An Inhibitor of p38 Mitogen-activated Protein Kinase Prevents Insulin-stimulated Glucose Transport but Not Glucose Transporter Translocation in 3T3-L1 Adipocytes and L6 Myotubes*

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The precise mechanisms underlying insulin-stimulated glucose transport still require investigation. Here we assessed the effect of SB203580, an inhibitor of the p38 MAP kinase family, on insulin-stimulated glucose transport in 3T3-L1 adipocytes and L6 myotubes. We found that SB203580, but not its inactive analogue (SB202474), prevented insulin-stimulated glucose transport in both cell types with an IC50 similar to that for inhibition of p38 MAP kinase (0.6 μM). Basal glucose uptake was not affected. Moreover, SB203580 added only during the transport assay did not inhibit basal or insulin-stimulated transport. SB203580 did not inhibit insulin-stimulated translocation of the glucose transporters GLUT1 or GLUT4 in 3T3-L1 adipocytes as assessed by immunoblotting of subcellular fractions or by immunofluorescence of membrane lawns. L6 muscle cells expressing GLUT4 tagged on an extracellular domain with a Myc epitope (GLUT4myc) were used to assess the functional insertion of GLUT4 into the plasma membrane. SB203580 did not affect the insulin-induced gain in GLUT4myc exposure at the cell surface but largely reduced the stimulation of glucose uptake. SB203580 had no effect on insulin-dependent insulin receptor substrate-1 phosphorylation, association of the p85 subunit of phosphatidylinositol 3-kinase with insulin receptor substrate-1, nor on phosphatidylinositol 3-kinase, Akt1, Akt2, or Akt3 activities in 3T3-L1 adipocytes. In conclusion, in the presence of SB203580, insulin caused normal translocation and cell surface membrane insertion of glucose transporters without stimulating glucose transport. We propose that insulin stimulates two independent signals contributing to stimulation of glucose transport: phosphatidylinositol 3-kinase leads to glucose transporter translocation and a pathway involving p38 MAP kinase leads to activation of the recruited glucose transporter at the membrane.

The phenomenon of insulin-stimulated glucose transporter (GLUT) translocation from an intracellular location to the plasma membrane has been demonstrated in several fat and muscle cell systems since its initial report in 1980 (1–3). Whether increased plasma membrane glucose transporter concentration can fully account for insulin-stimulated increases in glucose uptake is still being debated (4). It has been proposed that translocation of GLUTs might only account for as little as 30% of insulin-stimulated glucose transport (4), with the majority of insulin-stimulated glucose transport being due to changes in the intrinsic activity of GLUTs (5–7).

The intracellular signaling machinery employed by insulin in eliciting glucose transport has also been extensively investigated yet a precise understanding remains to be achieved (8). To date, only one signaling molecule involved in GLUT translocation has been unequivocally identified, i.e. the p85/p110 phosphatidylinositol (PI) 3-kinase (9). Debate currently exists on which insulin receptor substrates activate the PI 3-kinase pathway leading to GLUT translocation (10–12), as well as on whether the PI 3-kinase effector Akt/PKB is also required (13–15). There is no evidence for or against the participation of any insulin-dependent signaling pathway in regulation of the intrinsic activity of glucose transporters. The mitogen-activated protein kinase (MAPK) cascades represent one of the main intracellular signaling pathways stimulated by mitogenic and stress-inducing stimuli (16, 17) and their involvement in glucose transport depends on the tissue and the agonist analyzed (18). Three families of these proline-directed serine threonine protein kinases, which are activated by dual phosphorylation on threonine and tyrosine residues, have been identified (19). One of these is the p38 MAPK (also known as reactivating kinase, stress-activated protein kinase, and CSAID-binding protein) family, activation of which has been best characterized in response to stressors (such as UV light and hyperosmolarity) and cytokines (such as interleukin-1 or tumor necrosis factor-a) (20). We have shown that insulin can phosphorylate p38 in L6 myotubes (21, 22) and insulin has been shown to stimulate p38 activity in hepatoma cells (23), yet not in skeletal muscle (24).

A group of pyridinyl imidazole compounds were recently reported to bind to and potently inhibit p38 MAP kinase. One of these, SB203580, specifically inhibited the α and β isoforms of p38 MAP kinase with an in vitro IC50 of ~0.6 μM, while having no effect on the activity of 12 other closely related or prominent intracellular kinases (25). In KB cells, SB203580 prevented the stimulation of glucose transport elicited by the potent activator of p38 MAP kinase anisomycin and by IL-1, but had no effect on insulin-like growth factor 1-stimulated uptake.

1 The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; IRS-1, insulin receptor substrate-1; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; MKK, MAP kinase kinase; ATF-2, activating transcription factor-2; IL-1, interleukin-1; PBS, phosphate-buffered saline; PAG, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride.

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glucose transport (26). SB203580 also inhibited anisomycin-stimulated glucose transport in 3T3-L1 adipocytes (27), with an IC50 of <1 μM.

In the present study we assessed the effect of SB203580 and its inactive structural analogue, SB202474, on insulin-stimulated glucose transport. We report that SB203580 inhibits uptake of 2-deoxyglucose or 3-O-methylglucose in muscle and fat cells without interfering with translocation of GLUTs. This effect was not due to direct binding of SB203580 to GLUTs nor to a nonspecific effect on other signaling molecules. The results suggest that SB203580 attenuates the ability of insulin to stimulate the intrinsic activity of glucose transporter molecules recruited to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture solutions and supplements were obtained from Life Technologies, Inc. (Burlington, ON, Canada). 3T3-L1 cells were a kind gift from Dr. G. Holman (University of Bath, United Kingdom). L6 cells transfected with Myc-tagged GLUT4 were kindly provided by Dr. Y. Ebina (University of Tokushima, Japan). Human insulin (Humulin) was obtained from Eli Lilly Canada Inc. (Toronto, ON, Canada). Protein A and protein G-Sepharose were from Pharmacia (Uppsala, Sweden). Polyclonal anti-GLUT1 and anti-GLUT4 glucose transporter antisera was from East Acres Laboratories (South Bridge, MA). Polyclonal antibodies to Akt1 (C-20) and p38 MAP kinase and monoclonal antibody to Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Akt substrate peptide (Crosstide), monoclonal anti-phosphotyrosine, polyclonal anti-IRS-1, and Ak-β-2 and Ak-β-3 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). [γ-32P]ATP (600 Ci/mmol) and enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Oakville, ON, Canada). 2-O-Deoxy-[3H]glucose and 3-O-[3H]methylglucose were purchased from NEN (Mississauga, ON, Canada). ATP-2 peptide was from New England Biolabs (Mississauga, ON, Canada). Fluorescein isothiocyanate-conjugated donkey anti-rabbit and horseradish peroxidase-conjugated sheep anti-rabbit and anti-mouse antisera were from Jackson Immunoresearch (Baltimore Pike, PA). ProLong antifade mounting solution was from Molecular Probes (Eugene, OR). Purified 1-α-phatidylidyinositol (PI) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Oxalate-treated T/LC Silica Gel H plates (250 μm) were from Analytech (Newark, DE). α-Phe-γ-Lys dihydrochloride (C8) was obtained from Sigma. Okadaic acid (200-fold concentrated) was from Advanced Biochemicals (Burlington, MA). Triton X-100 (0.1%), 100 mM Na3VO4, 2 mM PMSF, 10% glycerol (v/v), and 1% Nonidet P-40 were as described previously (9). The monoclonal antibodies were a gift from Dr. Y. Ebina (University of Tokushima, Japan). Human muscle cells and L6 cells stably overexpressing GLUT4 tagged with a Myc epitope were grown and differentiated into myotubes as described previously (28). 3T3-L1 fibroblasts were differentiated into adipocytes as described previously (29). For transport studies cells were treated with trypsin and seeded in 12-well plates (2.5-cm diameter well) and maintained in growth medium as described above except supplemented with 2% fetal bovine serum. Cells were maintained under the same conditions in 6-well plates for preparation of whole cell lysates and in 10-cm diameter dishes for immunoprecipitations.

**Analysis of p38 MAP Kinase Phosphorylation**—Cells treated with insulin or SB203580 as indicated were lysed in 1 ml of lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl, 1 mM MgCl2, 1 mM CaCl2, 100 μM Na3VO4, 2 mM PMSF, 1% glycerol (v/v), and 1% Nonidet P-40 (v/v) (pH 7.5). Cell lysates were then centrifuged for 5 min at 12,000 rpm to remove cell debris and nuclei. Antiphosphotyrosine antibody (2 μg/condition) was then added to supernatants for overnight incubation under rotation at 4 °C, followed by addition of 30 μl of 10% (v/v) protein A-Sepharose for 1 h. The immunoprecipitation pellets were washed three times with PBS containing 0.1% Nonidet P-40 and 100 μM Na3VO4, solubilized in 30 μl of 2× Laemmli sample buffer, boiled for 5 min, and separated by 10% SDS-PAGE. Anti-p38 MAP kinase polyclonal antibody (1:1000 dilution) was added to the polyvinylidene difluoride membrane, followed by goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:5000 dilution) and protein was visualized by the enhanced chemiluminescence method.

**Assay of p38 MAP Kinase and Akt (PKB) Activity**—Immunoprecipitation of p38 MAP kinase and Akt isoforms and analysis of their kinase activity was performed in a similar fashion to that described previously for Akt (30). For both assays, cells were lysed with lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM Trition X-100 (v/v), 30 mM Na2PO4, 10 mM sodium fluoride, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 mM Na3VO4, 1 mM dithiothreitol, and 100 mM okadaic acid. Polyclonal anti-p38 MAP kinase antibody (2 μg/condition) precoupled to a mixture of protein A- and protein G-Sepharose (20 μl (100 μg/ml) each per condition) beads was added to 200 μg total cell protein. Anti-Akt and Akt3 antibodies were also precoupled to a mixture of protein A- and protein G-Sepharose (2 μg/condition) and added to 200 μg of total protein. Antibody coupled beads were washed twice with ice-cold PBS and once with icce-cold lysis buffer before use. Proteins were immunoprecipitated by incubating with the antibody bead complex for 2–3 h under constant rotation (4 °C). Immunocomplexes were isolated and washed 4 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% bovine serum albumin, 1 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 1 μM microcystin, and 100 mM okadaic acid) and twice with 1 ml of kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl2, and 1 mM dithiothreitol). The complexes were then incubated under constant agitation for 30 min at 30 °C with 30 μl of reaction mixture (10 μg of total cell protein and 2 μl of [γ-32P]ATP-2 as substrate in p38 MAP kinase assay) and 100 μl (Crossstat as substrate in Akt assays). Following the reaction, 30 μl of the supernatant was transferred onto Whatman p81 filter paper and washed 4 times for 10 min with 3 ml of 175 mM phosphoric acid and once with distilled water for 5 min. Filters were air-dried and then subjected to liquid scintillation counting.

**Determination of 2-Deoxyglucose and 3-O-Methylglucose Uptake**—L6 myotubes were deprived of serum for 5 h with α-minimal essential medium, 0.1% fetal bovine serum (v/v), and 25 mM glucose (serum-deprivation medium) while 3T3-L1 adipocytes were deprived of serum by incubation in Dulbecco’s modified Eagle’s medium for 2 h before experimental manipulations. 2-Deoxyglucose uptake measurements were carried out as described previously (31). Briefly, following all stimulations and incubations with inhibitors cell monolayers were washed twice with HEPES-buffered saline (140 mM NaCl, 20 mM Na-HEPES, 2.5 mM MgSO4, 1 mM CaCl2, 5 mM KCl, pH 7.4) and any remaining liquid was aspirated. Cells were then incubated for 5 min in HEPES-buffered saline containing 10 μM unlabeled 2-deoxyglucose and 10 μM β-2-deoxy-[3H]glucose (1 μCi/ml for L6 and 0.5 μCi/ml for 3T3-L1) in the absence of insulin. The reaction was terminated by washing three times with ice-cold 0.9% NaCl (w/v). Nonspecific uptake was determined in the presence of 10 μM cytochalasin B. Cell associated radioactivity was determined by lysing the cells with 0.05 n NaOH, followed by liquid scintillation counting. Total cellular protein was determined by the Bradford method (32). 3-O-Methylglucose uptake was measured in a similar fashion with the following differences: 50 μM 3-O-methylglucose (30 μl) was added to HEPES-buffered saline, allowed to occur for 30 s, a period over which 3-O-methylglucose uptake is known to be linear. After this time, cell monolayers were washed three times with 1 mM HgCl2 in saline solution before lysis with 0.05 n NaOH.

**Plasma membrane Lawns Formation and Immunofluorescence Labeling**—Differeniated 3T3-L1 adipocytes, grown on glass coverslips in 6-well dishes, were treated with CB203580 plus or minus insulin as described in the figure legends. Plasma membrane lawns (sheets) were prepared as described previously (33) with slight modifications. Following the various treatments, the cells were placed on ice and washed twice in ice-cold PBS. Hypotonic swelling buffer (23 mM KC1, 10 mM Na-HEPES, 2 mM MgCl2, 1 mM EGTA, pH 7.5) was added in three quick rinses. Five ml of breaking buffer (70 mM KC1, 30 mM Na-HEPES, 5 mM MgCl2, 3 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM pepstatin A, pH 7.5) were added to each well, and the solution was aspirated up and down using a 1.0-ml pipette to promote cell breakage. The coverslips were washed three times in breaking buffer and incubated with cold 3% paraformaldehyde in breaking buffer for 10 min on ice, followed by three 5 min washes in PBS. Excess fixative was quenched by a 5 min wash with 100 mM NH4Cl/PBS for 5 min, followed by three 5 min washes in PBS at room temperature. The lawns were subsequently blocked by a 1-h incubation in 5% goat serum in PBS at room temperature, then incubated with rabbit anti-GLUT4 antisera (1:150) for 30 min at room temperature and washed three times in PBS. Fluorescein isothiocyanate-conjugated donkey anti-rabbit antiserum (1:50) was added for 30 min then rinsed out with four washes with PBS and the coverslips mounted with Pro.
Long Antifade mounting solution. Confocal images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 63X objective. All images shown were collected under identical gain settings. Quantification was made using NIH Image software.

Fractionation of 3T3-L1 Adipocytes and Immunoblotting for GLUT4—Cellular fractionation was carried out essentially as described previously (34). Cells were homogenized in ice-cold 255 mM sucrose, 0.5 mM PMSF, 1 µM pepstatin A, 10 µM E-64, 1 mM EDTA, and 20 mM Na-HEPES (pH 7.4) and the homogenate centrifuged at 19,000 × g for 20 min. The resulting supernatant was centrifuged at 41,000 × g to pellet the crude plasma membranes; the supernatant from this step was centrifuged at 100,000 × g for 60 min to yield the low density microsomes. Crude plasma membranes were further purified by layering on a sucrose cushion (1.12 M sucrose, 1 mM EDTA, and 20 mM Na-HEPES, pH 7.4) and centrifuged at 100,000 × g in a Beckman SW-55 rotor for 60 min. The band at the interface was resuspended in homogenization buffer and pelleted at 40,000 × g for 20 min to yield purified plasma membranes. All fractions were resuspended in homogenization buffer to a final concentration of 2–10 mg/ml and stored at −80 °C.

Measurement of GLUT4myc Translocation in L6 Myotubes—The movement of Myc-tagged GLUT4 to the cell surface was measured by an antibody-coupled colorimetric assay (35) as follows. Quiescent L6 GLUT4myc cells treated as indicated in the legend for Table II were washed and 1 ml of 9E10 (1:100) was then added into the cultures at a dilution of 1:100 and incubated with 1% glycine in PBS at 4 °C for 10 min. The reaction was stopped by adding 0.25 ml of 3N HCl. The supernatant was collected and the pellet washed and 1 ml of o-phenylenediamine dihydrochloride reagent (0.4 mg/ml o-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydroperoxide in 0.05 M phosphate/citrate buffer) was added to each well for 10 min at room temperature. The resulting color was measured by incubation with 1% glycine in PBS at 4 °C for 10 min. The cells were blocked with 10% goat serum and 3% bovine serum albumin in PBS at 4 °C for at least 30 min. Primary antibody (anti-c-Myc, 9E10) was then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4 °C. The cells were extensively washed with PBS before introducing peroxidase-conjugated rabbit anti-mouse IgG (1:1000). After 30 min at 4 °C, the cells were extensively washed and 1 ml of o-phenylenediamine dihydrochloride reagent (0.4 mg/ml o-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydroperoxide in 0.05 M phosphate/citrate buffer) was added to each well for 10 min at room temperature. The reaction was stopped by addition of 0.25 ml of 3 N HCl. The supernatant was collected and the optical absorbance was measured at 492 nm.

Detection of IRS-1 Phosphorylation and Association of p85—3T3-L1 adipocytes were treated with 100 nM insulin for 5 min then IRS-1 was immunoprecipitated and tyrosine phosphorylation of IRS-1 and association of p85 were determined as described under “Experimental Procedures.” Results are from one representative experiment from at least three replicates.

RESULTS

Stimulation of Phosphorylation and Activity of p38 MAP Kinase by Insulin—Activation of p38 MAP kinase involves dual phosphorylation on tyrosine and threonine. Insulin stimulated phosphorylation of p38 MAP kinase in 3T3-L1 adipocytes as measured by phosphotyrosine immunoprecipitation followed by Western blotting with anti-p38 MAP kinase antibody (Fig. 1A). We have previously shown a similar result in L6 myotubes (22). SB203580, which interacts with the ATP-binding domain of p38 MAP kinase (37), did not affect the basal or insulin-stimulated level of p38 phosphorylation. An in vitro kinase assay was then used to measure the ability of p38 MAP kinase to phosphorylate one of its known natural substrates, ATF-2. Insulin caused an approximately 5-fold increase in p38-mediated ATF-2 phosphorylation and this effect was inhibited by SB203580 (Fig. 1B).

Effect of SB203580 and SB202474 on 2-Deoxyglucose and 3-O-Methylglucose Uptake—Fig. 2A shows a dose response of the effect of SB203580 on the uptake of 3H-labeled 2-deoxyglucose into 3T3-L1 adipocytes. The compound was given to cells for 20 min prior to addition of insulin. Treatment with the hormone alone for 30 min caused an increase in glucose uptake of over 4-fold (control, 7.4 ± 0.7 pmol/min/mg protein; insulin, 26.9 ± 4.1 pmol/min/mg protein (p < 0.05)). Pretreatment with a range of concentrations of SB203580 from 1 nM to 0.1 mM prevented stimulation by insulin in a dose-dependent fashion. The IC50 for this effect was calculated to be 0.75 µM. Fig. 2A also shows that, over the same range of concentrations, SB203580 had no effect on basal 2-deoxyglucose uptake in these cells. Fig. 2B shows that the dose-dependent inhibitory effect of SB203580 on insulin-stimulated 2-deoxy-D-glucose transport was also observed in L6 myotubes. In this case the IC50 was 0.1 µM and again SB203580 had no effect on basal glucose uptake.

Fig. 3 compares the effect of SB203580 with that of the structurally similar compound, SB202474, on insulin-stimulated 2-deoxyglucose and 3-O-methylglucose transport in 3T3-L1 adipocytes. SB202474 is unable to inhibit p38 MAP kinase and in this context is considered to be a functionally inactive analogue of SB203580 (38). As expected from results presented in Fig. 1A, SB203580 (10 µM) inhibited insulin-stimulated 2-deoxyglucose uptake by approximately 60% (p < 0.05) (Fig. 3A). However, SB202474 (10 µM) had no effect on the ability of insulin to stimulate 2-deoxyglucose uptake. Neither SB203580 nor SB202474 had any effect on basal 2-deoxyglucose transport level (Fig. 3A).

To explore whether the effect of SB203580 is on glucose transport activity and not on subsequent metabolism (phosphorylation) of the sugar, we analyzed its action on uptake of the nonmetabolizable glucose analogue, 3-O-methylglucose. Fig. 3B shows that SB203580 (10 µM) prevented the stimulation of 3-O-methylglucose uptake by insulin to a similar extent as it
prevented stimulation of 2-deoxyglucose uptake. The inactive analogue SB202474 had no effect on insulin-stimulated 3-O-methylglucose uptake. In the experiments described above cells were preincubated for 20 min with SB203580 before insulin stimulation. In order to test whether SB203580 has direct effects on the hexose transport process itself, cells were treated with or without insulin and SB203580 was added only during the 5-min period of the glucose uptake assay. By this protocol, SB203580 had no effect on either basal or insulin-stimulated 2-deoxyglucose uptake in either 3T3-L1 adipocytes or L6 myotubes (Table I).

Effect of SB203580 on Translocation of GLUT1 and GLUT 4 to the Plasma Membrane in Response to Insulin in 3T3-L1 Adipocytes—Two methods were used to assess the effect of SB203580 on insulin-induced glucose transporter translocation in 3T3-L1 adipocytes. First, the content of plasma membrane-associated glucose transporters was analyzed by preparation of plasma membrane lawns and subsequent immunofluorescence detection of GLUT1 and GLUT4. Fig. 4A shows an increase in the plasma membrane lawn content of both GLUT1 and GLUT4 in response to insulin, which was unaffected by preincubation of cells with SB203580. Quantification of these results gave the following values for GLUT4 levels on membrane lawns (in relative units): basal, 1.00 ± 0.11; insulin, 3.75 ± 0.40; insulin plus SB203580, 3.53 ± 0.14. The levels of GLUT1 on membrane lawns were as follows: basal, 1.00 ± 0.12; insulin, 1.75 ± 0.08; insulin plus SB203580, 1.92 ± 0.11. There was also no effect of SB203580 on GLUT1 and GLUT4 levels under basal conditions (results not shown).

Since the lack of effect of SB203580 on glucose transporter levels associated with the plasma membrane in the face of inhibition of glucose transport was not anticipated, we also analyzed the insulin-stimulated glucose transporter translocation by subcellular fractionation. This approach allowed us to test the effect of SB203580 on both the internal and the plasma membranes. Equal amounts of membrane protein from subcellular fractions isolated from cells treated with or without SB203580 and with or without insulin were analyzed by SDS-PAGE and immunoblotting (Fig. 4B). The results confirmed the observation made in plasma membrane lawns that SB203580 does not prevent the translocation of either GLUT1 or GLUT4 to the plasma membrane from intracellular stores.

Table I

| Condition | 2-Deoxyglucose uptake in 3T3-L1 adipocytes (pmol/mg/min) | 2-Deoxyglucose uptake in L6 muscle cells (pmol/mg/min) |
|-----------|----------------------------------------------------------|---------------------------------------------------------|
| Control   | 5.65 ± 0.54                                              | 3.36 ± 0.14                                             |
| SB203580  | 5.38 ± 0.17                                              | 3.48 ± 0.05                                             |
| Insulin   | 32.18 ± 2.78                                             | 6.31 ± 0.15                                             |
| Insulin + SB203580 | 27.41 ± 1.42                               | 6.18 ± 0.19                                             |
**SB203580 Inhibits Insulin-stimulated Glucose Transport**

A variety of stimuli initiate the sequence of hierarchical protein phosphorylation leading to activation of p38 MAP kinase, the most potent being stresses such as osmotic shock and ultraviolet radiation, as well as inflammatory cytokines (20). p38 MAP kinase is activated by phosphorylation on threonine and tyrosine residues. At least two MAP kinase kinases, MKK3 and MKK6, have been identified as the dual specificity kinases that phosphorylate and activate p38 MAP kinase (40). Previously identified MKK kinases (MEKK1–3) were found to be unable to activate MKK3/6; however, recently the MEKK homologue TAK-1 has been confirmed to activate MKK3/6 while both Ras- and p21-activated kinases have been implicated as functioning further upstream of MKK3/6 (20). Several cellular substrates have been identified to date for p38 MAP kinase, including MAP kinase-activated protein 2/3 and ATF2 (20). Both upstream regulation of p38 MAP kinase and downstream effectors seem to be determined in a cell-specific fashion. Four isoforms of p38 MAP kinase have been identified (p38α, β, γ, and δ) (41). Of these, p38α and p38γ are ubiquitously distributed and inhibited by SB203580 (42). In addition to these

**TABLE II**

|                | Effect of SB203580 on 2-deoxyglucose uptake | Effect of SB203580 on GLUT4 translocation to plasma membrane |
|----------------|-------------------------------------------|------------------------------------------------------------|
| Control        | 1.0 ± 0.03                                | 1.0 ± 0.04                                                |
| SB203580       | 0.91 ± 0.03                               | 1.03 ± 0.01                                               |
| Insulin        | 1.87 ± 0.05                               | 2.04 ± 0.05                                               |
| Insulin + SB203580 | 1.35 ± 0.09                                | 2.08 ± 0.03                                               |

**DISCUSSION**

The results presented so far suggest that Akt is activated by products of PI 3-kinase and several studies have suggested a role for Akt in insulin-stimulated glucose transport in 3T3-L1 adipocytes and L6 myotubes (14, 39). To examine the effect of SB203580 on stimulation of Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) by insulin these kinases were immunoprecipitated with isoform-specific antibodies and in vitro activity determined using Crosstide as substrate. We found that insulin stimulated all three isoforms in 3T3-L1 adipocytes and this stimulation was not affected by SB203580 (Fig. 5C). SB203580 also had no effect on basal Akt1, Akt2, or Akt3 activities.

**Effect of SB203580 on IRS-1 Phosphorylation, Association with the p85 Subunit of PI 3-Kinase and Stimulation of PI 3-Kinase Activity in 3T3-L1 Adipocytes—**An early step in the insulin signaling pathway necessary for stimulation of glucose transport is tyrosine phosphorylation of IRS molecules to activate PI 3-kinase. This pathway is required for translocation of GLUTs but it is not known if it can also regulate the intrinsic activity of GLUTs. We therefore examined the effect of SB203580 on the ability of insulin to phosphorylate IRS-1, in order to gain information on its mechanism of action leading to prevention of stimulation of glucose uptake by insulin. IRS-1 was immunoprecipitated from 3T3-L1 adipocyte cell lysates treated with and without insulin or SB203580, resolved by 7.5% SDS-PAGE, then subjected to Western blotting with anti-phosphotyrosine antibody. Fig. 5A shows that the insulin-induced tyrosine phosphorylation of IRS-1 was not affected by SB203580. The amount of p85 associated with IRS-1 immunoprecipitates was markedly increased by insulin and this effect was also not prevented by SB203580 (Fig. 5A). We next determined the effect of SB203580 on the stimulation of PI 3-kinase activity by insulin using an in vitro kinase assay to determine the ability of PI 3-kinase associated with IRS-1 in cell lysates to incorporate 32P into phosphatidylinositol. Fig. 5B shows that insulin activated PI 3-kinase activity approximately 10-fold and that SB203580 had no effect on insulin-stimulated or basal PI 3-kinase activity in IRS-1 immunoprecipitates (Fig. 5B).

**Effect of SB203580 on Insulin-stimulated Akt Activity—**Akt is activated by products of PI 3-kinase and several studies have suggested a role for Akt in insulin-stimulated glucose transport in 3T3-L1 adipocytes and L6 myotubes (14, 39). To examine the effect of SB203580 on stimulation of Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) by insulin these kinases were immunoprecipitated with isoform-specific antibodies and in vitro activity determined using Crosstide as substrate. We found that insulin stimulated all three isoforms in 3T3-L1 adipocytes and this stimulation was not affected by SB203580 (Fig. 5C). SB203580 also had no effect on basal Akt1, Akt2, or Akt3 activities.
SB203580 Inhibits Insulin-stimulated Glucose Transport

3T3-L1 adipocytes were treated with or without 10 μM SB203580 for 20 min followed by insulin treatment (100 nM) for 5 min where indicated. All assays were conducted as described under “Experimental Procedures.” A, phosphorylation of IRS-1 and association of the p85 subunit of PI 3-kinase with IRS-1. B, IRS-1-associated PI 3-kinase activity. C, activity of Akt1, Akt2, and Akt3. For A and C results are from one representative experiment from at least three replicates. The mean ± S.E. of three individual experiments is presented for B.

Regulation of p38 MAP kinase by insulin is an often overlooked signaling phenomenon and again control of p38 MAP kinase by this hormone appears to occur in a tissue-specific manner. It was established in Chinese hamster ovary cells that insulin-induced MKK3/6 phosphorylation and activation of p38 MAP kinase (44) and insulin stimulation of p38 MAP kinase was also demonstrated in hepatoma cells (23). Conversely, insulin decreased tyrosine phosphorylation of p38 and inhibited p38 activity in postmitotic fetal neurons where the hormone is a potent survival factor (45). Two studies have established activation of p38 MAP kinase by insulin in skeletal muscle (46, 47), yet in a third study no stimulation of p38 MAP kinase was detected (24). Less information is available on regulation of p38 MAP kinase activity by insulin in adipose cells. A preliminary communication reported that insulin stimulated p38 MAP kinase activity in 3T3-L1 fibroblasts and adipocytes (48). In the present study we further substantiate this finding and furthermore, show that p38 activation by insulin is inhibited by SB203580.

We have shown that SB203580, a cell permeant inhibitor of p38 MAP kinase, attenuates the stimulation of glucose transport by insulin. Translocation of GLUT1 and GLUT4 to the cell surface is undoubtedly an important component of the stimulation of glucose transport by insulin in 3T3-L1 adipocytes. However, some uncertainty still remains over whether enrichment of the plasma membrane with GLUTs can completely account for insulin-stimulated glucose uptake (4), and one estimate suggests that it contributes to only 30% of the total gain in glucose transport. We utilized two different approaches with 3T3-L1 adipocytes (preparation of plasma membrane lawns followed by immunoﬂuorescence or immunoblotting of subcellular fractions) to determine if the inhibition of insulin-stimulated glucose transport by SB203580 could be explained by a decreased amount of glucose transporters at the plasma membrane. Surprisingly, we discovered that in the presence of SB203580, insulin was able to elicit normal increases in the amount of GLUT1 and GLUT4 at the plasma membrane without a corresponding stimulation of glucose transport. Therefore, the results reveal a strategy to dissociate increased glucose transporter number at the cell surface from increased glucose transport activity.

One drawback with both of these approaches is that complete conﬁdence that glucose transporters are inserted into the plasma membrane bilayers is not assured. Indeed, some studies have postulated that in some instances glucose transporter-containing vesicles can be docked but occluded from the membrane (49, 50). Therefore, to determine if the newly arrived glucose transporters were properly inserted into the plasma membrane we utilized the recently characterized L6 muscle cells stably overexpressing GLUT4 that is tagged with a Myc epitope on the exofacial surface (35). These cells can be used to measure the actual incorporation of Myc-tagged GLUT4 into the plasma membrane by detecting exposure of the Myc epitope on the surface of intact cells. Importantly, insulin-induced translocation and incorporation of GLUT4myc into the plasma membrane was not affected by SB203580. Collectively, the results with 3T3-L1 adipocytes or L6-GLUT4myc cells suggest that insertion of glucose transporters into the plasma membrane in response to insulin is not sufﬁcient to account for the observed increased ﬂux of glucose across the membrane. Instead, another unidentified event or signal which is prevented by SB203580 is required to convert the newly arrived cell surface transporters into functional proteins after insertion into the plasma membrane. Given that the maximal effect of SB203580 (10 μM) was to inhibit insulin-stimulated glucose transport by around 50–70%, we suggest that full insulin-stimulated glucose transport may require a combination of increased GLUT translocation and increased intrinsic activity of GLUTs.

The results presented also show that insulin phosphorylated and activated p38 MAP kinase in 3T3-L1 adipocytes. As expected, SB203580 inhibited insulin-stimulated kinase activity but had no effect on insulin-stimulated phosphorylation, thus did not interfere with the upstream signaling pathway involved in activation of p38 MAP kinase by insulin. Based on its sensitivity to SB203580, a role for p38 MAPK has been suggested in the stimulation of glucose transport by anisomycin in 3T3-L1 adipocytes and interleukin-2 in KB cells (26, 27). Anisomycin

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2 P. Scherer, personal communication.
did not induce a redistribution of glucose transporters to the cell surface and hence it was proposed to stimulate the intrinsic activity of GLUT1 glucose transporters that are already present at the cell surface (27). Similarly, noradrenaline stimulates glucose transport into rat adipocytes by enhancing the functional activity of GLUT1, in this case via a cAMP-dependent mechanism (51). There is also precedence for activation of glucose transporters as part of the mechanism by which insulin stimulates glucose transport in fat cells (7, 52). A detailed study of this activation mechanism has been complicated by the fact that unlike anisomycin and noradrenaline, insulin causes a redistribution of glucose transporters to the cell surface. Hence, it has been difficult to clearly demonstrate the requirement for activation of transporters in the face of increased amounts of transporters on the cell surface. However, due to discrepancies in the degree of insulin-stimulated PI 3-kinase activity and glucose uptake in 3T3-L1 adipocytes it has recently been suggested that an additional pathway is necessary for full insulin-stimulated glucose transport (52). Moreover, we have recently shown that whereas membrane-permeant lipid products of PI 3-kinase cannot reproduce the insulin stimulation of glucose uptake, they effectively restore the inhibition of insulin action caused by wortmannin (53). These results also suggest that signals parallel to PI 3-kinase are necessary for the stimulation of glucose transport in 3T3-L1 adipocytes. In the present study we provide evidence to support this hypothesis and suggest that insulin stimulates both translocation and intrinsic activity of GLUTs. Our results are consistent with the hypothesis that the IRS → PI 3-kinase → Akt pathway is involved in GLUT translocation. We also suggest another parallel signaling pathway involving p38 MAP kinase, which culminates in increased intrinsic activity of GLUTs, required for full stimulation of glucose transport.

An alternative possibility is that SB203580 could bind directly to the newly recruited glucose transporters, thereby inhibiting their activity. Several years ago such a scenario was contemplated for the diterpine forskolin which inhibited insulin-stimulated glucose transport in a manner unrelated to its stimulation of adenylate cyclase (54). Indeed, forskolin was found to affect glucose transport by a direct interaction with glucose transporters since insulin-stimulated glucose transport was not altered by the presence of SB203580 during the 5-min transport assay. This result strongly suggests that SB203580 does not interact directly with glucose transporters. Instead, our results favor the interpretation that SB203580 inhibits insulin-dependent glucose transport by interfering with an intracellular signal. This conclusion is supported by the observations that: (a) the IC₅₀ of SB203580 for the inhibition of glucose transport in 3T3-L1 adipocytes (0.75 μM) was almost identical to that reported for specific inhibition of p38 MAP kinase (0.6 μM), and (b) an inactive structural analogue of SB203580 (SB202474) was without effect on insulin-stimulated glucose transport in 3T3-L1 adipocytes. These considerations place p38 MAP kinase in the insulin signal transduction pathway leading to the stimulation of glucose transport.

Transport of glucose into cells is followed almost immediately by its phosphorylation to produce glucose 6-phosphate. One possible explanation for our observations obtained measuring 2-deoxyglucose uptake could have been that SB203580 affects metabolism of this glucose analogue. To examine this possibility we assessed the effects of SB203580 on insulin-stimulated transport of the nonmetabolizable analog 3-O-methylglucose but obtained similar results to those seen with 2-deoxyglucose. This suggests that inhibition of glucose transport by SB203580 is manifest at the level of transport and not at the subsequent metabolism of glucose.

The lack of effect of SB203580 on insulin-stimulated translocation of GLUT1 and GLUT4 to the cell surface suggested that this inhibitor does not affect the previously characterized upstream signaling events required for this process. Indeed, the state of insulin-induced phosphorylation or activation of several elements of the insulin signaling pathway that are thought to be essential for insulin-dependent GLUT translocation were not affected by SB203580. Insulin-stimulated phosphorylation of IRS-1 was normal, as was association of the regulatory (p85) subunit of PI 3-kinase with IRS-1. Activation of PI 3-kinase and Akt1, Akt2, and Akt3 by insulin were also unaffected by SB203580. The inability of SB203580 to inhibit insulin-stimulated activation of all three isozymes of Akt also suggests that, contrary to previous suggestion (56), p38 MAPK is not in the signaling pathway leading to the activation of Akt in response to insulin.

In conclusion, we have shown that the inhibitor of p38 MAP kinase, SB203580, can prevent insulin-stimulated glucose uptake in 3T3-L1 adipocytes and L6 muscle cells. This inhibitory effect is not due to a nonspecific effect on insulin signaling pathways nor directly on glucose transporters. In addition, the action of SB203580 does not lie at the level of glucose metabolism. Interference with the translocation of glucose transporters is not the basis for the inhibitory effect of SB203580. We propose that glucose transporters are activated following their translocation to the plasma membrane and that this activation is prevented by SB203580, likely by its ability to inhibit insulin-dependent activation of p38 MAP kinase.

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