Sustained Exposure of L6 Myotubes to High Glucose and Insulin Decreases Insulin-Stimulated GLUT4 Translocation but Upregulates GLUT4 Activity

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Hyperglycemia and hyperinsulinemia are cardinal features of acquired insulin resistance. In adipose cell cultures, high glucose and insulin cause insulin resistance of glucose uptake, but because of altered GLUT4 expression and contribution of GLUT1 to glucose uptake, the basis of insulin resistance could not be ascertained. Here we show that GLUT4 determines glucose uptake in L6 myotubes stably overexpressing myc-tagged GLUT4. Preincubation for 24 h with high glucose and insulin (high Glc/Ins) reduced insulin-stimulated GLUT4 translocation by 50%, without affecting GLUT4 expression. Insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation, phosphatidylinositol 3-kinase activation, and Akt phosphorylation also diminished, as did insulin-mediated glucose uptake. However, basal glucose uptake rose by 40% without any gain in surface GLUT4. High Glc/Ins elevated basal p38 mitogen-activated protein kinase (MAPK) phosphorylation and activity, and a short inhibition of p38 MAPK with SB202190 corrected the rise in basal glucose uptake, suggesting that p38 MAPK activity contributes to this rise. We propose that in a cellular model of skeletal muscle, chronic exposure to high Glc/Ins reduced the acute, insulin-elicited GLUT4 translocation. In addition, basal state GLUT4 activity was augmented to partially compensate for the translocation defect, resulting in a more robust glucose uptake than what would be predicted from the amount of cell surface GLUT4 alone.
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In type 2 diabetes, there is a failure to increase glucose disposal into peripheral tissues in response to insulin, leading to chronically elevated levels of glucose in the circulation followed by a compensatory rise in insulin (1). The elevated glucose and insulin levels in turn exacerbate insulin resistance, contributing significantly to the pathogenesis of the disease (2). Attempts have been made in adipose cells in culture, to dissect out the mechanisms leading to this impaired insulin response of target tissue, using a high glucose and insulin model. In 3T3-L1 adipocytes and primary cultured rat adipocytes, 24- to 48-h pretreatment with high glucose and insulin resulted in decreases in insulin-stimulated glucose uptake, GLUT4 translocation to the cell surface, insulin receptor substrate (IRS)-1 tyrosine phosphorylation, and phosphatidylinositol (PI) 3-kinase activity (3–6). It is difficult to ascribe the insulin resistance to a single mechanism, because GLUT4 levels dropped in some of those studies, and GLUT1 also contributes to glucose uptake in 3T3-L1 adipocytes.

Although GLUT4 levels diminish in adipose cells of diabetic animals and humans, they are not altered in skeletal muscle (7–9). Therefore, the mechanisms leading to acquired insulin resistance may differ in muscle and fat tissues. To date, there are no studies of acquired insulin resistance in skeletal muscle cells that analyze the different steps governing glucose uptake. The objective of the present study was to analyze the effect of high glucose and insulin on GLUT4 translocation, glucose uptake, and the insulin signaling pathway, in a muscle cell line where GLUT4 is the predominant transporter. L6 myotubes overexpressing myc-tagged GLUT4 offer these possibilities, as well as afford the opportunity to compare GLUT4 translocation with glucose uptake in intact cell preparations, as required to assess possible changes in GLUT4 activity. Indeed, several studies suggest that GLUT4 activity may be regulated (10–15). Depending on the experimental technique used to measure GLUT4 translocation, the fold increase in translocation matches to different extents the fold increase in glucose uptake (10–12). In addition, several conditions lead to a dissociation of GLUT4 translocation from stimulation of glucose uptake (13–15). Recently, we demonstrated that SB203580, a selective inhibitor of p38 mitogen-activated protein kinase (MAPK) (16), decreased insulin-stimulated glucose uptake in rat skeletal muscle, L6 myotubes, and 3T3-L1 adipocytes without reducing GLUT4 translocation (17,18), raising the possibility that p38 MAPK can regulate the intrinsic activity of GLUT4. It is currently unknown whether GLUT4 activity or p38 MAPK is altered in insulin resistance. We report that 24-h pretreatment with 25 mmol/l glucose and 100 nmol/l insulin (high Glc/Ins) of L6-GLUT4myc myotubes activates p38 MAPK and stimulates basal-state glucose uptake mediated by GLUT4myc, without increasing cell surface GLUT4myc levels. The pretreatment also

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ATF, activating transcription factor; Glc/Ins, glucose/insulin; IC_{50}, half-maximal inhibitory concentration; IR, insulin receptor; IRS, insulin receptor substrate; JNK, Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; OPD, *o*-phenylenediamine dihydrochloride; PI, phosphatidylinositol.

causes a reduction in insulin-dependent GLUT4myc translocation and in the IRS-1–phosphatidylinositol 3-kinase–Akt pathway. The elevation in basal glucose uptake may be an adaptive change developed to counteract the defect in GLUT4 translocation.

RESEARCH DESIGN AND METHODS

Materials. Anti-GLUT1 and anti-GLUT4 antisera were raised in rabbits to peptides encompassing 12 COOH-terminal residues of each protein (19); anti-GLUT3 was generated using the 19 COOH-terminal peptides (20). Anti-p38 MAPK, anti-insulin receptor β (C-19), anti-myc (9E10) antibodies, and anti-p85 subunit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antibodies to p38 MAPK, Jun NH_2-terminal kinase (JNK), and activating transcription factor (ATF)-2 fusion protein were from New England Biolabs (Mississauga, ON, Canada). Anti-phosphotyrosine and anti–IRS-1 antibodies were from Upstate Biotechnology (Lake Placid, NY). *o*-Phenylenediamine dihydrochloride (OPD reagent) was from Sigma. SB202190 and blasticidin-HCl were from Calbiochem (La Jolla, CA). Indinavir was a gift from Dr. Ralph Germinario (Sir Mortimer B. Davis Jewish General Hospital, Montreal, Canada) and Dr. Nava Bashan (Ben-Gurian University, Béer-Sheva, Israel).

L6-GLUT1myc cells, L6-GLUT4myc cells, cell culture, and incubations. L6 muscle cells stably expressing myc-tagged GLUT4myc (L6-GLUT4myc cells) were described previously (21,22). L6 stably expressing GLUT1myc were prepared by cotransfection of L6 myoblasts with pCX2-GLUT1myc and pSV2-bsr, a blasticidin S deaminase expression plasmid, and selected with blasticidin-HCl. Glass cloning cylinders (Bellco Glass, Vineland, NJ) were used to select individual colonies for expansion. Each clonal line was maintained in 2 μg/ml blasticidin-HCl and tested for GLUT1myc expression and the ability to differentiate into myotubes. GLUT1 or GLUT4-myc myoblasts were differentiated into myotubes, pretreated with 25 mmol/l glucose and 100 nmol/l insulin (high Glc/Ins) for 24 h, and deprived of serum for 5 h in 5 mmol/l glucose before incubation with inhibitors, followed by acute insulin challenge. Inhibitors were administered in DMSO, with maximum concentration of 0.1% vol/vol in the incubation medium and without effect on the parameters measured.

Determination of 2-deoxyglucose uptake. 2-Deoxyglucose uptake measurements were carried out as described previously (18) for 5 min in HEPES-buffered saline containing 10 μmol/l 2-[^{3}H]deoxyglucose (0.5 μCi/ml) in the absence of insulin and inhibitors, unless otherwise indicated. Nonspecific uptake was determined in the presence of 10 μmol/l cytochalasin B and subtracted from all experimental values.

Measurement of GLUT4myc translocation in L6 myotubes. The amount of myc-tagged GLUT4 at the surface of intact cells was measured by an antibody-coupled colorimetric assay as validated previously (23). Briefly, monolayer of myotubes were exposed to anti-myc antibody (1:100) for 60 min, fixed with 4% paraformaldehyde for 10 min, and then incubated with peroxidase-conjugated donkey anti-mouse IgG (1:1,000) for 30 min, all at 4°C. Cells were washed extensively, and 1 ml of OPD reagent was added for 30 min at room temperature. The reaction was stopped by 0.25 ml of 3N HCl. The supernatant was collected, and the absorbance was measured at 492 nm. Nonspecific IgG binding, as measured by a peroxidase-conjugated anti-mouse IgG, was subtracted from experimental values.

Detection of total cellular p38 MAPK, protein phosphorylation, and assay of p38 MAPK activity. Total p38 MAPK protein and p38 MAPK phosphorylation and activity were measured as previously described (18) with the following modifications. For p38 MAPK activity, monoclonal mouse anti–phospho-p38 MAPK antibody covalently linked to Sepharose beads (New England Biolab) (20 μl/condition) was used to immunoprecipitate activated p38 MAPK from 200 μg total cell lysate. The complex was washed four times with 1 ml wash buffer and twice with 1 ml kinase buffer and then incubated under constant agitation for 30 min at 30°C with 30 μl reaction mixture (kinase buffer containing 200 μmol/l ATP and 2 μg recombinant ATF-2). Reaction was stopped by 50 μl of 2× Laemmli sample buffer, and supernatant was resolved by 10% SDS-PAGE. Phospho-ATF-2 was determined by immunoblotting with anti–phospho-ATF-2 antibody at 1:500 dilution.

Detection of total cellular GLUT1, GLUT3, and GLUT4 proteins. L6-GLUT4myc myoblasts were differentiated into myotubes in 10-cm plates, and total cellular membranes were prepared as described previously (24). Polyclonal primary antibody was used at 1:2,000 dilution.

Detection of insulin receptor and IRS-1 expression and phosphorylation and IRS-1–associated p85. Acute insulin stimulation (100 nmol/l for 5 min at 37°C) was followed by cell lysis, and whole-cell extracts were prepared from myotubes as described (18,25). Insulin receptor (IR) was immunoprecipitated from 500 μg protein, and the sample was boiled for 3 min before resolving the protein by 7.5% SDS-PAGE. IRS-1 was immunoprecipitated from 300 μg protein, and the immunoprecipitates were resolved by 7.5% SDS-PAGE. Tyrosine phosphorylation of IR and IRS-1 and the association of p85 with IRS-1 were determined by immunoblotting with anti-phosphotyrosine or anti-p85 antibodies at 1:2,000, 1:1,000, and 1:1,000 dilutions, respectively. Total IRS-1 content was determined in whole cell lysates, prepared by the same method as described above for detection of total cellular p38 MAPK.

PI 3-kinase activity assay. PI 3-kinase activity associated with phosphotyrosine immunoprecipitates was measured in vitro toward phosphatidylinositol as described previously (24)

Statistical analysis. Statistical analysis was performed using either unpaired Student's *t* test or ANOVA (Fisher, multiple comparisons), as applicable.

RESULTS

Exposure of L6-GLUT4myc myotubes to high Glc/Ins increased basal glucose uptake but decreased the acute insulin-mediated glucose uptake. We first examined whether muscle cells were susceptible to the effect of preincubation with high glucose and insulin. L6-GLUT4myc myotubes were incubated for 24 h with 25 mmol/l glucose and 100 nmol/l insulin and then for 5 h in serum-free medium (5 mmol/l glucose). Subsequently, the rates of hexose uptake in the basal state and after an acute (30-min) insulin challenge were determined (Fig. 1*A*). High Glc/Ins pretreatment increased basal 2-deoxyglucose uptake by 40% (control, 25.2 ± 1.7; high Glc/Ins, 34.9 ± 1.3 pmol · min^{-1} · mg^{-1} protein; $P < 0.005$). In control cells, acute insulin stimulation elevated 2-deoxyglucose uptake by 2.3-fold (basal, 25.2 ± 1.7; insulin, 58.7 ± 4.9 pmol · min^{-1} · mg^{-1} protein; Δ, 33.5 pmol · min^{-1} · mg^{-1} protein; $P = 0.0002$). In contrast, myotubes that were pretreated with high Glc/Ins for 24 h had a significantly smaller response to the acute insulin challenge, showing only a 1.6-fold increase in 2-deoxyglucose uptake (basal, 34.9 ± 1.3; insulin, 56.9 ± 3.1 pmol · min^{-1} · mg^{-1} protein; Δ, 22.0 pmol · min^{-1} · mg^{-1} protein; $P = 0.0002$).

Exposure of L6-GLUT4myc myotubes to high Glc/Ins decreased the acute insulin-mediated GLUT4 translocation. To elucidate the mechanism by which high Glc/Ins pretreatment altered glucose uptake, we measured surface GLUT4 levels under the various treatments. L6-GLUT4myc cells stably express GLUT4 tagged with an exofacial myc epitope (GLUT4myc). GLUT4myc segregates, cycles, and responds to insulin in a manner similar to endogenous GLUT4 (21–23). The amount of GLUT4myc incorporated into the plasma membrane was quantitated by immunologically labeling the myc epitope at the surface of intact cells. Acute insulin stimulation of untreated cells increased the amount of GLUT4myc at the cell surface by 2.3 ± 0.2-fold above basal ($P = 0.01$) (Fig. 1*B*). Preincubation of cells for 24 h with high Glc/Ins caused a 45% reduction in acute insulin-induced recruitment of GLUT4myc to the plasma membrane (basal, 0.9 ± 0.1; insulin, 1.7 ± 0.17; $P < 0.005$). However, high Glc/Ins treatment did not alter surface GLUT4myc under basal conditions (Fig. 1*B*), despite increasing 2-deoxyglucose uptake by 40% (Fig. 1*A*).

Total cellular content of GLUT1 but not GLUT4 was increased by chronic pretreatment with high Glc/Ins. In Fig. 1, we show that high Glc/Ins pretreatment caused a significant elevation of basal glucose uptake without increasing GLUT4myc translocation. These results suggest that GLUT4 may be hyperactive under these conditions or that upregulation of another transporter may account for

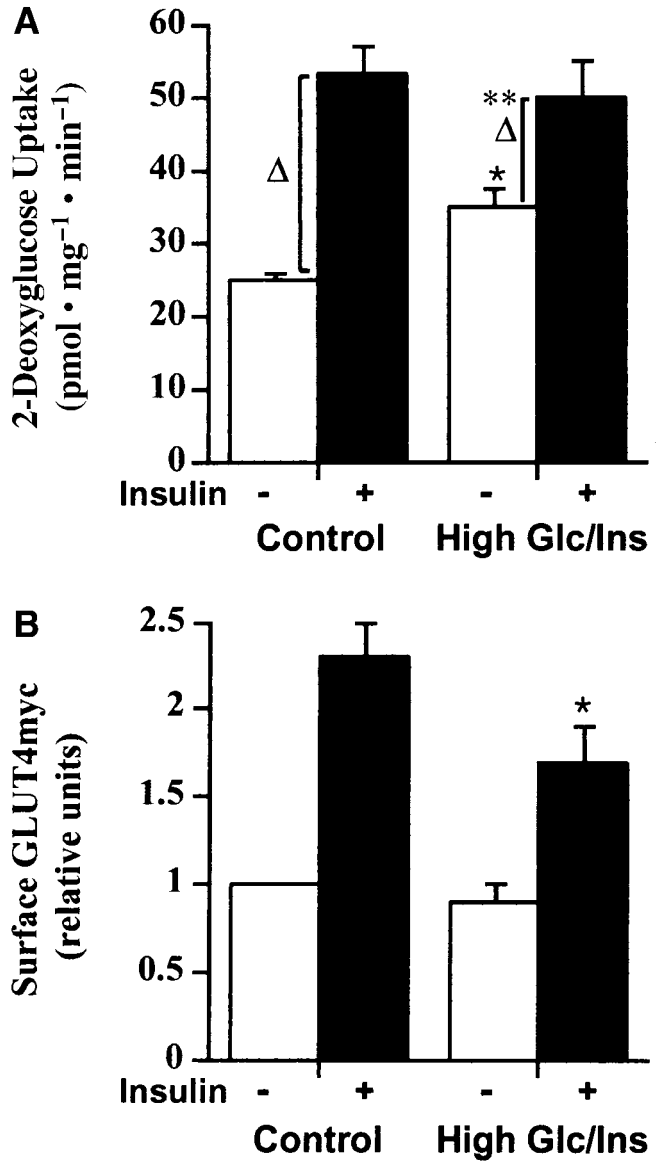

FIG. 1. Effect of chronic exposure of L6-GLUT4myc myotubes to high Glc/Ins on glucose uptake and GLUT4 translocation. Myotubes were incubated for 24 h in growth medium supplemented with 25 mmol/l glucose and 100 nmol/l insulin. Cells were depleted of insulin and serum in medium containing 5 mmol/l glucose for 5 h and then left untreated or stimulated for 30 min with 100 nmol/l insulin, followed by assessment of 2-deoxyglucose uptake (*A*) and cell surface GLUT4myc levels (*B*) in intact cells. Nonspecific antibody binding as measured by anti-mouse IgG alone was subtracted from all experimental values and then the amount of GLUT4myc at cell surface under each condition was expressed relative to that of control cells under basal conditions. Results are means ± SE of five experiments in which each condition was assayed in quadruplicate. *$P < 0.005$ vs. respective controls. **$P < 0.005$ vs. control cells. Δ, difference between control and acute insulin stimulation; □, basal; ■, acute insulin (100 nmol/l for 30 min).

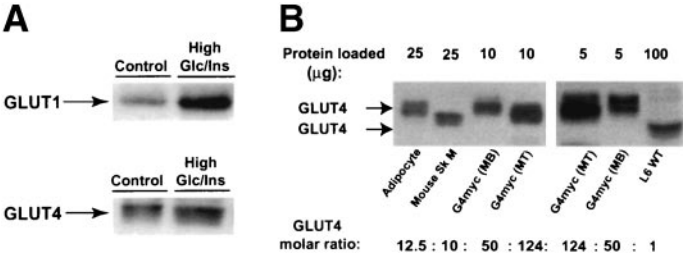

FIG. 2. In L6-GLUT4myc myotubes, GLUT4 is the predominant glucose transporter. *A*: L6-GLUT4myc myotubes were incubated in high Glc/Ins. Total cellular membranes were prepared, and 10 μg protein was immunoblotted for GLUT1 or GLUT4. A representative immunoblot of three separate experiments is shown. The levels of GLUT1 and GLUT4 cannot be directly compared because of the differences in antibody immunoreactivity. *B*: Total cellular membranes were prepared from 3T3-L1 adipocytes, mouse skeletal muscle, L6-GLUT4myc myoblasts (MB), L6-GLUT4myc myotubes (MT), and L6 parental myotubes (WT). Protein (5, 10, 25, or 100 μg, as indicated) was immunoblotted for GLUT4. The difference in amount of protein loaded was necessary to allow for quantitative comparison of GLUT4 in the parental L6 myotubes with GLUT4myc in the transfected cell line on the same SDS-PAGE. Using the FluorChem 8000 imaging system (Alpha Innotech), the optical density of each chemiluminescent protein band was measured within the linear range. The optical density relative to that obtained for L6 wild-type myocytes was then adjusted according to the amount of protein loaded on the gel to give the molar ratios of GLUT4 listed below the gel images. The lesser migration of GLUT4 in 3T3-L1 adipocytes in comparison to myocytes (26) and that of myoblasts in comparison to myotubes (27) have been previously reported.

the increased glucose uptake. To assess these possibilities, GLUT1 and GLUT4 levels were determined in total membranes isolated from L6-GLUT4myc myotubes pretreated with high Glc/Ins for 24 h. Total GLUT1 content was increased by 2.5 ± 0.5-fold ($P < 0.05$) (Fig. 2*A*). In contrast, high Glc/Ins pretreatment did not alter total GLUT4 content (Fig. 2*A*).

GLUT1 does not contribute to the increase in basal glucose uptake induced by high Glc/Ins. Because of the rise in GLUT1 protein, it was important to compare the level of GLUT4myc to that of the endogenous GLUT1. In parental L6 myotubes, the molar ratio of surface GLUT1 to GLUT4 is 1.0:0.8, as determined by quantitative photolabeling using azi-trifluroethylbenzoyl-bis-mannose-yloxypropylamine (ATB-BMPA) (28). In L6-GLUT4myc myotubes, the myc-tagged transporter is significantly overexpressed relative to the endogenous GLUT4. Figure 2*B* shows the molar ratio of GLUT4myc to that of GLUT4 in other cells and tissues expressing this transporter. GLUT1 expression is the same in GLUT4myc cells as in parental L6 cells. Thus, in L6-GLUT4myc cells, the ratio of GLUT4myc to GLUT4 to GLUT1 is 124:1:1.2. Hence, GLUT4myc is vastly the predominant glucose transporter in L6-GLUT4myc myotubes, and it is likely to be the transporter responsible for the increase in glucose uptake caused by high Glc/Ins (Fig. 1*A*). To further support this hypothesis, we used indinavir, a protease inhibitor that was shown to inhibit GLUT4 but not GLUT1 transporters directly (29,30). Figure 3*A* shows that when indinavir was present during the 2-deoxyglucose transport assay, there was a dose-dependent inhibition of 2-deoxyglucose uptake in the GLUT4myc but not in the GLUT1myc myotubes; a similar dose-response curve was observed after the cells were pretreated with high Glc/Ins. Taken together, these results suggest that GLUT4myc is the predominant glucose transporter in these cells responsible for glucose influx. Moreover, the high Glc/Ins effect is via GLUT4.

It was then of interest to explore whether GLUT1 activity is similarly regulated in cells where GLUT1 is the predominant route for glucose uptake. To this end, we used L6 muscle cells overexpressing GLUT1myc, where GLUT1myc expression is controlled by a constitutively active cytomegalovirus promoter, and therefore its levels are unlikely to change biosynthetically with high Glc/Ins. High Glc/Ins preincubation did not elevate the basal level of glucose uptake in L6-GLUT1myc myotubes (control, 20.6; high Glc/Ins, 21.3 pmol · min^{-1} · mg^{-1} protein) (Fig.

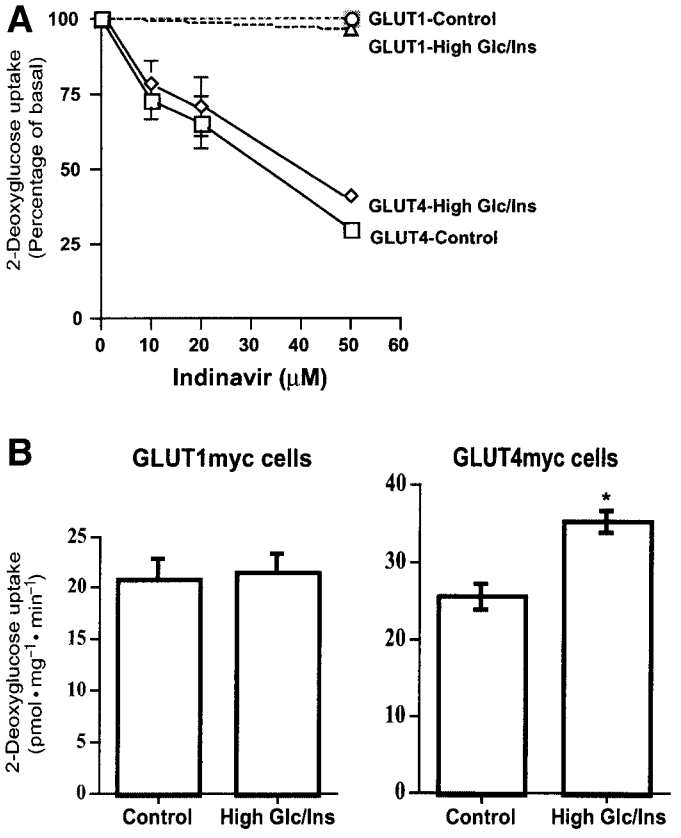

FIG. 3. GLUT4 but not GLUT1 mediates the increased basal glucose uptake in L6-GLUT4myc myotubes induced by high Glc/Ins. *A*: 2-Deoxyglucose uptake was measured in both control and high Glc/Ins–pretreated L6-GLUT4myc and L6-GLUT1myc cells, with 0–50 μmol/l indinavir present during the transport assay only. 2-Deoxyglucose uptake results are expressed as a percentage of the value obtained in the absence of indinavir. There was no statistical difference in the results between control and high Glc/Ins–pretreated cells within each cell type. *B*: L6-GLUT1myc myotubes were pretreated with high Glc/Ins for 24 h, and 2-deoxyglucose uptake was assessed. Results obtained in parallel using L6-GLUT4myc myotubes are presented for comparison. Results are means ± SE of five experiments in which each condition was assayed in quadruplicate. **P* < 0.005 vs. controls.

3*B*), suggesting that GLUT1 activity cannot be upregulated by high Glc/Ins. Collectively, these results highlight that functionally, GLUT4myc is responsible for both basal and acute insulin-stimulated glucose uptake in L6-GLUT4myc myotubes, and the basal state activity of GLUT4, but not of GLUT1, increases upon preincubation of myotubes with high Glc/Ins.

SB202190 restored basal glucose uptake in L6-GLUT4myc myotubes pretreated with high Glc/Ins to control levels. We have previously reported that an acute insulin challenge increases the activity of translocated GLUT4, an effect that is reduced by pretreating cells with p38 MAPK inhibitors (17). Therefore, we hypothesized that the activity of p38 MAPK contributes to the elevated basal glucose uptake observed following high Glc/Ins pretreatment. L6-GLUT4myc myotubes that were pretreated for 24 h with high Glc/Ins were incubated with an inhibitor of p38 MAPK for 20 min before assaying glucose uptake. Figure 4 shows that SB202190 restored the basal rate of glucose uptake in cells pretreated with high Glc/Ins, suggesting that p38 MAPK may regulate the basal state activity of GLUT4 in L6-GLUT4myc myotubes preincubated with high Glc/Ins.

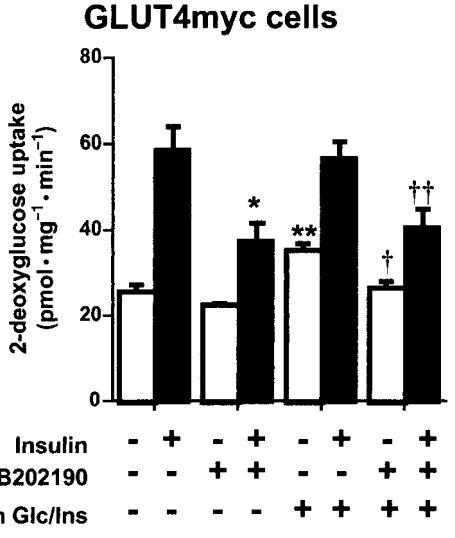

FIG. 4. SB202190 restored basal glucose uptake to control levels in L6-GLUT4myc myotubes pretreated with high Glc/Ins. Cells were incubated for 24 h in growth medium supplemented with 25 mmol/l glucose and 100 nmol/l insulin and then depleted of serum and insulin for 5 h and left untreated or treated with 10 μmol/l SB202190 for 50 min. Insulin (100 nmol/l) was added during the last 30 min of this incubation. 2-Deoxyglucose uptake was determined in the absence of SB202190. Results are means ± SE of four experiments in which each condition was assayed in quadruplicate. **P* < 0.01 vs. acute insulin without SB 202190. ***P* = 0.05 vs. control. †*P* = 0.05 vs. basal high Glc/Ins pretreatment. ††*P* = 0.001 vs. high Glc/Ins followed by acute insulin but without SB202190. □, basal; ■, acute insulin.

Insulin-activated but not basal p38 MAPK phosphorylation is attenuated by preexposure of L6-GLUT4myc myotubes to high Glc/Ins. The abrogation by SB202190 of the high Glc/Ins–induced elevation in basal glucose uptake in GLUT4myc myotubes suggested the participation of p38 MAPK. Hence, we determined the effect of high Glc/Ins on p38 MAPK phosphorylation and kinase activity in these cells. Activation of p38 MAPK by cytokines (31) and insulin (17) correlates with the dual phosphorylation of the enzyme on threonine 180 and tyrosine 182. Using antibodies that recognize p38 MAPK only when phosphorylated on both of these residues, pretreatment of myotubes with high Glc/Ins elevated basal p38 MAPK phosphorylation by 80% ($P < 0.05$) (Fig. 5*A*). The cellular content of p38 MAPK was also elevated by 25% by the high Glc/Ins pretreatment ($P < 0.05$, Fig. 5*B*).

We then determined the effect of high Glc/Ins treatment on p38 MAPK protein kinase activity, using an in vitro kinase assay. Consistent with the results of p38 MAPK phosphorylation, preincubation of cells with high Glc/Ins for 24 h increased basal p38 MAPK activity by 2.3 ± 0.3-fold ($P < 0.05$) (Fig. 5*C*). We also measured phosphorylation of JNK, another stress-activated MAPK, to determine if the effects of high Glc/Ins were specific for p38 MAPK. Treatment with high glucose and insulin for 24 h did not increase basal JNK phosphorylation (data not shown).

Chronic pretreatment with high Glc/Ins decreased the acute insulin-mediated tyrosine phosphorylation of insulin receptor, IRS-1, phosphotyrosine-associated PI 3-kinase activity, and Akt phosphorylation. We demonstrate in Fig. 1 that pretreatment of myotubes with high Glc/Ins reduced subsequent insulin-stimulated GLUT4 translocation by 45%. This result could arise from

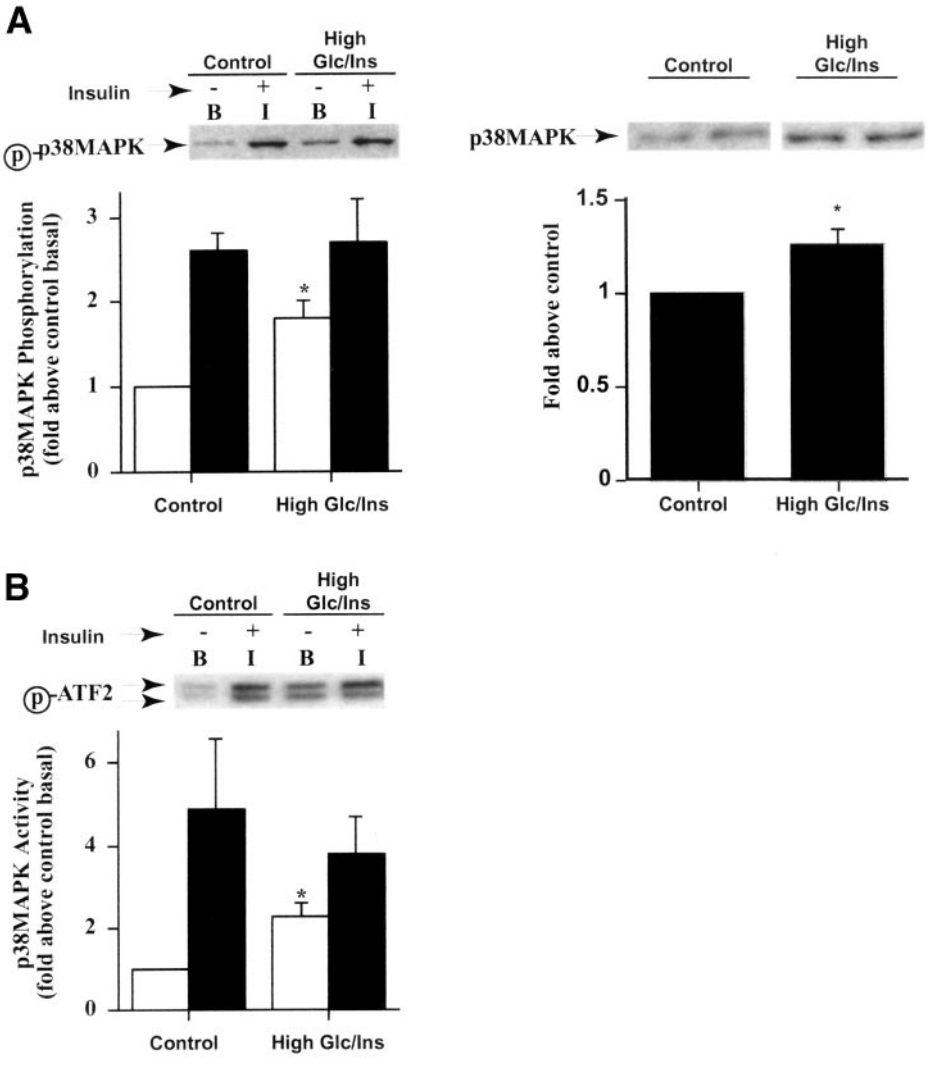

FIG. 5. High Glc/Ins elevates basal, but attenuates insulin-stimulated, p38MAPK phosphorylation and kinase activity. L6-GLUT4myc myotubes were incubated for 24 h in growth medium supplemented with 25 mmol/l glucose and 100 nmol/l insulin. Cells were depleted of serum and insulin for 5 h, then total cell lysates were prepared in Laemmli sample buffer, and 40 μg of protein was immunoblotted for phospho-p38 MAPK (*A*) and p38 MAPK (*B*). *C*: Cell lysate (300 μg) was immunoprecipitated with a monoclonal phospho-p38 MAPK antibody, followed by an in vitro kinase using ATF-2 fusion protein as the substrate. Results are means ± SE of three experiments. **P* < 0.05 vs. respective controls. □, basal; ■, acute insulin.

alterations in the signals thought to regulate GLUT4 translocation or from defects in the fusion machinery at the plasma membrane. Hence, we analyzed the effect of high Glc/Ins on tyrosine phosphorylation of the IR, IRS-1, activation of PI 3-kinase activity, and phosphorylation of Akt/protein kinase B. High Glc/Ins pretreatment of L6-GLUT4myc myotubes for 24 h led to a 38% and a 35% reduction in insulin receptor protein content (as determined by immunoprecipitation from cell lysate) and insulin-stimulated (5 min) tyrosine phosphorylation of insulin receptor ($P = 0.002$ and 0.003), respectively (Fig. 6*A*). Insulin stimulation (5 min) increased IRS-1 phosphorylation by fourfold in control cells ($P < 0.005$) (Fig. 6*B*), which was diminished by 65% after 24 h of high Glc/Ins pretreatment ($P < 0.005$). Concomitantly, there was a 50% ($P < 0.005$) reduction in total cellular IRS-1 protein (Fig. 6*B*). High Glc/Ins for 24 h also reduced IRS-1–associated p85 by 80% (Fig. 6*C*). Similarly, PI 3-kinase activity associated with anti-phosphotyrosine immunoprecipitates was also reduced by 40% in pretreated cells (Fig. 6*C*). As expected, acute insulin caused a robust phosphorylation of Akt at Thr 308 and Ser 473 in control cells, which was reduced by 60% and 50%, respectively, after 24-h preincubation with high Glc/Ins (Fig. 6*D*). Therefore, starting at the level of IR, we observed a downregulation to acute insulin effect on IR, IRS-1 phosphorylation, phosphotyrosine associated PI 3-kinase activity, and Akt phosphorylation.

DISCUSSION

Insulin resistance is a key feature of type 2 diabetes. In particular, it has been argued that elevated levels of glucose and insulin are a major cause for the development of secondary insulin resistance, but the molecular mechanisms remain obscure (32). Therefore, establishment of in vitro models of high glucose and insulin state has been pursued to allow understanding of the molecular basis of acquired insulin resistance. Previous studies in 3T3-L1 adipocytes and primary cultured adipocytes have shown

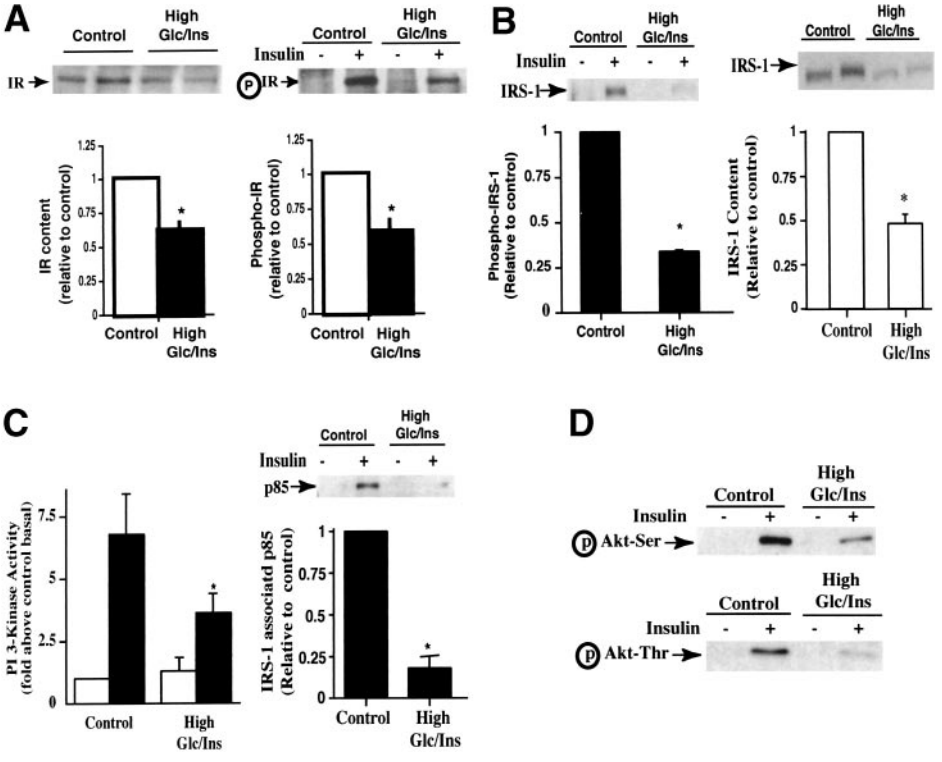

FIG. 6. Exposure of L6-GLUT4myc myotubes to high Glc/Ins decreases the acute insulin-mediated tyrosine phosphorylation of IR, IRS-1, IRS-1–associated p85, phosphotyrosine-associated PI 3-kinase activity, and Akt phosphorylation. L6-GLUT4myc myotubes were incubated for 24 h in control or high Glc/Ins medium. IR and IRS-1 were immunoprecipitated from 500 and 300 μg total cell lysate, respectively, and immunoblotted for IR and phospho-IR (*A*) and IRS-1–associated p85 (*C*). Total cell lysate (40 μg) was immunoblotted for IRS-1 (*B*) or phospho-Akt (*D*). *C*: In vitro phosphotyrosine-associated PI 3-kinase activity was measured in cell lysates as described in RESEARCH DESIGN AND METHODS. Results are expressed relative to the control cells, assigned a value of 1. Results are the means ± SE of three to four experiments. *$P < 0.005$ vs. control. □, basal; ■, acute insulin (100 nmol/l for 5–10 min).

that prolonged exposure to high concentrations of insulin and glucose resulted in increased basal glucose uptake and a decrease in acute insulin-mediated glucose transport, the latter attributed to reduced insulin-stimulated GLUT4 translocation (3–6,33). However, it remained possible that the insulin-resistant state may also be associated with diminished GLUT4 activity.

To our knowledge, there are no comprehensive accounts of the basis of acquired insulin resistance in muscle cells using a high glucose and insulin model, yet muscle tissue is a primary determinant of glycemic control in vivo. The aim of this study was to examine the alterations in signaling pathways regulating GLUT4 activity and GLUT4 translocation when a cell culture of muscle origin was exposed to sustained high levels of glucose and insulin. A muscle cell line where GLUT4 is the predominant transporter, L6 myotubes overexpressing myc-tagged GLUT4, offers the opportunity to compare GLUT4 translocation with glucose uptake in intact cell preparations, allowing us to assess possible changes in GLUT4 activity.

High Glc/Ins induces a GLUT4 translocation defect. We demonstrated in this study that 24 h of high glucose and insulin reduced insulin-stimulated GLUT4 translocation, with no significant change in cell surface GLUT4 at the basal state. This is consistent with previous reports of 50–100% reduction in GLUT4 translocation in 3T3-L1 adipocytes under similar conditions (4–6,33). To understand the mechanism of the translocation defect, we examined IR and IRS-1 phosphorylation, PI 3-kinase activity, and Akt phosphorylation. We found a 35% reduction in immunoprecipitable insulin receptor after 24 h of high insulin and glucose pretreatment in L6-GLUT4myc myotubes. The insulin-stimulated tyrosine phosphorylation of the receptor was similarly decreased by 35%. Similar observations have been made in insulin-resistant and diabetic humans (34,35). In agreement with Ricort et al. (5), we found that high Glc/Ins treatment also resulted in a significant reduction in insulin-stimulated IRS-1 tyrosine phosphorylation and total IRS-1 content. Reduced IRS-1 tyrosine phosphorylation has been reported in skeletal muscle of insulin-resistant Zucker rats (36) and in humans with type 2 diabetes (37,38) and obesity (39). Increased degradation of IRS-1 under chronic insulin treatment is one mechanism underlying insulin resistance (40). In our cellular model, a slight discrepancy between the percentage reduction of IRS-1 tyrosine phosphorylation (65% reduction) and reduction of IRS-1 content (50%) was observed. There are two possible explanations: first, the reduction in IRS-1 content was compounded with a reduction in IR phosphorylation and therefore activity, leading to a more significant reduction in IRS-1 phosphorylation than expected from the decrease in IRS-1 content. Second, there may be a separate defect in the insulin signaling pathway such that it impaired the tyrosine phosphorylation of the remaining IRS-1.

With the reduction in IRS-1 content and its phosphorylation after chronic exposure to high Glc/Ins, we predicted and observed a reduction in the acute insulin-dependent activation of PI 3-kinase. We also observed a 60% reduction in insulin-mediated Akt phosphorylation at threonine

308 and serine 473. Therefore, the defect in translocation may be explained in part by the alterations in signal transduction from IR to IRS-1 and then to PI 3-kinase and Akt, resulting in reduced GLUT4 translocation. In contrast, the reduction in GLUT4 translocation caused by high insulin and glucose using 3T3-L1 adipocytes was associated with reduced levels of total cellular GLUT4 (6,33).

GLUT4 is the determinant glucose transporter affected by high Glc/Ins. In L6-GLUT4myc myotubes pretreated with high Glc/Ins, there was an increase in total cellular GLUT1 content, which could potentially account for the increase in basal glucose uptake. Indinavir, an HIV protease inhibitor, has been associated with insulin resistance in humans and animals (29,30). Using *Xenopus* oocytes transfected with GLUT4 or GLUT1, Murata et al. (29) showed that indinavir inhibits GLUT4- but not GLUT1-mediated glucose transport, suggesting this as the basis of insulin resistance in vivo. Accordingly, we found profound inhibition of both basal and insulin-stimulated 2-deoxyglucose uptake in GLUT4myc but not in GLUT1myc cells. Indinavir effectively inhibited glucose uptake in L6-GLUT4myc cells pretreated with high Glc/Ins, indicating that this pretreatment did not alter the affinity of glucose transporters to indinavir. The elevation in basal glucose uptake caused by pretreatment with high glucose and insulin was entirely abolished by indinavir. These results pointed to GLUT4myc as the major glucose transporter in L6-GLUT4myc cells, and suggest that the increase in GLUT1 protein had no significant contribution to the observed increase in basal glucose uptake caused by high Glc/Ins pretreatment. Because GLUT4myc also overrides the endogenous GLUT4, the results in this study refer to the activity and translocation of GLUT4myc, in both basal and high glucose/insulin conditions.

GLUT4myc activation by high Glc/Ins. An insulin-stimulated increase in glucose uptake is the final outcome of any changes that affect the insulin signaling pathway. We found that in L6-GLUT4myc myotubes, 24-h exposure to high Glc/Ins resulted in a 40% increase in basal glucose uptake accompanied by a diminished net response to an acute insulin stimulation. Because the cell surface GLUT4 was not elevated at the basal state in the pretreated cells, the possibility that the intrinsic activity of GLUT4 was upregulated was explored.

An emerging body of literature suggests that GLUT4 translocation is probably not sufficient to fully account for the effect of insulin on glucose uptake (41). Differences are noted in the time course (17,42) and sensitivity to wortmannin (13,43) of insulin-dependent GLUT4 translocation and stimulation of glucose uptake in both 3T3-L1 adipocytes and GLUT4myc L6 myotubes. In each instance, substantial GLUT4 translocation occurred without commensurate increases in glucose uptake. Other studies have also alluded to a dissociation between GLUT4 translocation and the full insulin response of glucose uptake in vivo (14,15). Recently, we and others observed that SB203580, a selective inhibitor of p38MAPK (16), diminished insulin-stimulated glucose uptake by 30–60% in L6 myotubes (17) and 3T3-L1 adipocytes (18) without reducing GLUT4 translocation. These studies have led to the suggestion that insulin may increase the intrinsic activity of GLUT4 via a p38 MAPK-dependent mechanism. Indeed, insulin activates p38 MAPK in L6-GLUT4myc myotubes (17), 3T3-L1 adipocytes (18,44,45), rat skeletal muscle (46,47), and vascular smooth muscle (48). The precise role of p38 MAPK in insulin-stimulated glucose uptake needs further evaluation. In 3T3-L1 adipocytes, Kayali et al. (44) demonstrated that p38 MAPK was activated by insulin (2.7-fold), and insulin-stimulated glucose uptake was inhibited in a dose-dependent manner by SB203580 and SB202190. However, the half-maximal inhibitory concentration (IC_{50}) for inhibition of glucose transport was determined to be >10 μmol/l, greater than the reported half-maximal stimulatory concentration (EC_{50}) for inhibition of p38 MAPK (~0.5 μmol/l). Therefore, the authors concluded that p38 MAPK is not involved in insulin-stimulated glucose uptake in 3T3-L1 adipocytes. However, because SB203580 and SB202190 reduce insulin stimulation of glucose uptake by only 50% even at maximal concentrations, the IC_{50} for inhibition of glucose uptake is closer to 1–2 μmol/l (17). This value is very close to the IC_{50} for inhibition of phosphorylation of targets of p38 MAPK in intact cells (17). Therefore, the reduction in glucose uptake by SB202190 and SB203580 closely parallels that for p38 MAPK activity.

In the present study, we observed that with high Glc/Ins, basal-state glucose uptake was elevated without a concomitant increase in surface levels of GLUT4 and without contribution of GLUT1. We interpret these results to suggest that high Glc/Ins elevated the intrinsic activity of GLUT4. Therefore, we explored whether this elevation might be caused by a regulatory input via p38 MAPK. To this effect, we examined the status of p38 MAPK expression and activity in response to high Glc/Ins. This pretreatment caused a 25% gain in total cellular p38 MAPK, an 80% increase in p38 MAPK phosphorylation, and a 2.3-fold increase in the kinase activity measured in vitro. Moreover, when high Glc/Ins–pretreated L6 myotubes were treated briefly with SB202190, the elevation in basal glucose uptake was reversed to control levels. These results support the hypothesis that in response to the chronic high Glc/Ins environment, p38 MAPK is activated and may contribute to the increase in basal glucose uptake.

The magnitude of increase in p38 MAPK phosphorylation (85%) was greater than the increase in p38 MAPK content (25%), suggesting that there was also upregulation of the upstream kinases or downregulation of phosphatases, which would normally dephosphorylate p38 MAPK. Alterations in the p38 MAPK signal pathway is unlikely to be a generalized stress response, however, since there was no increase in phosphorylation of other known stress response kinases, such as JNK or the extracellular signal-regulated kinase (ERK) MAPK (results not shown). In contrast to basal p38 MAPK, high Glc/Ins treatment reduced the ability of insulin to acutely increase p38 MAPK phosphorylation, uncovering a correlation between reduced insulin-stimulated p38 MAPK activity and reduced insulin stimulation of glucose uptake in insulin-resistant states in muscle cells.

In conclusion, the results summarized in Table 1 suggest the following model of imposed insulin resistance in L6-GLUT4myc myotubes: high Glc/Ins for 24 h caused a defect in acute insulin-mediated IR and IRS-1 phosphorylation, PI 3-kinase activity, and Akt phosphorylation, lead-

TABLE 1
Effects of 24-h exposure of L6 myotubes to high glucose and insulin on insulin-mediated GLUT4 translocation, glucose uptake, and activation of the signal networks that regulate these processes

	Insulin-stimulated values (fold above respective basal)	
	Control	High Glc/Ins
Glucose uptake	2.3	1.6
p38 MAPK phosphorylation	2.6	1.5
p38 MAPK activity	4.7	1.6
Surface GLUT4myc levels	2.3	1.7
GLUT4 content*	1	1
IR content*	1	0.7
IR phosphorylation	1	0.7
IRS-1 phosphorylation	4	1.5
IRS-1 content*	1	0.5
PI-3 kinase activity	7	4.2
Akt phosphorylation (T308)	8.5	4
Akt phosphorylation (S473)	6	3

L6-GLUT4myc myotubes were treated with 25 mmol/l glucose and 100 nmol/l insulin (high Glc/Ins) for 24 h, followed by incubation in serum-free medium containing 5 mmol/l glucose for 5 h. Acute insulin challenge (100 nmol/l) was then given for 5 min for IRS-1 phosphorylation, 10 min for p38 MAPK phosphorylation and activity, and 30 min for GLUT4 translocation and glucose uptake assay. *Basal value only.

ing ultimately to a significant reduction in insulin-mediated GLUT4 translocation. We hypothesize that basal glucose uptake is elevated as an adaptive response, probably as a result of elevated p38 MAPK phosphorylation and activity. However, an additional defect was introduced into the p38 MAPK signal pathway by the high Glc/Ins pretreatment, resulting in a decrease in acute insulin-mediated gain in p38 MAPK phosphorylation and activity. These results support the tenet that p38 MAPK may be a regulator of glucose uptake and is altered in states of insulin resistance.

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