

# Enhanced Basal Activation of Mitogen-Activated Protein Kinases in Adipocytes From Type 2 Diabetes

## Potential Role of p38 in the Downregulation of GLUT4 Expression

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Serine and threonine kinases may contribute to insulin resistance and the development of type 2 diabetes. To test the potential for members of the mitogen-activated protein (MAP) kinase family to contribute to type 2 diabetes, we examined basal and insulin-stimulated Erk 1/2, JNK, and p38 phosphorylation in adipocytes isolated from healthy and type 2 diabetic individuals. Maximal insulin stimulation increased the phosphorylation of Erk 1/2 and JNK in healthy control subjects but not type 2 diabetic patients. Insulin stimulation did not increase p38 phosphorylation in either healthy control subjects or type 2 diabetic patients. In type 2 diabetic adipocytes, the basal phosphorylation status of these MAP kinases was significantly elevated and was associated with decreased IRS-1 and GLUT4 in these fat cells. To determine whether MAP kinases were involved in the downregulation of IRS-1 and GLUT4 protein levels, selective inhibitors were used to inhibit these MAP kinases in 3T3-L1 adipocytes treated chronically with insulin. Inhibition of Erk 1/2, JNK, or p38 had no effect on insulin-stimulated reduction of IRS-1 protein levels. However, inhibition of the p38 pathway prevented the insulin-stimulated decrease in GLUT4 protein levels. In summary, type 2 diabetes is associated with an increased basal activation of the MAP kinase family. Furthermore, upregulation of the p38 pathway might contribute to the loss of GLUT4 expression observed in adipose tissue from type 2 diabetic patients. *Diabetes* 52:634–641, 2003

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C/EBP, CCAAT-enhancer binding protein; ECL, enhanced chemiluminescence; IC<sub>50</sub>, half-maximal inhibitory concentration; JNK, Jun kinase; MAP, mitogen-activated protein; NF-1, nuclear factor 1; PI3, phosphatidylinositol 3; TNF, tumor necrosis factor.

The number of individuals afflicted with diabetes worldwide is estimated to double by 2025 (1 and references therein). Of these cases, the vast majority will be type 2 diabetes (1). Insulin resistance, the impaired ability for tissues to respond to insulin, may be the predominant defect leading to the development of impaired glucose tolerance and ultimately type 2 diabetes (1). The underlying cellular mechanisms leading to insulin resistance are unknown but are believed to be due to defects in insulin signal transduction and not to defects in the insulin receptor itself or insulin:insulin receptor binding.

IRS-1 plays a key role in insulin signal transduction, and GLUT4 is necessary for insulin-stimulated glucose uptake (2–5). IRS-1 and GLUT4 protein levels are diminished in the adipocytes of patients with type 2 diabetes (6–9). IRS-1 and GLUT4 protein levels are also decreased in the adipocytes from first-degree relatives of patients with type 2 diabetes (8). Experiments in transgenic mice have demonstrated the importance of these proteins in maintaining insulin sensitivity. Decreased GLUT4 expression, as either a heterozygous knockout or a GLUT4 gene ablation specifically in adipose tissue, leads to whole-body insulin resistance and eventually diabetes (4,5). Adipocytes isolated from IRS-1 knockout mice have a decreased insulin-stimulated rate of glucose uptake and diminished translocation of GLUT4 to the plasma membrane (2,3). Thus the loss of these proteins, specifically in adipose tissue, may be a significant contributor to the onset of insulin resistance and development of diabetes.

Serine and threonine kinases are believed to play an important role in the onset of insulin resistance by regulating IRS-1 and GLUT4 protein levels through both pre- and posttranslational mechanisms (6,10). Phosphorylation of IRS-1 on serine and/or threonine residues precedes the molecules' subsequent proteasomal degradation in insulin-resistant 3T3-L1 adipocytes (10,11). Not unexpectedly, insulin resistance in cells and animals is associated with increased serine kinase activity toward IRS-1 (12). Furthermore, serine/threonine kinases are often involved with the regulation of gene expression by serving as intermediates in signal transduction cascades that link extracellular and intracellular stimuli to the nucleus (13). Thus serine/

TABLE 1  
Characteristics of subjects

	Healthy	Type 2 diabetes
n (M, F)	7 (6, 1)	7 (4, 3)
Age (years)	40.3 ± 11.77	44.6 ± 17.6
BMI (kg/m <sup>2</sup> )	26 ± 5.42	33 ± 3.37*
Triglyceride content (μg/cell)	0.37 ± 0.107	0.57 ± 0.169
Blood glucose (mg/dl)	84.17 ± 10.13	202.33 ± 60.79*
Blood insulin (μU/ml)	8.12 ± 2.27	25.15 ± 15.23*

Data are means ± SD. \*Significant difference from healthy ( $P < 0.05$ ).

threonine kinases have the potential to contribute to the diminished GLUT4 mRNA levels in insulin-resistant adipose tissue.

Specifically, members of the mitogen-activated protein (MAP) kinase family of serine-threonine kinases may contribute to the development of insulin resistance. IRS-1 contains many potential MAP kinase phosphorylation sites (14), and it has been observed that certain MAP kinases are capable of phosphorylating IRS-1 (12,15,16). MAP kinases play important roles in the regulation of gene expression through the phosphorylation-mediated activation/inactivation of numerous transcription factors (13). Thus MAP kinases are theoretically capable of regulating IRS-1 and GLUT4 protein levels through pre- and post-translational mechanisms. Furthermore, insulin stimulation is capable of activating members of the MAP kinase family, specifically Erk 1/2, JNK, and p38, in various cell types (17–21). In human skeletal muscle, insulin signaling to Erk 1/2 is normal from patients with type 2 diabetes compared with healthy control subjects, despite a diminished insulin responsiveness of components required for insulin's metabolic effects (i.e., the phosphatidylinositol 3 [PI3] kinase and protein kinase B/AKT pathway) (22,23). However, the activation state and insulin responsiveness of the MAP kinases in adipose tissue from type 2 diabetic patients is unknown.

Herein, we compare and contrast the ability of insulin to stimulate the phosphorylation of the MAP kinases in isolated adipocytes from healthy individuals and patients with type 2 diabetes. An increased basal level of phosphorylation of Erk 1/2, JNK, or p38 was observed in adipocytes from type 2 diabetic subjects in association with a reduction in IRS-1 and GLUT4, suggesting that elevated MAP kinase activation may contribute to the type 2 diabetic condition. Furthermore, selective inhibitors of the p38 pathway, but not the Erk 1/2 or JNK pathway, attenuated the decrease in GLUT4 protein abundance in response to chronic insulin exposure of 3T3-L1 adipocytes, without affecting the insulin-stimulated loss of IRS-1. Taken together, these results—the increased phosphorylation of p38 in human adipocytes from type 2 diabetic subjects and the necessary role of p38 in insulin-stimulated downregulation of GLUT4—suggest that this kinase may play a key role in the development of insulin resistance and type 2 diabetes.

## RESEARCH DESIGN AND METHODS

**Human subjects.** Specimens of human subcutaneous adipose tissue were obtained from the abdominal region of nondiabetic and type 2 diabetic subjects (Table 1). Subjects were recruited from the Unit of Endocrinology and Diabetes at University Hospital San Martin (Corrientes, Argentina) and Northwestern University Medical School (Chicago, IL). Subjects signed an

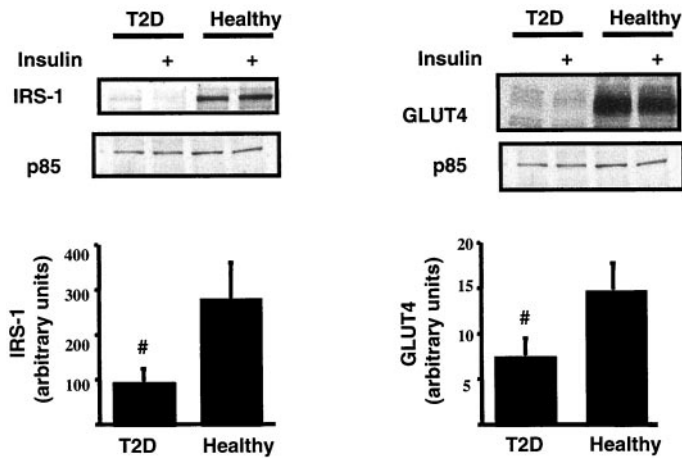
informed consent document indicating that they understood the risks and benefits of participation in this experiment. The experimental procedures were approved by the appropriate scientific review committees at University Hospital Corrientes and Northwestern University Medical School.

**Isolation and preparation of human adipocytes.** Adipose cells were prepared as described previously (7,24). Briefly, the adipocytes were isolated by digesting ~0.6 g tissue for 50 min at 37°C in medium 199 containing 25 mmol/l HEPES, 4% BSA, and 5.5 mmol/l glucose and collagenase (Sigma) at 0.8 mg/ml in a shaking water bath. Adipocyte cell size and number were determined as described previously (24). Isolated human adipocytes were distributed into plastic vials (12–15% cell suspension) in a final incubation volume of 400 μl. Cells were preincubated with or without 6.9 nmol/l insulin for 15 min, immediately separated by centrifugation through silicon oil, lysed in 0.4 ml lysis buffer containing 25 mmol/l Tris-HCl (pH 7.4), 0.5 mmol/l EGTA, 25 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/l NaF, 0.2 mmol/l leupeptin, 1 mmol/l benzamide, and 0.1 mmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and rocked for 40 min at 4°C. Detergent-insoluble material was sedimented by centrifugation at 12,000g for 10 min at 4°C. Protein concentration of the cell lysates was determined by bicinchoninic acid assay (Pierce Chemical, Rockford, IL). This insulin concentration (6.9 nmol/l) has been previously determined to achieve maximal insulin stimulation of human adipocytes (7).

**Immunoblotting.** To minimize any researcher bias, the researchers were blinded to the identity of the subjects from whom samples were taken. Total protein (10 μg) was separated on 10% acrylamide SDS-PAGE gels (BioRad) and transferred to nitrocellulose (Millipore). Membranes were blocked in 4% nonfat dry milk in TBS-T (Tris-buffered saline with 0.5% Tween-20) (Sigma), and primary antibodies were diluted in either 4% nonfat dry milk/TBS-T or 0.1% BSA/TBS-T. The blots were probed with various primary antibodies: phospho-specific antibodies toward Erk 1/2 and Jun kinase (JNK) (Promega, Madison, WI) phospho-specific antibodies to p38 MAP kinase (Cell Signaling Technologies, Beverly, MA); Erk 1/2, JNK, and p38 independent of phosphorylation state (Upstate Biotechnology, Lake Placid, NY; Santa Cruz Biotechnology, Santa Cruz, CA; and Cell Signaling Technologies, respectively); IRS-1, IRS-2, and the p85 regulatory subunit of PI3 kinase (whole antiserum) (Upstate Biotechnology); and GLUT4 (Chemicon, Temecula, CA). Membranes were washed thoroughly and incubated with donkey anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham). After washing, protein bands were visualized using enhanced chemiluminescence (ECL) (Amersham) and exposure to Hyperfilm ECL X-ray film (Amersham). Densitometric analysis of the bands was performed using a laser densitometer (PDS; Amersham) and analyzed by ImageQuant software (Amersham).

**p38 and JNK in vitro kinase assays.** The half-maximal inhibitory concentration (IC<sub>50</sub>) toward p38 and JNK of the compounds used in this experiment were determined using in vitro kinase assays. The new compounds (A-291077 and A-304000) are azazulene-based compounds designed to inhibit p38 MAP kinase. Recombinant p38 (α) or JNK were activated by incubation at 30°C for 1 h with recombinant MKK6 (4 mU) in 25 mmol/l HEPES (pH 7.4), 25 mmol/l β-glycerophosphate, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/l DTT, 25 mmol/l MgCl<sub>2</sub>, 5 mmol/l EDTA, 1 mmol/l NaF, and 50 μmol/l ATP. After activation of the kinase, activated p38 was incubated with 8 μg recombinant TRDH-ATF2 in 25 mmol/l HEPES (pH 7.4), 25 mmol/l β-glycerophosphate, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/l dithiothreitol (DTT), 25 mmol/l MgCl<sub>2</sub>, 5 mmol/l EDTA, and 1 mmol/l NaF. Reactions were started with the addition of [<sup>33</sup>P]ATP (10 μmol/l final; Amersham). Kinase reactions were allowed to proceed at 30°C for 1 h, after which the reactions were stopped with 5% phosphoric acid. The samples were transferred to filtration plates prewetted with 1% phosphoric acid and incubated for 15 min at room temperature. Vacuum filter plates were washed three times each with 1% phosphoric acid and counted in Microscint-20 on a Packard Topcount instrument.

**3T3-L1 cell culture.** 3T3-L1 cells were grown and maintained as fibroblasts in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) with high glucose containing 10% FBS (HyClone, Logan, UT) in a humidified atmosphere composed of 95% air and 5% CO<sub>2</sub>. Cells were differentiated into adipocytes by exposure to DMEM high glucose with 10% FBS, 0.4 μg/ml dexamethasone, 0.5 mmol/l isobutylmethylxanthine, and 5 μg/ml insulin. After 3 days, the medium was changed and the cells were maintained with DMEM containing 10% FBS. Day 10 adipocytes were starved for 2 h in DMEM/0.1% FBS before incubation in DMEM/0.1% FBS with or without insulin (1 μmol/l) for the time specified. In some cases, the cells were pretreated for 30 min before exposure to insulin with various reagents including SB203580, PD95085, A-291077, and A-304000. At the appropriate time, medium was aspirated from the cells and the cells were washed with ice-cold PBS. Subsequently, cells were lysed in lysis buffer [25 mmol/l Tris-HCl (pH 7.4), 0.5 mmol/l EGTA, 25 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/l NaF, 0.2 mmol/l leupeptin, 1 mmol/l benzamide, and 0.1



**FIG. 1.** IRS-1 and GLUT4 protein abundances are decreased in adipocytes isolated from patients with type 2 diabetes. Human adipocytes were isolated as described in RESEARCH DESIGN AND METHODS and stimulated with 6.9 nmol/l insulin for 15 min. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots were quantified using laser densitometry and are reported as integrated optical densities in arbitrary units. The representative immunoblots show data from one healthy individual and one patient with type 2 diabetes (T2D). The data presented are means and SDs for seven healthy and seven type 2 diabetic patients (see Table 1 for characteristics). #Significant difference from healthy ( $P < 0.05$ ).

mmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] (Calbiochem, La Jolla, CA) and rocked for 30 min at 4°C. Detergent-insoluble material was sedimented by centrifugation at 12,000g for 10 min at 4°C. Protein concentration of the cell lysates was determined by bicinchoninic acid assay (Pierce Chemical). Protein (20 µg) was separated on 7.5% SDS-PAGE gels (BioRad, Hercules, CA) and transferred to nitrocellulose. Western blotting was performed as described above.

**Statistical analysis.** Data are presented as means ± SD. Statistical significance was set at  $P < 0.05$ . Statistically significant differences in subject characteristics between healthy control subjects and type 2 diabetic patients were detected with an unpaired Student's two-tailed *t* test. Differences in phosphorylation status of the MAP kinases in the basal and insulin-stimulated state, as well as between healthy and type 2 diabetic patients, were determined using repeated measures ANOVA (JMP, version 3.1; SAS Institute, Cary, NC). A least significant difference test was used for posthoc analysis.

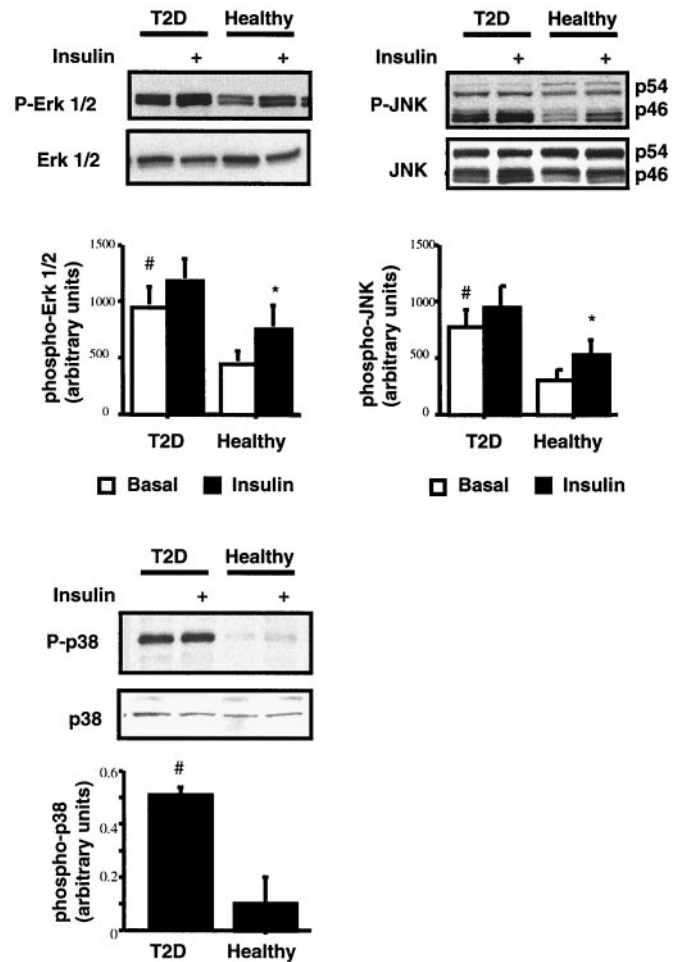
**RESULTS**

**Patient characteristics.** The type 2 diabetic patients examined in this study fit many of the previously described characteristics associated with obesity and type 2 diabetes, including BMI, blood glucose, and insulin concentrations (1,6–9). Type 2 diabetic patients had significantly greater blood glucose and blood insulin concentrations compared with healthy control subjects ( $P < 0.05$ ) (Table 1). Although the difference was not statistically significant ( $P = 0.06$ ), there was a tendency for adipocyte size to be greater in type 2 diabetic patients. BMI was greater in patients with type 2 diabetes compared with healthy control subjects. Because the control subjects were not matched for BMI, the following experiments cannot differentiate between the effects of obesity and type 2 diabetes on the parameters examined.

**IRS-1 and GLUT4 protein abundances are decreased in adipocytes isolated from patients with type 2 diabetes.** Insulin resistance in adipocytes is frequently characterized by diminished protein abundances of IRS-1 and GLUT4 (2–9). In the current report (Fig. 1), IRS-1 and GLUT4 protein abundances are decreased 70% and 50%, respectively, in adipocytes from type 2 diabetic patients

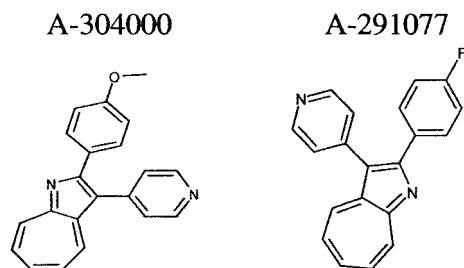
compared with healthy control subjects. This decrement is similar in magnitude to previously reported comparisons of IRS-1 and GLUT4 protein abundances in adipocytes from type 2 diabetic patients and healthy control subjects (6–9).

**Basal MAP kinase phosphorylation is increased in adipocytes isolated from patients with type 2 diabetes.** In adipocytes from healthy humans, 15 min of maximal insulin stimulation resulted in 1.8- and 2.0-fold elevated phosphorylation of Erk 1/2 and JNK, respectively (Fig. 2). p38 phosphorylation in response to insulin was observed in only two of seven healthy subjects examined. Interestingly, insulin treatment resulted in the phosphorylation of only a subset of JNK isoforms, specifically the p46 isoforms in both human adipocytes and 3T3-L1 adipocytes (data not shown). Although previous experiments have demonstrated JNK to be activated in response to insulin (17,18,20), this is the first report of an isoform-specific



**FIG. 2.** MAP kinase phosphorylation status in insulin-stimulated adipocytes from healthy humans and patients with type 2 diabetes. Human adipocytes were isolated as described in RESEARCH DESIGN AND METHODS and stimulated with 6.9 nmol/l insulin for 15 min. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with the indicated antibodies [Erk 1/2 (A), JNK (B), and p38 (C)]. Immunoblots were quantified using laser densitometry and are reported as integrated optical densities in arbitrary units. The representative immunoblots show data from one healthy individual and one patient with type 2 diabetes (T2D). The data presented are means and SDs for seven healthy and seven type 2 diabetic patients (see Table 1 for characteristics). #Significant difference from healthy ( $P < 0.05$ ); \*significant difference from the corresponding basal value ( $P < 0.05$ ).

TABLE 2  
 $IC_{50}$  (in  $\mu\text{mol/l}$ ) calculated from in vitro kinase assays



	A-304000	A-291007
p38	4.86	0.64
JNK	>50	3.47
Casein kinase 2	>50	>50
AKT 1	>50	>50
PKA	>50	7.3
PKC $\gamma$	>50	>50
MAPKAP 2	>50	>50
AMP kinase	>50	>50
c-Met	>50	>50
IGF receptor	>50	>50
Polo-like kinase	>50	>50
LCK kinase	>50	>50

response. Furthermore, this is the first report to describe the basal phosphorylation status and insulin responsiveness of members of the MAP kinase family in human adipocytes.

In contrast to healthy adipocytes, Erk 1/2 and JNK phosphorylation was not increased by insulin in type 2 diabetic adipocytes. The failure of insulin to increase MAP kinase phosphorylation may have been due to an elevated basal level of phosphorylation, which was significantly greater than in untreated healthy adipocytes. The most intriguing result, however, was the profound fivefold increase in p38 phosphorylation observed in the basal state of type 2 diabetic adipocytes. Insulin stimulation did not further increase p38 phosphorylation in type 2 diabetic adipocytes. The protein abundances of Erk 1/2, JNK, and p38 were not significantly different between healthy control subjects and patients with type 2 diabetes.

**MAP kinase inhibition has no effect on insulin-stimulated loss of IRS-1 protein abundance, but p38 is necessary for the loss of GLUT4.** To gain further insight into the potential mechanisms by which basal activation of MAP kinases in adipocytes may contribute to the development of insulin resistance, we systematically tested the role of Erk 1/2, JNK, and p38 using a panel of specific inhibitors in insulin-resistant 3T3-L1 adipocytes. 3T3-L1 adipocytes can be made insulin resistant through chronic exposure to insulin and elevated glucose concentrations (25–27). This model of insulin resistance mimics the expression pattern of human adipocytes in that there is a decreased protein abundance of IRS-1 and GLUT4 (10,11,25–27).

The panel of inhibitors used in this experiment included the MEK inhibitor PD98059 (to inhibit the Erk 1/2 pathway) as well as the p38 inhibitor SB203580 (28). In addition, we used two new compounds (Table 2) that show inhibitory properties toward p38 and JNK (A-291077)

or p38-alone (A-304000) (29). Table 2 presents the chemical structures and  $IC_{50}$  toward p38 and JNK as determined with in vitro kinase assays. The compound A-304000 is a selective inhibitor of p38, with little inhibitory activity toward JNK. Furthermore, these new compounds, specifically A-304000, exhibited minimal inhibitory activity in in vitro kinase reactions using AKT/PKB, PKA, MAPKAP2, casein kinase2, PKC $\gamma$ , AMP kinase, c-Met, IGF receptor, Polo-like kinase, and LCK kinase. Thus the pattern of inhibition achieved by this panel would test the contribution of Erk 1/2 (PD98059), JNK, and p38 (A-291077) and p38-alone (A-304000) in the downregulation of GLUT4 expression and/or IRS-1 protein abundance in a cell culture model of insulin resistance. The overlapping specificity of A-291077 and A-304000 would allow us to infer the role that either JNK or p38 may play in the insulin-stimulated loss of IRS-1 and/or GLUT4.

Chronic exposure to insulin in a high-glucose environment significantly decreased protein levels of IRS-1 and GLUT4, by 50% and 40%, respectively (Fig. 3). This effect was restricted to IRS-1 and GLUT4, as insulin exposure had no effect on the levels of p85 PI3 kinase or IRS-2 (data not shown) as previously reported (11). Treatment of the 3T3-L1 adipocytes with selective inhibitors of Erk 1/2, JNK, or p38 did not prevent the insulin-stimulated loss of IRS-1.

The insulin-stimulated loss of GLUT4 protein abundance was prevented only by inhibitors of p38 (Fig. 3). Chronic exposure of the 3T3-L1 adipocytes to insulin resulted in a significant decrease in GLUT4 protein levels. Treatment of the 3T3-L1 adipocytes with p38 inhibitors without insulin stimulation did not significantly alter the protein abundance of GLUT4. Treatment with 10  $\mu\text{mol/l}$  of the p38 selective inhibitors SB203580 or A-304000 did not prevent the insulin-stimulated downregulation of GLUT4. However, 50  $\mu\text{mol/l}$  of SB203580 or A-304000 resulted in a GLUT4 protein level that was not statistically different from that of untreated/unstimulated cells or cells treated with 50  $\mu\text{mol/l}$  of compound without insulin. These data indicate that inhibition of p38 is sufficient to prevent the insulin-stimulated downregulation of GLUT4. Pretreatment of the 3T3-L1 adipocytes with A-291077, an inhibitor of both JNK and p38, resulted in attenuation of the insulin-stimulated loss of GLUT4. This effect was observed at 10- and 50- $\mu\text{mol/l}$  concentrations of compound. By contrast, inhibition of the Erk 1/2 pathway with the MEK inhibitor PD98059 had no effect on the insulin-stimulated loss of GLUT4 protein.

A-304000 prevented the insulin-induced loss of GLUT4 protein abundance in a dose-responsive manner (Fig. 4), with a >40% recovery of GLUT4 protein abundance observed with 25  $\mu\text{mol/l}$  and a full recovery at 50  $\mu\text{mol/l}$ . Taken together, these results indicate that inhibition of p38, but not Erk 1/2, can prevent the insulin-induced downregulation of GLUT4.

**p38 phosphorylation is transiently increased by insulin in 3T3-L1 adipocytes.** Insulin stimulation resulted in an increased p38 phosphorylation by approximately two-fold at 5 and 10 min, before returning to baseline for the remainder of the experiment (Fig. 5A). Inhibition of p38 with A-304000 (Fig. 5B) or SB203580 (data not shown) prevented the insulin-induced p38 phosphorylation at 5

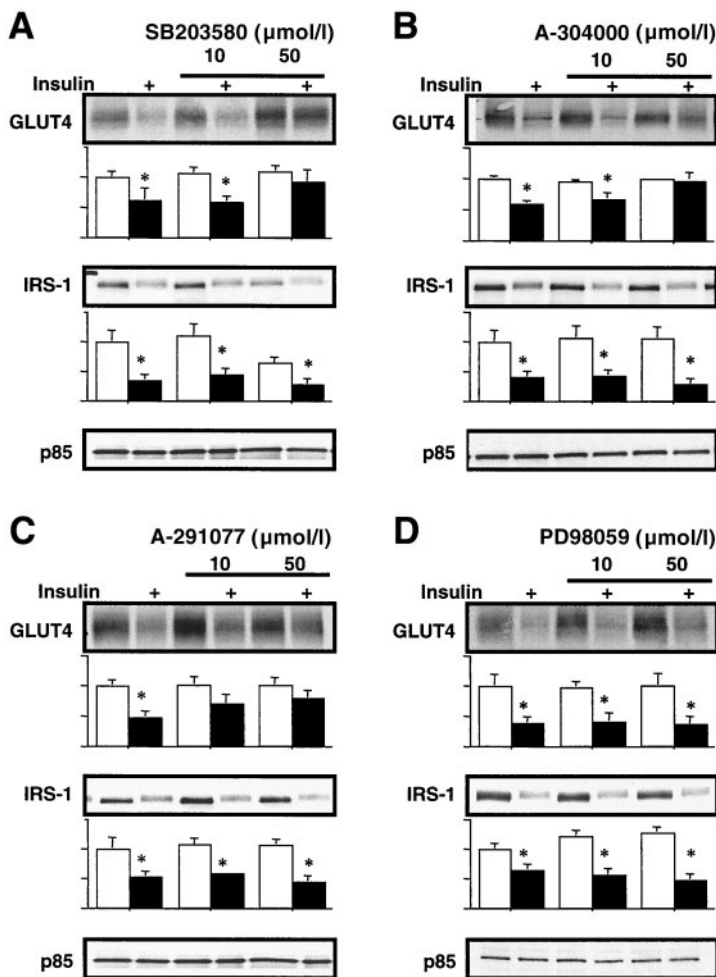


FIG. 3. MAP kinase inhibition has no effect on insulin-stimulated loss of GLUT4. 3T3-L1 adipocytes were pretreated with 10 or 50  $\mu\text{mol/l}$  SB20358 (A), A-304000 (B), A-291077 (C), or PD98059 (D) before stimulation with 1  $\mu\text{mol/l}$  insulin overnight. Cells were lysed, and total protein was subjected to 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots were quantified using laser densitometry and are reported as integrated optical densities in arbitrary units. Representative Western blots are shown for GLUT4, IRS-1, and p85. The corresponding bar graphs present the results as means and SDs of at least three independent experiments for GLUT4 and IRS-1 as indicated. The x-axis represents (from left to right) unstimulated cells (lane 1), cells treated with insulin overnight (lane 2), and cells pretreated with compound without or with insulin (lanes 3-6). The y-axis represents at least three independent observations expressed as arbitrary units. \*Significant difference from the corresponding basal value ( $P < 0.05$ ).

and 10 min of insulin stimulation. p38 protein levels were unaffected by insulin stimulation or the inhibitors.

**DISCUSSION**

This report is the first to describe the phosphorylation status and insulin responsiveness of the MAP kinases in human adipocytes from healthy control subjects and patients with type 2 diabetes. Members of the MAP kinase family have the potential to contribute to the perturbations in insulin signaling associated with type 2 diabetes. Thus we hypothesized that if one or more of the MAP kinases examined were involved with type 2 diabetes, there should be differential phosphorylation status of that particular MAP kinase between healthy adipocytes and type 2 diabetic adipocytes. In this report, we have demonstrated that basal MAP kinase phosphorylation, especially that of p38, is elevated in adipocytes from type 2 diabetic patients, as in previous experiments that have found elevated serine kinase activity in adipocytes from insulin-resistant animals (12), and our results are consistent with MAP kinases contributing to type 2 diabetes. The increased basal phosphorylation was most striking for p38, which exhibited a fivefold increase in adipocytes from type 2 diabetic patients compared with healthy control subjects. In contrast, Erk 1/2 and JNK phosphorylation was increased approximately twofold in adipocytes from type 2 diabetic patients compared with healthy control subjects.

The elevated basal phosphorylation state of members of

the MAP kinase family in adipocytes from type 2 diabetic subjects is associated with a reduction in IRS-1 and GLUT4 protein levels. In humans, diminished GLUT4 and IRS-1 protein abundance can predict the future development of type 2 diabetes (8,9), suggesting that dysregulation of the expression of these proteins may be an early step toward

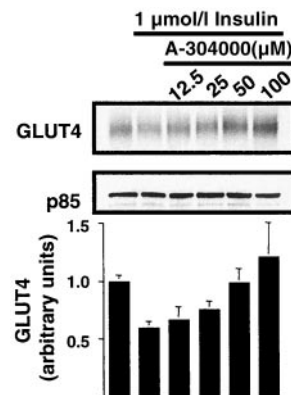
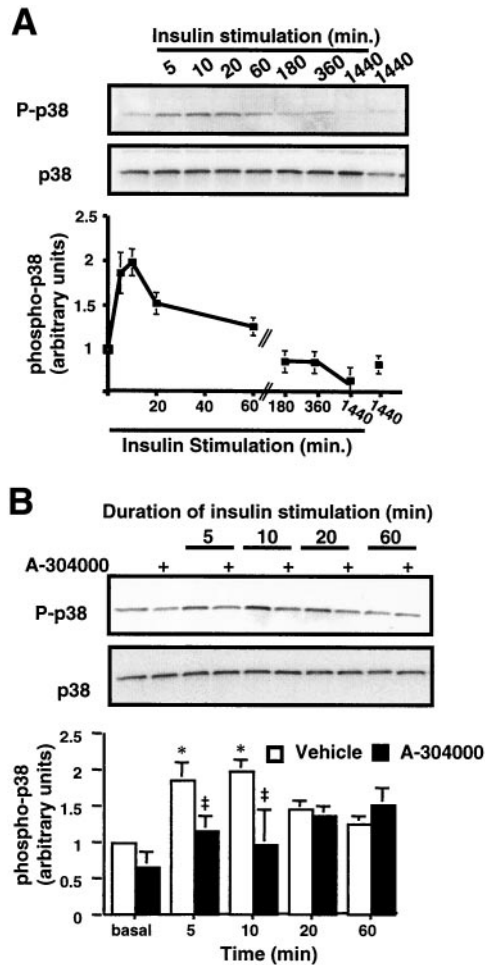


FIG. 4. Inhibition of p38 with A-304000 prevents the insulin-stimulated loss of GLUT4 protein abundance in a dose-responsive manner. 3T3-L1 adipocytes were pretreated with A-304000 at the indicated concentrations, before stimulation with 1  $\mu\text{mol/l}$  insulin overnight. Cells were lysed, and total protein was subjected to 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots were quantified using laser densitometry and are reported as integrated optical densities in arbitrary units. The data are presented as means and SDs from at least three independent experiments.



**FIG. 5.** p38 phosphorylation in response to insulin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated without or with A-304000 before stimulation with 1  $\mu\text{mol/l}$  insulin for the duration indicated. Cells were lysed, and total protein was subjected to 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots were quantified using laser densitometry and are reported as integrated optical densities in arbitrary units. The data were normalized to unstimulated cells and are presented as means and SDs from at least three independent experiments. \*Significant difference from the basal value ( $P < 0.05$ ); †significant difference from corresponding vehicle-treated value ( $P < 0.05$ ).

the development of type 2 diabetes. To begin to understand the mechanism by which activation of the MAP kinases might contribute to insulin resistance and type 2 diabetes, we systematically tested the role of these pathways in regulating IRS-1 and GLUT4 protein levels in a cell-culture model of insulin resistance. We used a panel of inhibitors that selectively inhibited Erk 1/2 (PD98059), p38 alone (A-304000), and p38 and JNK (A-291077) to test the contribution of all three MAP kinase pathways toward insulin-stimulated downregulation of IRS-1 and GLUT4. The insulin-stimulated loss of IRS-1 was not prevented by inhibition of any of the MAP kinase pathways examined, consistent with a prior publication that demonstrated that PD98059 has no effect on the insulin-stimulated loss of IRS-1 (11). These results expand on previous findings to rule out JNK and p38 as playing a role in IRS-1 degradation. Inhibition of mTOR with rapamycin has been previously shown to prevent serine phosphorylation and subsequent degradation of IRS-1 (11). Although these results indicate that these MAP kinases do not contribute

to insulin-induced IRS-1 degradation, we cannot rule out the possibility that they may be involved with serine phosphorylation of IRS-1, potentially leading to the development of insulin resistance.

The insulin-induced loss of GLUT4 protein abundance could be prevented by selective inhibitors of p38. This effect was also observed when both p38 and JNK were inhibited by A-291077, whereas inhibition of the Erk 1/2 pathway had no effect. Because the inhibition of both p38 and JNK simultaneously prevented the insulin-stimulated downregulation of GLUT4 in a manner similar to inhibition of p38 alone, we conclude that p38 is a major contributor to the insulin-stimulated loss of GLUT4. Although we cannot completely rule out the possibility that JNK and/or other kinases may be involved, our results are consistent with the findings of Fujishiro et al. (30), who demonstrated that constitutive activation of the MKK6-p38 pathway, but not the MKK7-JNK pathway, was sufficient for the downregulation of GLUT4 expression.

The role of p38 in the insulin-stimulated downregulation of GLUT4 is further supported by our use of two structurally distinct inhibitors, SB203580 and A-304000. These selective p38 inhibitors prevented the insulin-stimulated downregulation of GLUT4 with similar potencies. Although treatment with 50  $\mu\text{mol/l}$  SB203580 resulted in decreased protein levels of IRS-1, IRS-2, and p85 regardless of insulin stimulation, treatment did not decrease GLUT4 protein levels and prevented the insulin-stimulated loss of GLUT4. This suggests that high doses of SB203580 may be toxic to the cells but selectively preserve GLUT4 expression, consistent with p38 acting as a negative regulator of GLUT4 expression. These cytotoxic effects appear to be limited to SB203580 itself and are not related to inhibition of p38 per se, because the other p38 inhibitors (A-304000 and A-291077) did not affect IRS-1, p85, or IRS-2 levels. Thus, our results expand on the findings of Fujishiro et al. (30) by demonstrating that the p38 pathway is necessary for the insulin-stimulated downregulation of GLUT4. Furthermore, our observation that p38 phosphorylation is increased in adipocytes isolated from type 2 diabetic patients strongly supports a role for p38 to contribute to insulin resistance and the development of type 2 diabetes.

The 3T3-L1 adipocyte model used in this experiment (chronic exposure to high insulin and glucose concentrations) mimics very closely the conditions found in insulin-resistant human adipocytes. For example, chronic exposure of 3T3-L1 adipocytes to insulin and glucose results in 1) diminished insulin-stimulated glucose uptake, 2) failure of insulin to inhibit lipolysis, and 3) the loss of IRS-1 and GLUT4 (present data; 11,31). These similarities support the use of the 3T3-L1 adipocyte to test the role of p38 in the regulation of IRS-1 and GLUT4. Thus we can infer that activation of p38 observed in type 2 diabetic adipocytes may contribute to the low levels of GLUT4 observed in those cells.

The mechanisms by which p38 may regulate GLUT4 are currently unknown. In adipocytes from type 2 diabetic patients, p38 phosphorylation was increased, in association with reduced GLUT4 and IRS-1. However, unlike the human type 2 diabetic adipocytes, insulin stimulation of 3T3-L1 adipocytes resulted in a transient increase in p38

phosphorylation. Inhibition of p38 was able to prevent the loss of GLUT4; thus only a transient activation of p38 may be necessary for downregulation of GLUT4. Downregulation of GLUT4 mRNA transcription in response to insulin occurs within 2 h (25); thus it is possible that early changes in p38 activity may be capable of modulating GLUT4 expression. GLUT4 mRNA and protein are decreased in adipose tissue from type 2 diabetic and animal models of insulin resistance (9,32,33) and are also diminished in 3T3-L1 adipocytes treated with insulin (25–27), tumor necrosis factor (TNF)- $\alpha$  (34), and other factors (35,36). It has not yet been determined if p38 regulates GLUT4 expression through modifications of mRNA stability or mRNA transcription; however, many transcription factors are known to be downstream of the p38 pathway (13), consistent with gene transcription as a potential mechanism.

Many regions within the GLUT4 promoter have been identified as contributing to the downregulation of GLUT4 expression in response to insulin (37), TNF- $\alpha$  (38), and other factors (36,39). Nuclear factor 1 (NF-1) and Olf-1/early B-cell factor (O/E) are capable of binding to an element within the GLUT4 promoter that mediates the insulin and cAMP-induced downregulation of GLUT4 transcription (40,41). Alternatively, CCAAT-enhancer binding proteins (C/EBP) have been shown to play important roles in regulating GLUT4 expression during adipogenesis (42). NF-1 becomes phosphorylated in response to insulin (43); however, there are no data regarding the ability of p38 to phosphorylate NF-1. p38 has been shown to be capable of phosphorylating C/EBP  $\beta$  (44). Thus the potential exists for the p38 pathway to mediate repression of GLUT4 expression at the transcriptional level through directly modifying factors such as NF-1 and C/EBP  $\beta$ . We are currently pursuing further experimentation to test these possibilities.

The current report has not addressed the nature of the stimulus that may be responsible for the basal activation of p38 in human adipocytes. Because the human adipocytes used in the current experiment were isolated from abdominal subcutaneous biopsies, and thus underwent a substantial isolation process before insulin stimulation and examination, it is likely that any systemic factors that may have contributed to the elevated p38 phosphorylation were removed before analysis. Thus the elevated phosphorylation of p38 is likely due to biochemical characteristics intrinsic to type 2 diabetes adipocytes. Factors such as cAMP (35), oxidative stress (36), and numerous cytokines and interleukins (34) have been shown to decrease GLUT4 protein in 3T3-L1 adipocytes and activate p38 in other cell systems (36 and references therein). Future experiments will be necessary to further define the cause of p38 activation in type 2 diabetic adipocytes.

In conclusion, the current report has identified p38 as a potential candidate for mediating the loss of GLUT4 expression observed in adipocytes from type 2 diabetic subjects. This is based on an elevated basal phosphorylation status of p38 MAPK in human adipocytes from type 2 diabetic patients compared to healthy control subjects. Furthermore, we have demonstrated that p38 is necessary for the insulin-induced loss of GLUT4 expression in 3T3-L1 adipocytes. This may be of great importance because diminished GLUT4 expression results in the development

of insulin resistance, diabetes, and many related complications (4,5). In addition, the current experiment has ruled out the MAP kinases as contributing to the insulin-induced loss of IRS-1 protein abundance in 3T3-L1 adipocytes. Future work is necessary to define the mechanisms involved with the activation of p38 in insulin-resistant and diabetic adipocytes, as well as the connection between p38 activation and regulation of GLUT4 protein abundance.

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