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Reduction of Insulin-Stimulated Glucose Uptake in L6 Myotubes by the Protein Kinase Inhibitor SB203580 Is Independent of p38MAPK Activity

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Insulin increases glucose uptake through translocation of the glucose transporter GLUT4 to the plasma membrane. We previously showed that insulin activates p38MAPK, and inhibitors of p38MAPKα and p38MAPKβ (*e.g.* SB203580) reduce insulin-stimulated glucose uptake without affecting GLUT4 translocation. This observation suggested that insulin may increase GLUT4 activity via p38α and/or p38β. Here we further explore the possible participation of p38MAPK through a combination of molecular strategies. SB203580 reduced insulin stimulation of glucose uptake in L6 myotubes overexpressing an SB203580-resistant p38α (drug-resistant p38α) but barely affected phosphorylation of the p38 substrate MAPK-activated protein kinase-2. Expression of dominant-negative p38α or p38β reduced p38MAPK phosphorylation by 70% but had no effect on insulin-stimulated glucose uptake. Gene silencing via isoform-specific small interfering RNAs reduced expression of p38α or p38β by 60–70% without diminishing insulin-stimulated glucose uptake. SB203580 reduced photoaffinity labeling of GLUT4 by bio-LC-ATB-BMPA only in the insulin-stimulated state. Unless low levels of p38MAPK suffice to regulate glucose uptake, these results suggest that the inhibition of insulin-stimulated glucose transport by SB203580 is likely not mediated by p38MAPK. Instead, changes experienced by insulin-stimulated GLUT4 make it susceptible to inhibition by SB203580. (*Endocrinology* 146: 3773–3781, 2005)

GLUT4 IS THE main insulin-responsive glucose transporter (1, 2), and upon insulin stimulation, it is recruited from intracellular compartments to the plasma membrane (3–5). It is widely accepted that GLUT4 translocation is required for insulin-stimulated glucose uptake, but it has been debated whether translocation is the sole mechanism responsible for this outcome (6). Several studies have demonstrated that insulin-stimulated glucose uptake can be segregated from GLUT4 translocation based on differential sensitivities to insulin concentrations (7) and inhibition by the phosphatidylinositol 3-kinase inhibitor wortmannin (8, 9). These results suggested that GLUT4 translocation and the intrinsic activity of GLUT4 are differentially regulated (10).

p38MAPKs are serine/threonine kinases activated by environmental stressors (11), inflammatory cytokines (12), and protein synthesis inhibitors (13, 14) as well as growth factors (*e.g.* insulin, platelet-derived growth factor) (15, 16) and muscle contraction (17, 18). Four isoforms of p38MAPK have been identified: p38α (19) and p38β (20) are ubiquitously expressed, whereas p38γ (21) is highly expressed in skeletal muscle and p38δ in the lung and kidney (22). Full activation of p38MAPK requires dual phosphorylation on residues corresponding to threonine-180 and tyrosine-182 on p38α (12) catalyzed by dual-specificity MAPK kinases 3 and 6 (MKK3 and 6). Insulin-stimulated activation of p38MAPK has been observed in rat skeletal muscles (18), mouse brown adipocytes (23), 3T3-L1 adipocytes (15, 24), L6 muscle cells (7, 25), and human skeletal muscles (26) and adipocytes (27). Notably, p38MAPK inhibitors such as pyridinyl imidazole derivatives (SB203580 and SB202190) or azaazulene pharmacophores (A291077 and A304000) inhibit insulin-mediated glucose uptake (15, 18, 23–25, 28, 29) with no effect on GLUT4 translocation in 3T3-L1 adipocytes (15, 24), L6 myotubes (25), mouse brown adipocytes (23), and rat skeletal muscles (18). Collectively, these results have raised the hypothesis that insulin increases the intrinsic activity of GLUT4 via p38MAPK.

Although they have been shown to be highly potent and specific inhibitor of p38α and p38β (30, 31), recent studies have revealed some previously unknown targets of these drugs (32). When this manuscript was in progress, Holman and colleagues (33) suggested that SB203580 directly inhibits the endofacial glucose binding site of the transporter, based on the competitive effect of the drug on glucose uptake under equilibrium exchange but not zero-trans conditions. Of note, such inhibition of glucose uptake was reported only for the insulin-stimulated state. Moreover, in that and other studies, an analog of SB203580 that does not inhibit p38MAPK (SB202474) failed to inhibit glucose transport, thereby main-

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Abbreviations: DN, Dominant negative; DR, drug resistant; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; MK-2, MAPK-activated protein kinase-2; MKK, MAPK-kinase; NR, nonrelevant; si, small interfering; WT, wild type.

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taining the correlation between inhibition of p38MAPK and inhibition of glucose uptake. Thus, whether p38MAPK regulates the availability of glucose-binding sites and thus transport activity of GLUT4 deserves direct testing. The aim of this study was to use small interfering (si)RNA and p38MAPK mutants to examine the role of p38α and p38β in insulin-stimulated glucose uptake and GLUT4 translocation in muscle cells. We used adenovirus-mediated gene transfer to introduce either dominant-negative (DN) mutants of p38α or p38β (34) or drug-resistant (DR) p38α (32) into L6 myotubes to explore whether the SB203580-mediated reduction in insulin-stimulated glucose uptake occurs via inhibition of p38MAPK activity. In addition, we reduced p38α or p38β expression via siRNA gene silencing in an effort to determine which isoform is involved in insulin signaling. Finally, we examined the effect of SB203580 on Bio-LC-ATB-BMPA photolabeling (35). The photolabel binds to the exofacial glucose binding site of GLUT4, which becomes unavailable when the transporter is locked in the inner-facing configuration.

Materials and Methods

Materials

SB203580 was obtained from Calbiochem (San Diego, CA). Protease inhibitor cocktail, cytochalasin B, *O*-phenylenediamine dihydrochloride, 2-deoxyglucose, and α-FLAG antibodies were obtained from Sigma (St. Louis, MO). 2-Deoxy-D-[^{3}H]glucose was purchased from PerkinElmer Life Sciences (St. Laurent, Quebec, Canada). Streptavidin-immobilized agarose beads were obtained from Pierce Biotechnology (Rockford, Il). Bio-LC-ATB-BMPA was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). α-Phospho-p38 MAPK (recognizes all p38MAPK isoforms only when phosphorylated on residues corresponding to T180 and Y182 on p38α), α-phospho-MAPK-activated protein kinase-2 (MK-2; recognizes MK-2 when phosphorylated on T334), and α-p38 MAPKα antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Polyclonal α-*myc* antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat α-rabbit and sheep α-mouse antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Adenovirus particles encoding cDNAs for green fluorescent protein (GFP), DN-p38α (T180A, Y182F), DN-p38β (T180A, Y182F), and wild-type (WT)-p38α were prepared as previously described (36). DR-p38α (T180M, H107P, L108F) has been previously characterized (32). Synthetic siRNAs specific for rat p38MAPKα (AAG CAC GAG AAU GUG AUU GGU) and p38MAPKβ (AAG CAC GAG AAC GUC AUA GGA) isoforms were obtained from Dharmacon (Lafayette, CO).

Cell culture, lysates, and immunoblotting

GLUT4^{myc} cDNA was constructed by inserting the human c-*myc* epitope into the first ectodomain of GLUT4 and subcloned into the pCXN2 vector (37), stably transfected into L6 myoblasts, and characterized (38–40). L6-GLUT4^{myc} myoblasts were grown and differentiated into myotubes as previously described (41) and were deprived of serum for 3–5 h before all experimental procedures. Cell lysates and immunoblots were performed as previously described (7).

2-Deoxyglucose uptake and GLUT4^{myc} translocation

2-Deoxyglucose uptake was measured for 5 min at room temperature using 10 μM D-2-deoxyglucose (0.5 μCi/ml 2-deoxy-D-[^{3}H]glucose) as previously described (7). Uptake was terminated by rinsing the cells three times with 1 ml ice-cold 0.9% NaCl (wt/vol). GLUT4^{myc} translocation was assayed by a colorimetric method as previously described (15).

Construction and isolation of adenovirus particles and adenovirus infection

Adenovirus particles containing cDNAs encoding DR-p38α were constructed using the AdEasy I system (Stratagene, La Jolla, CA) (42). Briefly, cDNA encoding DR-p38α was subcloned into adenovirus shuttle vector (pAdTrack-CMV) and subsequently inserted into a plasmid (pAdEasy-I) encoding modified adenoviral genome by homologous recombination in *Escherichia coli* strain BJ5183. Subsequently recombined pAdEasy-I was transfected into HEK293AD cells. Adenovirus particles encoding all cDNAs used were harvested and purified by discontinuous cesium chloride gradient. The infectivity of adenovirus particles was determined using tissue culture infectious dose 50 criteria and then converted to plaque-forming units according to the AdEasy I system manual. Differentiating L6 myotubes were incubated with 2.5×10^5 PFU/well (24-well plate) adenovirus particles in antibiotic/antimycotic-free αMEM and 5 mM glucose for 4 h (in serum-free medium). Infection proceeded for 10–12 h (WT-p38α and DR-p38α adenovirus) or 18–24 h (DN-p38α, DN-p38β, and GFP adenovirus) before serum deprivation and experimentation.

siRNA transfection

Cells were incubated in antibiotic-free αMEM and 5 mM glucose, supplemented with 2% fetal bovine serum for 72 h, and then transfected with 500 nM siRNA of nonrelevant (NR) sequence or siRNA specific for p38α, p38β, or p38α and p38β together, using oligofectamine transfection reagent (Life Technologies, Carlsbad CA), 48 h and again 24 h before experimentation. Transfection involved incubation of L6 cells for 4 h in serum-free antibiotic/antimycotic-free αMEM containing 5 mM glucose for 4 h and the siRNA-oligofectamine mixture. Subsequently fetal bovine serum was added to each well to a final concentration of 2% and the cells were incubated until the next transfection or experiment.

Bio-LC-ATB-BMPA photolabeling of GLUT4^{myc}

The method of the bio-LC-ATB-BMPA photolabel (43) was adapted as follows: after experimental treatments, L6-GLUT4^{myc} myotubes were washed in 18 degree 10 mM HEPES buffer (pH 7.4), containing 140 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 2.5 mM NaH_2PO_4 and then exposed to 0.2 mM bio-LC-ATB-BMPA and irradiated for 1 min in a Rayonet photochemical reactor under 350 nm light. After washing in ice-cold HEPES buffer, cells were lysed in PBS containing 2% Thesit ($C_{12}E_9$) and centrifuged at 20,000 $\times$ *g* for 20 min at 4 degrees. Supernatants were incubated with immobilized streptavidin on 6% beaded agarose for 20 h at 4 degrees. Agarose beads were pelleted by centrifugation at 12,000 $\times$ *g* for 5 min, washed twice in PBS containing 1% (wt/vol) Thesit and twice in PBS containing 0.1% (wt/vol) Thesit. Precipitated proteins were solubilized in Laemmli sample buffer containing 8 M urea and 1 mM EDTA, heated at 98 C for 30 min, resolved by SDS-PAGE, and GLUT4^{myc} detected by immunoblotting with anti-*myc* antibodies.

Results

SB203580 inhibits insulin-stimulated glucose uptake in L6 muscle cells

In agreement with previous results (15), glucose uptake into L6 myotubes stimulated by either submaximal (10 nM) or maximal (100 nM) insulin doses was reduced by preincubation with SB203580 (Fig. 1A), whereas insulin-stimulated GLUT4 translocation was not affected by SB203580 at either insulin concentration (Fig. 1B). Notice that SB203580 is removed before all assays. Together the differential sensitivity of insulin-stimulated glucose uptake *vs.* GLUT4 translocation to SB203580 suggests that glucose uptake may require the participation of SB203580 sensitive target(s) independently of GLUT4 translocation.

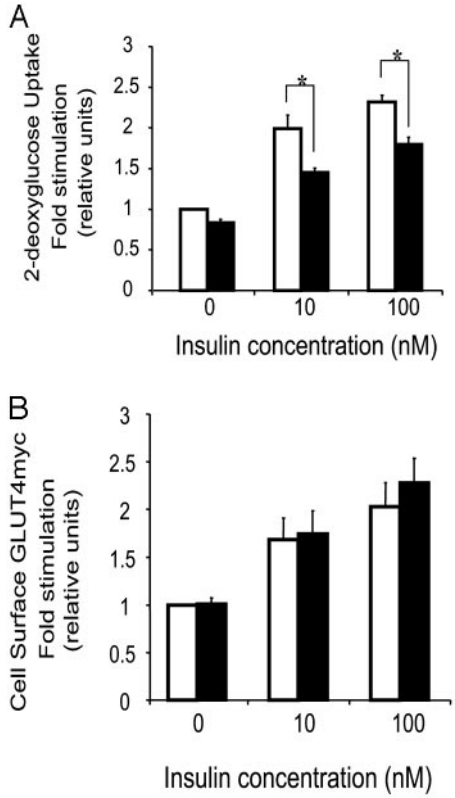

FIG. 1. SB203580 inhibits insulin-stimulated glucose uptake without affecting the amount of cell surface GLUT4. L6-GLUT4^{myc} myotubes were incubated with vehicle (*open bars*) or 10 μM SB203580 (*closed bars*) for 40 min in the absence or presence of insulin during the last 20 min of the incubation. A, Cells were washed three times in HEPES buffer to remove the inhibitor and 2-deoxyglucose uptake was measured as described in *Materials and Methods*. Results are expressed relative to the vehicle-treated basal condition and represent the mean ± SE of three independent experiments. Each condition was assayed in triplicate. B, GLUT4^{myc} translocation was performed as described in *Materials and Methods*. Values were normalized to the surface GLUT4^{myc} level of the vehicle-treated basal condition. Results shown are the mean ± SE of three independent experiments. Each condition was assayed in triplicate. *, $P < 0.05$

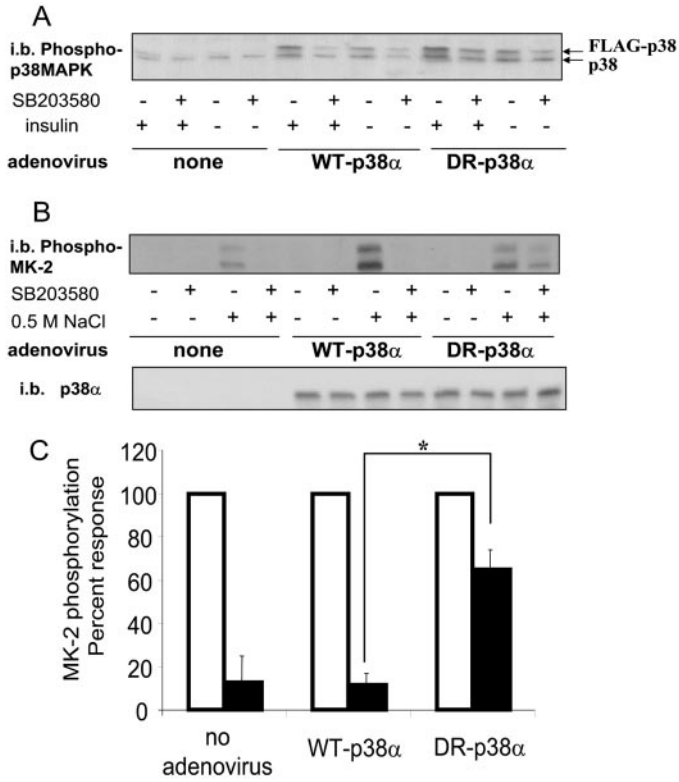

FIG. 2. DR-p38α in L6 myotubes protects hyperosmolarity-stimulated MK-2 phosphorylation from the effect of SB203580. L6 myotubes were infected with 2.5×10^5 PFU/well (24-well plate) of WT-p38α or DR-p38α adenovirus for 10–12 h, as described in *Materials and Methods*. L6-GLUT4^{myc} myotubes were preincubated with vehicle (*open bars*) or 10 μM SB203580 (*closed bars*) for 20 min. Then in the continued absence or presence of the drug, cells were stimulated with insulin (100 nM for 10 min) or hyperosmolar stress (0.5 M NaCl for 20 min) as indicated. Total cellular lysates were prepared in Laemmli sample buffer. Fifty micrograms of total protein were resolved on SDS-PAGE and immunoblotted for p38α, phospho-p38MAPK (T180/Y182) or phospho-MK-2 (T334), as indicated. Representative blots are shown in A and B. Seven immunoblots were scanned within the linear range to quantify MK-2 phosphorylation, and the results, expressed relative to the respective NaCl-treated condition are shown in C. *, $P < 0.05$, compared with the WT-p38α transfected, NaCl-, and SB203580-treated condition.

Effect of DR mutant of p38α on SB203580-mediated reduction in insulin-stimulated glucose uptake

SB203580 inhibits p38α and p38β with IC_{50} of 50 nM and 500 nM *in vitro* (31) and IC_{50} of 1.4 μM in intact cells including L6 myotubes (Ref. 9 and references within). Maximal inhibition of insulin-stimulated glucose uptake occurs at 10 μM SB203580; therefore, it is plausible that the effect might be mediated by a target other than p38α or p38β. To explore this possibility, WT- or DR-p38α was overexpressed in L6-GLUT4^{myc} myotubes via adenoviral delivery. We determined that infection of L6 myotubes with adenoviruses encoding either WT-p38α or DR-p38α resulted in a similar insulin-responsive increase in total phospho-p38MAPK (Fig. 2A). The gels show the slower migration of the FLAG-tagged transfected proteins, compared with the endogenous p38 MAPK. In these experiments the SDS-PAGE conditions were optimized to facilitate detection of the two bands, and this may explain why a doublet is now visible in the control, untransfected cells, potentially reflecting more than one isoform of the endogenous enzyme. Similar levels of p38MAPK phosphorylation were observed in cells overexpressing WT-p38α and DR-p38α. Determination of p38 MAPK action *in vivo* requires analysis of the phosphorylation of a known target, typically MK-2. Although MK-2 is phosphorylated in response to insulin in L6 myotubes, this largely involves the MEK1-Erk pathway (data not shown). Hence, as readout of the effectiveness of the WT- and DR-p38α, we examined the phosphorylation of MK-2 in response to another activator of p38MAPK-MK-2, namely hyperosmolar stress. In the presence of 10 μM SB203580, the hyperosmolarity-induced MK-2 phosphorylation was reduced by 89% in cells overexpressing WT-p38α and to a similar degree as in nontransfected cells. In contrast, 10 μM SB203580 reduced MK-2 phosphorylation by only 35% in cells overexpressing DR-p38α (Fig. 2, B and C). Because adenovirus infection efficiency was consistently around 80% (assessed by bicistronic expression of enhanced GFP AdEasy vector, data not shown), these results suggest

that SB203580 is largely unable to inhibit cellular p38α activity in cells overexpressing DR-p38α. Yet insulin-stimulated glucose uptake was inhibited by 10 μM SB203580 to a similar extent in cells overexpressing WT- and DR-p38α (Fig. 3).

Effect of DN mutants of p38α or -β on insulin-stimulated glucose uptake

To confirm the unexpected result obtained with the DR-p38α mutant, we sought to achieve inhibition of p38α and p38β activity by expressing DN mutants of each isoform. The DN-p38MAPK mutants are mutated at both of the phosphorylation sites (T180A, Y182F); therefore, they cannot be phosphorylated or activated by their upstream kinases, MKK3 and MKK6. Adenovirus-mediated infection of L6-GLUT4^{myc} myotubes resulted in a 50-fold overexpression of the DN-p38α or DN-p38β mutant relative to endogenous WT-p38α or p38β, as determined by immunoblotting of whole-cell lysates. In separate experiments, adenovirus infection efficiency was consistently 80% or higher, assessed by immunofluorescence microscopy (data not shown). Expression of either DN-p38α or DN-p38β reduced insulin-stimulated phosphorylation of p38MAPK by approximately 70% in whole-cell lysates (Fig. 4) and did not affect total GLUT4 protein amount in L6-GLUT4^{myc} myotubes (data not shown). Therefore, it appears that these mutants inhibit activation of p38MAPK by functionally sequestering MKK3 and/or MKK6, thereby preventing activation of downstream targets. Neither DN-p38α (Fig. 5A) nor DN-p38β (Fig. 5B) expression reduced glucose uptake stimulated by submaximal (10 nM) or maximal (100 nM) insulin doses. In addition, the inhibition of insulin-stimulated glucose uptake seen by pretreatment with 10 μM SB203580 occurred to similar extents in L6-GLUT4^{myc} myotubes expressing GFP, DN-p38α, or DN-p38β (34, 30, and 40%, respectively; data not shown). Hence, despite a marked reduction in insulin-stimulated p38MAPK phosphorylation by expression of DN-p38MAPKα/β, there was no concomitant reduction in insulin-stimulated glucose uptake.

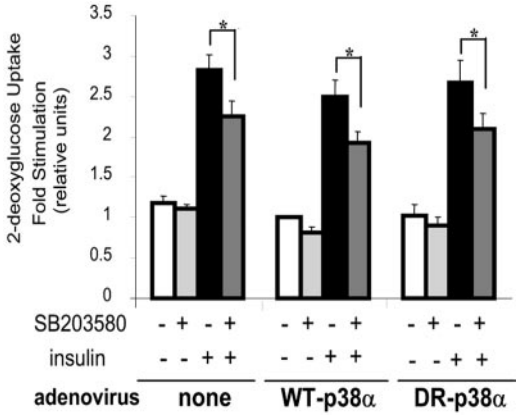

FIG. 3. DR-p38α does not prevent inhibition of insulin-stimulated glucose uptake by SB203580. L6-GLUT4^{myc} myotubes were infected with 2.5×10^5 PFU/well (24-well plate) of WT-p38α or DR-p38α adenovirus for 10–12 h and then incubated with 10 μM SB203580 (*striped bars*) or without the agent (*open bars*) for 40 min. During the last 20 min, myotubes were stimulated with 100 nM insulin for 20 min in the continued presence (*gray bars*) or absence (*closed bars*) of the inhibitor. 2-Deoxyglucose uptake was measured as described in *Materials and Methods*. Results are expressed relative to the WT-p38α-infected unstimulated condition and represent the mean ± SE of seven independent experiments. Each condition was assayed in triplicate. *, $P < 0.05$, compared with the corresponding WT-p38α-infected conditions.

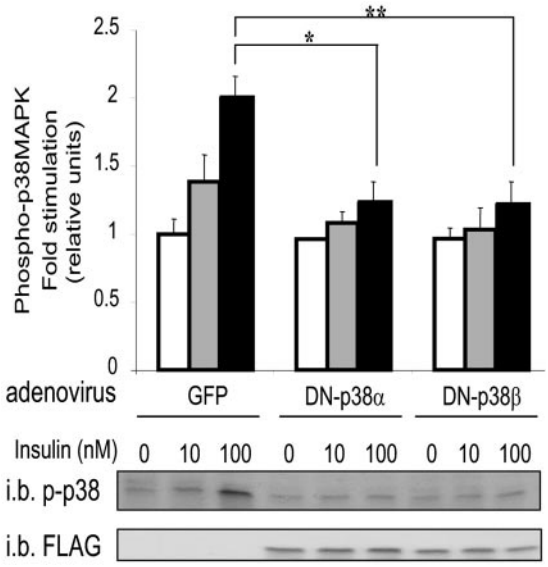

FIG. 4. DN-p38α or DN-p38β inhibit insulin-stimulated p38MAPK phosphorylation. L6-GLUT4^{myc} myotubes were infected with 2.5×10^5 PFU/well (24-well plate) of GFP, FLAG-DN-p38α, or FLAG-DN-p38β adenovirus for 18–24 h. Myotubes were left unstimulated (*open bars*) or stimulated with 10 (*gray bars*) or 100 nM insulin (*closed bars*) for 10 min. Total cellular lysates were prepared in Laemmli sample buffer, and 50 μg of total protein were resolved by SDS-PAGE and immunoblotted for phospho-p38MAPK. Immunoblots were scanned within the linear range to quantify protein phosphorylation, and the results of at least six independent experiments are expressed relative to the GFP-infected basal condition, as the mean ± SE. A representative immunoblot of phosphorylated p38MAPK is shown in the *top panel*, and a representative immunoblot (i.b.) showing expression of FLAG-DN-p38α and FLAG-DN-p38β in each condition, detected using FLAG-specific antibodies, is shown in the *bottom panel*. *, $P < 0.001$; **, $P < 0.01$, relative to the respective condition in GFP virus-infected cells.

Effect of siRNAs to p38 isoforms on insulin-stimulated glucose uptake

To complement the above analysis, we examined the effect of siRNA gene silencing to reduce p38α and/or p38β proteins on insulin-stimulated glucose uptake. Sequence-specific siRNAs were transfected into L6 myotubes on d 5, and experiments were performed on d 7 of myotube differentiation. A 65–70% reduction of p38α protein was achieved, as determined by immunoblotting whole-cell lysates (Fig. 6A). Reduction of p38α by siRNA did not appreciably affect expression of p38β (Fig. 6B). Because currently available antibodies specific to p38β are poor at detecting endogenous p38β protein in L6 myotubes, we used L6 cells stably expressing a FLAG-tagged DN-p38β and measured FLAG signal after siRNA transfection (Fig. 6B). p38β siRNA gene silencing achieved a knockdown of 63–74% of overexpressed p38β protein (Fig. 6B). We surmise that the endogenous p38β of L6-GLUT4^{myc} myotubes was reduced to a similar extent by

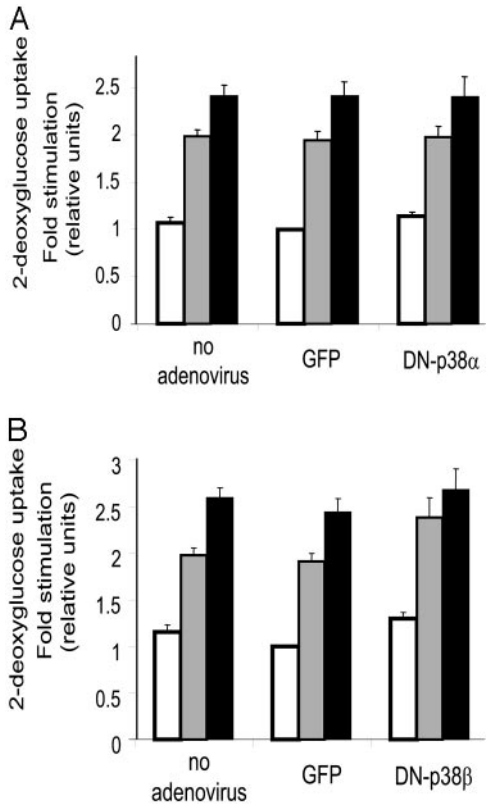

FIG. 5. Expression of DN-p38α or DN-p38β does not affect insulin-stimulated glucose uptake. L6-GLUT4^{myc} myotubes were infected with 2.5×10^5 PFU/well (24-well plate) of GFP and either FLAG-DN-p38α (A) or FLAG-DN-p38β (B) adenovirus for 18–24 h and then were left unstimulated (*open bars*) or stimulated with 10 (*gray bars*) or 100 nM (*closed bars*) insulin for 20 min. 2-Deoxyglucose uptake was measured as described in *Materials and Methods*. Results are expressed relative to the GFP-infected basal condition and represent the mean ± SE of six to seven independent experiments. Each condition was assayed in triplicate.

p38β siRNA. The reduction in p38β by siRNA did not appreciably affect expression of p38α (Fig. 6A), indicating specificity of each siRNA for its cognate sequence. In L6 myotubes transfected with siRNAs to both p38α and p38β, each isoform was reduced to the extents achieved when each siRNA was used separately (Fig. 6, A and B). In addition, insulin-stimulated p38 phosphorylation was markedly inhibited by siRNA gene silencing of p38α alone or by combined siRNA to p38α and p38β, whereas siRNA gene silencing of p38β was without effect on p38 phosphorylation (Fig. 6C). The total amount of GLUT4 in L6-GLUT4^{myc} myotubes was unaffected by siRNA gene silencing of p38 isoforms (data not shown). Importantly, the reductions in p38α, p38β, or p38α and p38β together (Fig. 7) were not accompanied by a drop in insulin-stimulated glucose uptake.

Effect of SB203580 on GLUT4^{myc} photolabeling

Bio-LC-ATB-BMPA contains a sugar-like moiety recognized by GLUTs and a reactive group that undergoes covalent insertion into neighboring atoms of the transporter on photoactivation (35). Thus, it is expected that the amount of photolabeled GLUT4 is an indication of the accessibility of the extracellular glucose binding site of GLUT4. In agreement with previous results in adipose cells (44–46), insulin caused an increase in the amount of bio-LC-ATB-BMPA photolabeled GLUT4^{myc} in L6 myotubes (Fig. 8). Pretreatment of cells with SB203580, followed by washout of the drug before bio-LC-ATB-BMPA incubation significantly reduced the amount of photolabeled transporter in the insulin-stimulated condition but was without effect in the basal state (Fig. 8). This results suggests that pretreatment of cells with SB203580 reduces the accessibility of the extracellular glucose binding site of insulin-stimulated GLUT4^{myc}. Because availability of the *myc* epitope to the extracellular medium was not affected by SB203580 (Fig. 1B), these results suggest that it is the extracellular availability of the glucose binding site, rather than of the entire transporter, that is reduced by the drug.

Discussion

It has been repeatedly observed that in muscle cells and adipocytes pretreated with specific inhibitors of p38MAPK such as pyridinyl imidazoles (SB203580 and SB202190) or azaazulene inhibitors (27) have reduced insulin-stimulated glucose uptake (30–40%) yet undiminished GLUT4 translocation (15, 23, 47). These results raised the hypothesis that insulin-stimulated glucose uptake requires both GLUT4 translocation and GLUT4 activation and that insulin-stimulated GLUT4 activation may involve a p38MAPK-dependent pathway (48). However, the hypothesis has largely relied on pharmacological inhibitors of p38MAPK.

Surprisingly, two studies employing DN mutants of p38MAPK to explore the regulation of glucose uptake have yielded contradicting results. Fujishiro *et al.* (28) showed that adenovirus-driven expression of DN-p38α in 3T3-L1 adipocytes elevated insulin-stimulated glucose uptake and that expression of DN mutants of upstream activators of p38MAPK (MKK3 and MKK6) elevated GLUT1 and diminished GLUT4 levels. DN-p38 MAPK may have had a similar effect, likely as a result of transcriptional input from the p38MAPK pathway on GLUT4 expression (27). In a related study from our group using 3T3-L1 adipocytes, induction of DN-p38 MAPK expression did not change GLUT1 or GLUT4 levels yet lowered insulin-stimulated glucose uptake (47). The amount of plasma membrane lawn-associated GLUT4 was also unchanged, but this approach does not distinguish GLUT4 vesicles docked/adhered from those fully inserted in the membrane. These considerations prompted us to reexamine the role of DN-p38 MAPK mutants in L6-GLUT4^{myc} cells, a stably transfected cell line in which GLUT4^{myc} insertion into the membrane can be accurately determined and in which GLUT4^{myc} biosynthesis is unlikely to be prone to regulation because it is driven by a heterologous promoter (37). Therefore, in these cells, GLUT4^{myc} expression cannot be regulated by p38MAPK input, and GLUT4^{myc} largely surpasses the expression and function of endogenous glucose transporters (2, 25). This cellular system was therefore selected to determine whether p38MAPK is required for insulin-stimulated glucose uptake in L6-GLUT4^{myc} myotubes using: 1) a mutant of p38α (DR-p38α) resistant to SB203580; 2) dominant-inhibitory mutants of p38α and p38β; and 3) siRNA against p38α and p38β.

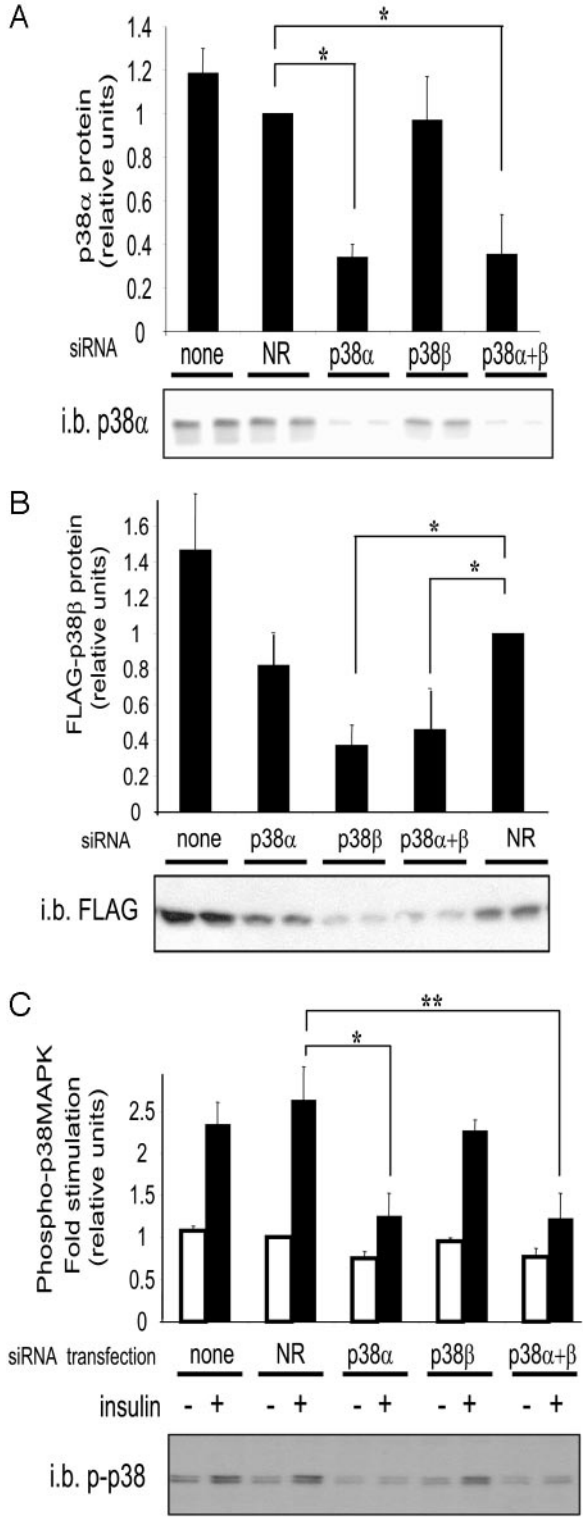

FIG. 6. p38MAPK isoform-specific siRNA reduces p38MAPK protein and insulin-stimulated p38MAPK phosphorylation. L6-GLUT4^{myc} myotubes were transfected with 500 nM of NR siRNA or p38α- or p38β-specific siRNA or both p38α- and p38β-specific siRNA together as described in *Materials and Methods*. Total cellular lysates were prepared in Laemmli sample buffer, and 50 μg of total protein were resolved by SDS-PAGE and immunoblotted as indicated. Each *panel* shows the averaged results of seven independent experiments and illustrates a representative gel. A, Immunodetection of p38α. B, Immunodetection of FLAG from L6-FLAG-DN-p38β. C, After transfection, L6-GLUT4^{myc} myotubes were either left unstimulated (*open bars*) or stimulated with 100 nM insulin (*closed bars*). Phospho-p38MAPK was detected by immunoblotting (i.b.). *, $P < 0.001$; **, $P < 0.01$ relative to insulin-treated NR-siRNA transfected cells.

The DR-p38MAPK has been previously used to determine whether SB203580-mediated effects occur via p38MAPK or other unknown SB203580 targets (32, 49, 50). Here we delivered WT- and DR-p38MAPK to L6 muscle cells, in which they were viably responsive to insulin, as judged from their phosphorylation in response to the hormone (Fig. 2A). Expression of DR-p38α in L6-GLUT4^{myc} myotubes reversed the SB203580-mediated inhibition of hyperosmolarity-induced MK-2 phosphorylation by 65% (Fig. 2). However, it did not prevent SB203580-mediated inhibition of insulin-stimulated glucose uptake (Fig. 3). The most obvious interpretation of these results is that SB203580-mediated inhibition of glucose uptake may not be due to inhibition of p38α and/or p38β by SB203580. Still it is also possible that p38MAPK is the target of SB203580, but an approximately 35% reduction in p38MAPK activity suffices to produce maximal inhibition of the p38MAPK-dependent component of insulin-stimulated glucose uptake. Alternatively, it is possible that the DR-p38α did not fully replace the endogenous p38α or p38β involved in regulating glucose uptake, even though DR-p38α was able to functionally replace the endogenous p38MAPK activity required for MK-2 phosphorylation.

To distinguish among these interpretations, we overexpressed DN mutants of p38α or p38β (DN-p38α or DN-p38β). Overexpression of either DN-p38α or DN-p38β reduced insulin-stimulated p38MAPK phosphorylation by 70–80% (Fig. 4) but did not lower glucose uptake stimulated by either 10 or 100 nM insulin (Fig. 5). Because each DN mutant reduced the total insulin-stimulated p38MAPK phosphorylation by 70–80%, these mutants likely inhibited signaling by both their cognate p38MAPK and other isoforms of p38MAPK. It is possible that the remaining 20–30% of p38MAPK activity suffices to stimulate GLUT4 activation. However, it is more likely that the remaining 20% p38MAPK phosphorylation arises from uninfected cells (see *Results*) and that, in infected cells, p38MAPK may not regulate GLUT4 activity. Instead, a different target may be responsible for the effects of SB203580.

To corroborate results obtained with the DN-p38 mutants, the expression of endogenous p38MAPK was diminished using siRNA against either p38α or p38β. Reducing the expression of endogenous p38α by 65% dropped insulin-stimulated p38MAPK phosphorylation by 70%. In contrast, reducing the expression of p38β by 60% did not affect insulin-stimulated p38MAPK phosphorylation (Fig. 6). These results suggest that in L6-GLUT4^{myc} myotubes, p38α is responsible for the majority of insulin-stimulated p38MAPK phosphorylation, even though both p38α and p38β are activated by insulin (15, 18). It is noteworthy that expression of DN-p38β resulted in a significant (~70%) inhibition of insulin-stimulated p38MAPK phosphorylation. Therefore, it is possible that this p38β mutant was able to also inhibit the activation of endogenous p38α, perhaps by sequestering an upstream

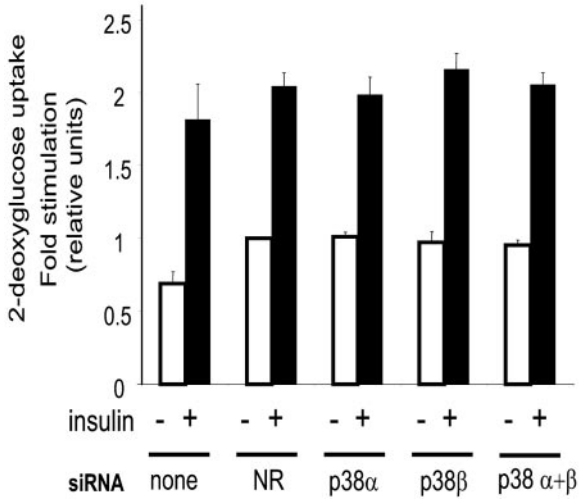

FIG. 7. p38-Isoform-specific siRNA has no effect on insulin-stimulated glucose uptake. L6-GLUT4^{myc} myotubes were transfected with 500 nM of NR siRNA, p38α- or p38β-specific siRNA, or both p38α- and p38β-specific siRNA together as described in *Materials and Methods*. After transfection, cells were left unstimulated (*open bars*) or stimulated with 100 nM insulin (*closed bars*) for 20 min as indicated, and 2-deoxyglucose uptake was measured as described in *Materials and Methods*. Results are expressed relative to the NR-siRNA transfected basal condition and represent the mean ± SE of seven independent experiments. Each condition was assayed in triplicate.

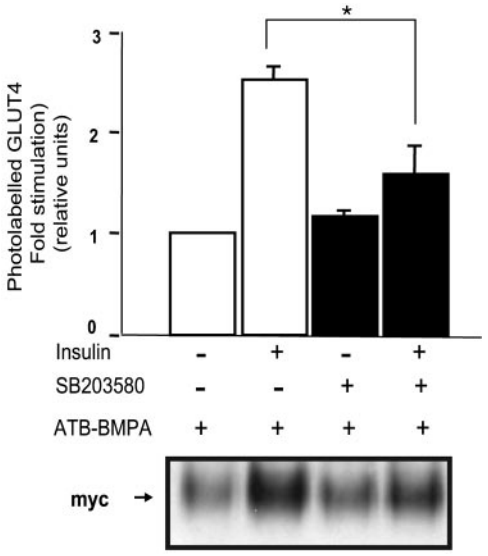

FIG. 8. SB203580 reduces insulin-stimulated but not basal ATB-BMPA photolabeling of cell surface GLUT4^{myc}. L6-GLUT4^{myc} myotubes were pretreated with 10 μM SB203580 for 20 min and then stimulated with insulin, as indicated, for 20 min in the continued presence of the inhibitor. SB203580 was then washed out before photolabeling of GLUT4 with biotin-conjugated ATB-BMPA and precipitation of the photolabeled transporters via immobilized streptavidin, as described in *Materials and Methods*. The amount of precipitated (photolabeled) GLUT4^{myc} was detected by immunoblotting with anti-*myc* antibody. A representative immunoblot is shown, and the results of n = 3 experiments are quantified. *, $P < 0.05$.

kinase common to both p38MAPK isoforms. In agreement with such an interpretation, overexpression of DN-p38α was shown to sequester the upstream kinase MKK3, thereby preventing the activation of another MKK3 substrate, Mirk (51). Consistent with the results obtained with DN-p38, reducing endogenous p38α or p38β by siRNA gene silencing had no effect on insulin-stimulated glucose uptake (Fig. 7). As in the case of L6 myotubes expressing DN-p38 mutants, siRNA gene silencing reduced only approximately 70% of p38MAPK activity, making it possible that the remaining small amount of active p38MAPK activity was sufficient to mediate maximal stimulation of GLUT4 activation. However, this argument is countered by the observation that in cells expressing DR-p38MAPK, glucose uptake was still reduced by SB203580, even though in the presence of the drug, the majority (65%) of p38MAPK was preserved. This result argues against a low level of p38MAPK sufficing to regulate glucose uptake.

The results obtained by three independent molecular methods to interfere with p38MAPK signaling did not interfere with submaximally or maximally stimulated glucose uptake. It may be possible that each of the DR-p38 and DN-p38 mutants and siRNA gene silencing of p38MAPK was unable to affect a small pool of p38MAPK that is responsible for SB203580-sensitive stimulation of GLUT4 activity in response to insulin. However, we recently observed that another pyridinyl imidazole, SB220025, inhibited p38MAPK as robustly as SB203580 (measured by hyperosmolarity-stimulated MK-2 phosphorylation) yet had no effect on insulin-stimulated glucose uptake (data not shown). Thus, the most likely interpretation of the collective results is that p38MAPK is not involved in insulin-stimulated glucose uptake and that the SB203580-mediated reduction in glucose uptake is due to an effect of SB203580 on another protein.

SB203580 binds to the ATP pocket of p38α and p38β (52) with a 1:1 stoichiometry. The specificity of several pyridinyl imidazoles has been tested in many studies, and the *in vitro* IC_{50} values range from 50 to 500 nM for p38α and p38β (31, 53), whereas p38γ (21) and p38δ (22) are insensitive to these inhibitors. In addition, the *in vivo* specificity of SB203580 has been validated in several systems using the DR-p38α mutant used in this study (11, 32, 49, 50). Notably, however, selective outcomes were shown to be independent of participation of p38MAPK (50). Moreover, very recently, Godl *et al.* (54) detected several previously unknown targets of SB203580 in HeLa cells. Cell extracts from these cells were screened for proteins binding to SB203580 using affinity chromatography coupled to mass spectrometry. Several proteins not previously known to be targets of SB203580 were identified, some with IC_{50} values similar to or lower than p38MAPK, including rip-like interacting caspase-like apoptosis-regulatory protein kinase, CK1, cyclin G-associated kinase, protein kinase Nβ, Janus kinase 1, and c-Jun N-terminal kinase (JNK)1/2. Indeed, under certain *in vitro* reaction conditions, the IC_{50} of JNK1 and JNK2 can be 13 and 0.7 μM, respectively, making SB203580 a much more potent inhibitor of JNK1/2 than previously reported. However, the JNK inhibitor SP600125 had virtually no effect on insulin-stimulated glucose uptake in L6 myotubes (Niu, W., and A. Klip, unpublished observations), rat neonatal myotubes (55), or primary rat adipocytes (56). Therefore, the possibility that SB203580 inhibited insulin-stimulated glucose uptake and GLUT4 ac-

tivation via protein(s) other than p38MAPK and JNK seems possible, and future work seeking to identify such protein(s) would be of great interest. In support of this interpretation, Kayali *et al.* (29) observed discrepancies between the dose of SB203580 required to reduce insulin-stimulated glucose uptake in 3T3-L1 adipocytes and the EC_{50} of inhibition of activating transcription factor-2 activity recovered *in vitro*.

Alternatively, it is also plausible that the SB203580-mediated inhibition of insulin-stimulated glucose uptake is due to direct inhibition of GLUT4 by the compound. However, this is unlikely because SB203580 has no effect on basal glucose uptake (15, 23), which is mediated by GLUT4 in L6-GLUT4^{myc} myotubes (2). Moreover, in our experimental protocol, SB203580 is washed away before the assay of glucose uptake. However, difficulties in removing the compound from cells has been described (33), raising the possibility that some quantity of SB203580 is still present in the cells at the time of measurement of glucose transport. Holman and colleagues (33) recently suggested that SB203580 may interact with insulin-stimulated GLUT4 based on its competitive action on equilibrium-exchange glucose uptake in insulin-stimulated rat adipocytes. Such an effect required loading of the cells with SB203580 and presence of the compound during the transport assay. Those results are compatible with either SB203580 acting on a target that reduces GLUT4 affinity of the intracellular glucose-binding site (such as an endogenous inhibitory protein) or SB203580 selectively binding to and directly inhibiting the insulin-stimulated configuration of GLUT4. In either case, SB203580 would diminish glucose uptake through GLUT4 in the insulin-stimulated but not the basal state.

To better understand the nature of the SB203580-mediated reduction in glucose uptake, we examined the effect of SB203580 on photoaffinity labeling of GLUT4^{myc} by bio-LC-ATB-BMPA (Fig. 8). As for glucose uptake, SB203580 reduced ATB-BMPA photolabeling of GLUT4 in insulin-stimulated but not basal conditions. Because SB203580 does not reduce the amount of cell surface transporters detected via the *myc* epitope (Fig. 1), the drug appears to reduce the accessibility of the extracellular glucose-binding site of cell surface GLUT4^{myc}. During the preparation of this manuscript, Bazuine *et al.* (57) observed a similar effect of SB203580 on ATB-BMPA photolabeling of GLUT4^{myc} in insulin-stimulated 3T3-L1 adipocytes. This reduction is likely not due to SB203580 binding to the extracellular hexose-binding site of GLUT4 because the drug inhibits hexose influx noncompetitively (58). Instead, the SB203580-dependent reduction in GLUT4^{myc} photolabeling at the extracellular site could be envisaged to occur concomitantly with the decreased affinity of the intracellular glucose binding site, potentially even as a consequence of it. Because insulin does not affect the Michaelis constant of GLUT4 for hexose entry, these results also suggest that stimulation with insulin may alter the rate of turnover of GLUT4 between outward- and inward-facing conformations.

In summary, pretreatment of L6-GLUT4^{myc} myotubes with the pyridinyl imidazole SB203580 inhibited insulin-stimulated glucose uptake without decreasing GLUT4 translocation. The expression of DR-p38α did not rescue the SB203580-mediated inhibition of glucose uptake, and reduction of endogenous p38α and p38β activities by either dominant-negative mutants of p38α and p38β or siRNA-mediated gene silencing had no effect on insulin-stimulated glucose uptake. These results support the hypothesis that insulin-mediated glucose uptake and GLUT4 translocation can be segregated and that a p38α/β-independent, SB203580-sensitive target or GLUT4 conformation may be required for insulin-stimulated activation of GLUT4 but not for GLUT4 translocation.

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