Dissection of stress-activated glucose transport from insulin-induced glucose transport in mammalian cells using wortmannin and ML-9

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The signalling pathways responsible for the activation of glucose transport by insulin and by metabolic stress in mammalian cells were studied in Clone 9 cells and 3T3-L1 adipocytes. Exposure of both cell types to azide or insulin markedly increased their glucose uptake capacity ($V_{\max}$) without affecting their apparent affinity for glucose (K_m). The effects of azide and insulin were not additive. Wortmannin, a selective inhibitor of phosphatidylinositol (PI) 3-kinase, did not affect stimulation of transport by azide but inhibited insulin-induced glucose transport with a K_i of

< 10 nM. ML-9, a putative mitogen-activated protein kinase inhibitor, was equipotent in its inhibition of azide- and insulin-stimulated glucose transport. These findings suggest that multiple signalling cascades are involved in the stimulation of glucose transport in mammalian cells and that PI 3-kinase, an essential link in the pathway by which insulin stimulates glucose transport, is not necessary for the activation of glucose uptake by metabolic stress.

INTRODUCTION

The transport of glucose across the cell membrane, the rate-limiting step for its utilization in most mammalian cells, is acutely stimulated by anabolic hormones such as insulin and growth factors, and also by catabolic challenges (stress) such as exercise, viral infection, heat shock or exposure to metabolic poisons [1,2]. The early phases of glucose transport activation by anabolic and catabolic stimuli are very similar. The effects are completed in less than 60 min and do not require newly synthesized proteins; both responses are characterized by an increase in uptake capacity ($V_{\max}$) without significant changes in the apparent affinity (K_m) of the transporters for sugars. In rat adipocytes and mouse 3T3-L1 adipocytes, the best-characterized insulin-sensitive systems, the $V_{\max}$ change results primarily from translocation of sugar transporters from an intracellular membrane compartment to the cell surface, although a small part of the transport stimulation probably reflects enhanced intrinsic activity of the carriers (reviewed in [2–4]). Experiments in heart [5] and skeletal muscle [6], BHK cells [7], Clone 9 cells [8,9], L6 myoblasts and 3T3-L1 adipocytes [10] suggest that both translocation and alteration of intrinsic activity of individual transporters are mechanisms by which metabolic stress can regulate sugar transport. Further evidence for similarities in the regulation of transport by stress and by anabolic hormones is provided by the finding that polymyxin B, an antibiotic that displaces Ca^{2+} from anionic phospholipids, inhibits both insulin-activated and hypoxia-activated glucose uptake in muscle [11].

These common features support the notion that insulin- and stress-induced glucose transport share a common regulatory pathway starting downstream of the insulin receptor [12]. The present study was designed to investigate this hypothesis in two cell types, one very sensitive to metabolic stress (Clone 9 cells) and one specially sensitive to insulin (3T3-L1 adipocytes). In

particular, we have taken advantage of the recent discovery of two potent inhibitors of the stimulation of glucose uptake by insulin, wortmannin [13–15] and ML-9 [16].

EXPERIMENTAL

Materials

2-Deoxy-D-[G- 3H]glucose (8.1 Ci/mmol) was purchased from DuPont–New England Nuclear. Affinity-purified antibodies against the C-terminal peptides of rat GLUT1 (residues 477–492) and GLUT4 (residues 494–509) were prepared as previously described [17]. Insulin, azide, wortmannin, ML-9, tissue-culture reagents and standard chemicals were purchased from Sigma.

Cell culture

Cells were obtained from the European Collection of Animal Cell Cultures. Clone 9 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 50 units/ml penicillin–streptomycin at 37 °C in 9.3% CO_2 . Cultures were split 1:10 into 6-well (35 mm diam.) plates every week and the medium replaced every 48 h. Cells were studied 24–48 h after reaching confluence. 3T3-L1 fibroblasts were differentiated into adipocytes in 6-well plates as previously described [18] except that the period of incubation with insulin, dexamethasone and isobutylmethylxanthine, and that of the subsequent exposure to insulin alone, was 72 h rather than 48 h. With this protocol, routinely > 95% of the cells displayed fat droplets at the end of the differentiation process. Protein content was determined by the method of Lowry et al. [19] using BSA as a standard.

Abbreviations used: GLUT, glucose transporter isoform; MAP kinase, mitogen-activated protein kinase; PI, phosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; KRH, Krebs–Ringer–Hepes saline solution; PM lawns, isolated plasma membrane lawns.

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Sugar transport assays

Clone 9 cell and 3T3-L1 adipocyte culture supernatants were replaced by fresh medium 48 h prior to uptake measurements. 3T3-L1 cells were pre-incubated for 2 h with serum-free DMEM followed by the appropriate treatments as indicated in the Figure legends. Monolayers were then rinsed with Krebs–Ringer–Hepes saline solution (KRH; 136 mM NaCl, 20 mM Hepes, 4.7 mM KCl, 1.25 mM MgSO_4 , 1.25 mM CaCl_2 , pH 7.4). The uptake of 0.2 mM 2-deoxy- ^3H glucose (0.25 $\mu\text{Ci}/\text{ml}$) was measured at 37 °C in KRH containing the same additions (e.g. azide) as were present during the experimental treatment period. Uptake was terminated by washing the monolayers three times with 2 ml of ice-cold phosphate-buffered saline (PBS) and the radioactivity was extracted into 1 ml of 1% Triton X-100. Under these conditions, 2-deoxyglucose uptake was linear for up to 20 min in both cell types. Non-mediated sugar uptake was estimated in the presence of 20 μM cytochalasin B and found to be similar [4 ± 0.1 ($n = 8$) pmol/min per mg of protein] in both cell types. No significant changes in non-mediated sugar uptake were observed in the presence of 5 mM azide or 160 nM insulin. Six-well plates contained approximately 10^6 cells per well, and 220 ± 5 ($n = 36$) and 863 ± 15 ($n = 36$) μg of protein per well for Clone 9 cells and mature 3T3-L1 adipocytes respectively.

PM lawns

Isolated plasma membrane lawns (PM lawns) were prepared by the method of Robinson et al. [20]. In brief, cells were grown and differentiated on ethanol-sterilized glass coverslips (No1 Chance Propper Ltd) in 6-well plates. After experimental treatments, coverslips were quickly washed in PBS and then immersed for 30 s in 0.5 mg/ml poly-L-lysine in PBS. Cells were swollen by three 10-s incubations in hypotonic buffer (one-third $\times$ sonication buffer), transferred to sonication buffer (70 mM KCl, 30 mM Hepes, 5 mM MgCl_2 , 3 mM EGTA, pH 7.5) and sonicated for 10 s (one-third maximum power under a 1-cm-diam. probe). Cells were then fixed for 20 min in 4% paraformaldehyde in PBS and washed three times with PBS. Excess fixative was quenched by immersion for 15 min in 100 mM glycine in PBS. Fixed PM lawns were stored up to 1 month in 0.02% sodium azide in PBS without loss of immunoreactivity. For immunostaining (performed at 37 °C), PM lawns were blocked by incubation for 1 h with 5% FBS in PBS, followed by 2 h incubation with a suitable dilution of primary antibody in PBS. Bound antibody was detected by incubation for 1 h with an 1:100 dilution of a goat anti-(rabbit IgG) fluorescein conjugate (Sigma). Finally, coverslips were washed and mounted in Citifluor anti-fade medium (Citifluor, U.K.) for examination using a Leitz confocal microscope.

RESULTS

Stress- and insulin-stimulated sugar uptake in Clone 9 cells and 3T3-L1 adipocytes

Azide, a metabolic poison that stops the mitochondrial synthesis of ATP by blocking electron transfer from cytochrome oxidase to oxygen, stimulated 2-deoxyglucose uptake in Clone 9 cells in a dose- and time-dependent manner (Figure 1). Previous work on these cells in which 3-O-methyl-D-glucose was used as uptake tracer [21] suggested that this phenomenon involved solely changes in transport capacity without increase in affinity for sugars. Table 1 shows that such is also the case when 2-deoxyglucose is used as uptake tracer: azide increased the V_{max} for uptake more than 7-fold while the K_m was slightly, although not significantly, increased. This experiment is relevant because

Figure 1 Time- and concentration-dependence of the stimulation of 2-deoxyglucose uptake by azide in Clone 9 cells

(a) Cells were exposed to 5 mM azide (○) or 1 μM insulin (●) for the periods shown. Uptake of 0.2 mM 2-deoxyglucose was then measured over 5 min. (b) Cells were exposed for 90 min to increasing concentrations of azide. Mean $\pm$ S.E.M. (triplicates). Similar results were obtained in at least two separate experiments.

the phosphorylation of the latter to 2-deoxyglucose 6-phosphate could potentially be impaired in cells exposed to azide, thus affecting the rate-limiting step for sugar uptake. The time profile of the transport stimulation parallels the recovery of cell ATP levels in the continuous presence of azide in Clone 9 cells [22] or of 2,4-dinitrophenol in L6 myotubes [10].

Clone 9 cells also exhibited insulin-sensitive 2-deoxyglucose uptake (Figure 1a and Table 1). The effect of insulin was, as in other systems studied [2,3], a V_{max} effect. Smaller than the response to azide, the response to insulin in these cells displayed a similar time course with $t_{1/2}$ of approximately 45 min, much slower than in fat cells such as 3T3-L1 adipocytes ($t_{1/2} < 6$ min [2,3]). Interestingly, the effect of insulin on transport in Clone 9 cells was not additive to that of azide (Table 2). To examine whether this was also the case in a cell type more sensitive to insulin, we next investigated 3T3-L1 adipocytes. As previously described by many workers (reviewed in [2,3]), we found that the effect of insulin on sugar uptake by 3T3-L1 cells involved an increase in the V_{max} for transport (Table 1). Exposure of these cells to azide also caused a significant increase in the rate of sugar uptake which, as in Clone 9 cells, proved to be a V_{max} effect (Table 1). However, as for the Clone 9 cells, the effects of azide and insulin on hexose uptake by 3T3-L1 adipocytes were not additive (Table 2).

Effects of wortmannin and ML-9 on stress- and insulin-stimulated sugar uptake

Wortmannin, a fungal metabolite that selectively inhibits the enzyme phosphatidylinositol (PI) 3-kinase, potentially inhibited

Table 1 $V_{\max}$ and K_m of glucose uptake by Clone 9 cells and 3T3-L1 adipocytes

Cells were incubated for 90 min in DMEM plus the indicated experimental treatments. 2-Deoxy- ^3H glucose uptake was measured in duplicate in the presence of 0, 0.3, 0.9, 2.7, 8.1 and 24.1 mM glucose. Glucose uptake was calculated from the molar ratio between tracer and unlabelled sugar. $V_{\max}$ (pmol/min per dish) and K_m (mM) were obtained by fitting the data to a rectangular hyperbola using the non-linear regression programme Enzfitter (Elsevier, Biosoft), and are expressed as mean $\pm$ S.E.M. (number of separate experiments). *, $P < 0.05$ when compared with basal using ANOVA and Tukey–Kramer's test; †, $P > 0.05$. No significant differences were found in the protein content of cells exposed to azide or insulin.

	$V_{\max}$ (pmol/min per dish)			K_m (mM)		
	Basal	Azide (5 mM)	Insulin (160 nM)	Basal	Azide (5 mM)	Insulin (160 nM)
Clone 9 cells	300 $\pm$ 26 (4)	2300 $\pm$ 750 (4)*	1300, 1160*	1.0 $\pm$ 0.1 (4)	2.1 $\pm$ 0.5 (4)†	1.0, 1.6†
3T3-L1 adipocytes	1150 $\pm$ 220 (4)	3600 $\pm$ 700 (3)*	6500 $\pm$ 780 (3)*	1.0 $\pm$ 0.3 (4)	1.2 $\pm$ 0.2 (3)†	1.0 $\pm$ 0.3 (3)†

Table 2 Lack of additivity of azide-activated and insulin-stimulated 2-deoxyglucose uptake

Cells were incubated for 90 min in DMEM plus the indicated experimental treatments. Uptake of 0.2 mM 2-deoxy- ^3H glucose was measured over 5 min as described in the Experimental section. Mean $\pm$ S.E.M. (three separate experiments).

	2-Deoxyglucose uptake (-fold stimulation over basal)	
	Clone 9 cells	3T3-L1 adipocytes
Azide (5 mM)	3.6 $\pm$ 0.6	2.6 $\pm$ 0.7
Insulin (160 nM)	2.7 $\pm$ 0.4	7.7 $\pm$ 2.0
Azide + insulin	3.6 $\pm$ 0.2	5.4 $\pm$ 1.1

insulin stimulation of 2-deoxyglucose uptake in both Clone 9 cells and 3T3-L1 adipocytes (Figure 2). In separate experiments, the concentrations of wortmannin capable of inhibiting 50% of insulin-stimulated sugar uptake (IC_{50}) were 7 and 8 nM for Clone 9 cells, and 6 and 8 nM for 3T3-L1 adipocytes. In some experiments, a minor fraction of insulin-activated uptake in both Clone 9 and 3T3-L1 cells was resistant to concentrations of wortmannin as high as 2.5 μM (Figures 2c and 2d). This finding cannot have stemmed from loss of reversibly bound inhibitor by dilution during the transport assay, because wortmannin was also included in the uptake medium. A possible explanation is the reported instability of wortmannin during long incubations in neutral aqueous solutions [23]. However, this seems unlikely given our finding that the apparent potency of wortmannin (K_i) was not decreased in such experiments.

In contrast to its inhibition of insulin-stimulated glucose uptake, wortmannin did not affect azide-activated 2-deoxyglucose uptake in either cell type (Figure 2). The small difference observed in azide-stimulated uptake in the absence and presence of wortmannin can be accounted for by an effect of the inhibitor on basal uptake. Interestingly, in cells stimulated simultaneously with azide and insulin, wortmannin did not inhibit 2-deoxyglucose transport (Figure 2).

Wortmannin consistently inhibited basal sugar uptake in both cell types (Figure 2). Because other workers have reported a lack of effect of the inhibitor on basal glucose uptake [14,15], we tried several modifications of the uptake protocol in order to investigate this phenomenon further. Longer pre-incubation with serum-free medium (up to 24 h) or pre-incubation with 0.5% FBS in DMEM [24] did not affect the wortmannin-sensitive component of the basal uptake (results not shown). Neither did reducing (12 h) or increasing (up to 96 h) the time between the last change of culture medium and uptake measurements. How-

ever, exposing the cells for 2 h to uptake medium (KRH) supplemented with 25 mM glucose, rather than to serum-free DMEM, totally removed the wortmannin-sensitive fraction of the basal uptake (results not shown). We conclude that one of the components of DMEM directly stimulates glucose uptake via a wortmannin-sensitive pathway in both cell types.

To investigate the mechanism by which azide and insulin stimulate glucose transport in Clone 9 cells, we employed the PM lawn approach to assess the amount of GLUT1 at the cell surface (GLUT1 is the only isoform known to be expressed in these cells). In the experiment summarized in Figure 3, Clone 9 cells were treated with azide and wortmannin, and then their cell membranes were isolated as PM lawns and screened for GLUT1 immunoreactivity. Although this approach is not quantitative, there was no clearly discernible difference between the amount of transporter at the cell surface in the presence and absence of azide (with or without wortmannin), despite the approx. 7-fold increase in transport $V_{\max}$ brought about by the poison. These findings agree with the results of Shetty et al. who, using alternative approaches, also showed a lack of correlation between translocation of GLUT1 and the increase in $V_{\max}$ induced by azide [9]. It is therefore clear that activation of glucose transport by stress in Clone 9 cells involves an increase in transporter intrinsic activity with no translocation of GLUT1. Similarly, no obvious increase in GLUT1 immunoreactivity was found in PM lawns prepared from Clone 9 cells exposed to insulin (results not shown).

In 3T3-L1 adipocytes treated with azide, neither GLUT1- nor GLUT4-immunoreactivity associated with plasma membranes showed any apparent increase (Figure 4). This observation was particularly clear for GLUT4, which was almost undetectable in the plasma membranes of either basal or azide-stimulated cells. Such findings are consistent with the results of Bashan et al. [10], who, using immunoblot analysis of 3T3-L1 plasma membrane fractions, found no increase in GLUT4 and only a 25% increase in GLUT1 immunoreactivity after 4 h of exposure to 0.5 $\mu\text{g}/\text{ml}$ rotenone which is also an inhibitor of oxidative phosphorylation. In contrast to the lack of effect of azide, the levels of both GLUT1 and GLUT4 were seen to be increased in PM lawns prepared from adipocytes exposed to insulin (Figure 4). The prominent nature of the observed changes in staining intensity, particularly in the case of GLUT4, confirms the suitability of the PM lawn method for detecting changes in cell-surface-transporter density.

ML-9, a compound originally described as a myosin light chain kinase inhibitor, inhibits both the stimulation of 2-deoxyglucose transport and the translocation of GLUT1 and GLUT4 induced by insulin in 3T3-L1 adipocytes [16]. The dose-response curve for these effects of ML-9 ($\text{IC}_{50} = 27 \mu\text{M}$) is

Figure 2 Effect of wortmannin on 2-deoxyglucose uptake by Clone 9 cells and 3T3-L1 adipocytes

Upper panels: uptake of 0.2 mM 2-deoxyglucose by Clone 9 cells (a) and 3T3-L1 adipocytes (b) which had been exposed for 90 min to 5 mM azide or 1 μ M insulin, in the presence (hatched bars) or absence (open bars) of 250 nM wortmannin. Mean $\pm$ S.E.M. of five separate experiments in Clone 9 cells and three in 3T3-L1 adipocytes. Lower panels: concentration dependence of the effect of wortmannin on the uptake of 0.2 mM 2-deoxyglucose measured in the basal condition (■), in the presence of 5 mM azide (○), 1 μ M insulin (●) or azide plus insulin (★) in Clone 9 cells (c) and 3T3-L1 adipocytes (d).

Figure 3 Effect of wortmannin on the abundance of plasma membrane-associated GLUT1 in Clone 9 cells

Clone 9 cells were exposed for 90 min to DMEM only (a), DMEM plus 5 mM azide (b), or DMEM containing 5 mM azide and 250 nM wortmannin (c). PM lawns were then prepared and stained for GLUT1 as described in the Experimental section. Microscope detection parameters were kept the same for all Figures. Bars are 25 μ m.

essentially superimposable upon that of its *in vitro* inhibition of mitogen-activated protein kinase (MAP kinase) activity [16]. Recent data on the activation of protein phosphatase-1 by insulin and phorbol esters in L6 myoblasts are also consistent with MAP kinase being a target for ML-9 [25]. Figure 5 shows that 50 μ M ML-9 inhibited the stimulation of 2-deoxy-D-glucose uptake by azide in both Clone 9 cells and 3T3-L1 adipocytes. The effect was found to be dose-dependent and 100 μ M ML-9 almost

Figure 4 Effect of azide and insulin on the abundance of plasma membrane-associated glucose transporters in 3T3-L1 adipocytes

3T3-L1 adipocytes were exposed for 30 min to DMEM only (a,d), DMEM plus 5 mM azide (b,e) or DMEM plus 1 μ M insulin (c,f). PM lawns were then prepared and stained for GLUT1 (a-c) or GLUT4 (d-f) as described in the Experimental section. Microscope detection parameters were kept the same for all Figures except for the inserts, which show Figures (d) and (e) scanned at a higher gain in order to demonstrate that the lack of staining did not reflect the absence of cell membranes on the coverslip. Bars are 25 μ m.

Figure 5 Effect of ML-9 on 2-deoxyglucose uptake by Clone 9 cells and 3T3-L1 adipocytes

The uptake of 0.2 mM 2-deoxyglucose was measured in Clone 9 cells (a) and 3T3-L1 adipocytes (b) that had been exposed 90 min to DMEM only (basal), DMEM plus 5 mM azide or DMEM plus 1 μ M insulin, in the presence (hatched bars) or absence (open bars) of 50 μ M ML-9. Mean $\pm$ S.E.M. of five separate experiments in Clone 9 cells and three in 3T3-L1 adipocytes.

Table 3 Effect of external glucose on the response to azide and insulin in Clone 9 cells

Cells were pre-incubated for 90 min with azide or insulin in KRH buffer in the absence or presence of 25 mM glucose. Uptake of 0.2 mM 2-deoxy-[3 H]glucose was then measured over 5 min as described in the Experimental section. Mean $\pm$ S.E.M. (triplicate). *, uptake in azide-treated cells was significantly higher ($P < 0.05$, Student's t test) in cells pre-incubated with glucose. Similar results were obtained in a separate experiment.

	2-Deoxyglucose uptake (pmol/min per dish)	
	KRH	KRH + 25 mM glucose
Basal	44 $\pm$ 5	34 $\pm$ 3
Azide (5 mM)	108 $\pm$ 7	208 $\pm$ 9*
Insulin (160 nM)	118 $\pm$ 8	105 $\pm$ 26

completely blocked the stimulation of transport by azide, insulin or both agents (results not shown). ML-9 has previously been reported to have no effect on basal sugar uptake, indicating that this compound does not affect the transport mechanism itself [16]. The inhibition of basal uptake seen in the present study (Figure 5) may reflect our pre-incubation of cells in DMEM medium, which partially stimulates transport, rather than in the Krebs-Ringer phosphate buffer used by other workers [16]. Thus, it is possible that ML-9 inhibits the small DMEM-induced

component of the basal uptake that is also sensitive to wortmannin (see above).

Effect of extracellular glucose depletion on stress- and insulin-stimulated glucose uptake

In cells exposed to metabolic inhibitors, the activation of glucose uptake becomes evident about 10–20 min after poisoning, just after the intracellular ATP concentration reaches its nadir [10,22]. An AMP-activated protein kinase has been suggested to mediate several other cellular responses to stresses such as heat shock and arsenite, which cause ATP depletion and consequent elevation of AMP levels [26]. In order to assess the possible involvement of such a kinase in the effect of stress on sugar uptake, the effect of omitting glucose from the medium during exposure of Clone 9 cells to azide or insulin was examined. This omission is likely to increase the cell [AMP]/[ATP] ratio. As shown in Table 3, incubation in the absence of glucose for 90 min had no significant effect on either the basal rate of sugar uptake or the stimulation produced by insulin. Surprisingly, however, the omission of glucose from the medium significantly diminished the stimulatory effect of azide.

DISCUSSION

Recent research has begun to reveal that multiple, interconnected, phosphorylation cascades mediate the regulatory effects of growth factors on diverse biochemical pathways such as protein synthesis [27,28], gene transcription [29], and lipid and glycogen metabolism [14,30–32]. In the present study, we have used wortmannin and ML-9, two pharmacological tools that have proven useful for the delineation of insulin- and other growth factor-activated cascades, in order to examine the possibility that the pathways by which metabolic stress and insulin stimulate glucose uptake share common features.

Wortmannin, a potent inhibitor of PI 3-kinase, has been shown in several previous studies to inhibit the stimulation of glucose transport by insulin in highly insulin-sensitive cells such as adipocytes [13–15]. These findings suggest that activation of PI 3-kinase plays a vital role in the pathway by which glucose transport is stimulated by insulin. However, although activation of the kinase is essential for the effect of insulin, recent studies have shown that it is not sufficient for stimulation of transport in 3T3-L1 adipocytes, suggesting that additional, unknown signalling pathways must also be involved [33]. In the present study we have extended the observation that wortmannin inhibits insulin stimulation of transport to a cell type that is poorly insulin sensitive but very sensitive to metabolic stress. Although no direct measurements of PI 3-kinase activity have been reported for Clone 9 cells, several lines of evidence suggest that the observed effect of wortmannin in these epithelial cells is due to inhibition of PI 3-kinase activity. First, the IC_{50} value for wortmannin inhibition of insulin-stimulated 2-deoxyglucose uptake in Clone 9 cells was essentially identical to those for wortmannin inhibition of insulin-stimulated sugar uptake in CHO cells [13], rat adipocytes [14] and 3T3-L1 adipocytes (this study and [15]). Secondly, in these and other cell types such as neutrophils [34] and RBL-2H3 cells [35], PI 3-kinase activity is known to be inhibited by wortmannin in the same, low nanomolar, concentration range. Thirdly, no other kinase has been shown to be inhibited by nanomolar concentrations of wortmannin, despite intensive investigation [13,14,34].

In contrast to the effect of wortmannin on insulin-stimulation of transport, we found that this reagent had no effect on the stimulation of transport by azide either in highly insulin-sensitive cells (3T3-L1 adipocytes) or in cells that are particularly sensitive

to stress (Clone 9 cells). The results of parallel experiments on insulin stimulation of transport in both cell types showed that the lack of effect of wortmannin on activation of transport by stress did not reflect insensitivity of the cells to wortmannin *per se*. We therefore conclude that PI 3-kinase activation does not play a part in the activation of glucose uptake by metabolic stress, in either Clone 9 cells or 3T3-L1 adipocytes, and that both cell types thus possess multiple pathways regulating glucose transport. The existence of multiple wortmannin-sensitive and -insensitive regulatory pathways in a single cell type has been reported previously: in brown adipocytes the stimulation of glucose transport by insulin is wortmannin-sensitive whereas that by noradrenaline is not [36].

The different mechanisms of transport stimulation (i.e. translocation versus augmented intrinsic activity) also point towards separate pathways for the effects of stress and insulin (Figures 3 and 4). Thus, whereas insulin induces translocation of intracellularly sequestered transporters to the cell surface, with perhaps only a 2-fold increase in the intrinsic activity of individual carriers [2–4], the effects of azide (and rotenone [10]) appear to be due almost exclusively to changes in transporter intrinsic activity. However, the notion of totally independent pathways appears to be at odds with our observation that the effects of azide and insulin are not additive in either 3T3-L1 or Clone 9 cells (Table 2). The similar potency shown by ML-9 in its inhibition of both effects also suggests the involvement of a common step which may represent MAP kinase activation [16,25].

These apparently discrepant data may eventually be reconciled by a model in which multiple, parallel events rather than a linear signal transduction pathway are involved in transport stimulation. In such a model the step inhibited by ML-9 might play a permissive role in glucose transport stimulation by both metabolic stress and insulin. Additional approaches, such as the development of more specific kinase inhibitors, will be required for the further investigation of these phenomena. Similarly, the initial signalling events specifically activated by azide in its stimulation of glucose transport remain unclear. Several kinases and phosphatases have been implicated in other responses to cellular stress including an AMP-activated protein kinase [26]; a heat shock- and oxidative stress-induced protein-tyrosine phosphatase [37]; p54s, a subfamily of c-Jun kinases activated by heat shock, protein synthesis inhibitors and tumour necrosis factor- α [38]; and p38, a kinase activated by UV light, osmotic stress and inflammatory cytokines [39]. Whether any of these are involved in the stimulation of glucose transport by metabolic stress is unclear, but in the light of the effect of the putative MAP kinase inhibitor ML-9, it is noteworthy that the p54s and p38 are members of the superfamily of MAP kinases [39].

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