A Dominant-negative p38 MAPK Mutant and Novel Selective Inhibitors of p38 MAPK Reduce Insulin-stimulated Glucose Uptake in 3T3-L1 Adipocytes without Affecting GLUT4 Translocation*

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Participation of p38 mitogen-activated protein kinase (p38) in insulin-induced glucose uptake was suggested using pyridinylimidazole p38 inhibitors (e.g. SB203580). However, the role of p38 in insulin action remains controversial. We further test p38 participation in glucose uptake using a dominant-negative p38 mutant and two novel pharmacological p38 inhibitors related to but different from SB203580. We present the structures and activities of the azaazulene pharmacophores A291077 and A304000. p38 kinase activity was inhibited in vitro by A291077 and A304000 (IC50 = 0.6 and 4.7 μM). At higher concentrations A291077 but not A304000 inhibited JNK2α (IC50 = 3.5 μM). Pretreatment of 3T3-L1 adipocytes and L6 myotubes expressing GLUT4myc (L6-GLUT4myc myotubes) with A291077, A304000, SB202190, or SB203580 reduced insulin-stimulated glucose uptake by 50–60%, whereas chemical analogues inert toward p38 were ineffective. Expression of an inducible, dominant-negative p38 mutant in 3T3-L1 adipocytes reduced insulin-stimulated glucose uptake. GLUT4 translocation to the cell surface, immunodetected on plasma membrane lawns of 3T3-L1 adipocytes or on intact L6-GLUT4myc myotubes, was not altered by chemical or molecular inhibition of p38. We propose that p38 contributes to enhancing GLUT4 activity, thereby increasing glucose uptake. In addition, the azaazulene class of inhibitors described will be useful to decipher cellular actions of p38 and JNK.

The p38 mitogen-activated protein kinases (p38), also referred to as stress-activated protein kinases-2, are a family of proline-directed serine/threonine kinases (1, 2). At least four isoforms, the products of different genes, have been cloned and are 60–70% identical in their amino acid sequence. The most commonly used nomenclature of these isoforms are p38α (3, 4), p38β (5, 6), p38γ (7, 8), and p38δ (9, 10). A splice variant of the p38β, referred to as p38β2, has also been described (11). Northern blot analysis has shown a wide tissue distribution of these isoforms, although p38β and p38γ are preferentially expressed in skeletal muscle (5, 9). In addition to stressors, members of this family of protein kinases can also be activated by growth factors (12–15).

Full activation of p38 by pro-inflammatory cytokines requires phosphorylation of Thr-180 and Tyr-182 found within a TGY tripeptide motif in the activation loop of the kinase (16). This double phosphorylation is catalyzed by the dual-specific MAPK kinases MKK3 and MKK6 and possibly via auto-phosphorylation (17). It is remarkable that stimuli that increase p38 phosphorylation such as insulin-like growth factor-1 (18), muscle contraction (19–21), lipoic acid (22), 5-aminooimidazole-4-carboxamide ribonucleoside (23), pro-inflammatory cytokines (18), protein synthesis inhibitors (24, 25), hyperosmolar stress (26), and preconditioning (ischemia/reperfusion) (27) also elevate glucose uptake. Importantly, the pyridinylimidazole inhibitor of p38, SB203580, reduced the stimulation of glucose uptake by all of the above stimuli including insulin in skeletal muscle and/or various cell lines, suggesting that p38 may be a component in the signaling pathway leading to the stimulation of glucose uptake (18, 19, 22, 23, 25, 28).

Insulin stimulates glucose uptake in mature skeletal muscle, adipose tissue, and insulin-responsive cells in culture by recruiting glucose transporter 4 (GLUT4) to the plasma membrane. Interestingly, although SB203580 reduced insulin-mediated glucose in 3T3-L1 adipocytes and L6 muscle cells, it did not diminish GLUT4 translocation to the plasma membrane (13, 14). This reduction in glucose uptake by SB203580 was because of a decrease in the transport Vmax with no effect on the apparent Km for glucose (14). Based on these results, we proposed that insulin activates GLUT4 via a p38-dependent signaling pathway. In contrast to these reports, a recent study (29) reported that SB203580, but not adenoviral driven expression of a dominant-negative p38, decreased insulin-induced glucose uptake in 3T3-L1 adipocytes. These conflicting results

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The abbreviations used are: MAPK, mitogen-activated protein kinase; ATF2, activating transcription factor-2; CREB, cAMP-response element binder; IPTG, isopropyl-thio-beta-D-galactoside; GLUT, glucose transporter; MKK, MAPK kinase; DTT, dithiothreitol; PBS, phosphate-buffered saline.
promoted us to re-examine the role of p38 in the stimulation of glucose uptake by insulin. To this end, a dominant-negative p38 mutant under the influence of an inducible promoter was expressed in 3T3-L1 adipocytes. In addition, two novel inhibitors of p38 that are structurally different from the pyridine imidazoles were identified and used to determine their effect on the stimulation of glucose uptake in L6 cells expressing Myc-tagged GLUT4 and 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Activating transcription factor-2 (ATF2) fusion protein, phospho-specific antibodies to ATF2 (Thr-71) and p38 (Thr-180 and Tyr-182), and anti-p38 antibody were purchased from New England Biolabs (Beverly, MA). Anti-c-Myc (9E10) antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GLUT1 and anti-GLUT4 C-terminal antibodies were from BioGenes (Senden, NH), Mono- clonal anti-FLAG antibody (M2), cytochalasin B, and forskolin were from Sigma. Partially purified rabbit MKK6 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Isopropyl-thio-β-D-galactoside (IPTG) was obtained from Fisher. SB202190, SB203580, and phospho-specific CREB antibody (S133) were purchased from Calbiochem. Goat anti-rabbit IgG conjugated to horseradish peroxidase was pur- chased from Promega. 3T3L1 cells were obtained from the American Type Culture Collection (Rockville, MD). 3T3-L1 adipocytes were purchased from Clontech. 3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD).

Generation of a 3T3-L1 Cell Line Stably Expressing an Inducible Dominant-negative p38 MAPK Mutant (p38AGF-3T3-L1 Cells)—Cells were generated and described previously (31). Briefly, full-length mouse p38α was obtained by PCR using a 3T3-L1 fibroblast library as a template (32). The product was cloned into pCG7 vector as a HindIII fragment. This construct served as the template to replace threonine 180 and tyrosine 182 with alanine and phenylalanine, respectively. The mutant obtained, p38AGF, was also used in previous studies (33–35). A C-terminal FLAG tag was introduced to distinguish between endoge nous and transfected p38. The resulting fragment was cloned into vector pOP13 (Stratagene) as a Not1 fragment. The p38 insert was sequenced on both strands to confirm the mutation. This system allows transfection and transfected p38. The resulting fragment was cloned into vector pOP13 (Stratagene) as a Not1 fragment. The p38 insert was sequenced on both strands to confirm the mutation. This system allows transfection and transfection of fibroblasts into adipocytes was initiated 2 days following transfection.

Results were normalized to that of control in each experiment.

Detection of CREB and p38 Phosphorylation—Lysates were prepared as described (14) from 3T3-L1 adipocytes. Following appropriate incubations, cells were lysed on ice with 2× Laemmlapipe sample buffer supplemented with 7.5% β-mercaptoethanol (v/v), phosphatase inhibitors (1 mM Na3VO4 and 100 μM okadaic acid), and protease inhibitors (1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM E-64, 1 μM aprotinin, 1 mM leupeptin, and 0.5 mM pepstatin A, and 1 μM DTT) supplemented with 15% glycerol and 10 mM HEPES (pH 7.5) supplemented with 1 mM DTT, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin, and 10 μM micropressin was added, and the solution was aspirated up and down using a 1-ml pipette to promote cell breakage. The coverslips were washed twice with breaking buffer and incubated with cold 3% paraformaldehyde (v/v) in PBS for 10 min on ice, followed by three washes in PBS. Excess fixative was quenched with 50 mM NH4Cl in PBS for 5 min, followed by three washes with PBS at room temperature. The plasma membrane layers were subsequently blocked by a 1-h incubation in 5% goat serum (v/v) in PBS at room temperature and then incubated for 60 min with anti-GLUT1 or anti-GLUT4 C-terminal an- tibodies (1:250 dilution each) at room temperature and washed three times in PBS. Fluorescein isothiocyanate- or Cy3-conjugated species-specific secondary antibodies (1:250) were added for 45 min and then washed four times with PBS. Coverslips were placed on microscope slides with mounting solution (Dako). Images of plasma membrane lawns ("sheets") were obtained using a Leica DMIRB inverted fluores- cence microscope with a 40× objective. All images were collected under identical gain settings using Winview software and processed with Adobe Photoshop software for figure production. NIH Image software was used to quantify fluorescence intensity. For quantification, the fluorescence/unit area of each lawn was measured in several fields containing multiple lawns. Results were normalized to that of control in each experiment.

Detection of Cell Surface GLUT4myc—GLUT4myc levels at the cell surface were measured by an antibody-coupled optical assay as described previously (38). Following treatments, myotubes were washed once with PBS, fixed for 5 min with 3% paraformaldehyde (v/v) at room temperature, and then neutralized with 1% glycinate (v/v) in PBS at 4 °C for 10 min. Monolayers were blocked with 10% (v/v) goat serum and 3% bovine serum albumin (w/v) in PBS at 4 °C for at least 30 min and then incubated with anti-c-Myc antibody (9E10, 1:100) at 4 °C for 60 min. Myotubes were then incubated with peroxidase-conjugated donkey anti-mouse IgG (1:100) at 4 °C for 30 min. Following removal of the antibody, cells were incubated for 20 min at room temperature with 1 mL of OPD reagent (0.4 mg/mL O-phenylenediamine dihydrochloride and 0.4 mg/mL urea hydroperoxide in 0.05× phosphate-citrate buffer) per well. The reaction was stopped by adding 0.25 mL of 3 M HCl. Optical absorbance of the supernatant was measured at 492 nm.

Detection of CREB and p38 Phosphorylation—Lysates were prepared as described (14) from 3T3-L1 adipocytes. Following appropriate incubations, cells were lysed on ice with 2× Laemmmlapipe sample buffer supplemented with 7.5% β-mercaptoethanol (v/v), phosphatase inhibitors (1 mM Na3VO4 and 100 μM okadaic acid), and protease inhibitors (1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM E-64, 1 μM aprotinin, 1 mM leupeptin, and 0.5 mM pepstatin A, and 1 μM DTT) supplemented with 15% glycerol and 10 mM HEPES (pH 7.5) supplemented with 1 mM DTT, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin, and 10 μM micropressin was added, and
The inhibitory activity and potency of the compounds were evaluated using an in vitro kinase assay. Inactive baculovirus-expressed human p38 was activated by partially purified rabbit MKK6 enzyme and then preincubated in vitro with increasing concentrations of A291077, A304000, A305267, or SB203580. Kinase activity toward ATF2 was then determined. The results are shown in Fig. 1. A291077 and A304000 reduced p38 activity in vitro with IC50 values of 0.6 and 4.7 μM, respectively. The structural analogue A305267, however, had no inhibitory action toward p38. SB203580 inhibited p38 in vitro with IC50 of 0.3 μM. We also analyzed the effects of these compounds on the activity of the closely related MAPK, JNK2α (Fig. 1). Compared with inhibition of p38, A291077 showed a 6-fold lower potency for JNK2α, inhibiting the enzyme with IC50 of 3.5 μM in vitro. Similarly, SB203580 showed a 5-fold lower potency for JNK2α, inhibiting this kinase in vitro with IC50 of 1.4 μM. In contrast, A304000 reduced JNK2α activity only at much higher concentrations than needed to inhibit p38 (only 37% inhibition at 10 μM). A305267 (up to 100 μM) did not affect JNK2α activity. Several other protein kinases including protein kinase B/Akt, casein kinase-2, and MAPK-activated protein kinase-2 (MAPKAPK2) were relatively insensitive to inhibition by A291077 and A304000 (Table I). These results suggest that A291077 and A304000 are potent inhibitors of p38.

A291077 and A304000 Prevent Activation of the p38 Pathway by Insulin in Intact Cells—The results in Table I showed that A291077 and A304000 inhibit p38 in vitro. To determine whether these agents also inhibit p38 signaling in intact cells, we monitored their effects on phosphorylation of the transcription factor CAMP-response element binder (CREB), a downstream effector of p38. Phosphorylation of CREB induced by insulin, fibroblast growth factor, or ultraviolet radiation is abrogated by pretreating cells with 10 μM SB203580 (14, 45, 46). Ultraviolet radiation-induced CREB phosphorylation is inhibited by SB203580 in cells expressing wild type p38 but not in cells expressing an SB203580-resistant p38 mutant, confirming that CREB phosphorylation is p38-dependent (46). L6-GLUT4 myotubes were treated with 20 μM A291077 or A304000 for 20 min and then stimulated with insulin for 10 min in the absence or presence of insulin, in the continued presence of the azaazulene compounds. Cell extracts were resolved by SDS-PAGE and then immunoblotted for phosphorylated CREB using an antibody that recognizes CREB only when phosphorylated on S133. The results are shown in Fig. 2. Insulin treatment increased CREB phosphorylation by 2.8 ± 0.2-fold above basal values. Pretreatment of myotubes with either A291077 or A304000 completely inhibited insulin-induced CREB phosphorylation (A291077 + insulin, 0.8 ± 0.3-fold; A304000 + insulin, 0.9 ± 0.2-fold above basal values). A
similar inhibition of insulin-dependent CREB phosphorylation was previously obtained with SB202190 and SB203580 (14). These results confirm that A291077 and A304000 inhibit p38 in intact cells. Insulin-induced CREB phosphorylation was also completely inhibited by SB203580 and SB202190 in 3T3-L1 adipocytes (data not shown).

A291077 and A304000 Reduce Insulin-mediated Glucose Uptake but Not GLUT4 Translocation in L6-GLUT4myc Myotubes—A291077 and A304000 were used to analyze further the role of p38 in the stimulation of glucose uptake by insulin in L6-GLUT4myc muscle cells. These cells express GLUT4 with an exofacial Myc epitope tag. GLUT4 molecules that are fully immunologically reacting the cell surface with anti-Myc antibody, followed by a secondary antibody-linked enzymatic assay (38). Moreover, in these cells GLUT4myc determines both the basal and insulin-dependent glucose uptake with virtually no contribution of GLUT1 to glucose flux (47). Myotubes were pretreated for 20 min with the indicated concentrations of A291077 or A304000 and stimulated with insulin for another 20 min in the presence of these agents. Unstimulated (basal) cells were incubated for 40 min with the same compounds. In all cases, the medium was changed rapidly, and 2-deoxyglucose uptake and GLUT4myc translocation were assessed in the absence of any drug (Fig. 3). A statistically significant reduction of insulin-mediated 2-deoxyglucose uptake was obtained with 20 μM A291077 (Fig. 3C, insulin, 2.2 ± 0.9; A291077 + insulin, 16.6 ± 1.2, values in pmol/min/mg protein, p < 0.001). Similarly, insulin-mediated 2-deoxyglucose uptake was diminished significantly by 10 or 20 μM A304000 (Fig. 3B and D, insulin, 23.5 ± 0.8; 10 μM A304000 + insulin, 17.0 ± 1.9, p < 0.01; 20 μM A304000 + insulin, 14.2 ± 1.1, p < 0.001, values in pmol/min/mg protein). Basal 2-deoxyglucose uptake was not altered significantly by A291077. Although this parameter was slightly reduced by A304000, the change was not statistically significant (p > 0.05). A291077 and A304000 diminished the net insulin response of glucose uptake (i.e. the insulin-dependent component) by over 50%. Importantly, A305267, the inactive analogue that does not inhibit p38 MAPK activity, had only a minimal effect on insulin stimulation of glucose uptake when tested through the same protocol. Insulin stimulation of glucose uptake was 2.3 ± 0.2-fold in untreated L6 myotubes, n = 7, compared with 1.9, 1.9, and 2.0-fold in myotubes pretreated with 10, 20, or 30 μM A305267, respectively (results of two experiments each performed in triplicate).

We then examined the effect of the p38 MAPK inhibitory azauzulene on GLUT4 translocation to the plasma membrane. Insulin treatment increased the amount of GLUT4myc at the surface of intact cells by 2.6 ± 0.2-fold above basal levels. Pretreatment of cells with 20 μM A291077 or A304000 did not alter basal surface GLUT4 levels or insulin-induced GLUT4 translocation (values relative to control, unstimulated cells: A291077, 1.3 ± 0.2-fold; A291077 + insulin, 3.1 ± 0.3-fold; A304000, 1.2 ± 0.2-fold; A304000 + insulin: 2.8 ± 0.3-fold, Fig. 3B and D). These results contrast with the significant reduction in insulin-stimulated glucose uptake and demonstrate that glucose uptake, but not GLUT4 translocation, is reduced by A291077 and A304000.

In all the experiments described above, 2-deoxyglucose uptake was assayed in the absence of inhibitors. Nonetheless, to determine whether A291077 or A304000 may lower insulin-dependent glucose uptake by inhibiting GLUT4 directly, 2-deoxyglucose uptake was determined in the presence of A291077 or A304000, in basal or insulin-stimulated myotubes that were not pretreated with any inhibitors. Unlike other known inhibitors of GLUT4 such as cytochalasin B, forskolin, and indinavir (48, 49), the presence of 20 μM A291077 or A304000 exclusively in the transport assay did not alter glucose uptake measured in basal or insulin-treated cells (Table II). These results suggest that A291077 and A304000 reduced insulin-stimulated glucose uptake by preventing a permissive signal, rather than through a direct effect on GLUT4.
Inhibition of Insulin-induced Glucose Uptake by A291077, A304000, SB202190, and SB203580 in 3T3-L1 Adipocytes—
3T3-L1 adipocytes are widely used to study insulin action. Here we compared the effects of A291077 and A304000 on insulin-induced glucose uptake in 3T3-L1 adipocytes with those of SB202190 and SB203580. Adipocytes were treated for 40 min with MeSO4 alone or with increasing concentrations of each drug. Insulin (100 nM) was added during the last 20 min of this incubation. 2-Deoxyglucose uptake was then measured in the absence of inhibitors (Fig. 4). A291077 reduced insulin-stimulated glucose uptake in a dose-dependent manner (Fig. 4A). A statistically significant reduction in insulin action was obtained with 5 μM A291077 (values in pmol/min/mg protein, basal, 4.5 ± 0.7; A291077, 5.5 ± 1.2; insulin, 32.7 ± 1.3; A291077 + insulin, 22.6 ± 1.0; p < 0.05 for insulin versus A291077 + insulin). The maximum inhibition of insulin-stimulated glucose uptake obtained was ~60%, at a concentration of 20 μM (A291077, 4.1 ± 0.9; A291077 + insulin, 14.6 ± 2.5 pmol/min/mg protein, p < 0.001). No further reduction was obtained with 30 μM A291077 (p > 0.05). Basal glucose uptake was not affected significantly by A291077 at any of the concentrations used (Fig. 4A, p > 0.05). Similar to results obtained with A291077, A304000 led to a dose-dependent reduction in insulin-stimulated glucose uptake (Fig. 4B). A statistically significant reduction in insulin-stimulated glucose uptake was obtained with 10 μM A304000. The maximum reduction in insulin action was ~55%. A304000 did not cause any statistically significant decrease in basal glucose uptake at any of the concentrations used (Fig. 4B, p > 0.05). Importantly, A305267, the analogue of A304000 and A291077 that is inert toward p38 MAPK, was without effect on insulin-stimulated glucose uptake when given through the same protocol as the enzymatic inhibitors (values in pmol/min/mg protein, basal, 3.2 ± 0.4; 30 μM A305267, 3.7 ± 0.6; insulin, 19.5 ± 3.6; 30 μM A305267 + insulin, 20.3 ± 1.1). Moreover, addition of the p38-inhibitory A291077 exclusively to the transport solution for up to 20 min caused only a minimal reduction in glucose uptake into 3T3-L1 adipocytes in two experiments (values in pmol/min/mg protein, basal, 9.3; basal + 20 μM A291077, 8.21). Under these conditions, the bona fide inhibitor of glucose transporters, cytochalasin B, practically obliterated glucose uptake (results not shown).

Pretreatment of 3T3-L1 adipocytes with SB202190 (Fig. 4C) or SB203580 (Fig. 4D) also caused a dose-dependent decrease in insulin-induced 2-deoxyglucose uptake. A statistically significant reduction in insulin-stimulated glucose uptake was obtained with 1 μM SB202190 or SB203580 (values in pmol/min/mg protein, SB202190, 4.6 ± 0.3; SB202190 + insulin, 33.8 ± 2.3; SB203580, 4.7 ± 0.3; SB203580 + insulin, 34.4 ± 0.7). The highest concentration of SB202190 or SB203580 used here (10 μM) lowered the stimulation of glucose uptake by 59 and 53%, respectively (values in pmol/min/mg protein, SB202190, 4.5 ± 0.3; SB202190 + insulin, 19.6 ± 1.0; SB203580, 4.3 ± 0.2; SB203580 + insulin, 21.4 ± 1.6). Basal glucose uptake was not significantly altered by either SB202190 (Fig. 4C, p > 0.05) or SB203580 (Fig. 4D, p > 0.05). At much higher concentrations of SB203580 (50–100 μM) a further drop in insulin-mediated glucose uptake was observed previously (13). This inhibition appeared to arise from a secondary action of the drug based on the bimodal shape of the dose-response curve (13).

p38 Inhibitors Do Not Reduce Insulin-mediated GLUT4 Translocation in 3T3-L1 Adipocytes—The above results illustrate that inhibition of p38 using A291077, A304000, SB202190, or SB203580 correlates with a reduction in insulin-mediated glucose uptake in 3T3-L1 adipocytes. We have shown previously that SB203580 did not reduce insulin-stimulated

| Condition | Insulin-stimulated 2-deoxyglucose uptake |
|-----------|------------------------------------------|
| 1. Control | 100                                      |
| 2. A291077 pretreatment | 50 ± 6                         |
| 3. A304000 pretreatment | 54 ± 4                         |
| 4. A291077 in transport assay only | 100 ± 8                    |
| 5. A304000 in transport assay only | 98 ± 7                    |
| 6. Cytochalasin B in transport assay only | 20 ± 3                    |
| 7. Forskolin in transport assay only | 28 ± 4                    |
| 8. Indinavir in transport assay only | 28 ± 11                   |
GLUT4 translocation in 3T3-L1 adipocytes (13). We next assessed the effect of A291077, A304000, SB202190, or SB203580 on insulin-mediated GLUT4 translocation using plasma membrane lawns. This approach has been widely used to score and quantitate GLUT4 translocation in 3T3-L1 adipocytes (42, 50, 51). Plasma membrane lawns were prepared from 3T3-L1 adipocytes that were treated for 20 min with Me$_2$SO only or with 10 μM SB202190 or SB203580, or 30 μM A291077 or A304000, prior to insulin treatment for an additional 20 min. GLUT4 presence on the lawns was detected by indirect immunofluorescence using an anti-GLUT4 C-terminal antibody. Representative experiments are shown in Fig. 5, A–C. The fluorescence signal under the different conditions was quantitated, and the results of several experiments are shown in Fig. 5D. Insulin treatment increased the amount of GLUT4 on plasma membrane lawns by 2.9 ± 0.4-fold, relative to basal state levels (assigned a value of 1.0). Pretreatment of cells with 30 μM A291077 or A304000 prior to insulin stimulation did not cause any significant reduction in the plasma membrane GLUT4 levels (A291077 + insulin, 3.0 ± 0.7-fold; A304000 + insulin, 3.0 ± 0.4-fold, compared with control cells in the basal state). Similarly, 10 μM SB202190 or SB203580 did not decrease the amount of GLUT4 found in plasma membrane lawns (SB202190 + insulin, 2.9 ± 0.4-fold; SB203580 + insulin, 2.8 ± 0.5-fold, compared with control cells in the basal state). Taken together, these results suggest that p38 may be part of the signaling pathway utilized by insulin to activate GLUT4 without affecting its translocation to the cell surface.

Expression of a Dominant-negative p38 Mutant (p38AGF) in 3T3-L1 Adipocytes—To further verify that the inhibitory effects of the various drugs on glucose uptake was mediated via inhibition of p38, we used a genetic approach. A p38 mutant (p38AGF) in which the regulatory phosphorylation sites have been replaced with alanine and phenylalanine, respectively, (p38AGF) in which the regulatory phosphorylation sites have been replaced with alanine and phenylalanine, respectively, has been used widely to investigate p38 function in vivo (33–35). We analyzed the participation of p38 in insulin action using a 3T3-L1 cell line that stably expressed this mutant p38 cDNA under the control of the lac operon. An advantage of this system is that it allows relatively tight repression of the transgene in the absence of the inducer, IPTG. Treatment of cells with IPTG overrides this transcriptional repression, thereby allowing expression of the construct. These cells, referred to here as p38AGF-3T3-L1 were described previously (31).

Three strategies were used to demonstrate expression of FLAG-tagged p38AGF, induced in fully differentiated adipocytes. Cell extracts prepared from p38AGF-3T3-L1 adipocytes...
that were treated for 48 h with 5 mM IPTG were immunoblotted for p38 (Fig. 6A, top panel) or for the FLAG tag (Fig. 6A, middle panel). Two representative immunoblots are shown for each experiment. Immunoblotting with an anti-p38 antibody demonstrated a 3.1 ± 0.6-fold increase in p38 protein expression in cells that were treated with IPTG (Fig. 6A, top panel). Fig. 6A, middle panel, shows an anti-FLAG immunoblot of cell lysates prepared from IPTG-treated p38AGF-3T3-L1 adipocytes (lanes 3 and 4). No band was detected in untreated p38AGF-3T3-L1 adipocytes, as expected (Fig. 6A, middle panel, 1st and 2nd lanes). To confirm that FLAG expression detected in Fig. 6A, middle panel, was due to an increase in the expression of the FLAG-tagged p38AGF, we immunoblotted anti-FLAG immunoprecipitates for p38. As shown in Fig. 6A, bottom panel, treatment of p38AGF-3T3-L1 adipocytes with IPTG increased the amount of p38 MAPK that could be immunoprecipitated with anti-FLAG antibody.

Expression of p38AGF in 3T3-L1 Adipocytes Prevents Insulin-stimulated CREB Phosphorylation—To determine whether p38AGF could dominantly inhibit endogenous p38, we examined the effect of expression of this mutant on insulin-induced CREB phosphorylation. p38AGF-3T3-L1 adipocytes were left untreated or treated for 48 h with IPTG and then stimulated with insulin for 10 min. Whole cell extracts were immunoblotted for phosphorylated CREB. Insulin treatment increased CREB phosphorylation by 2.2 ± 0.2-fold relative to basal levels in p38AGF-3T3-L1 adipocytes that were not treated with IPTG (Fig. 6B, p < 0.05). Insulin treatment also increased CREB phosphorylation in wild type, untransfected 3T3-L1 adipocytes by 2.6 ± 0.4-fold, p < 0.05. Expression of p38AGF by IPTG treatment had no significant effect on basal CREB phosphorylation (1.2 ± 0.1-fold relative to basal, Fig. 6B, p > 0.05). However, expression of p38AGF abrogated insulin-induced CREB phosphorylation (1.0 ± 0.3-fold above basal, Fig. 6B). These results suggest that p38AGF acts as a dominant-negative inhibitor of endogenous p38 signaling.

To gain some insight into possible mechanism(s) via which the p38AGF mutant exerted its dominant-negative action, we determined its effect on insulin-stimulated phosphorylation of endogenous p38. p38AGF-3T3-L1 adipocytes were left untreated or treated for 48 h with IPTG followed by 10 min of insulin stimulation. Whole cell extracts were immunoblotted for phosphorylated p38. In untreated p38AGF-3T3-L1 adipocytes, insulin for 10 min increased p38 phosphorylation by 1.9 ± 0.2-fold relative to basal levels. Induction of p38AGF expression did not reduce insulin-induced phosphorylation of endogenous p38 (2.0 ± 0.4-fold above basal). Similarly, basal p38 phosphorylation was not affected significantly by expression of p38AGF (1.3 ± 0.3-fold above basal, p > 0.05). These results suggest that p38AGF does not prevent signaling from upstream kinases. Instead, it may exert its dominant-negative effect by competing effectively with endogenous p38 for access to substrates.

Inhibition of Insulin-stimulated Glucose Uptake in 3T3-L1 Adipocytes following Induction of p38AGF Expression—To explore further the relationship between p38 and insulin-induced glucose uptake in 3T3-L1 adipocytes, we determined the effect of expressing p38AGF on insulin-induced glucose uptake. p38AGF-3T3-L1 adipocytes were treated for 48 h with IPTG followed by insulin for 20 min. 2-Deoxyglucose uptake was then measured for 5 min in the absence of IPTG. As shown in Fig. 7A, induction of p38AGF expression reduced insulin stimulation of glucose uptake by 39.0 ± 0.3% (−IPTG, 16.1 ± 0.8; +IPTG, 11.4 ± 0.5 pmol/min/mg protein, p < 0.01). Treatment of cells with IPTG for 48 h had no statistically significant effect on basal glucose uptake (−IPTG, 6.4 ± 0.3; +IPTG, 5.5 ± 0.4 pmol/min/mg protein, Fig. 7A, p > 0.05). No further reduction in insulin-stimulated glucose uptake was obtained when p38AGF-3T3-L1 adipocytes expressing the dominant-negative p38 mutant were also pretreated with 10 μM SB203580 (data not shown). To control for nonspecific effects of IPTG, 3T3-L1 adipocytes that do not harbor the p38AGF transgene were treated with IPTG for 48 h, prior to measurement of glucose uptake. This treatment had no effect on basal or insulin-stimulated glucose uptake (data not shown). These results suggest that SB203580 and the p38AGF mutant most likely inhibited the same signal needed for the stimulation of glucose uptake by insulin.

Expression of p38AGF Has No Effect on GLUT4 Translocation—The possibility that expression of p38AGF might have affected GLUT4 translocation was tested using the plasma membrane lawn technique. Quantitation of GLUT4 content on plasma membrane lawns is shown in Fig. 7B. Insulin treatment caused a 2.0 ± 0.2-fold increase in plasma membrane GLUT4 content in p38AGF-3T3-L1 adipocytes not treated with IPTG. Insulin-dependent GLUT4 translocation was not altered by induction of p38AGF expression (2.0 ± 0.3-fold increase relative to basal). Similarly, IPTG treatment did not alter the amount of GLUT4 detected on plasma membrane lawns in the basal state (Fig. 7B). The findings that inducible expression of dominant-negative p38AGF and diverse pharmacological inhibitors of p38 reduced insulin-stimulated 2-deoxyglucose uptake without affecting GLUT4 translocation support the concept that insulin increased the intrinsic activity of GLUT4 via a p38-dependent signal.

**DISCUSSION**

It is widely recognized that insulin stimulates glucose uptake via the rapid recruitment of GLUT4 to the surface of skeletal muscle and adipose tissue. However, many studies have shown that the extent of GLUT4 recruitment to the cell surface in response to an acute insulin challenge is less than the extent of the increase in glucose uptake (Table III). This
discrepancy between GLUT4 translocation and the stimulation of glucose uptake by insulin has been demonstrated in skeletal muscle (52–56), isolated rat and human adipocytes (57–61), and L6 myotubes overexpressing GLUT4myc (14). Furthermore, insulin-stimulated insertion of GLUT4 into the plasma membrane of isolated rat adipocytes (60) and L6 myotubes (14) occurs with a half-time of 1 and 2.5 min, respectively, whereas the half-time for the stimulation of glucose uptake in the same studies was 2 and 6 min, respectively. In both studies, GLUT4 translocation was recorded by labeling the transporter from the extracellular side, ensuring detection of only GLUT4 molecules fully inserted into the plasma membrane. These conditions and other strategies listed in Table IV suggest that translocation of GLUT4 to the cell surface does not suffice to achieve maximum stimulation of glucose uptake by insulin. One possibility is that insulin leads to activation of the translocated transporters.

Very little is known about the signaling pathway leading to GLUT4 activation. Based on the use of SB203580, we proposed that p38 may participate in the stimulation of glucose uptake in 3T3-L1 adipocytes (13). The role of p38 in inflammatory and stress responses is well defined (1), yet little is known about the role of this family of kinases in growth factor-regulated signaling pathways and metabolism. By using multiple approaches, we and others have demonstrated insulin-mediated phosphorylation and activation of p38 in rat skeletal muscle (15, 19), L6 myotubes (14), 3T3-L1 adipocytes (13, 63), Chinese hamster ovary cells overexpressing the insulin receptor (64), and vascular smooth muscle cells (65). In the present study, the hypothesis that activation of p38 is a permissive signal for the stimulation of glucose uptake by insulin was tested. Here we characterize two new p38 inhibitors that are structurally different from the prototypical SmithKline Beecham p38 inhibitor SB203580 (3). A291077 and A304000 inhibited p38 in vitro with IC_{50} values of 0.6 and 4.7 μM, respectively. The closely related MAPK-JNK2α was also sensitive to inhibition by higher concentrations of A291077 and SB203580 than are required for inhibition of p38 in vitro. However, A304000 was very specific for p38, not inhibiting JNK2α. Other protein kinases such as casein kinase-2, MAPKAPK2, cAMP-dependent protein kinase, protein kinase B/Akt, and protein kinase C were not affected by either A291077 or A304000. These results suggest that A291077 and A304000 are inhibitors of p38 and can be used to decipher the precise biological role of this protein.

Insulin-stimulated glucose uptake was reduced to a similar extent by A291077 and A304000 in 3T3-L1 adipocytes and L6-GLUT4myc myotubes. In sharp contrast, A305267, a structural analogue of A291077 and A304000 with no inhibitory action toward p38, did not lower insulin-mediated glucose uptake in 3T3-L1 adipocytes or L6-GLUT4myc myotubes. This selectivity displayed by the azaurazole compounds was also displayed by the pyridine imidazoles, because SB202474, an inactive structural analogue of SB203580 without inhibitory action on p38 (3), did not affect the stimulation of glucose uptake by insulin in 3T3-L1 adipocytes (13) or L6 myotubes (14). Importantly, translocation of GLUT4 to the plasma membrane was not altered by any of these drugs, whether active or inactive toward p38. It is unlikely that any of the p38 inhibitory agents interfered with GLUT4 insertion into the plasma membrane because GLUT4 translocation in L6-GLUT4myc myotubes was measured as exposure of the extracellular Myc epitope. It is also unlikely that the inhibition of insulin-dependent glucose uptake is due to a direct effect of these drugs on GLUT4 to curb its activity directly, because of the following observations.

1) Basal glucose uptake, which like insulin-stimulated uptake is mediated by GLUT4myc in L6GLUT4myc myotubes, was not affected. Unstimulated (basal state) L6 myotubes or 3T3-L1 adipocytes were exposed to the drugs for the same time as the cells receiving insulin. In both conditions (basal and insulin-stimulated), the compounds were removed prior to assaying glucose uptake.

2) Inclusion of any of the four p38 inhibitors in the transport assay only, without any pretreatment, had virtually no effect on basal or insulin-stimulated glucose uptake. Under identical conditions, agents that interact directly with GLUT4 such as

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**Table III**

Examples of discrepancies between GLUT4 translocation and glucose uptake in response to insulin in skeletal muscle and adipose cells

| Species                  | Measurement                  | Half-time (min) | Ref. |
|-------------------------|------------------------------|----------------|-----|
| Rat muscle              | Basal glucose uptake         | 1.8            | 67  |
| Rat muscle              | Insulin-stimulated glucose   | 2.5            | 67  |
| Mouse muscle            | Basal glucose uptake         | 1.4            | 70  |
| Human muscle            | Basal glucose uptake         | 1.7            | 70  |
| Human muscle            | Insulin-stimulated glucose   | 1.3            | 74  |
| Human muscle            | Insulin-stimulated glucose   | 2.2            | 77  |
| Human muscle            | Insulin-stimulated glucose   | 2.4            | 79  |
| Human muscle            | Insulin-stimulated glucose   | 2.4            | 79  |
| Human muscle            | Insulin-stimulated glucose   | 1.6            | 75  |
| Human muscle            | Insulin-stimulated glucose   | 2.9            | 76  |
| Human muscle            | Insulin-stimulated glucose   | 2.4            | 78  |
| Human muscle            | Insulin-stimulated glucose   | 2.4            | 78  |

* A number of other studies using affinity photolabeling of GLUT4 found less divergence between translocation and glucose uptake. However, because activity photolabels bind to the active site of GLUT4, it is conceivable that they preferentially label the more active transporters exposed to the cell surface (78, 80). See “Discussion” for additional references.
cytochalasin B, indinavir, and forskolin completely abrogated the stimulation of glucose uptake (Table II).

3) Two distinct families of agents (pyridinylimidazoles and azaazulenes) gave similar results, and within them only those compounds with inhibitory action on p38 MAPK reduced the ability of insulin to stimulate glucose uptake.

4) The reduction in insulin stimulation of glucose uptake displayed by p38 MAPK inhibitors was seen in mature myotubes but not in undifferentiated myoblasts (66). Yet in both instances, GLUT4myc dictated glucose uptake suggesting that actions other than direct interaction with GLUT4 are responsible for the diminished response. Consistent with this observation, p38 MAPK was activated by insulin only in mature myotubes and adipocytes but not in myoblasts or fibroblasts (66).

Nonetheless, the possibility remains that the reduction in insulin stimulation of glucose uptake caused by p38 MAPK inhibitors was brought about through mechanisms other than via inhibition of p38 MAPK. For this reason, a molecular approach complemented the chemical study.

We used a dominant-negative p38 mutant to further investigate the role of p38 in the stimulation of glucose uptake by insulin. The p38AGF mutant has been used previously to study the ability of insulin to stimulate glucose uptake. The p38AGF mutant has been used previously to study the role of p38 in the stimulation of glucose uptake by insulin stimulation of glucose uptake caused by p38 MAPK (66).

Inhibition by pyridinylimidazoles

Inhibition by azaazulenes

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p38 MAPK Inhibitors and GLUT4 Activity

| GLUT4 translocation | Glucose uptake | Ref. |
|---------------------|---------------|-----|
| L6 myotubes         |               |     |
| Time course of insulin action | $t_{1/2}$ 2.5 min | $t_{1/2}$ 6 min | 14 |
| Susceptibility to wortmannin | IC$_{50}$ 43 nm | IC$_{50}$ 3 nm | 81 |
| Inhibition by pyridinylimidazoles | No | No | 40–60% |
| Inhibition by azaazulenes | No | 50% | This study |
| RAT adipocytes      |               |     |
| Time course of insulin action | $t_{1/2}$ 1 min | $t_{1/2}$ 2 min | 60 |
| 3T3-L1 adipocytes  |               |     |
| Susceptibility to wortmannin | IC$_{50}$ 80 nm | IC$_{50}$ 6 nm | 62 |
| Inhibition by pyridinylimidazoles | No | No | 40–60% |
| Inhibition by azaazulenes | No | 50–60% | This study |

$^a$ SB203580 or SB202190 (10 µM in preincubation medium, absent during uptake assay).

$^b$ A304000 or A291077 (20 µM in preincubation medium, absent during uptake assay).
A Dominant-negative p38 MAPK Mutant and Novel Selective Inhibitors of p38 MAPK Reduce Insulin-stimulated Glucose Uptake in 3T3-L1 Adipocytes without Affecting GLUT4 Translocation

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