered by muscle contraction and different from that triggered by insulin, since the effects of maximum contraction and hypoxia are not additive, whereas those of contraction and insulin are [23–25]. However, the differences or similarities in the mechanisms of action of insulin, hypoxia or contraction have been difficult to dissect, since until now there were no approaches available to prevent specifically one of the responses. We showed previously that DNP, like hypoxia, imposes a metabolic challenge to muscle cells in culture, the former by uncoupling and the latter by inhibiting the aerobic mitochondrial ATP production, without affecting cell viability [15,16]. Recent studies have identified three elements as requisites for insulin stimulation of glucose transport: tyrosine kinase activity [1], activation of PI3k [3–8] and the actin filament cytoskeleton [11]. Therefore, in the present study we examined the potential role of these elements in the stimulation of glucose transport by DNP in L6 muscle cells in culture.

Figure 1 shows that insulin and DNP stimulate glucose

Figure 1 Time course of stimulation of glucose transport by insulin and DNP

Myotubes were serum-depleted for 5 h, then incubated for different periods of time with either 100 nM insulin (○) or 0.5 mM DNP (●). Immediately after these incubations, the cells were washed twice and glucose transport was measured. Results are expressed as change above the value of the untreated control, given the value of 1. Basal uptake was 5.5 ± 0.8 pmol/min per mg of protein ($n = 6$).

Figure 2 Effect of insulin and DNP on glucose-transporter content at the plasma membrane

Serum-depleted myotubes were stimulated for 30 min with or without 100 nM insulin or 0.5 mM DNP and subjected to subcellular fractionation to obtain purified plasma membranes. Portions (20 μ g of protein) of purified plasma membranes (PM) from control (C), insulin-treated (I) and DNP-treated (D) myotubes were subjected to immunoblotting using anti-GLUT1, anti-GLUT3 and anti-GLUT4 specific antibodies (dilutions: 1/1000, 1/300 and 1/1000 respectively). The immunoblots of four independent experiments were quantified, and their values are expressed relative to the untreated control, given the value of 1 (* P < 0.05 and ** P < 0.01 relative to untreated control.)

	Plasma-membrane glucose-transporter content		
	Control	Insulin	DNP
GLUT1	1.00 $\pm$ 0.0	1.39 $\pm$ 0.19*	1.30 $\pm$ 0.10*
GLUT3	1.00 $\pm$ 0.0	1.28 $\pm$ 0.04**	1.04 $\pm$ 0.12
GLUT4	1.00 $\pm$ 0.0	1.74 $\pm$ 0.46*	1.51 $\pm$ 0.19*

transport in a time-dependent manner, but with a different time course. Insulin caused a fast increase in glucose uptake that reached a maximum (2-fold increase) within 10 min of addition of the hormone. The elevated glucose transport rate was maintained at a plateau for at least 1 h. In contrast, DNP stimulated glucose transport with a slower time course and continued to accelerate the transport rate during the first 1 h, reaching a maximum of about 3 times the basal rate of transport. We have observed previously that DNP [16] and insulin [26,27] stimulate glucose uptake by two mechanisms: an early one independent of ongoing protein synthesis, involving glucose transporter translocation from internal stores to plasma membrane, and a late one, beginning 1 h after addition of the compounds, which involves GLUT1 gene expression. Our present interest was to compare the signals that lead to the early stimulation of glucose transport by insulin and DNP. Therefore, in all subsequent experiments we investigated only the effects of these agents during the first 30 min of incubation. In this time period, both compounds increased glucose transport to a similar extent. Figure 2 compares the effects of insulin and DNP on the amount of glucose transporters in isolated plasma membranes detected by immunoblotting. As seen previously [11,19,26], insulin increased significantly the content of the three glucose transporter isoforms (GLUT1, GLUT3 and GLUT4) at the plasma membrane. In contrast, DNP caused significant recruitment to the

Figure 3 Effect of insulin and DNP on protein tyrosine phosphorylation

Serum-depleted myotubes were incubated with or without 100 nM insulin (I) or 0.5 mM DNP (D) for 5 min. Immediately after, treated and untreated control (C) cells were washed twice with ice-cold PBS and lysed as described in the Experimental section. Portions (50 μ g of lysate protein) from cells in the three conditions were subjected to SDS/PAGE and immunoblotted using anti-phosphotyrosine-specific antibody (anti-P-Y; dilution 1/5000), detected by the ECL method. One experiment representative of three independent ones is shown. The migration of two major tyrosine-phosphorylated proteins, IRS-1 and MAPK, is indicated, as determined from parallel immunoblots (not shown).

plasma membrane of only GLUT1 and GLUT4, but not the GLUT3 isoform. These results indicate that glucose transporter isoforms are differentially regulated, and that GLUT1 and GLUT4 can be recruited to the plasma membrane by two different stimuli. The inability of GLUT3 glucose transporters to be mobilized to the cell surface in response to uncoupling of the mitochondrial ATP production could, at least partly, explain the slower stimulation of glucose transport in response to DNP compared with insulin (Figure 1). The DNP-dependent recruitment of GLUT1 and GLUT4 to the plasma membrane was associated with a decrease in these transporters in the internal membranes (results not shown).

The mechanisms whereby DNP or hypoxia mediate their effects on glucose transport remains unknown. In L6 myotubes treated with DNP, a transient fall in the cellular ATP levels was detected, but the cellular ATP levels recover, probably by the engagement of anaerobic metabolism [16]. This phenomenon may be of signalling relevance, but the pathway by which changes in ATP levels could be sensed by the glucose transport system has not been described. Since the insulin signalling cascade is initiated by tyrosine phosphorylation of the receptor and IRS-1, leading to activation of PI3k, we investigated whether these reactions participate in the action of DNP. Figure 3 shows that stimulation of myotubes with 100 nM insulin for 5 min caused a rapid and large increase in tyrosine phosphorylation of several proteins, notably of polypeptides of 42, 44 and 175 kDa. The latter was previously identified by parallel immunoblotting and immunoprecipitation as IRS-1 [11] and, in results not shown, we identified the 42 and 44 kDa polypeptides as MAPK. In contrast with insulin, stimulation of myotubes with 0.5 mM DNP for 5 min (Figure 3) or for 1 h (results not shown) did not alter the protein tyrosine-phosphorylation pattern of IRS-1, MAPK or any other protein in myotube lysates (Figure 3).

Table 1 Effect of wortmannin (a) and cytochalasin D (b) on the insulin- and DNP-induced stimulation of glucose transport

Serum-depleted myotubes were incubated for 40 min with or without 100 nM wortmannin (a), or for 2 h with or without 1 μ M cytochalasin D (b). Insulin (100 nM) or DNP (0.5 mM) was added in the last 30 min of these incubations, and glucose transport (measured as ^3H -2DG uptake) was immediately determined. The results of 6–7 independent experiments are expressed relative to the basal rate of transport in untreated myotubes. The averaged basal glucose transport rate of untreated cells was 6.23 ± 0.35 pmol/min per mg of protein. * $P < 0.001$ relative to insulin-stimulated cells.

		Basal	Insulin	DNP	Stimulation above basal	
					Insulin	DNP
(a)	Control	1.00 ± 0.00	2.10 ± 0.07	2.15 ± 0.12	1.09 ± 0.07	1.15 ± 0.11
	Wortmannin	0.62 ± 0.06	$0.80 \pm 0.19^*$	1.84 ± 0.25	$0.23 \pm 0.09^*$	1.21 ± 0.18
(b)	Control	1.00 ± 0.00	2.05 ± 0.21	2.13 ± 0.06	1.05 ± 0.21	1.13 ± 0.06
	Cytochalasin D	1.03 ± 0.09	$1.18 \pm 0.17^*$	1.94 ± 0.03	$0.07 \pm 0.04^*$	0.90 ± 0.07

Figure 4 Effect of insulin and DNP on the organization of the myotube actin network

Serum-depleted myotubes were grown on glass coverslips and preincubated with or without 100 nM wortmannin (WM) for 10 min. The cells were then stimulated with either 100 nM insulin (ins.) or 0.5 mM DNP for 30 min, fixed, permeabilized and incubated with rhodamine-labelled phalloidin (1 unit/coverslip), as described in the Experimental section, before examination under a Nikon inverted fluorescence microscope.

The role of PI3k in the stimulation of glucose transport by DNP was subsequently assessed by using wortmannin, a specific inhibitor of the enzyme. Table 1(a) shows the effects of 100 nM wortmannin on the basal rate of glucose transport and its stimulation by insulin or DNP. We have recently detected that this concentration of wortmannin inhibits both basal and insulin-stimulated PI3k activity detected in anti-p85 and anti-IRS-1 immunoprecipitates, as well as basal glucose transport activity and its stimulation by insulin [10]. Wortmannin treatment alone significantly detected the basal rate of glucose transport by 38%. In the absence of wortmannin, both insulin and DNP stimulated glucose transport by 2-fold. Treatment of myotubes with 100 nM wortmannin for 10 min before addition of insulin, followed by

co-incubation of the cells with both insulin and wortmannin for 30 min, markedly inhibited the ability of the hormone to increase the rate of glucose transport. In contrast, pretreatment of myotubes with wortmannin, followed by incubation with both wortmannin and DNP, did not alter the response of glucose transport to the DNP challenge. These results suggest that PI3k plays a central role in the regulation of glucose transporter traffic by insulin, but not by DNP. The present study indicates that not all intracellular traffic of glucose transporters in muscle cells depends on protein tyrosine phosphorylation and PI3k activity, and that alternative regulation of glucose transporter traffic may exist.

We have previously shown that in L6 myotubes [11], as in

fibroblastic cells [12,13], insulin induces a rapid reorganization of the actin network involving bundling of polymerized actin under the plasma membrane. Figure 4 examines the effect of insulin and DNP on the organization of the actin network and the dependence of this response on PI3k. In unstimulated serum-deprived myotubes, phalloidin labelled a large number of distinct actin fibres running along the entire length of the cells. Incubating myotubes with insulin for 30 min produced a dramatic reorganization of the actin network, manifested as extensive bundling of actin structures. Several areas of continued arrays of actin bundling could be detected in most insulin-stimulated myotubes. Using video-enhanced fluorescence and differential interference contrast microscopy, we have seen that actin reorganization sites co-localize with membrane ruffles that appear as soon as 20 s after addition of insulin (T. Tsakiridis, K. Foskett and A. Klip, unpublished work). These results were confirmed by confocal-microscopy observations, which showed that actin bundles and membrane ruffles project the myotube plasma membrane above the resting cell surface. Activation of PI3k in response to insulin and growth factors appears to be required for the induction of actin reorganization and membrane ruffling in fibroblastic cells [13]. In agreement with those results, we observed that wortmannin (100 nM) alone had no effect on actin fibres in control L6 myotubes (results not shown), but the drug totally abolished the insulin-induced actin bundling (Figure 4). In contrast with insulin, incubation of myotubes with DNP for the same time period (30 min) did not alter the actin network. The results in Figure 4 suggest that actin reorganization and membrane ruffling do not occur concomitantly with the elevation of glucose transport by all stimuli, and instead they may be associated specifically with the action of insulin and other agents that activate PI3k.

In order to investigate whether intact actin filaments are required for the activation of glucose transport by DNP, as they are for insulin, cytochalasin D was used to disassemble the myotube actin network. Table 1(b) compares the sensitivity of the stimulation of glucose transport by insulin and DNP to the action of cytochalasin D. Myotubes were incubated for 2 h with 1 μ M cytochalasin D, conditions required to achieve complete disassembly of the actin network [11]. During the last 30 min of the 2 h period, the cells were incubated with either insulin (100 nM) or DNP (0.5 mM). Cytochalasin D completely abolished the insulin-induced stimulation of transport, but was unable to affect the stimulation of transport by DNP, suggesting that, unlike insulin, the stimulation of glucose transport by DNP does not utilize the actin network.

In conclusion, insulin and DNP appear to generate independent signals to stimulate glucose transport and glucose-transporter recruitment to the plasma membrane. GLUT1 and GLUT4 are mobilized by both signals, whereas GLUT3 is mobilized only by insulin. Protein tyrosine phosphorylation, PI3k activity and the actin network participate only in the

insulin-regulated pathway of stimulation of glucose transport, but not in that activated by DNP.

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