MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes

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Harmon, Anne W., David S. Paul, and Yashomati M. Patel. MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 287: E758–E766, 2004.—In 3T3-L1 adipocytes, insulin activates three major signaling cascades, the phosphoinositide 3-kinase (PI3K) pathway, the Cbl pathway, and the mitogen-activated protein kinase (MAPK) pathway. Although PI3K and Cbl mediate insulin-stimulated glucose uptake by promoting the translocation of the insulin-responsive glucose transporter (GLUT4) to the plasma membrane, the MAPK pathway does not have an established role in insulin-stimulated glucose uptake. We demonstrate in this report that PI3K inhibitors also inhibit the MAPK pathway. To investigate the role of the MAPK pathway separately from that of the PI3K pathway in insulin-stimulated glucose uptake, we used two specific inhibitors of MAPK kinase (MEK) activity, PD-98059 and U-0126, which reduced insulin-stimulated glucose uptake by ~33 and 50%, respectively. Neither MEK inhibitor affected the activation of Akt or PKCζ/δ, downstream signaling cascades in the PI3K pathway. Inhibition of MEK with U-0126 did not prevent GLUT4 from translocating to the plasma membrane, nor did it inhibit the subsequent docking and fusion of GLUT4-myc with the plasma membrane. MEK inhibitors affected glucose transport mediated by GLUT4 but not GLUT1. Importantly, the presence of MEK inhibitors only at the time of the transport assay markedly impaired both insulin-stimulated glucose uptake and MAPK signaling. Conversely, removal of MEK inhibitors before the transport assay restored glucose uptake and MAPK signaling. Collectively, our studies suggest a possible role for MEK in the activation of GLUT4.

p44/p42 mitogen-activated protein kinase; glucose transporter 4; glucose transport; U-0126; PD-98059

THE BINDING OF INSULIN TO ITS RECEPTOR TRIGGERS MULTIPLE SIGNALING PATHWAYS THAT PARTICIPATE BROADLY IN CELLULAR GROWTH AND DIFFERENTIATION AND IN THE METABOLISM OF LIPIDS AND GLUCOSE. TO MAINTAIN GLUCOSE HOMEOSTASIS, INSULIN NOT ONLY INHIBITS THE PRODUCTION OF GLUCOSE IN THE LIVER BUT ALSO STIMULATES THE TRANSPORT OF GLUCOSE INTO MUSCLE AND ADIPOSE TISSUE FOR SUBSEQUENT USE OR STORAGE. AUTO Phosphorylation of the insulin receptor tyrosine kinase results in the recruitment and tyrosine phosphorylation of substrates and the activation of three major pathways, the phosphoinositide 3-kinase (PI3K) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the Cbl pathway (see review in Ref. 3). PI3K catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate, or PIP3, an allosteric activator of phosphoinositide-dependent kinase (PDK). Targets of PDK include Akt and the atypical PKC isoforms, which, when activated via phosphorylation, stimulate the translocation of the insulin-responsive glucose transporter (GLUT4)-containing vesicles to the plasma membrane. Phospholipase C-γ1 (PLC-γ1), another downstream target of PI3K in insulin signaling, also facilitates GLUT4 translocation (13, 25). Although the relative contributions of PLC-γ1, Akt, and atypical PKCs to GLUT4 translocation remain controversial, studies have demonstrated the necessity of PI3K activity for insulin-stimulated glucose uptake (6, 43).

Insulin also stimulates glucose transport via a PI3K-independent pathway. The adapter protein Cbl-associated protein (CAP) recruits Cbl to the insulin receptor for tyrosine phosphorylation (32). The Cbl-CAP complex then localizes to lipid rafts, where recruitment of an adapter protein, CRKII, and a guanine nucleotide exchange factor, C3G, leads to the activation of TC10, a rho family protein required for insulin-stimulated GLUT4 translocation (7). TC10 then interacts with Exo70, one of the components of the exocyst complex that targets GLUT4 to the plasma membrane (21).

Insulin also initiates the MAPK cascade (see review in Ref. 2), in which a series of phosphorylation events results in the activation of p44/p42 MAPK by the upstream kinase, MAPK kinase (MEK). Although the PI3K pathway (6, 43) and the Cbl-CAP pathway (7) have gained wide acceptance as critical components in insulin-stimulated glucose uptake, the MAPK pathway does not have an established role in mediating the metabolic effects of insulin in adipocytes. Several researchers have reported the existence of cross talk between the PI3K pathway and the MAPK pathway. Importantly, inhibition of PI3K prevents full activation of p44/p42 MAPK in several cell types, including 3T3-L1 adipocytes (39) and human hepatoma cells (28). By showing that PI3K inhibitors affect the MAPK pathway as well as the PI3K pathway, these studies raise the possibility that the MAPK cascade does indeed contribute to insulin-stimulated glucose uptake.

In this report, we showed that, in 3T3-L1 adipocytes, the PI3K inhibitor naringenin blocked insulin-induced MAPK phosphorylation, and we also confirmed that the well-established PI3K inhibitor wortmannin inhibited the MAPK pathway. To investigate the role of the MAPK pathway separately from that of the PI3K pathway in insulin-stimulated glucose uptake, we used two specific inhibitors of MEK activity, PD-98059 and U-0126. Inhibition of MEK with either compound markedly reduced insulin-stimulated glucose uptake. Importantly, neither MEK inhibitor affected the PI3K pathway, as shown by activation of the downstream signaling molecules Akt and PKCζ/δ. Inhibition of MEK did not affect GLUT4 translocation, as shown by immunofluorescent microscopy. In 3T3-L1 cells expressing myc-tagged GLUT4, an analysis of

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cell-surface GLUT4-myc showed that disruption of the MAPK pathway did not affect the proper docking or fusion of GLUT4-myc with the plasma membrane. Direct inhibition of MAPK signaling during a glucose transport assay adversely affected GLUT4-mediated glucose uptake in response to insulin. Our results therefore suggest a role for MEK in insulin-stimulated glucose uptake, possibly in the activation of GLUT4.

MATERIALS AND METHODS

Reagents. All tissue culture materials were from Gibco (Grand Island, NY). Insulin was obtained from Roche Diagnostics (Indianapolis, IN). Dexamethasone, methylisobutylxanthine, naringenin, PD-98059, and anti-goat and anti-rabbit IgG peroxidase-conjugated secondary antibodies were from Sigma (St. Louis, MO). A p38 MAPK assay kit and phospho-Akt (Ser473), phospho-Akt (Thr476), Akt, phospho-Erk5 (Thr218/Tyr220), phospho-glycogen synthase kinase (GSK)-3β (Ser9), phospho-p44/42 MAP kinase (Thr202/Tyr204), p44/42 MAP kinase, phospho-MEK1/2 (Ser187/192), MEK1/2, and phospho-PKCα (Thr410/415) antibodies were obtained from Cell Signaling Technology (Beverly, MA). U-0126 was from Promega (Madison, WI). Alexa Fluor 594 donkey anti-goat IgG was from Molecular Probes (Eugene, OR). The enhanced chemiluminescence (ECL) detection kit and horseradish peroxidase-conjugated secondary antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell culture. 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum until confluent (day 2) and maintained for an additional 2 days (until day 0). Differentiation was induced on day 0 by the addition of 0.5 mM methylisobutylxanthine (M), 0.5 μM dexamethasone (D), 10 μg/ml insulin (I), and 10% fetal bovine serum (FBS) in DMEM. On day 3, the MDI medium was replaced with DMEM-10% FBS, which was changed every 2 days thereafter until analysis. Cytoplasmic triglyceride droplets became abundant between days 4 and 5, and by day 7, the cells were fully differentiated (16).

Immunoblot analysis. 3T3-L1 adipocytes, treated as indicated in the figure legends, were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate (Na3VO4) and placed immediately in lysis buffer containing 25 mM HEPES, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 2% glycerol, 5 mM NaF, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaPPI, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin (23). Lysates were gently mixed for 20 min at 4 °C and then centrifuged at 6,000 × g for 20 min at 4 °C to pellet insoluble material. The supernatant was retained for subsequent analysis. Protein concentrations of whole cell lysates were determined using the bicinchoninic acid method (Pierce Chemical). Samples were heated for 5 min at 95 °C, separated by 10% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting as previously described (26). Immunoblots were developed with the ECL kit.

Glucose uptake assay. Glucose uptake assays were performed on fully differentiated 3T3-L1 adipocytes (days 7 and 8). Briefly, preadipocytes were cultured in 6-well plates and induced to differentiate using the MDI protocol we have described. Adipocytes were serum-starved for 4 h and pretreated for 15 min with 0.1% DMSO (vehicle), PD-98059, or U-0126, as indicated in the legends of Figs. 1–8. After pretreatment, adipocytes were washed twice with 37 °C Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing (in mM) 128 NaCl, 4.7 KCl, 1.65 CaCl2, 2.5 MgSO4, and 5 NaHPO4 and then were placed in KRP buffer that contained vehicle PD-98059, or U-0126. Adipocytes were either untreated (basal) or treated with insulin (0.1 nM or 100 nM) for 10 min, followed by the addition of 2-[1-14C]deoxy-o-glucose (2-[14C]DOG; 0.1 μCi/well; NEN) and 5 mM glucose for an additional 10 min at 37 °C. Cells were then washed three times with ice-cold PBS and solubilized in 0.5 M NaOH and 0.1% SDS. Samples were assayed for 2-[14C]DOG uptake as disintegrations per minute per milligram protein.

p38 MAPK activity assay. 3T3-L1 adipocytes were serum-starved for 4 h, pretreated with 0.25% DMSO (vehicle) or 25 μM U-0126 for 15 min, and left untreated or stimulated with 100 nM insulin for 10 min. The activity of p38 MAPK was assayed with an assay kit (Cell Signaling Technology, catalog no. 9820) according to the manufacturer’s instructions. Briefly, active p38 MAPK was immunoprecipitated from cell extracts with an immobilized phospho-p38 MAPK (Thr180/Tyr182) antibody. The immune complex was washed thoroughly and resuspended in a kinase buffer containing ATP and activating transcription factor-2 (ATF-2), alone or in the presence of vehicle or 25 μM U-0126. Phosphorylation of ATF-2 was analyzed by Western blotting with a phosphospecific anti-ATF-2 antibody.

Immunofluorescence. 3T3-L1 preadipocytes were cultured on sterilized glass coverslips and induced to differentiate via the MDI protocol. Fully differentiated adipocytes were serum-starved for 4 h in the presence of 25 μM U-0126 or 0.25% DMSO and treated according to the glucose uptake protocol (omitting 2-[14C]DOG). After treatment, cells were fixed with 2% buffered paraformaldehyde, permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) for 5 min on ice, incubated with goat anti-GLUT4 antibody (Santa Cruz Biotechnology), washed with PBS, and incubated with an anti-goat AlexaFlour 594-labeled antibody (Molecular Probes). Treated coverslips were mounted on glass slides and viewed at ×60 with a Zeiss LSM 110 fluorescent microscope.

RESULTS

Impaired phosphorylation of p44/p42 MAPK by PI3K inhibitors. Recently, we identified the flavonone naringenin as a PI3K inhibitor, as shown by an in vitro kinase assay and impaired phosphorylation of the downstream signaling molecule Akt (18). To determine whether naringenin, like other PI3K inhibitors (39), adversely affects the MAPK pathway, we examined its effect on the phosphorylation (activation) status of p44/p42 MAPK. Although p44/p42 MAPK remained unphosphorylated under basal conditions, both MAPKs exhibited strong phosphorylation in response to insulin (Fig. 1). Naringenin inhibited insulin-stimulated MAPK phosphorylation without affecting the phosphorylation of the upstream kinases, MEK1/2 (Fig. 1). For comparison, we examined the effects of wortmannin, a widely used PI3K inhibitor, on the MAPK pathway. Wortmannin not only blocked the insulin-induced phosphorylation of Akt and PKCα but also reduced the phosphorylation of p44/p42 MAPK and MEK1/2 after insulin stimulation (Fig. 2). Although the PI3K pathway remains an undisputed facilitator of insulin-stimulated glucose uptake, the MAPK pathway does not have a clear role in this process. In agreement with a previous report (39), we have shown that PI3K inhibitors affect not only the PI3K pathway but also the MAPK pathway, which may suggest a role for the MAPK pathway in insulin-stimulated glucose uptake.

Inhibition of glucose uptake by MEK inhibitors. Although Lazar et al. (27) previously used 10 μM PD-98059 to demonstrate that insulin-stimulated glucose uptake in 3T3-L1 adipocytes...
cytes does not require MEK activity, researchers now routinely use 50 μM PD-98059 to achieve a more complete inhibition of MEK activity (8, 30, 42). To determine the effect of 50 μM PD-98059 on insulin-stimulated glucose uptake in 3T3-L1 adipocytes, we pretreated serum-starved adipocytes for 15 min with DMSO (vehicle) or 50 μM PD-98059 and then performed glucose uptake assays using 2-[14C]DOG. In response to insulin, glucose uptake increased 10-fold over basal levels (Fig. 3A). Treatment with PD-98059 inhibited insulin-stimulated glucose uptake by ~33% compared with the vehicle control (Fig. 3A). Although PD-98059 had no effect on the PI3K pathway, as shown by the phosphorylation status of the downstream signaling molecules Akt and PKCζ/λ, it did impair the MAPK pathway by reducing the phosphorylation of p44/p42 MAPK and the upstream kinases MEK1/2 (Fig. 3B). As an inhibitor of MEK1 phosphorylation (1), PD-98059 reduces, but does not completely prevent, the phosphorylation of p44/p42 MAPK. The MEK inhibitor U-0126, on the other hand, blocks the activity of both MEK1 and MEK2 without affecting their phosphorylation status (14). As the more potent MEK inhibitor, U-0126 should be more effective than PD-98059 at reducing insulin-stimulated glucose uptake. To test this hypothesis, we pretreated serum-starved adipocytes for 15 min with vehicle or U-0126 and then performed glucose uptake assays using 2-[14C]DOG. As shown in Fig. 4A, U-0126 inhibited insulin-stimulated glucose uptake in a dose-dependent manner, with 25 μM U-0126 inhibiting glucose uptake by >50% compared with the vehicle control. We observed a similar inhibition of insulin-stimulated 3-O-methylglucose transport in the presence of vehicle or 50 μM PD-98059 (A) or analyzed by SDS-PAGE and then immunoblotted with the indicated antibodies (B). Results are means ± SE of 3 independent experiments.

![Fig. 1. Effect of naringenin on the MAPK pathway. Serum-starved 3T3-L1 adipocytes were pretreated with 0.1% DMSO vehicle (V) or 100 μM naringenin (N) for 4 h and were then left untreated or stimulated with 100 nM insulin for 10 min. Cell lysates were analyzed by SDS-PAGE and then immunoblotted with antibodies against phospho-p44/p42 MAPK, p44/p42 MAPK, phospho-MEK1/2, or MEK1/2. Results are representative of 3 independent experiments.](image1)

![Fig. 2. Effect of wortmannin on insulin-induced signaling events. 3T3-L1 adipocytes were pretreated with 0.1% DMSO vehicle (V) or 100 nM wortmannin (W) for 15 min and were then left untreated or stimulated with 100 nM insulin for 10 min. Cell lysates were analyzed by SDS-PAGE and then immunoblotted with the indicated antibodies. Results are representative of 3 independent experiments.](image2)

![Fig. 3. Effect of PD-98059 on insulin-stimulated glucose uptake. Serum-starved 3T3-L1 adipocytes were pretreated with 0.1% DMSO vehicle or PD-98059 for 15 min. Cells were then left untreated or stimulated with 100 nM insulin for 10 min and assayed for 2-[14C]deoxy-D-glucose (2-[14C]DOG) uptake in the presence of vehicle or 50 μM PD-98059 (A) or analyzed by SDS-PAGE and then immunoblotted with the indicated antibodies (B). Results are means ± SE of 3 independent experiments.](image3)
U-0126 also inhibits other kinases in 3T3-L1 adipocytes. To determine the specificity of U-0126 for MEK1/2 in 3T3-L1 adipocytes, we examined the activity of several other kinases. Davies et al. (9) found that U-0126 reduced the in vitro kinase activity of p38 MAPK by 25%. In contrast, studies in rat cardiomyocytes (45) and in human airway smooth muscle cells (17) have shown that U-0126 selectively inhibits p44/p42 MAPK activity without affecting p38 MAPK activity. To determine whether U-0126 inhibits the activity of p38 MAPK in response to insulin in adipocytes, we conducted an in vitro p38 MAPK assay. Serum-starved adipocytes were pretreated for 15 min with vehicle or 25 µM U-0126 and then stimulated with insulin for 10 min. Active p38 MAPK was immunoprecipitated from cell extracts, and in vitro kinase assays were performed in the presence and absence of vehicle or U-0126 with ATF-2 fusion protein as a substrate. Phosphorylation of ATF-2 increased in response to insulin and remained unaffected by either pretreatment with U-0126 or the addition of U-0126 in vitro (Fig. 5A). Examination of the phosphorylation status of endogenous p38 MAPK and ATF-2 in cell extracts confirmed that U-0126 did not alter the activity of p38 MAPK (Fig. 5B).

Mody et al. (29) have shown that U-0126 inhibits the EGF-induced activation of ERK5 in HeLa cells. To determine whether U-0126 affects the insulin-stimulated activation of ERK5 in 3T3-L1 adipocytes, we analyzed whole cell lysates for phosphorylation of ERK5 under the basal condition and after stimulation with insulin in the presence and absence of U-0126. We observed a very low basal level of ERK5 phosphorylation that increased dramatically after insulin stimulation (Fig. 5C). Importantly, U-0126 had no effect on the insulin-stimulated phosphorylation of ERK5 (Fig. 5C).

U-0126 may also affect the activity of a third enzyme, Akt (PKB). Davies et al. (9) found that U-0126 reduced the in vitro kinase activity of Akt by ~20%. Although we showed that U-0126 had no effect on the ability of PI3K to phosphorylate Akt (Fig. 4B), the possibility exists that U-0126 may prevent the kinase activity of Akt, regardless of its phosphorylation status. Therefore, we examined the phosphorylation of glycogen synthase kinase-3β (GSK-3β), a substrate of Akt. We detected a very low basal level of GSK-3β phosphorylation that increased dramatically in response to insulin; however,

**Fig. 4.** Effect of U-0126 on insulin-stimulated glucose uptake. Serum-starved 3T3-L1 adipocytes were pretreated with 0.1% DMSO vehicle or 5, 10, or 25 µM U-0126 for 15 min. Cells were then left untreated or stimulated with 100 nM insulin for 10 min and assayed for 2-14CDOG uptake in the presence of vehicle or 5, 10, or 25 µM U-0126 (A) or analyzed by SDS-PAGE and then immunoblotted with the indicated antibodies (B). Results are means ± SE of 3 independent experiments.

**Fig. 5.** Effect of U-0126 on activity of p38 MAPK, MEK5, and Akt. Serum-starved 3T3-L1 adipocytes were pretreated with 0.25% DMSO vehicle or 25 µM U-0126 for 15 min. Cells were then left untreated or stimulated with 100 nM insulin for 10 min. A: lysates were assayed for p38 MAPK activity by immunoprecipitating the active kinase and analyzing phosphorylation of the substrate activating transcription factor (ATF)-2 alone or in the presence of vehicle or U-0126. B: activity of p38 MAPK in cell extracts was evaluated by Western blot analysis of phospho-p38 and phospho-ERK5. C: activities of MEK5 and Akt were evaluated by Western blot analysis of phospho-MEK1/2 and phospho-GSK-3β, respectively.
U-0126 had no effect on the phosphorylation of GSK-3β in response to insulin (Fig. 5C). Thus, although U-0126 has exhibited nonspecific inhibition of both Akt and p38 MAPK in vitro kinase assays and has inhibited EGF-induced ERK5 phosphorylation in HeLa cells, we have shown that, in adipocytes stimulated with insulin, U-0126 functions as a selective inhibitor of MEK1/2 that has no effect on the activation or activity of these other kinases.

No involvement of MEK in GLUT4 translocation, docking, or fusion with the plasma membrane. To determine how MEK facilitates insulin-stimulated glucose uptake, we examined the effect of U-0126 on the translocation of insulin-responsive GLUT4 vesicles to the plasma membrane. 3T3-L1 adipocytes were untreated (basal), treated with insulin, or treated with insulin plus U-0126. After treatment, cells were fixed and incubated with a GLUT4 antibody. Immunofluorescence microscopy revealed that GLUT4 vesicles were localized primarily to the perinuclear region under the basal condition (Fig. 6A). Insulin stimulated a marked increase in GLUT4 translocation to the plasma membrane, as exhibited by the increase in fluorescent staining at the cell membrane (Fig. 6B). The presence of U-0126 had no effect on insulin-stimulated GLUT4 translocation (Fig. 6, compare B and C).

After translocating to the plasma membrane, GLUT4 vesicles must then dock and fuse with the plasma membrane. To determine whether MEK participates in GLUT4 docking and fusion, we assessed the effect of U-0126 on the functional insertion of GLUT4 into the plasma membrane in 3T3-L1 adipocytes stably expressing a c-myc epitope in an exofacial domain of GLUT4 (3T3-L1-GLUT4-myc); these cells exhibit a robust increase in insulin-stimulated glucose uptake (22). 3T3-L1-GLUT4-myc adipocytes serum-starved in the presence of vehicle or U-0126 were stimulated with 100 nM insulin for 30 min and then analyzed for the presence of myc-tagged GLUT4 at the cell surface by use of a colorimetric assay that provides a quantitative measure of GLUT4 translocation and evidence of functional insertion into the plasma membrane (38, 44). Figure 7 shows that insulin stimulated a 2.3-fold increase in the level of myc-tagged GLUT4 on the cell surface compared with the basal level, which was normalized to 1. Inhibition of MEK with U-0126 did not affect the proper insertion of GLUT4-myc into the plasma membrane. Collectively, our results indicate that MEK does not mediate GLUT4 translocation, docking, or fusion with the plasma membrane.

Direct effect of MEK on GLUT4-mediated glucose transport. In the basal state, GLUT1 is present both at the cell surface and in intracellular sites, but GLUT4 remains sequestered almost entirely within the cell (see review in Ref. 36). Although insulin stimulates a dramatic increase in glucose uptake primarily via the GLUT4 transporter, GLUT1 also responds to insulin with a two- to threefold increase in translocation to the plasma membrane (4). To address the possibility that MEK inhibitors influence the glucose uptake activity of GLUT1 as well as GLUT4, we examined the effect of 50 μM PD-98059 or 25 μM U-0126 on glucose uptake in the absence of insulin and found no difference compared with the vehicle-treated control (Fig. 8A). Correspondingly, we found no difference in basal phosphorylation of p44/p42 MAPK or Akt in the presence of MEK inhibitors (data not shown). To assess the relative contributions of GLUT1 and GLUT4 toward insulin-stimulated glucose uptake, we stimulated the cells with 0.1 nM insulin, which recruits only GLUT4, not GLUT1, to the plasma membrane (33). In the presence of 0.1 nM insulin, 50 μM PD-98059 and 25 μM U-0126 inhibited GLUT4-mediated glucose uptake by ~40 and 55%, respectively, compared with that in vehicle-treated control cells (Fig. 8A).
To determine whether the MEK inhibitors affect GLUT4 directly, we omitted the pretreatment step and added the inhibitors only during the glucose uptake assay. Serum-starved adipocytes were stimulated with insulin for 10 min. Inhibitors were added immediately before 2-[14C]DOG, and glucose uptake was measured 10 min later. Both MEK inhibitors exerted a marked effect on glucose transport, with each inhibiting glucose uptake by ~40% (Fig. 8B). Importantly, both MEK inhibitors blocked insulin-stimulated phosphorylation of p44/p42 MAPK under the glucose uptake assay conditions (Fig. 8C). The finding that both MEK inhibitors acted rapidly and required no pretreatment step suggested that an equally rapid restoration of MEK activity may occur after the removal of the inhibitors. A previous study has shown that 100 nM insulin had no effect on insulin-stimulated glucose uptake (27). When we pretreated serum-starved adipocytes with 10 or 50 μM PD-98059 and compared insulin-stimulated glucose uptake in the presence and absence of the inhibitor, we found that the absence of PD-98059 during the assay restored glucose uptake to control values (Fig. 8D). Correspondingly, the removal of PD-98059 from insulin-stimulated cells restored MEK activity, as shown by the phosphorylation status of p44/p42 MAPK (Fig. 8E). Taken together, our results suggest that for maximal glucose transport, GLUT4 requires sustained activation of MEK.

**DISCUSSION**

Recent studies have identified several key proteins that participate in insulin-dependent but PI3K-independent GLUT4 translocation, including the GTP-binding protein TC10 (7) and Exo70, a component of the exocyst complex that appears to target GLUT4 vesicles to sites of fusion at the plasma membrane (21). The mechanisms involved in the PI3K-dependent pathways, however, remain unclear: no targets have been identified for PKCζ/λ or PLCγ in insulin-stimulated glucose transport. Akt phosphorylates a Rab-GTPase-activating protein designated AS160 (35), but its downstream targets have yet to be identified. The MAPK pathway, although strongly activated by insulin, does not have an established role in glucose trans-
port. In 1995, Lazar et al. (27) used the MEK1 inhibitor PD-98059 to demonstrate the lack of involvement of the MAPK pathway in insulin-stimulated glucose uptake. Although 10 μM PD-98059 prevented the insulin-stimulated increase in MAPK activity in an in vitro kinase assay, it had no significant inhibitory effect on insulin-stimulated glucose uptake in living cells. Interestingly, in another report, the same investigators claimed that PD-98059 has an IC₅₀ of 10 μM (12). The use of 10 μM PD-98059 may therefore allow 50% of MAPK activity to occur in vivo. The subsequent availability of phosphospecific antibodies to p44/p42 MAPK has revealed that much higher concentrations of PD-98059 are needed to inhibit p44/p42 MAPK phosphorylation. Consequently, many researchers now routinely use 50 μM PD-98059 for inhibition of MEK1 activity (8, 30, 42).

Our study is the first to show that MEK participates in insulin-stimulated glucose transport, as determined by both 2-deoxy-D-glucose and 3-O-methylglucose uptake assays. We found significant reduction in insulin-stimulated glucose uptake with two different MEK inhibitors, PD-98059 and U-0126. PD-98059, less active against MEK2 than against MEK1, binds to inactive MEK and prevents its phosphorylation by the upstream kinase Raf (1). Although two previous studies (27, 40) found that pretreatment with PD-98059 did not inhibit insulin-stimulated glucose uptake, neither included PD-98059 at the time of the actual uptake assay. We found that PD-98059 must be present during the assay; pretreatment alone had no effect on insulin-stimulated glucose uptake or MEK activity.

Because our studies showed that PD-98059 failed to prevent p44/p42 MAPK phosphorylation even at 50 and 100 μM doses, we used U-0126, a potent inhibitor of both MEK1 and MEK2. We found that U-0126 inhibited insulin-stimulated p44/p42 MAPK phosphorylation and glucose uptake in a dose-dependent manner. Widely regarded as a specific inhibitor of MEK activity, U-0126 does not impair the activities of protein kinase C, Abl, Raf, MEKK, ERK, JNK, MKK-3, MKK-4/SEK, MKK-6, Cdk2, or Cdk4 (14). As expected, we showed that neither PD-98059 nor U-0126 affected the activation of PKCζ/α or Akt. Although Davies et al. (9) found that U-0126 inhibited p38 MAPK activity in vitro, other studies have determined that U-0126 selectively inhibits p44/p42 MAPK activity in rat cardiomyocytes (45) and in human airway smooth muscle cells (17) without affecting p38 MAPK activity. We confirmed that U-0126 had no effect on p38 MAPK activity in adipocytes and also demonstrated that it had no effect on the activities of MEK5 and Akt, as shown by the phosphorylation status of their downstream targets, ERK5 and GSK-3β, respectively. Even though 25 μM U-0126 had no effect on the major kinases outlined above, the possibility exists that at higher concentrations it nonspecifically alters the activities of other enzymes; increasing the concentration of U-0126 to 50 μM continued to reduce insulin-stimulated glucose uptake even after complete inhibition of p44/p42 MAPK phosphorylation (data not shown).

Although inhibition of PI3K-dependent and Cbl-dependent pathways prevents the insulin-induced exocytosis of GLUT4, inhibitors of p38 MAPK activity have no effect on GLUT4 translocation, docking, or fusion with the plasma membrane but nonetheless reduce insulin-stimulated glucose uptake by ~50% (38, 41). Studies with L6 myotubes stably expressing GLUT4 with an exofacial Myc epitope show that GLUT4 fuses with the plasma membrane in the presence of p38 MAPK inhibitors and that these inhibitors have no direct effect on GLUT4 itself (41). In addition, Somwar et al. (37) observed a delay between GLUT4 exocytosis and insulin-stimulated glucose uptake in L6-GLUT4-myc myotubes. This time delay suggests that activation of GLUT4 precedes glucose transport. Consequently, Somwar et al. (38) have proposed that the p38 MAPK pathway participates in the insulin-induced activation of GLUT4. In accord with these studies, we have found that inhibition of the classical p44/p42 MAPK pathway also inhibits insulin-stimulated glucose uptake without affecting GLUT4 exocytosis. We found that insulin stimulated a 2.3-fold increase in myc-tagged GLUT4 at the cell surface and a 10-fold increase in glucose uptake; these results are nearly identical to those published by Kanai et al. (22) and by Calderhead and Lienhard (5), who noted the discrepancy between the translocation of labeled glucose transporters and the rate of 2-deoxyglucose uptake. Our studies support the hypothesis that insulin not only stimulates GLUT4 translocation but also increases the intrinsic activity of the transporter (5). We propose that two pathways facilitate insulin-induced GLUT4 activation: the p38 MAPK pathway and the p44/p42 MAPK pathway. In vitro kinase activity assays have demonstrated that insulin activates both pathways in adipocytes (34, 41). Importantly, insulin stimulates an 18-fold increase in p44/p42 MAPK activation as well as a 2- to 3-fold increase in the activation of p38 MAPK (25).

Unlike p38 MAPK inhibitors, which have no direct effect on the glucose transport process itself (41), MEK inhibitors impair glucose uptake when present only during the transport assay. This finding suggests that the p38 MAPK pathway is not involved in insulin-stimulated glucose uptake by mechanisms other than inhibition of the MAPK pathway, if we assume that MEK inhibitors have no effect after the initiation of the cascade. We found, however, that the addition of each inhibitor to an active insulin-signaling cascade effectively prevented the phosphorylation of p44/p42 MAPK. Taken together, these findings suggest that full activity of the GLUT4 transporter also requires the activity of the MAPK cascade.

A number of researchers have indirectly examined the role of the MAPK pathway in insulin-stimulated GLUT4 translocation by introducing mutated Ras into 3T3-L1 cells. Ras is a small GTP-binding protein that mediates insulin-induced activation of the p44/p42 MAPK cascade (10). Several studies have shown that dominant-negative N17-ras has no effect on GLUT4 translocation (19, 20, 31) and are thus consistent with our findings. However, other studies (11, 15, 24) demonstrate that N17-ras has no effect on insulin-stimulated glucose uptake. These studies predate the availability of phosphospecific antibodies to p44/p42 MAPK and therefore do not exclude the possibility that insulin stimulated a sufficient degree of MEK activity in these cells by an activator other than Ras. We have shown that the PI3K inhibitors naringenin (Fig. 1) and wortmannin (Ref. 39 and Fig. 2) inhibit the insulin-stimulated phosphorylation of p44/p42 MAPK and that MEK inhibitors have no effect on the PI3K pathway (Figs. 3 and 4). Cross talk between the PI3K pathway and the MAPK pathway may therefore allow insulin to activate MEK in N17-Ras cells.

In summary, we have shown that inhibition of MEK activity reduced insulin-stimulated glucose uptake without affecting...
the activities of PI3K or p38 MAPK, other kinases known to participate in this process. Inhibition of MEK had no effect on the translocation of GLUT4 or on its functional insertion into the plasma membrane. Collectively, our studies suggest that MEK promotes the activation of GLUT4.

GRANTS

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