Stimulation of MAPK Cascades by Insulin and Osmotic Shock

Lack of an Involvement of p38 Mitogen-Activated Protein Kinase in Glucose Transport in 3T3-L1 Adipocytes

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Osmotic shock and insulin stimulate GLUT4 translocation and glucose transport via mechanisms that are for the most part distinct yet convergent. In this article, we investigated the effect of osmotic shock and insulin on the activation of the mitogen-activated protein kinase (MAPK) cascades in differentiated 3T3-L1 adipocytes. The MAPKs are activated by phosphorylation on conserved tyrosine and threonine residues. Both sorbitol and insulin strongly stimulated extracellular regulated kinase (ERK) 1 and 2 phosphorylation (8- and 18-fold, respectively). In contrast, c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) phosphorylation was stimulated only by sorbitol (sevenfold) and not by insulin. Phosphorylation of p38 MAPK was stimulated strongly by sorbitol (22-fold) but weakly by insulin (2.7-fold). Measurement of intrinsic JNK and p38 MAPK activity confirmed the phosphorylation studies. JNK and p38 MAPK were activated only significantly by sorbitol. The MAPKs are phosphorylated by dual-specificity kinases (mitogen-activated ERK-activating kinase [MEK] or MAPK kinase [MKK]). As expected, sorbitol and insulin both stimulated MEK phosphorylation. MKK4 was phosphorylated only in response to sorbitol, and neither of the stimuli caused phosphorylation of MKK3 or 6. To determine the functional significance of the observed activation of p38 MAPK in response to insulin and osmotic shock, we used three pyridinyl imidazole p38 MAPK inhibitors, SB203580, SB202190, and PD169316. Insulin and osmotic shock–stimulated glucose transport was not inhibited by any inhibitor at concentrations that were shown to block p38 MAPK activity. Furthermore, activation of the p38 MAPK pathway by treatment of cells with anisomycin did not stimulate glucose transport. These results suggest that activation of the p38 MAPK pathway is not involved in the stimulation of glucose transport. Diabetes 49:1783–1793, 2000

Mitogen-activated protein kinase (MAPK) cascades comprise one of the major signaling systems by which cells transduce and integrate diverse intracellular signals. The three subfamilies of the MAPKs are the extracellular regulated kinases (ERKs), the c-Jun NH2-terminal kinases (JNK)/stress-activated protein kinases (SAPK) and the p38 MAPKs (1–3). The ERKs are strongly activated by polypeptide growth factors and phorbol esters but are weakly activated by environmental stresses, such as osmotic or heat shock, UV light, and inhibitors of protein synthesis. In contrast, JNK and p38 MAPKs are strongly activated by cytokines and adverse stimuli, but are poorly activated by growth factors.

All MAPKs are activated by phosphorylation on both threonine and tyrosine residues within the motif Thr-Xaa-Tyr. The Xaa represents Glu in the ERK subfamily, Pro in the JNK subfamily, and Gly in the p38 MAPK subfamily. Both the threonine and tyrosine residues are phosphorylated by a dual-specificity kinase or MAPK (mitogen-activated ERK-activating kinase [MEK] or MAPK kinase [MKK]). The central residue in the Thr-Xaa-Tyr motif allows for selective activation by different MKKs, such that MEK1 and MEK2 selectively phosphorylate and activate the ERKs; MKK4/SAPK kinase (SEK) 1 and MKK7 phosphorylate and activate JNK; MKK3 and MKK6 phosphorylate and activate p38 MAPK (4–9). Despite the prevailing view of the selectivity of these kinases, a growing body of evidence indicates that these pathways overlap. In a study examining interleukin-1β–induced cyclooxygenase-2 expression in rat renal mesangial cells, Guan et al. (10) have found that overexpressing the dominant negative form of MKK4 inhibited both JNK and p38 MAPK phosphorylation in their system. This finding indicates that MKK4 can act upstream of p38 in certain systems (10). In another study, the investigators coexpressed p38 and MKK3, MKK4, and MKK6 in 293 cells and found that, although MKK3 and MKK6 were the dominant regulators, MKK4 could also phosphorylate p38 (11).
In insulin signaling, the MAPK pathway has primarily been associated with the regulation of mitogenesis (12). A coherent body of experimental evidence exists to support the conclusion that the stimulation of the Ras-Raf-MAPK pathway is not sufficient for the acute regulation of the metabolic effects of insulin. Inhibition of this pathway by PD98059 did not abrogate insulin stimulation of glucose uptake or glycogen synthase in 3T3-L1 adipocytes, L6 muscle cells, or rat diaphragm (13,14).

With the cloning of stress MAPKs (JNK and p38 MAPKs), there has been renewed interest in the involvement of MAPK cascades in the metabolic actions of insulin (15). Moxham et al. (16) reported a rapid activation of JNK in skeletal muscle in response to insulin administration, which they suggest may activate glycogen synthase. Similarly, in vivo administration of insulin to mice resulted in a rapid activation of p38 and JNK in skeletal muscle, with a simultaneous increase in glycogen synthase activity (17). The results are not conclusive, however, as Napoli et al. (18) reported that neither JNK phosphorylation nor JNK activity is stimulated by insulin in rat skeletal muscle. Conversely, overexpression of the insulin receptor in Chinese hamster ovary (CHO) cells allowed activation of JNK by insulin (19) and, finally, insulin induction of p38 MAPK activity has recently been reported in L6 cells and 3T3-L1 adipocytes (20).

The major metabolic effect of insulin in target tissues is the stimulation of glucose transport. The precise cellular events that lead to insulin-stimulated glucose transport have not been delineated because transport involves a complex vesicular trafficking pathway with many unknown components. Several important signaling events have been identified. After the binding of insulin to the receptor, the β-subunit is activated by autophosphorylation. The activated receptor can phosphorylate a number of substrates such as insulin receptor substrate (IRS)-1, IRS-2, IRS-3, IRS-4, Gab1, and Shc (21). The kinase activity of the receptor has been demonstrated to be essential for GLUT4 translocation. Adipocytes transfected with insulin receptors mutated in the ATP-binding site of the tyrosine kinase domain were unable to stimulate GLUT4 translocation in response to insulin (22). Secondly, there is compelling evidence for the involvement of phosphatidylinositol 3-kinase (PI3K) in the activation and/or targeting of glucose transport in response to insulin. Pharmacological inhibitors of PI3K activity, microinjection of dominant negative proteins derived from its regulatory domain, and overexpression of a constitutively active PI3K have demonstrated that PI3K is necessary for the metabolic actions of insulin (23).

Three different families of proteins that lie downstream of PI3K have been implicated in insulin-stimulated glucose transport. The serine/threonine kinase Akt/protein kinase B (PKB), the atypical protein kinase C (PKC), and the γ isoforms of phospholipase C (PLC) have all been shown to participate. All of these signaling proteins are activated directly by the lipid products of PI3K. The two families of kinases are regulated by phosphoinositide-dependent protein kinase (PDK) 1, which itself is regulated by 3'-phosphoinositides lipids (24). Evidence of an increase in glucose transport and GLUT4 translocation to the plasma membrane in muscle and fat cells overexpressing either wild-type or constitutively active Akt/PKB-α (25,26) led to the suggestion that PKB may be involved. The demonstration of an increased association of Akt2/PKB-β with GLUT4 in response to insulin and colocalization of the two molecules in GLUT4 vesicles has strengthened potential Akt/PKB involvement in GLUT4 translocation (27). However, only a modest 20% reduction in insulin-stimulated glucose transport was observed in rat adipose cells expressing a dominant negative kinase-inactive mutant of Akt/PKB, which was able to inhibit other Akt-dependent signaling pathways (28). A more disabled Akt/PKB, which additionally contains mutations at the two major phosphorylation sites, is effective at attenuating GLUT4 translocation, but conclusions drawn from this mutant are clouded by the fact that it may function as a pseudo-substrate inhibitor of the upstream kinase PDK1 (29). For the atypical PKCs, insulin has been observed to activate PKC-ζ and PKC-λ in 3T3-L1 (30) and rat adipocytes (31). Moreover, overexpression of a kinase-deficient mutant of PKC-λ resulted in a 50% reduction in insulin-stimulated glucose transport (32). We recently reported that the insulin receptor interacts with PLC-γ1 and that the disruption of this interaction blocks GLUT4 translocation (33). Inhibition of phospholipase activity has also been shown to decrease maximal glucose transport in 3T3-L1 cells stimulated by insulin or by epidermal growth factor (EGF) in 3T3-L1 cells overexpressing the EGF receptor (34). These three pathways, lying downstream of PI3K, may be complementary as none of the dominant-negative mutants block glucose transport as effectively as inhibition of PI3K itself.

Osmotic shock is also known to stimulate glucose transport, albeit less effectively than insulin. Although the downstream effectors are identical, the upstream signaling pathways appear to be distinct. For instance, osmotic shock, unlike insulin, does not require the PI3K pathway for translocation of GLUT4 and stimulation of glucose transport (35). In contrast, inhibition of PLC activity completely abolishes osmotic shock–stimulated glucose transport but has only a modest effect on insulin-stimulated transport. Two groups have shown that the stimulation of glucose transport by osmotic shock, like that for insulin, is tyrosine kinase–dependent (36,37). Osmotic shock induced the phosphorylation of several proteins, including Gab1, PLC-γ, Cbl, p130cas, and a calcium-dependent tyrosine kinase. Downstream of PI3K, osmotic shock and insulin have opposing effects on Akt/PKB phosphorylation (38). Osmotic shock does not signal through PI3K unlike insulin and, consequently, does not activate Akt/PKB; however, the effects of the two signaling pathways are not additive. Pretreatment of 3T3-L1 adipocytes with sorbitol before stimulation with insulin prevented the normal stimulation of glucose transport. Mechanistically, this finding was explained by the dephosphorylation of Akt/PKB by sorbitol (39).

Although upstream signaling pathways may be different, they must converge at some point to cause GLUT4 translocation and/or activate transporters already resident in the plasma membrane. The finding that both osmotic shock and insulin can both stimulate stress-activated kinases suggests that this might be the point of convergence. We undertook the present study to delineate the response of the growth factor and stress-activated MAPKs and their upstream MKKs to insulin and osmotic stress in the 3T3-L1 adipocytes.
replaced with DMEM high glucose containing 10% FCS, 2 mMol/l GLUTA-MAX (12-Alanyl-l-glutamine), 50 µM penicillin, and 50 µg/ml streptomycin. The cells were plated into 6- or 12-well dishes at day 7 postdifferentiation, and the medium was changed every 2 days until days 12–15.

Assessment of ERK, p38, and JNK phosphorylation. 3T3-L1 adipocytes were maintained in 10% FCS in DMEM/5 µmol/l glucose medium. Cells were stimulated with 16.7 µmol/l insulin or 500 µmol/l sorbitol for 2–30 min or with 10 µg/ml anisomycin for 15 min at 37°C. They were then lysed in radiolimmunoprecipitation assay (RIPA) buffer containing 20 mMol/l Tris, pH 7.5, 1 mMol/l EDTA, 140 mMol/l NaCl, 1% Nonidet P-40, 1 mMol/l sodium orthovanadate, 1 mMol/l phenethylsulfonyl fluoride, 50 µMol/l sodium fluoride, and 10 µg/ml aprotinin. After centrifugation, the fat was aspirated and the pellet was discarded. Equal fractions of total cell lysates were mixed with 2× SDS sample buffer. The samples were boiled and the proteins resolved on a 10% SDS-PAGE gel. After being transferred to polyvinylidene fluoride (PVDF) paper, the proteins were immunoblotted with rabbit polyclonal antibodies to active ERK1, ERK2, or JNK (New England Biolabs, Beverly, MA). The anti-active ERK antibody was generated using a dually phosphorylated peptide derived from the catalytic region of MAPK, corresponding to Thr183 and Tyr185 of the mammalian ERK2 enzyme and used at a dilution of 1:20,000. The anti-active p38 MAPK antibody was generated using a dually phosphorylated peptide derived from the catalytic core of the active form of p38 MAPK, corresponding to Thr182 and Tyr185 of the mammalian p38 enzyme, and used at a dilution of 1:20,000. The anti-active JNK antibody was generated using a synthetic phospho-Thr-Pro-Tyr region peptide derived from the catalytic region of JNK2, corresponding to the active form of JNK, corresponding to the mammalian JNK2 enzyme, and used at a dilution of 1:5,000.

Assessment of MEK, MKK3/MKK6, and SEK1/MKK4 phosphorylation. 3T3-L1 adipocytes were treated with 500 µmol/l sorbitol, 0.2–100 µmol/l insulin, 50 µg/ml platelet-derived growth factor (PDGF) or 100 ng/ml IGF-1 for 5–15 min at 37°C. Cells were lysed in RIPA buffer as described in the previous section. After solubilization, centrifugation, and boiling, the proteins were resolved by SDS-PAGE electrophoresis and electrotransferred to PVDF membranes. The membranes were immunoblotted with antibodies to phospho-MEK, phospho-SEK1/MKK4, phospho-MKK3/6, and MKK3 protein according to the manufacturer’s recommendations (New England Biolabs). Phospho-MEK antibodies were generated using a synthetic phospho-Ser217/221 peptide corresponding to residues 214–226 around Ser217/221 of human MEK1 and used at a dilution of 1:10,000. Phospho-SEK1/MKK4 antibodies were generated using a synthetic phospho-Thr peptide corresponding to residues around Thr223 of human SEK1/MKK4 and used at a dilution of 1:2,000. Phospho-MKK3/6 antibodies were produced against a synthetic phospho-Ser189 peptide corresponding to residues around Ser189/207 of human MKK3 and used at a dilution of 1:2,000. MKK3 antibodies were generated against a synthetic peptide corresponding to residues 296–309 of human MKK3 and used at a dilution of 1:10,000.

Measurement of p38 and JNK activity. 3T3-L1 adipocytes were treated with 16.7 µmol/l insulin or 500 µmol/l sorbitol at 37°C for 30 min. Cells were then lysed with a lysis buffer containing 20 mMol/l Tris, pH 7.5, 150 mMol/l NaCl, 1 mMol/l EDTA, 1 mMol/l EGTA, 1% Triton X-100, 2.5 mMol/l sodium pyrophosphate, 1 mMol/l β-glycerophosphate, 1 mMol/l sodium orthovanadate, 1 µg/ml leupeptin, and 1 mMol/l phenethylsulfonyl fluoride. To measure JNK activity, a Jun/ATF-2 fusion protein, it was pulled down on glutathione-sepharose beads by centrifugation at 1,200g. The pellets were washed twice with the lysis buffer and twice with kinase buffer containing 25 mMol/l Tris, pH 7.4, 5 mMol/l β-glycerophosphate, 2 mMol/l DTT, 0.1 mMol/l sodium orthovanadate, and 10 mMol/l MgCl2. The kinase assay was performed in a volume of 50 µl in kinase buffer containing 100 µCi of [γ-32P]ATP. Upon termination with SDS sample buffer, the samples were boiled, and the proteins resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were immunoblotted with a phospho-JNK antibody (New England Biolabs). Activated p38 MAPK was immunoprecipitated from cell lysates by incubation with the immobilized antibody on agarose (New England Biolabs). Activated p38 MAPK was then washed with lysis and kinase buffers as previously described and were allowed to phosphorylate in the presence of 200 µCi/ATP for 30 min at 30°C using the GST–ATF-2 fusion protein as substrate. Phosphorylation of the ATF-2 fusion proteins was assessed as previously described.

Measurement of MAPK-activated protein kinase-2 activity. 3T3-L1 adipocytes were pretreated with DMSO, 5 µmol/l SB202190, or 500 µmol/l PD169316 for 20 min and then stimulated with 16.7 µmol/l insulin or 500 µmol/l sorbitol for 5 min at 37°C. Cells were lysed in a buffer containing 25 mMol/l HEPS, pH 7.4, 0.3 mMol/l NaCl, 20 mMol/l β-glycerophosphate, 0.2 mMol/l EDTA, 0.1% Triton X-100, 0.5 mMol/l DTT, 0.1 mMol/l orthovanadate, 1 mMol/l phenethylsulfonyl fluoride, and 2 µg/ml leupeptin. After centrifugation at 14,000g, the fat was aspirated and the lysates containing 250 µg protein were immunoprecipitated with 2 µg of MAPK-activated protein (MAPRAP) kinase–polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) for 2 h at 4°C. Subsequently, the immune complexes were bound to Protein A/G-agarose beads for 1 h at 4°C. After centrifugation, the immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer containing 25 mMol/l HEPS, pH 7.4, 0.3 mMol/l NaCl, 25 mMol/l β-glycerophosphate, 0.1 mMol/l orthovanadate, and 2 mMol/l DTT. The MAPKAP kinase-2 assay was performed using Hsp27 (2 µg) (StressGen Biotechnologies, Victoria, BC, Canada) as a substrate in a volume of 30 µl of kinase buffer containing 10 µCi of [γ-32P]ATP in the presence of 100 µmol/l cold ATP for 30 min at 30°C. The reactions were terminated by the addition of SDS sample buffer. The phosphorylation of Hsp27 was visualized after SDS-PAGE gel electrophoresis by autoradiography, quantified by densitometry, and analyzed using NIH image software.

2-Deoxyglucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were seeded into 12-well plates 7 days after differentiation. Transport assays were performed between days 10 and 14. Cells were maintained in 10% FCS DMEM/5 µmol/l glucose for 12–16 h before the assay. Cells were incubated in a Krebs-Ringer phosphate (KRP)–HEPS buffer containing 0.1% bovine serum albumin, 5 mMol/l glucose, 2 mMol/l pyruvate, and 25 mMol/l HEPS, pH 7.4, at 37°C for 30 min and were then pretreated with inhibitors for 20 min. Cells were stimulated for 30 min with increasing concentrations of insulin, 500 µmol/l sorbitol, or 10 µg/ml anisomycin. Uptake was initiated by the addition of 0.1 µCi of 2-deoxy-2-[1,2-3H]glucose (NEN Life Sciences Products, Boston, MA) and then terminated after 10 min by the addition of 0.1 mMol/l EDTA. The glucose uptake was terminated by boiling all cells in 1 N NaOH. After neutralization, radioactivity was measured by scintillation counting and corrected for total cellular protein.

Assessment of PKB/Akt phosphorylation. 3T3-L1 adipocytes were starved overnight and pretreated with DMSO, 1 µmol/l, 5 µmol/l, 10 µmol/l, or 20 µmol/l of SB202190 for 20 min, and then stimulated with 16.7 µmol/l insulin or 500 µmol/l sorbitol at 37°C. Cells were lysed in RIPA buffer containing 20 mMol/l Tris, pH 7.5, 1 mMol/l NaCl, 1% Nonidet P-40, 1 mMol/l orthovanadate, 1 mMol/l phenethylsulfonyl fluoride, 50 µmol/l sodium fluoride, and 10 µg/ml aprotinin. After centrifugation, the fat was aspirated and the pellet discarded. Equal fractions of total cell lysates were mixed with 2× SDS sample buffer. The samples were boiled and the proteins resolved on a 10% SDS-PAGE gel. After being transferred to PVDF paper, the proteins were immunoblotted with rabbit polyclonal antibodies to phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, MA). The anti–phospho-Akt antibody was generated using a serine phosphorylated peptide corresponding to residues around Ser473 of mouse Akt and used at a dilution of 1:100,000.

Statistical analysis. Results were analyzed by analysis of variance (ANOVA), and groups were compared using the Student-Newman-Keuls post-test or Welch’s t test assuming Gaussian distributions with different SDs for the data.

RESULTS
Time course of stimulation of p38 MAPK, JNK, and ERK1/ERK2 phosphorylation by sorbitol. Osmotic shock has been shown to stimulate p38 MAPK and JNK activity in many cell systems. The first set of experiments in this study focused on the effect of sorbitol on p38, JNK, and MAPK phosphorylation in differentiated 3T3-L1 adipocytes. After
being treated with 500 mmol/l sorbitol for 2, 5, 10, 15, or 30 min at 37°C, cells were lysed in RIPA buffer, and whole-cell lysates were immunoblotted for activated p38 using an antibody to dually phosphorylated (Thr180/Tyr182) p38 MAPK. Sorbitol caused a very strong phosphorylation of p38 that was detectable as early as 2 min (3.6-fold), was maximal at 10 min (24-fold), and was maintained for at least 30 min (22-fold) (Fig. 1, top lane). The membranes were blotted to show equal protein loading using an antibody to unphosphorylated p38 protein. Stimulation of JNK phosphorylation was assessed in parallel samples using an antibody to dually phosphorylated (Thr183/Tyr185) JNK. Again, sorbitol caused a strong phosphorylation of JNK that was first seen at 10 min (fourfold) and increased at 30 min (sevenfold) (Fig. 1). Although ERK1 and ERK2 are primarily activated by growth factors and phorbol esters, they can also be stimulated by stressful stimuli (40). We therefore documented the time course of ERK1 and ERK2 phosphorylation in response to 500 mmol/l sorbitol in our system using an antibody to dually phosphorylated (Thr183/Tyr185) ERK (Fig. 1, bottom lane). Sorbitol activated ERK1 and -2 with a time course similar to that seen for p38 MAPK. ERK2 phosphorylation was elevated eightfold after 5 min, and this level was maintained for 30 min. Thus, sorbitol strongly stimulates the phosphorylation of all three subfamilies of MAPKs in 3T3-L1 adipocytes.

**Time course of stimulation of p38 MAPK, JNK, and ERK1/ERK2 phosphorylation by insulin.** 3T3-L1 adipocytes were treated with 16.7 nmol/l insulin for 2, 5, 10, 15, or 30 min at 37°C and lysed in RIPA buffer. The proteins in the lysates were resolved by SDS-PAGE and transferred onto PVDF membranes. Stimulation of MEK phosphorylation was assessed using an antibody to phosphorylated (Ser217/221) MEK. Both sorbitol and insulin stimulated MEK phosphorylation strongly (8-fold and 10-fold, respectively), which is consistent with our findings of ERK1 and ERK2 phosphorylation by both ligands (Fig. 3, top lane). This observation corresponded to respective 2.6- and 3.3-fold increases in MEK activity when assayed using recombinant kinase inactive ERK2 (data not shown). The JNKs have been shown to be phosphorylated and activated by either MKK4 or MKK7. Consequently, stimulation of MKK4 phosphorylation was assessed using an antibody to phosphorylated (Thr182/Tyr184) ERK1 and MKK4. Sorbitol treatment resulted in stimulation of MKK4 phosphorylation in 3T3-L1 adipocytes, and, as expected, insulin did not result in MKK4 stimulation, which is in agreement with the observation that the JNKs were not phosphorylated by insulin (Fig. 3, middle lane).

The p38 MAPKs have been shown to be phosphorylated by either MKK3 or MKK6. Therefore, MKK3 and MKK6 phosphorylation was assessed using an antibody that recognizes the phosphorylated forms of both proteins (Ser189/211). Phosphorylation of MKK3 or MKK6 was not observed in response to either sorbitol or insulin treatment in 3T3-L1 adipocytes (Fig. 3, middle panel). This finding was surprising given the robust stimulation of p38 phosphorylation by sorbitol, and suggests that MKK4 is the upstream activator of p38.
as has been shown in other systems. The membrane was stripped and rebotted for native MKK3 to demonstrate equal loading. To show that the antibody to phospho-MKK3/6 is functional, 3T3-L1 adipocytes were stimulated with other agonists. Cells were treated for 10 min with PDGF (50 ng/ml), IGF-1 (100 ng/ml), 500 mmol/l sorbitol, or 16.7 mmol/l insulin. Whole-cell extracts were immunoblotted using the phospho-MKK3/6 antibody. Both PDGF and IGF-1 caused phosphorylation of MKK6 (1.9-fold and 2.7-fold, respectively) but not MKK3 (Fig. 3, bottom lane). Similarly, p38 phosphorylation was stimulated 2.9-fold by PDGF and 2-fold by IGF-1 (data not shown).

**Stimulation of JNK and p38 activities.** It is possible that the threonine and tyrosine phosphorylation of JNK and p38 observed with the antibodies does not reflect true activation of the kinases. Therefore, JNK and p38 activities were determined using a combination of pull-down and immunoprecipitation coupled with in vitro kinase assays. 3T3-L1 adipocytes were treated with 500 mmol/l sorbitol or 16.7 mmol/l insulin for 30 min at 37°C and lysed in a buffer containing 1% Triton. A GST fusion protein containing amino acids 1–90 of ATF-2 was used to pull down the kinases that can phosphorylate ATF-2. The kinase assay was then performed using exogenous GST–ATF-2 as substrate for the p38 MAPKs. Consequently, a GST-fusion protein containing amino acids 1–90 of ATF-2 was used to pull down the kinases that can phosphorylate ATF-2. The kinase assay was then determined using an in vitro kinase assay as for the c-jun fusion protein in the previous section. Sorbitol stimulated ATF-2 kinase activity 16.5-fold, whereas insulin stimulated activity 6-fold (Fig. 4, middle panel). However, ATF-2 can be phosphorylated by other members of the MAPK family (41–44), so to measure p38 activity directly, cell lysates were immunoprecipitated using antibodies to activated p38. The kinase assay was then performed using exogenous GST–ATF-2 fusion protein as substrate, as before. Insulin does not stimulate p38 activity but sorbitol stimulates activity 7-fold (Fig. 4, bottom panel). Thus, osmotic shock activates both JNK and p38, but insulin activates neither kinase.

**Inhibition of insulin and osmotic shock–stimulated glucose transport by inhibitors of PI3-kinase and PLC.** As reviewed in the introduction, the involvement of PI3-kinase in insulin-stimulated glucose transport has been established by many groups. This lipid kinase does not appear to be involved in osmotic shock–stimulated glucose transport. We have previously demonstrated a role for PLCγ in insulin-stimulated glucose transport, and Ueno et al. (36) have recently suggested PLC may be involved in the stimulation of glucose transport by osmotic shock in 3T3-L1 adipocytes. Therefore, the next experiment was designed to compare insulin- and sorbitol-stimulated 2-deoxyglucose uptake and to assess the involvement of PI3-kinase and PLC using pharmacological inhibitors of these enzymes, wortmannin and U73122, respectively. Consistent with our previous studies, 10 µmol/l U73122, a non-specific PLC inhibitor, resulted in 50% inhibition of insulin-stimulated 2-deoxyglucose uptake and 100 nmol/l Wortmannin, a PI3-kinase inhibitor,
Inhibition of insulin and osmotic shock–stimulated glucose transport by pyridinyl imidazoles. Because both osmotic shock and insulin stimulate glucose transport and both treatments also stimulate p38, albeit very weakly in the case of insulin, it is conceivable that insulin and osmotic shock share this pathway to stimulate glucose transport. The pyridinyl imidazoles SB203580, SB202190, and PD169316, which bind the ATP binding pocket of p38 MAPK, have been used as specific inhibitors of p38 in many cell systems (3). To assess the involvement of the p38 pathway in insulin and osmotic shock–stimulated glucose transport, 3T3-L1 adipocytes were pretreated with increasing concentrations of these inhibitors before stimulation with insulin or sorbitol. Insulin-stimulated glucose transport was inhibited in a dose-dependent manner by SB203580 and SB202190 (Fig. 6, top and middle panels). The EC_{50} for inhibition of transport was >10 µmol/l for both inhibitors, which is much higher than the accepted EC_{50} for inhibition of p38 (~0.5 µmol/l). In contrast, no significant inhibition was observed with the third inhibitor PD169316 even at a concentration of 5 µmol/l, which is 50 times the EC_{50} for inhibition of p38 (Fig. 6, bottom panel). Basal and osmotic shock–stimulated glucose transport were not inhibited significantly by any of the p38 inhibitors.

Stimulation of MAPKAP kinase-2. Because the p38 inhibitors do not appreciably inhibit glucose transport, it is important to demonstrate that they are able to inhibit a bona fide p38 response in intact cells. One of the downstream targets of p38 is MAPKAP kinase-2. This kinase has been shown to phosphorylate Hsp27 in vivo and in vitro. We wanted to determine, firstly, whether osmotic shock and insulin would stimulate MAPKAP kinase-2 and, secondly, whether the inhibitors of p38 could block stimulation of MAPKAP kinase-2. 3T3-L1 cells were pretreated with DMSO, 5 µmol/l SB203580, or 500 nmol/l PD169316 for 20 min and were stimulated with either 16.7 nmol/l insulin or 500 nmol/l sorbitol for 5 min at 37°C. Cell lysates were immunoprecipitated with MAPKAP kinase-2 antibody, and an in vitro kinase assay performed using Hsp27 as substrate. Reaction products were analyzed by SDS-PAGE gel electrophoresis followed by autoradiography. We found a significant stimulation of MAPKAP kinase-2 in response to sorbitol but a complete lack of stimulation in response to insulin (Fig. 7). This finding confirms that osmotic shock but not insulin activates the p38 pathway and is in agreement with our previous immunoblotting and kinase results. It was interesting to note that MAPKAP kinase-2 is more sensitive to p38 inhibition than glucose transport. Pretreatment of cells for 20 min with 5 µmol/l SB203580 or 500 nmol/l PD169316 blocked the sorbitol stimulation of MAPKAP kinase-2 to basal levels (Fig. 7). The sensitivity of MAPKAP kinase-2 to SB203580 and PD169316 indicates that these inhibitors are able to block p38 MAPK at doses that have no effect on 2-deoxyglucose transport.

Inhibition of insulin-stimulated serine phosphorylation of PKB/Akt by inhibitors of p38 MAPK. Because the pyridinyl imidazoles inhibited insulin-induced glucose uptake at concentrations of ~10–20 µmol/l, we were concerned about possible nonspecific effects of these inhibitors. Recently, Lali et al. (45) have reported that sorbitol resulted in an inhibition of insulin-stimulated glucose transport (39). Thus, the effects of osmotic shock and insulin on glucose transport are not independent, and both signals are likely to converge on a common element to mediate transport.

FIG. 5. PI3-kinase and PLC inhibitors block insulin and osmotic shock–stimulated 2-deoxyglucose uptake. A: 3T3-L1 adipocytes (12 to 15 days postdifferentiation) were incubated in KRP-HEPES buffer for 30 min and pretreated with 10 µmol/l U73122 (U7), 100 nmol/l Wortmannin (wort) or vehicle for 20 min (top panel). After a 30-min stimulation with 8.4 nmol/l insulin or 500 nmol/l sorbitol, 0.1 µCi of [3H]2-DG was added to the cells, and the uptake assay was terminated after 10 min by the addition of 0.1 mmol/l phloretin. Cells were solubilized, and radioactivity was measured by liquid scintillation counting. The mean results from three experiments are expressed as percent maximal transport (± SE). Asterisks indicate statistical significance (P < 0.05) relative to insulin or sorbitol alone. B: Subsequently, cells were stimulated simultaneously with 500 nmol/l sorbitol and increasing concentrations of insulin for 30 min (bottom panel). 2-Deoxyglucose uptake was assessed as above. The results from three experiments are expressed as absolute transport (± SE). Asterisks indicate statistical significance (P < 0.05) relative to basal transport.

completely abolished insulin-stimulated glucose transport (Fig. 5, top panel). Osmotic shock–stimulated 2-deoxyglucose uptake was reduced to basal levels by the PLC inhibitor; however, Wortmannin did not reduce transport significantly. Thus, the relative importance of PI3-kinase and PLC signaling for insulin and osmotic shock is reversed, with PI3-kinase being predominant for insulin and PLC predominant for osmotic shock.

We wanted to determine whether the effects of osmotic shock and insulin were additive. To address this question, differentiated 3T3-L1 adipocytes were treated with increasing concentrations of insulin in the presence or absence of 500 nmol/l sorbitol. Insulin alone causes a dose-dependent increase in 2-deoxyglucose transport (Fig. 5, bottom panel). Sorbitol alone also causes a significant increase in 2-deoxyglucose transport. Addition of increasing concentrations of insulin in the presence of sorbitol did not lead to a stimulation of glucose transport over that seen with sorbitol alone (Fig. 5, bottom panel). These data are consistent with the results reported by Chen (39), in which pretreatment with sorbitol alone also causes a significant increase in 2-deoxyglucose transport. These data are consistent with the observation that pretreatment with sorbitol resulted in an inhibition of insulin-stimulated glucose transport (39). Thus, the effects of osmotic shock and insulin on glucose transport are not independent, and both signals are likely to converge on a common element to mediate transport.

Inhibition of insulin and osmotic shock–stimulated glucose transport by pyridinyl imidazoles. Because both osmotic shock and insulin stimulate glucose transport and both treatments also stimulate p38, albeit very weakly in the case of insulin, it is conceivable that insulin and osmotic shock share this pathway to stimulate glucose transport. The pyridinyl imidazoles SB203580, SB202190, and PD169316, which bind the ATP binding pocket of p38 MAPK, have been used as specific inhibitors of p38 in many cell systems (3). To assess the involvement of the p38 pathway in insulin and osmotic shock–stimulated glucose transport, 3T3-L1 adipocytes were pretreated with increasing concentrations of these inhibitors before stimulation with insulin or sorbitol.

Insulin-stimulated glucose transport was inhibited in a dose-dependent manner by SB203580 and SB202190 (Fig. 6, top and middle panels). The EC_{50} for inhibition of transport was >10 µmol/l for both inhibitors, which is much higher than the accepted EC_{50} for inhibition of p38 (~0.5 µmol/l). In contrast, no significant inhibition was observed with the third inhibitor PD169316 even at a concentration of 5 µmol/l, which is 50 times the EC_{50} for inhibition of p38 (Fig. 6, bottom panel). Basal and osmotic shock–stimulated glucose transport were not inhibited significantly by any of the p38 inhibitors.

Stimulation of MAPKAP kinase-2. Because the p38 inhibitors do not appreciably inhibit glucose transport, it is important to demonstrate that they are able to inhibit a bona fide p38 response in intact cells. One of the downstream targets of p38 is MAPKAP kinase-2. This kinase has been shown to phosphorylate Hsp27 in vivo and in vitro. We wanted to determine, firstly, whether osmotic shock and insulin would stimulate MAPKAP kinase-2 and, secondly, whether the inhibitors of p38 could block stimulation of MAPKAP kinase-2. 3T3-L1 cells were pretreated with DMSO, 5 µmol/l SB203580, or 500 nmol/l PD169316 for 20 min and were stimulated with either 16.7 nmol/l insulin or 500 nmol/l sorbitol for 5 min at 37°C. Cell lysates were immunoprecipitated with MAPKAP kinase-2 antibody, and an in vitro kinase assay performed using Hsp27 as substrate. Reaction products were analyzed by SDS-PAGE gel electrophoresis followed by autoradiography. We found a significant stimulation of MAPKAP kinase-2 in response to sorbitol but a complete lack of stimulation in response to insulin (Fig. 7). This finding confirms that osmotic shock but not insulin activates the p38 pathway and is in agreement with our previous immunoblotting and kinase results. It was interesting to note that MAPKAP kinase-2 is more sensitive to p38 inhibition than glucose transport. Pretreatment of cells for 20 min with 5 µmol/l SB203580 or 500 nmol/l PD169316 blocked the sorbitol stimulation of MAPKAP kinase-2 to basal levels (Fig. 7). The sensitivity of MAPKAP kinase-2 to SB203580 and PD169316 indicates that these inhibitors are able to block p38 MAPK at doses that have no effect on 2-deoxyglucose transport.
SB203580 could inhibit PKB phosphorylation in interleukin-2–stimulated T-cells at concentrations as low as 5 µmol/l. In their study, both PKB and PDK1 activity were inhibited by SB203580. Therefore, the effect of SB203580, SB202190, and PD169316 on insulin-stimulated PKB/Akt phosphorylation was examined (Fig. 8). As expected, insulin caused a robust stimulation of serine phosphorylation of PKB/Akt. Pretreatment with increasing doses of all three inhibitors resulted in a reduction in insulin-stimulated serine phosphorylation (Fig. 8).

Protein synthesis inhibitors as stimulators of stress MAPKs in 3T3-L1 adipocytes. Lastly, many cellular stresses are known to activate the p38 pathway. Protein synthesis inhibitors like anisomycin inhibit cell proliferation not only by inhibiting protein synthesis but also by eliciting a ribotoxic stress response through activation of JNK and p38 MAPKs resulting in apoptosis in many cell types (46). Anisomycin at a concentration of 300 µmol/l for 4–6 h has also been reported to stimulate glucose transport in 3T3-L1 adipocytes. This stimulation occurred in the absence of alterations in glucose transporter number in the plasma membrane fraction (47). Another study reported a stimulatory effect of 0.3 µmol/l anisomycin on GLUT1 within 30 min in the absence of protein synthesis (48). This early activation of transport was transient and sensitive to inhibitors of p38 MAPK.

We therefore determined which subfamilies of MAPKs are stimulated by anisomycin in 3T3-L1 adipocytes. Phosphorylation of ERKs, JNKs, and p38 was assessed by immunoblotting whole-cell extracts with antibodies to dually phosphorylated ERK, JNK, and p38 as before. Anisomycin, unlike sorbitol and insulin, did not stimulate ERK or JNK phosphorylation (Fig. 9, top and middle panels). Anisomycin did cause a dose-dependent increase in p38 phosphorylation (fivefold and ninefold at doses of 10 ng/ml and 10 µg/ml anisomycin, respectively). As expected, sorbitol stimulated p38 phosphorylation strongly and insulin weakly (Fig. 9, bottom panel). Interestingly, none of the MKks tested (MEK, MKK4, or MKK3/6) were phosphorylated in response to anisomycin (data not shown).

Given the robust stimulation of p38 phosphorylation by anisomycin in 3T3-L1 cells, we wanted to determine whether anisomycin could also stimulate glucose transport. We therefore compared insulin-, sorbitol-, and anisomycin-stimulated 2-deoxyglucose uptake. In these experiments, 8.4 nmol/l insulin and 500 mmol/l sorbitol stimulated glucose transport 5-fold and 2.5-fold, respectively (Fig. 10). Anisomycin treatment at 10 or 100 µg/ml (data not shown) for 30 min did not result in a stimulation of glucose transport. Thus, activation of p38 alone is not sufficient for glucose transport.
DISCUSSION

Our working model for the regulation of the MAPK cascades in 3T3-L1 adipocytes is shown in Fig. 11. Expectedly, osmotic shock is the most potent stimulator of the stress MAPK pathway and activates the JNKs, p38s, and ERKs very strongly. Insulin stimulates the ERKs predominantly, has no effect on JNK, and causes a weak phosphorylation of p38. However, this slight phosphorylation does not correspond to an activation of the kinase as assayed by immune-complex/kinase assay or activation of MAPKAP kinase-2 in vivo. Anisomycin stimulates p38 but not JNK or ERK phosphorylation. At the level of the MKKs, sorbitol stimulates MKK4 and MEK but not MKK3 or MKK6. It is likely that the activation of MKK4 is sufficient to phosphorylate both the JNKs and p38s. Insulin activates only MEK and not MKK3, 4, or 6, which is consistent with the exclusive activation of the ERKs. PDGF and IGF1 were observed to stimulate MKK6 and also p38. Anisomycin does not stimulate MKK3, 4, or 6, yet activates p38 very strongly, suggesting the intermediacy of another unidentified MKK.

Recently, it has been reported that insulin stimulates p38 MAPK in L6 myoblasts and 3T3-L1 adipocytes (20). Our findings are apparently contradictory. We found no activation of p38 when we used an immunoprecipitation/kinase assay, despite a small but reproducible phosphorylation detected by immunoblotting. More importantly, we did not observe any activation of MAPKAP kinase-2 in vivo with insulin. This kinase is the only known direct in vivo substrate for p38 and thus is arguably the best measure of p38 activation in vivo. In the report by Sweeney et al. (20), phosphorylated p38 was detected by immunoprecipitation with an antiphosphotyrosine antibody followed by immunoblotting with a p38 MAPK antibody. It may be that the p38 is phosphorylated only on tyrosine 182 and would be recognized by anti-phosphotyrosine antibodies but not by the anti–dually phosphorylated p38 antibodies used in our study. In addition, the p38 kinase

FIG. 8. P38 inhibitors reduce insulin-stimulated serine phosphorylation of PKB/Akt. 3T3-L1 adipocytes were starved overnight, pretreated with increasing concentrations of SB203580 (data not shown), SB202190, or PD169316 for 20 min, and then stimulated with 16.7 mmol/l insulin for 10 min at 37°C. Whole-cell lysates were immunoblotted for phosphoAkt (Ser473).

FIG. 9. Anisomycin treatment causes phosphorylation of p38 MAPK but not ERK or JNK. 3T3-L1 adipocytes were treated with 10 ng/ml or 10 µg/ml anisomycin, 500 mmol/l sorbitol, or 16.7 mmol/l insulin for 15 min at 37°C. Whole-cell lysates were immunoblotted for phospho-ERK, phospho-JNK, and phospho-p38 MAPK (top to bottom panels, respectively).

FIG. 10. Acute anisomycin treatment does not stimulate 2-deoxyglucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated in KRP-HEPES buffer for 30 min and stimulated for 30 min with 8.4 mmol/l insulin, 500 mmol/l sorbitol, or 10 µg/ml anisomycin, 0.1 µCi of [3H]2-DOG was added to the cells, and the assay was terminated after 10 min by the addition of 0.1 mmol/l phloretin. Cells were solubilized, and radioactivity was measured by liquid scintillation counting. Transport is expressed as percent maximum (mean ± SE). Asterisks indicate statistical significance (P < 0.05) compared with basal.

FIG. 11. Summary of MAPK cascades activated in response to sorbitol, insulin IGF1, PDGF, and anisomycin in 3T3-L1 adipocytes.
assays were performed on total p38 immunoprecipitates in the previous study, whereas only the dually-phosphorylated form of p38 was precipitated in the assay used here. The previous study did not contain a positive control for activation of the stress-activated MAPKs, so in the present study we included a known activator of the stress-activated MAPKs. Osmotic stress caused by treatment with 500 mmol/l sorbitol activates JNK and p38 very strongly (20–30-fold); the activation of p38 by insulin is an order of magnitude lower than this classical stress.

A further discrepancy between the studies is the concentration of insulin. In our glucose transport studies, maximal transport is achieved using 8.3 nmol/l insulin. Stimulation with 100 nmol/l insulin in the earlier study may well activate IGF1 receptors. There are several reports of IGF1 stimulation of p38 MAPK (49,50), and we have observed a phosphorylation of MKK6 and p38 MAPK in response to IGF1 stimulation of 3T3-L1 adipocytes. IGF1 also stimulates glucose transport as efficiently as insulin in these cells (data not shown).

Sweeney et al. (20) reported that inhibition of p38 using the pyridinyl imidazole SB203580 causes a 60% inhibition of insulin-stimulated glucose transport in the face of normal GLUT4 and GLUT1 translocation. The EC50 for inhibition of transport corresponded very closely with the known EC50 for inhibition of p38. We extended these experiments and used two additional p38 inhibitors, SB202190 and PD169316. Because osmotic shock and insulin both stimulate glucose transport, primarily by different pathways, we postulated that they might utilize the p38 MAPK pathway as a common component of the regulation of glucose transport. However, using the three cell-permeable inhibitors SB203580, SB202190, and PD169316, we were unable to inhibit glucose transport at concentrations close to the EC50 for inhibition of p38 MAPK. We verified that the inhibitors were indeed blocking p38 in vivo by measuring MAPKAP kinase-2 activity. Both SB203580 and PD169316 blocked activation of MAPKAP kinase-2 at concentrations that did not inhibit glucose transport.

It is noteworthy that we do observe inhibition of glucose transport at higher concentrations of SB203580 and SB202190. In particular, concentrations of SB203580 and SB202190 >10–20 µmol/l caused a significant repression in insulin-stimulated, but not basal or osmotic shock–stimulated, glucose transport. In contrast, the more potent inhibitor PD169316 had no effect on glucose transport even at concentrations 50 times the EC50 for p38 inhibition. The pyridinyl imidazole SB203580 has been used in many experiments to demonstrate an involvement of p38 MAPK in many biological processes (3). However, data has been emerging that SB203580 also binds other enzymes, including cyclo-oxygenases 1 and 2 (51), Raf (52,53), and JNK (54). In most cases, SB203580 inhibits enzyme activity; however, a 25-fold increase in c-Raf activity has also been reported. The co-crystallization of p38 MAPK in complex with pyridinyl imidazoles underscored the importance of residue Thr106 in the ATP-binding pocket for the inhibition of the enzyme. Subsequently, Eyers et al. (55) have made SB203580-resistant p38 mutants that have the Thr 106 mutated to Met. Conversely, mutation of the corresponding residue in the JNKs and ERK2 confers increased sensitivity to SB203580 (55–57). It is possible that the decrease in glucose transport observed at higher concentrations of inhibitor may reflect inhibition of other signaling proteins. In fact we did observe an inhibition of insulin-stimulated serine phosphorylation of PKB/Akt at concentrations of SB203580 and SB202190 >10 µmol/l. Diminished serine phosphorylation of PKB/Akt reflects an inhibition of PDK1 activity. Because both PKB/Akt and the atypical PKCs are downstream of PDK1 and both are considered to be involved in insulin-stimulated glucose transport, the inhibition of glucose transport in response to the higher concentrations of pyridinyl imidazoles may be explained by a reduction in PDK1 activity. Clearly, caution needs to be observed interpreting data based on SB203580 or SB202190 at concentrations >10 times the IC50 (0.3–0.5 µmol/l) required to inhibit p38 MAPK.

Alternatively, activation of glucose transport may involve isoforms of p38 that are less susceptible to inhibition by SB203580. Indeed, p38δ and p38γ (58) have been reported to be more resistant to SB203580 and SB202190. Fukuwatari et al. (59) have recently reported that in isolated rat skeletal muscle insulin did not increase p38 α, β, or γ kinase activities whereas sorbitol stimulated p38 α and β activities. It is known that p38 activity is modulated during 3T3-L1 adipocyte differentiation and drops dramatically during the later stages of differentiation (60). Interestingly, differentiation is inhibited by SB203580, and it has been suggested that the p38α, p38β1, and p38β2 isoforms may be involved in adipocyte differentiation. It is also likely that different isoforms of p38 are expressed at different times during differentiation. We have, however, performed experiments on cells at different points of differentiation (days 8–15) and obtained identical results. It is possible that differences in subclones of the 3T3-L1 adipocyte cell line differ in the repertoire of p38 isoforms expressed and thus differ in sensitivity to inhibition. Further experiments are planned to investigate signaling by individual isoforms of p38.

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