GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase

Romel SOMWAR^{*†}, David Y. KIM^{*}, Gary SWEENEY^{*1}, Carol HUANG^{*‡}, Wenyan NIU^{*}, Crina LADOR^{*}, Toolsie RAMLAL^{*} and Amira KLIP^{*†2}

^{*}Programme in Cell Biology, Hospital for Sick Children, 555 University Avenue, Toronto, ON, Canada, M5G 1X8, [†]Department of Biochemistry, University of Toronto, Toronto, ON, Canada, M5S 1A8, and [‡]Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada, M5S 1A8

We previously reported that SB203580, an inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), attenuates insulin-stimulated glucose uptake without altering GLUT4 translocation. These results suggested that insulin might activate GLUT4 via a p38 MAPK-dependent pathway. Here we explore this hypothesis by temporal and kinetic analyses of the stimulation of GLUT4 translocation, glucose uptake and activation of p38 MAPK isoforms by insulin. In L6 myotubes stably expressing GLUT4 with an exofacial Myc epitope, we found that GLUT4 translocation ($t_{1/2} = 2.5$ min) preceded the stimulation of 2-deoxyglucose uptake ($t_{1/2} = 6$ min). This segregation of glucose uptake from GLUT4 translocation became more apparent when the two parameters were measured at 22 °C. Preincubation with the p38 MAPK inhibitors SB202190 and SB203580 reduced insulin-stimulated transport of either 2-deoxyglucose or 3-*O*-methylglucose by 40–60 %. Pretreatment with SB203580 lowered the apparent transport V_{max} of insulin-mediated 2-deoxyglucose and 3-*O*-methylglucose without any significant change in the

apparent K_m for either hexose. The IC_{50} values for the partial inhibition of 2-deoxyglucose uptake by SB202190 and SB203580 were 1 and 2 μ M respectively, and correlated with the IC_{50} for full inhibition of p38 MAPK by the two inhibitors in myotubes (2 and 1.4 μ M, respectively). Insulin caused a dose- ($EC_{50} = 15$ nM) and time- ($t_{1/2} = 3$ min) dependent increase in p38 MAPK phosphorylation, which peaked at 10 min (2.3 ± 0.3 -fold). *In vitro* kinase assay of immunoprecipitates from insulin-stimulated myotubes showed activation of p38 α (2.6 ± 0.3 -fold) and p38 β (2.3 ± 0.2 -fold) MAPK. These results suggest that activation of GLUT4 follows GLUT4 translocation and that both mechanisms contribute to the full stimulation of glucose uptake by insulin. Furthermore, activation of GLUT4 may occur via an SB203580-sensitive pathway, possibly involving p38 MAPK.

Key words: intrinsic activity, K_m , pyridinylimidazole, temperature, V_{max} .

INTRODUCTION

Translocation of the glucose transporter GLUT4 to the plasma membrane is a prerequisite for the stimulation of glucose uptake by insulin in muscle and fat tissues [1,2]. However, several studies have shown that GLUT4 translocation alone cannot quantitatively account for the stimulation of glucose uptake in skeletal muscle [3–9] and rat adipocytes [10–12]. These studies demonstrate a consistent discrepancy between the fold stimulation in the rate of glucose uptake into the tissue and the fold translocation of GLUT4. To explain this difference between the magnitude of GLUT4 translocation and glucose transport, it was proposed that insulin may also activate GLUT4, i.e. increase the turnover number of the transporter. This concept is supported further by the following observations. (i) Transporters in isolated intracellular membranes of rat adipocytes did not transport glucose as effectively as transporters isolated from the plasma membrane, even when a 10-fold excess of intracellular membrane transporters was compared [10]. (ii) Insulin decreased the activation energy needed for the transport of glucose, measured in plasma membranes of rat adipocytes [10]. (iii) Low concentrations of wortmannin (< 10 nM) prevented the stimulation of glucose uptake by insulin in 3T3-L1 adipocytes without diminishing GLUT1 or GLUT4 translocation [13]. (iv) We have shown that

the pyridinylimidazole derivative SB203580, an inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), reduced the stimulation of glucose uptake by insulin in 3T3-L1 adipocytes without diminishing the appearance of GLUT1 or GLUT4 at the cell surface [14]. Taken together, these results strongly support the notion that the intrinsic activity of glucose transporters may be regulated by insulin.

The signalling pathway regulating the intrinsic activity of glucose transporters remains largely undefined. Based on the results with SB203580 discussed above, we proposed that p38 MAPK lies in a signalling pathway which regulates the stimulation of glucose transport via modulation of transporter activity [14]. Although SB203580 is selective for p38 MAPK [15], it remained possible that this compound exerted its effect on glucose uptake by other mechanisms. In this study we further explored the role of p38 MAPK in the stimulation of glucose uptake in L6 myotubes expressing Myc-tagged GLUT4, using the two p38 MAPK inhibitors SB203580 and SB202190, as well as the inactive structural analogue SB202474. In addition, we performed temporal analysis of glucose uptake, GLUT4 translocation and activation of p38 MAPK. We report here that insulin-stimulated GLUT4 translocation precedes the full stimulation of glucose uptake. Insulin-activated p38 α and p38 β MAPK and the stimulation of 2-deoxyglucose or 3-*O*-methylglucose

Abbreviations used: ATF-2, activating transcription factor-2; CREB, cAMP-response-element binding protein; p38 MAPK, p38 mitogen-activated protein kinase; PKB, protein kinase B; PI 3-kinase, phosphoinositide 3-kinase.

¹ Present address: Department of Biology, 110 Farquarson, York University, 4700 Keele Street, Toronto, ON, Canada, M3J 1P3.

² To whom correspondence should be addressed (e-mail amira@sickkids.on.ca).

uptake was reduced by preincubation with SB202190 and SB203580, without any effect on GLUT4 translocation. The reduction in insulin-stimulated hexose uptake was due to a reduction in the transport $V_{\max}$, without any effect on the K_m . Prolonged exposure of unstimulated cells to these compounds and their presence in the transport assay solution did not reduce basal glucose uptake. We conclude that insulin activates GLUT4, probably utilizing a p38 MAPK-dependent signal.

MATERIALS AND METHODS

Materials

Activating transcription factor-2 (ATF-2) fusion protein, phospho-specific antibodies to Akt (Thr-308 or Ser-473) and p38 MAPK and anti-p38 MAPK α antibody were purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-phospho-specific cAMP-response-element binding protein (CREB) antibody, SB202190, SB202474 and SB203580 were from Calbiochem (San Diego, CA, U.S.A.). Antibodies to Akt1/protein kinase B α (PKB α ; D-17), p38 α , p38 β and c-Myc (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies to Akt2/PKB β and Akt3/PKB γ , and Crosstide (nine amino acid Akt/PKB α substrate derived from glycogen synthase kinase-3), were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Goat anti-rabbit IgG conjugated to horseradish peroxidase was purchased from Pierce (Rockford, IL, U.S.A.).

L6-GLUT4myc cell line and cell culture

GLUT4myc cDNA was constructed by inserting the human c-Myc epitope (14 amino acids) into the first ectodomain of GLUT4 and then subcloning the construct into the pCXN2 vector as described earlier [16]. The plasmid was stably transfected into L6 myoblasts as described previously [17,17a]. The resulting L6-GLUT4myc cells were characterized previously [18–20]. Myoblasts were maintained in α -minimal essential medium supplemented with 10 % fetal bovine serum (v/v) in a humidified atmosphere of 95 % air/5 % CO₂ at 37 °C and were differentiated into myotubes by growing in α -minimal essential medium supplemented with 2 % fetal bovine serum (v/v). Cells were deprived of serum for 4 h prior to all experimental manipulations. Inhibitors were administered in DMSO and the maximum concentration of the solvent in the incubation medium did not exceed 0.1 % (v/v). This concentration of vehicle was without effect on any of the parameters measured.

Assays of 2-deoxyglucose and 3-O-methylglucose uptake

All inhibitors were administered to myotubes for 40 min and insulin (100 nM) was added during the last 20 min of this incubation. Cells were washed twice and hexose transport was assayed in Hepes-buffered saline solution (140 mM NaCl, 20 mM Hepes/Na, 2.5 mM MgSO₄, 1 mM CaCl₂ and 5 mM KCl, pH 7.4) as described previously [21]. Uptake measurements were carried out in the absence of inhibitors, unless indicated otherwise. For temporal analysis, cells were stimulated with insulin (100 nM) for the times indicated in Figure 1 (see below) and then glucose uptake (0.01 mM 2-deoxyglucose, 1 μ Ci/ml) was measured for 30 s in the continued presence of insulin. In experiments in which the effect of temperature was analysed, all incubations and glucose-uptake (0.01 mM 2-deoxyglucose, 1 μ Ci/ml) measurements were carried out at either 22 or 37 °C for 2 min in the continued presence of insulin. For kinetic analysis, 2-deoxyglucose uptake was measured in Hepes-buffered saline solution containing 0.01–5 mM 2-deoxyglucose (4 μ Ci/ml)

for 2 min, in the absence of insulin. 3-O-Methylglucose uptake was measured in Hepes-buffered saline solution containing 0.01–1.0 mM 3-O-methylglucose (8 μ Ci/ml) for 30 s [22]. Non-specific uptake was determined in the presence of 10 μ M cytochalasin B and was subtracted from all values.

Determination of cell-surface GLUT4myc

GLUT4myc levels at the cell surface were measured by an antibody-coupled optical assay as described previously [18]. Following treatments, myotubes were washed once with PBS, fixed for 3 min with 3 % paraformaldehyde (v/v) at room temperature and then neutralized with 1 % glycine (w/v) in PBS at 4 °C for 10 min. Monolayers were blocked with 10 % (v/v) goat serum and 3 % BSA (w/v) in PBS at 4 °C for at least 30 min and then incubated with anti-c-Myc antibody (9E10, 1:100) at 4 °C for 60 min. Myotubes were then incubated with peroxidase-conjugated donkey anti-mouse IgG (1:1000, 4 °C) for 30 min. Following removal of secondary antibody, cells were incubated for 20 min at room temperature with 1 ml/well of OPD reagent (0.4 mg/ml *O*-phenylenediamine dihydrochloride/0.4 mg/ml urea hydrogen peroxide in 0.05 M phosphate/citrate buffer). The reaction was stopped by adding 0.25 ml of 3 M HCl. Optical absorbance of the supernatant was measured at 492 nm.

Detection of Akt/PKB CREB and p38 MAPK phosphorylation

Lysates were prepared as described previously with modifications [23]. For time-course studies cells were treated with 100 nM insulin for the times indicated in the Figure legends. To determine the effects of SB202190 and SB203580 on Akt and CREB phosphorylation, cells were preincubated with these drugs for 20 min prior to insulin treatment for 10 min, in the continued presence of the drugs. Following appropriate incubations, cells were lysed on ice with 150 μ M 2 $\times$ Laemmli sample buffer supplemented with 7.5 % β -mercaptoethanol, phosphatase inhibitors (1 mM Na₂VO₄ and 100 nM okadaic acid) and protease inhibitors (1 mM benzamidine, 0.2 mM PMSF, 10 μ M E-64, 1 μ M pepstatin A and 1 μ M leupeptin), passed five times through a 24-gauge syringe and heated for 15 min at 65 °C. Total protein (50 μ g) was resolved by SDS/PAGE and immunoblotted with the respective phospho-specific antibody.

Immunoblotting

Phospho-specific primary antibodies were used at a 1:500 dilution. Anti-p38 MAPK antibody was used at a 1:1000 dilution. Goat anti-rabbit IgG conjugated to horseradish peroxidase was used as a secondary antibody at a 1:15000 dilution. Proteins were detected by the enhanced chemiluminescence method. Immunoblots were exposed to X-ray film to produce bands within the linear range and then quantitated using NIH Image (National Institutes of Health software).

Immunoprecipitation and assay of p38 MAPK activity

Anti-p38 α (rabbit polyclonal) or anti-p38 β (goat polyclonal) MAPK antibodies (2 μ g/condition) were adsorbed to Protein A- or Protein G-Sepharose beads, respectively, by incubating for 2 h at 4 °C. Adsorbed beads were washed twice with 1 ml of ice-cold PBS and once with 1 ml of ice-cold lysis buffer [50 mM Hepes, pH 7.6, 150 mM NaCl, 10 % glycerol (v/v), 1 % Triton X-100 (v/v), 30 mM sodium pyrophosphate, 10 mM NaF and 1 mM EDTA] supplemented with 1 mM dithiothreitol, phosphatase inhibitors (1 mM Na₂VO₄ and 100 nM okadaic acid) and protease inhibitors (1 mM benzamidine, 0.2 mM PMSF, 10 μ M E-64, 1 μ M pepstatin A and 1 μ M leupeptin). Lysates

were vortexed for 1 min, passed five times through a 24-gauge syringe and centrifuged for 5 min at 12000 *g* and 4 °C. p38 MAPK was immunoprecipitated by incubating 250 µg of total cellular protein for 2–3 h with the adsorbed Sepharose beads. Immunocomplexes were isolated and washed four times with 1 ml of wash buffer [25 mM Hepes, pH 7.8, 10% glycerol (v/v), 1% Triton X-100 (v/v), 0.1% BSA (w/v) and 1 M NaCl] supplemented with 1 mM dithiothreitol, 0.2 mM PMSF and phosphatase inhibitors (1 mM Na₂VO₄ and 10 nM okadaic acid) and twice with 1 ml of kinase buffer (50 mM Tris/HCl, pH 7.5 and 10 mM MgCl₂) supplemented with 1 mM Na₂VO₄ and 10 nM okadaic acid. Immunocomplexes were then incubated for 30 min at 30 °C with 30 µl of reaction mixture (kinase buffer containing 2 µg of ATF-2, 150 µM ATP and 2 µCi of [γ -³²P]ATP per condition) on a platform shaker. The reaction was stopped by addition of 30 µl of 2× Laemmli sample buffer and heating for 30 min at 37 °C. Samples were centrifuged for 5 min (12000 *g*), and 40 µl of the supernatant was then resolved by SDS/PAGE (10% gel) and electrotransferred on to PVDF membranes. The amount of radiolabelled phosphate transferred on to the substrate was determined by exposing the PVDF membrane to a PhosphorImager cassette and quantified using a Molecular Dynamics PhosphorImager System (Sunnyvale, CA, U.S.A.).

Immunoprecipitation and assay of Akt/PKB protein kinase activity

Immunoprecipitation of Akt isoforms and kinase assay were performed as described previously [24]. Antibodies to Akt1, 2 or 3 (2 µg/condition) were adsorbed to a mixture of Protein A– or Protein G–Sepharose beads. Cells were lysed following all incubations and 250 µg of total cellular protein was immunoprecipitated for 2 h at 4 °C. Crosstide (150 µg/condition) was used as the Akt substrate. Non-specific activity, determined as activity associated with irrelevant IgG, was subtracted from each value.

Statistical analysis

Results were analysed by ANOVA and groups were compared using the Dunnett or Tukey post-tests.

RESULTS

GLUT4 translocation precedes the full stimulation of glucose transport by insulin

L6-GLUT4myc cells stably express GLUT4 tagged with a Myc epitope (GLUT4myc) which is exofacial when GLUT4 is fully inserted into the plasma membrane [17,17a,19]. In L6-GLUT4myc myotubes GLUT4myc is the predominant glucose transporter. Ectopically expressed GLUT4myc segregates, cycles and responds to insulin in a manner similar to endogenous GLUT4 [19,20]. Therefore, these cells provide a good system where GLUT4 is the major glucose transporter in which to study glucose uptake. The amount of GLUT4 that is fully incorporated into the plasma membrane is easily detected and quantified by immunological labelling of the Myc epitope at the surface of intact cells. We performed a detailed temporal analysis of the stimulation of GLUT4 translocation and glucose uptake. Myotubes were treated with 100 nM insulin for 2–20 min and then the amount of GLUT4myc at the cell surface was determined. GLUT4 concentration at the cell surface was increased rapidly by insulin with an estimated $t_{1/2}$ of 2.5 min (Figure 1A). The maximum stimulation observed was 2.8 ± 0.2 -fold above basal attained at 7 min, and stimulation stabilized at a value of 2.5 ± 0.1 -fold above basal at 10 min. This difference between

Figure 1 GLUT4 translocation precedes the stimulation of glucose transport by insulin

(A) L6-GLUT4myc myotubes were treated for the indicated time periods with 100 nM insulin at 37 °C. Cell-surface GLUT4myc level (■) was then determined in intact cells. In parallel, 2-deoxyglucose uptake (○) was measured, in the continued presence of insulin at 37 °C. The time of insulin treatment shown does not include the time of the uptake assay. Results are means $\pm$ S.E.M. from nine (GLUT4 translocation) or five (2-deoxyglucose) experiments. Glucose uptake or GLUT4myc translocation is expressed relative to the respective basal values. (B) Insulin stimulation and uptake measurements were carried out at 37 °C (left-hand panel) or 22 °C (right-hand panel). Results are means $\pm$ S.E.M. from three (22 °C) or five (37 °C) experiments. Filled bars, surface GLUT4myc levels; empty bars, 2-deoxyglucose uptake. Glucose uptake and GLUT4myc translocation are expressed relative to respective basal values.

GLUT4 translocation at these two time points was consistently observed but not statistically significant ($P > 0.05$). The rate of 2-deoxyglucose uptake was measured in parallel to surface GLUT4 levels (Figure 1A). Insulin-stimulated 2-deoxyglucose uptake was delayed compared with GLUT4 translocation. Insulin treatment increased 2-deoxyglucose uptake maximally at 20 min by 2.4 ± 0.1 -fold above basal (8.1 ± 0.2 compared with 19.1 ± 1 pmol/min per mg of protein, basal versus insulin respectively). The estimated $t_{1/2}$ was approx. 6 min. This delay of at least 2 min between the arrival of GLUT4 at the plasma membrane and any observed increase in intracellular glucose suggests the existence of an inactive GLUT4 intermediate. Activation of this GLUT4 intermediate may be necessary for the subsequent stimulation of glucose uptake. A similar observation was also made in rat adipocytes where GLUT4 translocation was detected using the ATB-BMPA [2-*N*-4-(1-azido-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-manose-4-yl)-2-propylamine] photolabel [12].

The results described above were performed in myotubes that were incubated at 37 °C. A previous report suggested that glucose transport activity in isolated rat adipocytes is influenced by the temperature at which this parameter is measured [10]. Hence, we compared the stimulation of GLUT4 translocation and glucose uptake by insulin at different temperatures. Cells were treated with insulin at either 22 °C (Figure 1B, right-hand panel) or

37 °C (Figure 1B, left-hand panel) and GLUT4 translocation and 2-deoxyglucose uptake were determined. At the lower temperature glucose uptake did not reach a maximum, even after 30 min of insulin treatment, whereas GLUT4myc exposure at the surface was already maximal at 20 min (Figure 1B, right-hand panel). GLUT4 translocation at 22 °C was also slowed down early during insulin stimulation (results not shown). However, maximal GLUT4 translocation was achieved following 20 min of insulin treatment at either 22 or 37 °C. These results suggest that GLUT4 activation is reduced at low temperatures.

SB202190 and SB203580 reduce the stimulation of 2-deoxyglucose and 3-O-methylglucose uptake by insulin

SB202190 and SB203580 are pyridinylimidazole derivatives that are widely used as selective inhibitors of p38 MAPK [15,25]. These two compounds inhibit the activity of p38 α and p38 β MAPK without consequence to the γ and δ isoforms [26,27]. SB202474 is a structurally related analogue of SB202190 and SB203580, and is a poor inhibitor of p38 MAPK [28]. Here we determined the effect of preincubation of myotubes with 10 μ M SB202190, SB203580 or SB202474 on the stimulation of glucose uptake and GLUT4 translocation by insulin. This concentration is reported to fully inhibit the enzymes in intact cells [25]. The effect of these drugs on 2-deoxyglucose uptake is shown in Figure 2(A). Insulin treatment caused a 2.2 ± 0.1 -fold increase in 2-deoxyglucose uptake (basal, 7.9 ± 0.1 ; insulin, 17.8 ± 0.8 pmol/min per mg of protein). Preincubation of myotubes with 10 μ M SB202474 did not cause a significant reduction in basal or insulin-stimulated 2-deoxyglucose uptake (SB202474, 7.5 ± 0.5 ; SB202474+insulin, 17 ± 0.8 pmol/min per mg of protein). In contrast, the two active compounds reduced the stimulation of 2-deoxyglucose uptake by insulin significantly. SB202190 reduced insulin-induced 2-deoxyglucose uptake by $64 \pm 9\%$ (SB202190, 8.6 ± 0.2 ; SB202190+insulin, 12.2 ± 1.1 pmol/min per mg of protein). Similarly, SB203580 reduced insulin-induced uptake by $61 \pm 1\%$ (SB203580, 7.9 ± 0.3 ; SB203580+insulin, 11.6 ± 0.5 pmol/min per mg of protein).

To explore whether the inhibitory effect of SB203580 on 2-deoxyglucose was due to inhibition of glucose transport activity itself, and not to secondary effects on post-transport glucose utilization, we measured 3-O-methylglucose uptake in cells that were exposed to 10 μ M SB203580. Insulin increased 3-O-methylglucose uptake by approx. 2.7 ± 0.3 -fold (basal, 62 ± 7 ; insulin, 171 ± 15 pmol/min per mg of protein). This effect of insulin on 3-O-methylglucose uptake was reduced by $56 \pm 9\%$ by SB203580 (SB203580+insulin, 116 ± 16 pmol/min per mg of protein). These results suggest that the effect of the inhibitors of p38 MAPK was due to inhibition of glucose-transport activity and not to alteration in post-transport glucose utilization.

SB203580 lowers the $V_{\max}$ of insulin-stimulated glucose uptake

It is now well established that insulin increases glucose uptake by increasing the $V_{\max}$ of transport without changing the K_m of the transporters for glucose in adipocytes [10] and skeletal muscle [29]. Here we performed kinetic analysis of 2-deoxyglucose and 3-O-methylglucose transport in L6-GLUT4myc myotubes to determine which kinetic parameter was altered by SB203580. Hexose transport was analysed as a function of the concentration of either 2-deoxyglucose (Figure 2B) or 3-O-methylglucose (Figure 2C). Both 2-deoxyglucose uptake and 3-O-methylglucose uptake displayed saturation kinetics. Data were linearized by the method of Eadie-Hofstee [30]. This analysis assumes that

Figure 2 SB202190 and SB203580 reduce insulin-stimulated glucose uptake by reducing the transport $V_{\max}$

(A) Cells were treated for 20 min with DMSO vehicle alone or with 10 μ M SB202474 (inactive analogue), SB202190 or SB203580 prior to insulin treatment for an additional 20 min. 2-Deoxyglucose was then determined. Empty bars, basal; filled bars, insulin treatment. * $P < 0.05$, compared with basal; # $P < 0.01$, compared with insulin-treated control. (B, C) L6-GLUT4myc myotubes were left untreated or treated with 10 μ M SB203580 for 20 min prior to insulin treatment for an additional 20 min. Transport of 2-deoxyglucose (B) or 3-O-methylglucose (C) were then determined in the presence of increasing concentrations of the respective glucose analogue. Results are means $\pm$ S.E.M. from 2–5 experiments. Each condition was assayed in triplicate. □, Basal; ○, insulin; ●, SB203580 + insulin.

hexose transport displays simple hyperbolic kinetics. Apparent $V_{\max}$ and K_m values were determined and are shown in Table 1. The apparent K_m and $V_{\max}$ values for zero-trans influx of 2-

Table 1 $V_{\max}$ and K_m values for hexose uptake in L6-GLUT4myc myotubes

Cells were treated with SB203580 for 20 min prior to insulin treatment for an additional 20 min. Hexose uptake was then measured as a function of substrate concentration. $V_{\max}$ (pmol/min per mg of protein) and K_m (mM) values were obtained for individual experiments by Eadie–Hofstee analysis of experimental data [30]. Results are the means $\pm$ S.E.M. or S.D. from 2–5 experiments. There were three replicates of each condition per experiment.

	2-Deoxyglucose uptake		3- <i>O</i> -Methylglucose uptake	
	K_m	$V_{\max}$	K_m	$V_{\max}$
Basal	1.9 ± 0.4	2140 ± 428	0.5 ± 0.1	420 ± 151
Insulin	1.7 ± 0.1	5567 ± 620	0.8 ± 0.2	1553 ± 215
SB203580 + insulin	1.7 ± 0.1	3104 ± 471	0.9 ± 0.2	1135 ± 73

Figure 3 GLUT4 translocation is not affected by SB202190 or SB203580

Surface GLUT4myc levels were determined in cells that were treated as described for Figure 2(A). Cell-surface GLUT4myc levels are expressed relative to basal levels. Results are means $\pm$ S.E.M. from three experiments. Empty bars, basal; filled bars, insulin.

deoxyglucose under basal conditions were 1.9 ± 0.4 mM and 2140 ± 428 pmol/min per mg of protein, respectively. Insulin increased the apparent transport $V_{\max}$ for 2-deoxyglucose uptake by approx. 2.6-fold (5567 pmol/min per mg of protein) without altering the apparent K_m (1.7 ± 0.1 mM). Pretreatment of cells with SB203580 reduced the apparent transport $V_{\max}$ of insulin-mediated 2-deoxyglucose transport by approx. 60% (3104 ± 471 pmol/min per mg of protein) without any change in the apparent K_m (1.7 ± 0.1 mM).

The apparent K_m and $V_{\max}$ values for zero-trans influx of 3-*O*-methylglucose uptake under basal conditions were 0.5 ± 0.1 mM and 420 ± 151 pmol/min per mg of protein, respectively (Table 1). Insulin increased the apparent $V_{\max}$ by 3.5-fold (1553 ± 215 pmol/min per mg of protein) without any significant change in the apparent K_m for 3-*O*-methylglucose (0.8 ± 0.2 mM). Similar to 2-deoxyglucose uptake, pretreatment of cells with SB203580 reduced the apparent transport $V_{\max}$ of insulin-mediated 3-*O*-methylglucose to 1135 pmol/min per mg of protein. This effect occurred without any significant change in the apparent K_m for this hexose. The compounds had no effect on the kinetics of basal glucose uptake (results not shown). These results suggest that pyridinylimidazole inhibitors of p38 MAPK reduce insulin-mediated glucose uptake by decreasing the carrying capacity of GLUT4 without altering the affinity of the transporter for glucose. Since the compounds were not present in the transport assay, these effects are probably due to actions on the ability of insulin to stimulate glucose uptake.

Table 2 Glucose uptake is not affected by the presence of SB202190 or SB203580 in the transport assay

Cells were treated for the indicated time periods with 10 μ M SB202190 or SB203580. 2-Deoxyglucose transport was then determined in the absence or continued presence of the indicated inhibitor for an additional 10 min. Results are the means $\pm$ S.E.M. from three experiments in which each condition was assayed in triplicate determinations. 2-Deoxyglucose uptake in untreated cells was 7.9 ± 0.1 pmol/min per mg of protein.

Time with drug (min)	2-Deoxyglucose uptake (pmol/min per mg of protein)	
	SB202190	SB203580
0	7.9 ± 0.9	7.3 ± 1.2
5	7.3 ± 1.1	8.4 ± 0.6
10	7.4 ± 0.7	7.6 ± 0.1
15	7.2 ± 0.05	7.6 ± 0.8
20	7.1 ± 1.1	7.7 ± 0.5

Insulin-stimulated GLUT4 translocation is not reduced by SB202190 or SB203580

We measured cell-surface GLUT4 levels to determine whether the reduction in glucose uptake observed in the presence of the p38 MAPK inhibitors was due to lower GLUT4 translocation. Similar to the results shown in Figure 1(A), 20 min of insulin treatment caused a 2.5 ± 0.2 -fold increase in plasma membrane GLUT4myc (Figure 3). This effect of insulin on GLUT4 translocation was not affected by preincubation of cells with 10 μ M SB202190 or SB203580 (SB202190 + insulin, 2.7 ± 0.2 -fold; SB203580 + insulin, 3.0 ± 0.4 -fold). Similarly, the stimulation of GLUT4 translocation by insulin remained unaffected by up to 50 μ M of either inhibitor (SB202190, 1.3 ± 0.2 -fold; SB202190 + insulin, 2.7 ± 0.3 -fold; SB203580, 1.3 ± 0.1 -fold; SB203580 + insulin, 2.9 ± 0.4 -fold). Hence, the reduction in insulin-stimulated glucose uptake did not arise from a decrease in the number of cell-surface-associated GLUT4 molecules. These results are consistent with the interpretation that in addition to GLUT4 translocation, insulin increases glucose uptake by increasing GLUT4 activity and that such activation was prevented by inhibitors of p38 MAPK.

SB202190 and SB203580 do not reduce 2-deoxyglucose uptake when present in the transport assay

The results in Figure 2 suggest that the inhibitory effect of SB202190 and SB203580 on insulin-stimulation of glucose uptake could be due to the ability of these drugs to inhibit p38 MAPK since the inactive analogue SB202474 had no effect. However, it is also possible that the inhibitory agents exerted their action by directly inhibiting GLUT4. We have shown previously that including SB203580 during the 5 min transport assay alone had no effect on basal or insulin-stimulated 2-deoxyglucose transport, suggesting that the compound does not interact directly with GLUT4 [14]. In case the lack of effect of SB203580 observed by this protocol was because the drug needed more than 5 min to interact with GLUT4, we determined the effect of prolonged incubation with SB202190 and SB203580 on 2-deoxyglucose uptake. Myotubes were preincubated with each of the two agents for 0–20 min and then the rate of 2-deoxyglucose uptake was measured for 10 min in the continued presence of each drug. By this protocol, neither SB202190 nor SB203580 (10 μ M) affected glucose uptake, even after 20 min of preincubation followed by 10 min of uptake measured in the presence of each inhibitor (Table 2). These results suggest that the effect of SB202190 and

Figure 4 Insulin increases p38 MAPK phosphorylation

Lysates were prepared from cells that were treated with (A) the indicated concentrations of insulin for 10 min or (B) 100 nM insulin for the indicated time periods. Total protein (50 μ g) was resolved by SDS/PAGE (10% gel) and immunoblotted with a phospho-specific p38 MAPK antibody. A representative immunoblot is shown for each time point. Phosphorylated p38 MAPK was quantified and is shown in the accompanying graphs. All values are expressed relative to basal phosphorylation. Results are means $\pm$ S.E.M. from four experiments.

SB203580 on insulin-stimulation of glucose uptake was not due to a direct interaction with GLUT4.

Insulin increases p38 MAPK phosphorylation

Activation of p38 MAPK by cytokines requires dual phosphorylation by specific kinases (MAPK kinase 3/6) on Thr-180 and Tyr-182 found within a TGY motif [31]. Detecting the phosphorylation at these sites has become a convenient way to measure p38 MAPK activation. To explore the effect of insulin on p38 MAPK, we monitored enzyme phosphorylation using an antibody which recognizes only the phosphorylated protein [31]. A detailed analysis of the dose-dependent and temporal effects of insulin on enzyme phosphorylation is shown in Figure 4. L6-GLUT4myc myotubes were treated with increasing concentrations of insulin for 10 min and p38 MAPK phosphorylation determined. Immunoblots of several experiments were quantified and the results are illustrated in Figure 4(A). Insulin elicited a dose-dependent phosphorylation of p38 MAPK, reaching a maximum by 50 nM (2.2 ± 0.3 -fold). The EC_{50} for this effect of insulin was approx. 15 nM. Myotubes were also treated for 2–15 min with 100 nM insulin. Immunoblots of several experiments were quantified and the results are also illustrated in

Figure 5 Activation of p38 α and p38 β MAPK by insulin

Total cell extracts were prepared from cells that were left untreated or were treated with 100 nM insulin for the indicated time periods. p38 α (A) or p38 β (B) MAPK were immunoprecipitated and enzyme activities were determined by an *in vitro* kinase assay using ATF-2 as substrate. Results represent the means $\pm$ S.E.M. from four experiments.

Figure 4(B). Induction of p38 MAPK phosphorylation by insulin was rapid and transient. This parameter was significantly elevated following 2 min of insulin stimulation (1.5 ± 0.1 -fold, $P < 0.01$), reached maximum at 5–10 min (2.3 ± 0.3 -fold) and declined by 15 min (Figure 4A). The $t_{1/2}$ for the stimulation of p38 MAPK phosphorylation by insulin was approx. 3 min.

p38 MAPK α and β isoforms are activated by insulin

The phospho-specific p38 MAPK antibody is expected to recognize all isoforms of the enzyme. Since the two pyridinyl-imidazole derivatives that reduced glucose uptake (Figure 2) inhibit p38 α and p38 β MAPK, we monitored activation of these two isoforms by insulin in L6-GLUT4myc myotubes. Enzyme activity was assayed *in vitro* and results are shown in Figure 5. Activation of each isoform was rapid and displayed similar time courses. Both enzymes were activated maximally within 5 min, paralleling enzyme phosphorylation. Maximal activation of p38 α and p38 β MAPK were 2.6 ± 0.2 and 2.3 ± 0.2 -fold, respectively.

Insulin induces phosphorylation of CREB, a p38 MAPK effector

The transcription factor CREB is a well-characterized p38 MAPK effector [25,32,33]. Therefore, we monitored phosphorylation of CREB as an index of *in vivo* activation of p38 MAPK by insulin. Phosphorylation of CREB was determined by immunoblotting with a phospho-specific CREB antibody and the results are shown in Figure 6. CREB phosphorylation in four individual immunoblots was quantified and is expressed in the graph (Figure 6). As observed for p38 MAPK phosphorylation, a significant increase in CREB phosphorylation was observed

Figure 6 Insulin increases phosphorylation of the p38 MAPK effector CREB

Cells were treated for the indicated time periods with 100 nM insulin. Lysates were prepared and 50 μ g of total protein was separated by SDS/PAGE (10% gel) and immunoblotted with a phospho-specific CREB antibody. A representative immunoblot of the time course of CREB phosphorylation is shown. Immunoblots were quantified and the amounts of phosphorylated CREB are shown in the graph. Results are the means $\pm$ S.E.M. from four experiments.

within 5 min of insulin treatment (1.7 ± 0.1 -fold) and reached a maximum by 10 min (2.4 ± 0.2 -fold). Phosphorylation remained elevated for at least 15 min.

Glucose uptake and p38 MAPK activity are reduced with similar potencies by SB202190 and SB203580

CREB phosphorylation was utilized to determine the IC_{50} for inhibition of p38 MAPK by SB202190 and SB203580 in intact cells. Myotubes were treated for 20 min with various concentrations of either SB202190 or SB203580 and then insulin was added for an additional 10 min. Whole-cell extracts were prepared and immunoblotted for phosphorylated CREB. Phosphorylation detected in individual immunoblots was quantified and the results are shown in Figure 7 (left-hand panels). Insulin-stimulated CREB phosphorylation in the absence of any inhibitor (2.6 ± 0.1 -fold) was set at 100%. This response was abolished by SB202190 (Figure 7A, left-hand panel) and SB203580 (Figure 7B, left-hand panel) with IC_{50} values of 2 and 1.4 μ M, respectively.

We have previously reported that inhibition of insulin-stimulated 2-deoxyglucose uptake in L6 myotubes and 3T3-L1 adipocytes was complete and occurred within an expanded range of SB203580 concentrations (0.01–100 μ M). The range of these dose-response curves and their apparent bimodal shape prompted us to re-examine the effectiveness of preincubation with SB202190 and SB203580 to inhibit insulin-induced glucose uptake. Pretreatment of myotubes with SB202190 caused a dose-dependent decrease in insulin-stimulated 2-deoxyglucose uptake (Figure 7A, right-hand panel). However, maximum inhibition of approx. 40% was reached at 2.5 μ M and remained stable in the presence of up to 10 μ M SB202190. The IC_{50} for this partial reduction caused by SB202190 was approx. 1 μ M. No further

Figure 7 SB202190 and SB203580 reduce CREB phosphorylation and glucose uptake with similar potencies

Insulin-stimulated CREB phosphorylation and 2-deoxyglucose uptake were determined in cells that were treated with SB202190 (A) or SB203580 (B) as described for Figures 5 and 2, respectively. Insulin-stimulated CREB phosphorylation (left-hand panels) or 2-deoxyglucose uptake (right-hand panels) in the absence of inhibitors are expressed as 100%. All other values are expressed relative to these values. Results are means $\pm$ S.E.M. from three (CREB phosphorylation) or four–five (2-deoxyglucose uptake) experiments. * $P < 0.01$, compared with insulin-stimulated value in the absence of any inhibitor.

Figure 8 Phosphorylation and activation of Akt isoforms by insulin is not affected by SB202190 or SB203580

Cells were treated for 20 min with DMSO vehicle alone or 10 μ M SB202190 or SB203580 prior to stimulation with 100 nM insulin for an additional 10 min. **(A)** Lysates (50 μ g) were immunoblotted for Akt phosphorylated at Thr-308 (upper immunoblot) or Ser-473 (lower immunoblot). Similar results were obtained in two experiments. **(B)** Akt 1, 2 or 3 were immunoprecipitated and kinase activity determined by an *in vitro* kinase assay using Crossslide as the substrate. All values are expressed relative to basal kinase activity, which was assigned a value of 1. Results are the means $\pm$ S.E.M. from three experiments in which each condition was assayed in duplicate.

reduction was observed at concentrations up to 25 μ M (results not shown), beyond which we suspect that non-specific effects of the drug may occur. Pretreatment with SB203580 also resulted in a maximum of 40% reduction of insulin-stimulated glucose uptake, achieved with 5 μ M. The IC_{50} for this partial inhibition of glucose uptake by SB203580 was approx. 2 μ M (Figure 7B, right-hand panel). The IC_{50} values calculated for the maximally attainable inhibition of glucose uptake by SB202190 and SB203580 correlated closely with the corresponding IC_{50} values for full inhibition of CREB phosphorylation.

Early insulin signals are not affected by SB202190 or SB203580

We have previously reported that 10 μ M SB203580 did not reduce insulin-stimulated tyrosine phosphorylation of insulin-receptor substrate-1 (IRS-1), the association of phosphoinositide 3-kinase (PI 3-kinase) with IRS-1, PI 3-kinase activity or activation of Akt1, 2 or 3 in 3T3-L1 adipocytes [14]. Similarly, activation of Akt by hyperosmolarity or heat shock was not affected by SB203580 [34]. In L6 myotubes, activation of p70 ribosomal protein S6 kinase by insulin was also not reduced by 10 μ M SB203580 [24]. More recently, it was shown that 3-phosphoinositide-dependent kinase-1 activity remained unaffected by *in vitro* incubation with SB202190 or SB203580 [35]. In contrast to these results, it was reported that SB203580 abolished interleukin-1-induced Akt phosphorylation in T-lymphocytes with an IC_{50} value of approx. 5 μ M [36]. Given the apparent cell-specific differences, we determined the effect of 10 μ M SB202190 or SB203580 on insulin-stimulated phosphorylation of Akt at Thr-308 and Ser-473 and activation of the three Akt isoforms expressed in L6 myotubes. Akt phosphorylation was detected using antibodies that recognize the enzyme phosphorylated at either Thr-308 or Ser-473. Figure 8(A) shows that treatment of L6-GLUT4myc myotubes with insulin for

10 min caused a substantial increase in Akt phosphorylation on Thr-308 and Ser-473. Neither SB202190 nor SB203580 had any effect on these parameters. The activation of the three Akt isoforms by insulin was also not affected by 10 μ M SB202190 or SB203580 (Figure 8B). The lack of effect of SB202190 and SB203580 on Akt activation is consistent with the observation that these inhibitors have no effect on surface GLUT4 levels (see Figure 3) since GLUT4 translocation may require Akt in L6 cells [37].

DISCUSSION

Discrepancy between GLUT4 translocation and the stimulation of glucose uptake

Numerous studies have concluded that the cell-surface content of GLUT4 does not correlate quantitatively with the magnitude of insulin-induced glucose uptake [3–7,12]. This discrepancy between glucose uptake and GLUT4 translocation may be reconciled if the intrinsic activity of GLUT4, i.e. the glucose-transport activity of each transporter molecule, were also elevated by insulin. Providing experimental evidence in support of this phenomenon has required a system that allows accurate detection and quantification of GLUT4 at the cell surface and parallel measurements of glucose uptake. Using L6 myotubes which express Myc-tagged GLUT4 we report here that GLUT4 is fully inserted into the plasma membrane at least 2 min before an increase in glucose uptake is observed. Glucose uptake continued to lag behind GLUT4 translocation until 20 min after initiation of insulin treatment, when a maximal rate of uptake was observed. This dissociation in time between GLUT4 translocation and the stimulation of glucose uptake became even more apparent at 22 $^{\circ}$ C. At this temperature, full translocation had already occurred at 20 min yet the stimulation of glucose

uptake remain depressed even after 30 min of insulin treatment. The exacerbated discrepancy between GLUT4myc translocation and glucose uptake observed at 22 °C suggests that the activation step is more temperature-sensitive than the translocation. These differences between glucose uptake and GLUT4 translocation cannot be ascribed to a slow fusion step of docked/occluded vesicles because we define translocation as exposure of the Myc epitope at the cell surface. Further evidence that GLUT4 is activated following translocation is supported by the observation that transporters in the low-density microsomes isolated from rat adipocytes transport glucose much less efficiently than plasma-membrane glucose transporters [10]. Taken together, these observations suggest that GLUT4 at the cell surface may require activation before it can transport glucose to its maximum capacity. In L6 myotubes overexpressing GLUT4myc and in rat adipocytes, GLUT4 markedly predominates over GLUT1 and the latter transporter is deemed unlikely to contribute significantly to insulin-stimulated glucose uptake [38,39]. GLUT1 also appears to be 'silent' in chronically stimulated L6 myotubes [39a]. Moreover, L6-GLUT4myc myotubes have a higher sensitivity to forskolin, compared with parental L6 myotubes (C. Huang, W. Niu and A. Klip, unpublished work), suggesting that GLUT4 is the predominant transporter in this cell line. Nevertheless, we cannot completely eliminate the possibility that the low amount of endogenous GLUT1 may also contribute to glucose uptake.

Insulin stimulation of glucose uptake, but not GLUT4 translocation, is reduced by pyridinylimidazoles that inhibit p38 MAPK

Here we show that preincubation of L6 myotubes with the p38 MAPK inhibitors SB202190 and SB203580 reduced insulin-stimulated 2-deoxyglucose and 3-*O*-methylglucose uptake without impinging on GLUT4 translocation. This observation is similar to our previous study in 3T3-L1 adipocytes in which SB203580 reduced the stimulation of 2-deoxyglucose and 3-*O*-methylglucose uptake without affecting GLUT1 or GLUT4 translocation [14]. Both studies imply that insulin regulates the transport capacity of GLUT4. In the current study we demonstrate further that the effect of SB203580 on insulin-stimulated hexose uptake was due to a reduction in the apparent $V_{\max}$ for transport of either 2-deoxyglucose or 3-*O*-methylglucose. The drug had no effect on the apparent K_m for either hexose.

Incubation of L6 myotubes with 10 μ M SB202190 or SB203580 for up to 20 min and subsequent measurement of basal glucose uptake in the continued presence of each agent for 10 min did not affect 2-deoxyglucose uptake. Therefore, it is unlikely that the reduction in glucose uptake could be due to any non-specific interaction of SB202190 or SB203580 with GLUT4. Agents such as protease inhibitors [40,41] and forskolin [42], which bind to GLUT4 and inhibit its transport function, act rapidly. Moreover, SB202474, a structural analogue of SB202190 and SB203580 that does not modulate p38 MAPK activity, had no effect on the stimulation of 2-deoxyglucose uptake by insulin.

Reduction of insulin-stimulated glucose uptake by pharmacological inhibitors of p38 MAPK correlates with inhibition of p38 MAPK *in vivo*

In Figure 7 we show a partial reduction in insulin-stimulated glucose uptake that resembled a single exponential curve using concentrations of up to 10 μ M SB202190 or SB203580. The IC_{50} values for the maximally attainable inhibition of glucose uptake by SB202190 or SB203580 were 1 and 2 μ M, respectively. These values are higher than the IC_{50} for inhibition of p38 MAPK *in*

vitro [28]. However, when given to intact cells, these agents compete with ATP for binding to p38 MAPK and the intracellular concentration of ATP is higher than the concentration of ATP in the assays used to characterize these inhibitors [35]. To obtain a more accurate reflection of the sensitivity of p38 MAPK to the pyridinylimidazole derivatives in intact cells, we determined the IC_{50} values for inhibition of p38 MAPK by SB202190 and SB203580 *in vivo*. An informative way of detecting p38 MAPK activity in intact cells is to monitor the activation of CREB, a known p38 MAPK effector [25,32,33]. Using phosphorylation of CREB as an index of p38 MAPK activity *in vivo* we found that SB202190 and SB203580 inhibited p38 MAPK with IC_{50} values of 2 and 1.4 μ M, respectively. Therefore, the reduction of insulin-stimulated GLUT4 activity by SB202190 and SB203580 correlates closely with inhibition of p38 MAPK *in vivo*.

We have demonstrated previously that SB203580 did not affect upstream signalling events such as activation of PI 3-kinase and Akt in 3T3-L1 adipocytes [14]. In the present study we extend these observations to show that SB202190 and SB203580 did not reduce Akt activation by insulin in L6-GLUT4myc myotubes. Taken together, these data demonstrate that these pyridinylimidazole derivatives do not have rapid effects directly on the glucose-transport system or on the signalling network responsible for the translocation and docking/fusion of GLUT4 with the plasma membrane. Therefore, the reduction in GLUT4 activity caused by SB202190 and SB203580 is possibly due to a specific effect on p38 MAPK-dependent signalling. In support of this conclusion, we have recently observed that expression of a dominant-negative p38 MAPK mutant under the control of an inducible promoter in 3T3-L1 adipocytes [43] reduced the insulin-dependent stimulation of glucose uptake [44].

Insulin increases p38 MAPK phosphorylation and activates p38 α and p38 β MAPK

To date, four isoforms of p38 MAPK encoded by different genes have been identified. They have been designated p38 α , p38 β , p38 δ and p38 γ MAPK [45]. Northern-blot analysis showed a wide tissue distribution of the four isoforms [46]. L6 myotubes express p38 α , p38 β and p38 γ MAPK (R. Somwar and A. Klip, unpublished work). In the present study we measured activation of p38 MAPK by insulin using three different approaches. First, we performed dose-dependent and temporal analyses of p38 MAPK phosphorylation. The dose-dependent increase in p38 MAPK phosphorylation that was observed parallels the stimulation of glucose uptake caused by insulin in these cells [19]. A similar increase in p38 MAPK phosphorylation has been demonstrated in skeletal muscle of rat [47,48] and mouse [49] and in 3T3-L1 adipocytes [14,50,51], Chinese hamster ovary cells [23] and vascular smooth-muscle cells [52]. In contrast to these studies, Goodyear et al. [53] did not observe an increase in p38 MAPK phosphorylation in rat mixed hindlimb muscle. It is possible that the stimulation could have been missed because of the prolonged insulin treatment (20–30 min) and the transient nature of p38 MAPK's response to insulin (Figures 4 and 5, and [48]).

Secondly, we observed that insulin activated p38 α and p38 β MAPK using an *in vitro* kinase assay. Similar to p38 MAPK phosphorylation, the time courses of activation of p38 α and p38 β MAPK preceded the stimulation of glucose uptake. This result is consistent with the hypothesis that activation of p38 MAPK could be a permissive signal for the stimulation of glucose uptake by insulin. Insulin also activates p38 MAPK in extensor digitorum longus [47], gastrocnemius and quadriceps [47] muscles of the rat and mouse mixed hindlimb muscle [49]. In

contrast, Kayali et al. [51] concluded that insulin did not increase the kinase activity of p38 MAPK in 3T3-L1 adipocytes. However, this conclusion was based on measurement of p38 MAPK activity at 30 min of insulin treatment although the same study showed that insulin-stimulated p38 MAPK phosphorylation (2.2-fold above basal) reached a maximum between 5 and 10 min and returned to basal levels by 30 min. In all instances, the insulin-dependent stimulation of p38 MAPK phosphorylation or activity is lower than the effects of hyperosmolar stress, suggesting the possibility that insulin may engage only a subset of p38 MAPK molecules.

Conclusions

In summary, the observation that GLUT4 translocation preceded the stimulation of glucose uptake by insulin in L6 myotubes suggests that insulin increases glucose uptake by first increasing GLUT4 translocation and then further activating the transporters. Insulin treatment caused activation of p38 α and p38 β MAPK with time courses that paralleled the stimulation of glucose uptake. Two pharmacological inhibitors of p38 MAPK reduced the stimulation of glucose uptake with IC₅₀ values that correlate with the inhibition of p38 MAPK in intact myotubes, yet insulin-induced GLUT4 translocation was not affected. These results constitute the basis for the hypothesis that GLUT4 activation may be regulated by a p38 MAPK-dependent signalling pathway.

We thank Dr Philip J. Bilan for critical assessment of this manuscript. This work was supported by a research grant from the Canadian Diabetes Association to A.K.R.S. was supported by a Doctoral Award from the Canadian Institute for Health Research. D.Y.K. was supported by summer studentship from the Research Institute Training Center at the Hospital for Sick Children. G.S. was supported by a joint Post Doctoral Fellowship from the Banting and Best Diabetes Center at the University of Toronto and Novo Nordisk Canada. W.N. was supported by the Visiting Scientist Program at the Hospital for Sick Children.

REFERENCES

- Kahn, B. B. (1996) Lilly lecture 1995. Glucose transport: pivotal step in insulin action. *Diabetes* **45**, 1644–1654
- Klip, A. and Marette, A. (1992) Acute and chronic signals controlling glucose transport in skeletal muscle. *J. Cell. Biochem.* **48**, 51–60
- Hansen, P. A., Wang, W., Marshall, B. A., Holloszy, J. O. and Mueckler, M. (1998) Dissociation of GLUT4 translocation and insulin-stimulated glucose transport in transgenic mice overexpressing GLUT1 in skeletal muscle. *J. Biol. Chem.* **273**, 18173–18179
- Marette, A., Richardson, J. M., Ramlal, T., Balon, T. W., Vranic, M., Pessin, J. E. and Klip, A. (1992) Abundance, localization, and insulin-induced translocation of glucose transporters in red and white muscle. *Am. J. Physiol.* **263**, C443–C452
- Goodyear, L. J., Hirshman, M. F., Smith, R. J. and Horton, E. S. (1991) Glucose transporter number, activity, and isoform content in plasma membranes of red and white skeletal muscle. *Am. J. Physiol.* **261**, E556–E561
- Garvey, W. T., Maianu, L., Zhu, J. H., Brechtel-Hook, G., Wallace, P. and Baron, A. D. (1998) Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J. Clin. Invest.* **101**, 2377–2386
- King, P. A., Horton, E. D., Hirshman, M. F. and Horton, E. S. (1992) Insulin resistance in obese Zucker rat (fa/fa) skeletal muscle is associated with a failure of glucose transporter translocation. *J. Clin. Invest.* **90**, 1568–1575
- Kahn, B. B., Simpson, I. A. and Cushman, S. W. (1988) Divergent mechanisms for the insulin resistant and hyperresponsive glucose transport in adipose cells from fasted and refed rats. Alterations in both glucose transporter number and intrinsic activity. *J. Clin. Invest.* **82**, 691–699
- Zierler, K. (1998) Does insulin-induced increase in the amount of plasma membrane GLUTs quantitatively account for insulin-induced increase in glucose uptake? *Diabetologia* **41**, 724–730
- Joost, H. G., Weber, T. M. and Cushman, S. W. (1988) Qualitative and quantitative comparison of glucose transport activity and glucose transporter concentration in plasma membranes from basal and insulin-stimulated rat adipose cells. *Biochem. J.* **249**, 155–161
- Malide, D., Ramm, G., Cushman, S. W. and Slot, J. W. (2000) Immunoelectron microscopic evidence that GLUT4 translocation explains the stimulation of glucose transport in isolated rat white adipose cells. *J. Cell Sci.* **113**, 4203–4210
- Clark, A. E., Holman, G. D. and Kozka, I. J. (1991) Determination of the rates of appearance and loss of glucose transporters at the cell surface of rat adipose cells. *Biochem. J.* **278**, 235–241
- Hausdorff, S. F., Fingar, D. C., Morioka, K., Garza, L. A., Whiteman, E. L., Summers, S. A. and Birnbaum, M. J. (1999) Identification of wortmannin-sensitive targets in 3T3-L1 adipocytes. Dissociation of insulin-stimulated glucose uptake and GLUT4 translocation. *J. Biol. Chem.* **274**, 24677–24684
- Sweeney, G., Somwar, R., Ramlal, T., Volchuk, A., Ueyama, A. and Klip, A. (1999) An inhibitor of p38 MAPK mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. *J. Biol. Chem.* **274**, 10071–10078
- Caenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**, 229–233
- Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M. and Ebina, Y. (1993) Direct demonstration of insulin-induced GLUT4 translocation to the surface of intact cells by insertion of a c-myc epitope into an exofacial GLUT4 domain. *J. Biol. Chem.* **268**, 14523–14526
- Kishi, K., Muramoto, N., Nakaya, Y., Miyata, I., Hagi, A., Hayashi, H. and Ebina, Y. (1998) Bradykinin directly triggers GLUT4 translocation via an insulin-independent pathway. *Diabetes* **47**, 550–558
- Erratum (1998) *Diabetes* **47**, 1170
- Wang, Q., Khayat, Z., Kishi, K., Ebina, Y. and Klip, A. (1998) GLUT4 translocation by insulin in intact muscle cells: detection by a fast and quantitative assay. *FEBS Lett.* **427**, 193–197
- Ueyama, A., Yaworsky, K. L., Wang, Q., Ebina, Y. and Klip, A. (1999) GLUT-4myc ectopic expression in L6 myoblasts generates a GLUT-4-specific pool conferring insulin sensitivity. *Am. J. Physiol.* **277**, E572–E578
- Li, D., Randhawa, V. K., Patel, N., Hayashi, M. and Klip, A. (2001) Hyperosmolarity reduces GLUT4 endocytosis and increases its exocytosis from a vAMP2-independent pool in L6 muscle cells. *J. Biol. Chem.* **276**, 22883–22891
- Somwar, R., Sweeney, G., Ramlal, T. and Klip, A. (1998) Stimulation of glucose and amino acid transport and activation of the insulin signaling pathways by insulin lispro in L6 skeletal muscle cells. *Clin. Ther.* **20**, 125–140
- Klip, A., Logan, W. J. and Li, G. (1982) Hexose transport in L6 muscle cells. Kinetic properties and the number of [³H]cytochalasin B binding sites. *Biochim. Biophys. Acta* **687**, 265–280
- Lee-Kwon, W., Park, D., Baskar, P. V., Kole, S. and Bernier, M. (1998) Antiapoptotic signaling by the insulin receptor in Chinese hamster ovary cells. *Biochemistry* **37**, 15747–15757
- Somwar, R., Sumitani, S., Taha, C., Sweeney, G. and Klip, A. (1998) Temporal activation of p70 S6 kinase and Akt1 by insulin: PI 3-kinase-dependent and -independent mechanisms. *Am. J. Physiol.* **275**, E618–E625
- Eyers, P. A., van den Ijssel, P., Quinlan, R. A., Goedert, M. and Cohen, P. (1999) Use of a drug-resistant mutant of stress-activated protein kinase 2a/p38 MAPK to validate the *in vivo* specificity of SB 203580. *FEBS Lett.* **451**, 191–196
- Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C. and Young, P. R. (1997) Novel homologues of CSBP/p38 MAPK MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem. Biophys. Res. Commun.* **235**, 533–538
- Jiang, Y., Li, Z., Schwarz, E. M., Lin, A., Guan, K., Ulevitch, R. J. and Han, J. (1997) Structure-function studies of p38 MAPK mitogen-activated protein kinase. Loop 12 influences substrate specificity and autophosphorylation, but not upstream kinase selection. *J. Biol. Chem.* **272**, 11096–11102
- Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W. et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature (London)* **372**, 739–746
- Hansen, P., Gulve, E., Gao, J., Schluter, J., Mueckler, M. and Holloszy, J. (1995) Kinetics of 2-deoxyglucose transport in skeletal muscle: effects of insulin and contractions. *Am. J. Physiol.* **268**, C30–C35
- Palmer, T. (1991) *Understanding Enzymes*, Ellis Horwood Limited, West Sussex
- Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 MAPK mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270**, 7420–7426
- Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P. and Comb, M. J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAPK MAP kinase and MAPKAP kinase-2. *EMBO J.* **15**, 4629–4642

- 33 Iordanov, M., Bender, K., Ade, T., Schmid, W., Sachsenmaier, C., Engel, K., Gaestel, M., Rahmsdorf, H. J. and Herrlich, P. (1997) CREB is activated by UVC through a p38 MAPK/HOG-1-dependent protein kinase. *EMBO J.* **16**, 1009–1022
- 34 Shaw, M., Cohen, P. and Alessi, D. R. (1998) The activation of protein kinase B by H_2O_2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem. J.* **336**, 241–246
- 35 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- 36 Lali, F. V., Hunt, A. E., Turner, S. J. and Foxwell, B. M. (2000) The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 MAPK mitogen-activated protein kinase. *J. Biol. Chem.* **275**, 7395–7402
- 37 Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R. and Klip, A. (1999) Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* **19**, 4008–4018
- 38 Palfreyman, R. W., Clark, A. E., Denton, R. M., Holman, G. D. and Kozka, I. J. (1992) Kinetic resolution of the separate GLUT1 and GLUT4 glucose transport activities in 3T3-L1 cells. *Biochem. J.* **284**, 275–282
- 39 Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A. and Cushman, S. W. (1990) Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J. Biol. Chem.* **265**, 18172–18179
- 39a Khayat, Z. A., Tsakiridis, T., Ueyama, A., Somwar, R., Ebina, Y. and Klip, A. (1998) Rapid stimulation of glucose transport by mitochondrial uncoupling depends in part on cytosolic Ca^{2+} and cPKC. *Am. J. Physiol.* **275**, C1487–C1497
- 40 Aiello, L. P., Wessling-Resnick, M. and Pilch, P. F. (1986) Dipeptide metalloendoprotease substrates are glucose transport inhibitors and membrane structure perturbants. *Biochemistry* **25**, 3944–3950
- 41 Murata, H., Hruz, P. W. and Mueckler, M. (2000) The mechanism of insulin resistance caused by HIV protease inhibitor therapy. *J. Biol. Chem.* **275**, 20251–20254
- 42 Klip, A., Ramlal, T., Douen, A. G., Bilan, P. J. and Skorecki, K. L. (1988) Inhibition by forskolin of insulin-stimulated glucose transport in L6 muscle cells. *Biochem. J.* **255**, 1023–1029
- 43 Engelman, J. A., Lisanti, M. P. and Scherer, P. E. (1998) Specific inhibitors of p38 MAPK mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J. Biol. Chem.* **273**, 32111–32120
- 44 Somwar, R., Niu, W., Kim, D., Rhandawa, V., Ramlal, T., Sweeney, G. and Klip, A. (2001) In VIIIth International Symposium on Insulin Receptors and Insulin Action, Geneva, Switzerland
- 45 Cohen, P. (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.* **7**, 353–361
- 46 Wang, X. S., Diener, K., Manthey, C. L., Wang, S., Rosenzweig, B., Bray, J., Delaney, J., Cole, C. N., Chan-Hui, P. Y., Mantlo, N. et al. (1997) Molecular cloning and characterization of a novel p38 MAPK mitogen-activated protein kinase. *J. Biol. Chem.* **272**, 23668–23674
- 47 Somwar, R., Perreault, M., Kapur, S., Taha, C., Sweeney, G., Ramlal, T., Kim, D. Y., Keen, J., Cote, C. H., Klip, A. and Marette, A. (2000) Activation of p38 MAPK mitogen-activated protein kinase α and β by insulin and contraction in rat skeletal muscle: potential role in the stimulation of glucose transport. *Diabetes* **49**, 1794–1800
- 48 Moxham, C. M., Tabrizchi, A., Davis, R. J. and Malbon, C. C. (1996) Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin *in vivo*. *J. Biol. Chem.* **271**, 30765–30773
- 49 Guo, J. H., Wang, H. Y. and Malbon, C. C. (1998) Conditional, tissue-specific expression of Q205L Galphai2 *in vivo* mimics insulin activation of c-Jun N-terminal kinase and p38 MAPK kinase. *J. Biol. Chem.* **273**, 16487–16493
- 50 Chen, D., Elmendorf, J. S., Olson, A. L., Li, X., Earp, H. S. and Pessin, J. E. (1997) Osmotic shock stimulates GLUT4 translocation in 3T3L1 adipocytes by a novel tyrosine kinase pathway. *J. Biol. Chem.* **272**, 27401–27410
- 51 Kayali, A. G., Austin, D. A. and Webster, N. J. (2000) Stimulation of MAPK cascades by insulin and osmotic shock: lack of an involvement of p38 MAPK mitogen-activated protein kinase in glucose transport in 3T3-L1 adipocytes. *Diabetes* **49**, 1783–1793
- 52 Igarashi, M., Yamaguchi, H., Hirata, A., Daimon, M., Tominaga, M. and Kato, T. (2000) Insulin activates p38 MAPK mitogen-activated protein (MAP) kinase via a MAP kinase kinase (MKK) 3/MKK 6 pathway in vascular smooth muscle cells. *Eur. J. Clin. Invest.* **30**, 668–677
- 53 Goodyear, L. J., Chang, P. Y., Sherwood, D. J., Dufresne, S. D. and Moller, D. E. (1996) Effects of exercise and insulin on mitogen-activated protein kinase signaling pathways in rat skeletal muscle. *Am. J. Physiol.* **271**, E403–E408

Received 14 March 2001/19 July 2001; accepted 4 September 2001