

# Activation of p38 Mitogen-Activated Protein Kinase $\alpha$ and $\beta$ by Insulin and Contraction in Rat Skeletal Muscle

## Potential Role in the Stimulation of Glucose Transport

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The stress-activated p38 mitogen-activated protein kinase (MAPK) was recently shown to be activated by insulin in muscle and adipose cells in culture. Here, we explore whether such stimulation is observed in rat skeletal muscle and whether muscle contraction can also affect the enzyme. Insulin injection (2 U over 3.5 min) resulted in increases in p38 MAPK phosphorylation measured in soleus (3.2-fold) and quadriceps (2.2-fold) muscles. Increased phosphorylation (3.5-fold) of an endogenous substrate of p38 MAPK, cAMP response element binder (CREB), was also observed. After *in vivo* insulin treatment, p38 MAPK $\alpha$  and p38 MAPK $\beta$  isoforms were found to be activated (2.1- and 2.4-fold, respectively), using an *in vitro* kinase assay, in immunoprecipitates from quadriceps muscle extracts. *In vitro* insulin treatment (1 nmol/l over 4 min) and electrically-induced contraction of isolated extensor digitorum longus (EDL) muscle also doubled the kinase activity of p38 MAPK $\alpha$  and p38 MAPK $\beta$ . The activity of both isoforms was inhibited *in vitro* by 10  $\mu$ mol/l SB203580 in all muscles. To explore the possible participation of p38 MAPK in the stimulation of glucose uptake, EDL and soleus muscles were exposed to increasing doses of SB203580 before and during stimulation by insulin or contraction. SB203580 caused a significant reduction in the insulin- or contraction-stimulated 2-deoxyglucose uptake. Maximal inhibition (50–60%) occurred with 10  $\mu$ mol/l SB203580. These results show that p38 MAPK $\alpha$  and  $\beta$  isoforms are activated by insulin and contraction in skeletal muscle. The data further suggest that activation of p38 MAPK may participate in the stimulation of glucose uptake by both stimuli in rat skeletal muscle. *Diabetes* 49:1794–1800, 2000

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ATF, activating transcription factor; CREB, cAMP response element binder; DTT, dithiothreitol; EDL, extensor digitorum longus; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride.

**T**ranslocation of glucose transporters in muscle and fat cells is a prerequisite for full stimulation of glucose uptake by insulin (1,2). Activation of several lipid and serine/threonine kinases in an orderly fashion is believed to be required for this translocation. Arrival of GLUT4 at the plasma membrane in rat skeletal muscle (3), L6 muscle cells (4), isolated rat adipocytes (5), and 3T3-L1 adipocytes (6) is contingent on prior activation of phosphatidylinositol 3-kinase (PI3K). Multiple approaches, such as the use of the pharmacological agents wortmannin and LY294002 (7), the use of dominant inhibitory constructs, and the introduction of antibodies by microinjection (8), support this requirement of PI3K. Obliteration of the activity of the PI3K effector Akt/protein kinase B by expression of a mutant that is kinase-inactive and inactivatable prevented the insulin-induced accumulation of plasma membrane GLUT4 in L6 muscle cells (9). In addition, expression of a kinase-inactive Akt mutant in rat adipocytes (10) and introduction of inhibitory peptides into 3T3-L1 adipocytes (11) also reduced GLUT4 levels at the plasma membrane. Another downstream effector of PI3K, the atypical protein kinase C- $\lambda$ , also contributes to the translocation of GLUT4 in response to insulin in 3T3-L1 adipocytes (12,13).

Despite the aforementioned information, recent studies by us and others have suggested that insulin-stimulated GLUT4 translocation in rat skeletal muscle (14), L6 muscle cells (15), and 3T3-L1 adipocytes (8,15) is not sufficient to achieve the maximal increase in glucose uptake. Therefore, these studies proposed that the intrinsic activity of the translocated GLUT4 is subject to regulation. Unlike GLUT4 translocation, very little is known about the signals controlling GLUT4 catalytic activity. An inhibitor of p38 mitogen-activated protein kinase (MAPK), SB203580, reduced the stimulation of glucose uptake by insulin in 3T3-L1 adipocytes and L6 muscle cells (15). GLUT4 exposure at the cell surface was not affected by SB203580 in L6 muscle cells expressing a cDNA encoding a GLUT4 molecule with a myc epitope tag at the first exofacial loop. This result was confirmed in 3T3-L1 adipocytes by subcellular fractionation and immunofluorescent detection of GLUT1 and GLUT4 on plasma membrane lawns (15). Therefore, we proposed that p38 MAPK functions in a signaling pathway that may modulate the catalytic activity of glucose transporters in cells in culture.

It is not known whether p38 MAPK contributes to the stimulation of glucose uptake by insulin or contraction in mature skeletal muscle, the principal site of glucose disposal *in vivo*. To address this question, it is important to determine which p38 MAPK isoforms are activated by these stimuli. Using several different *in vivo* and *in vitro* assays to monitor enzyme phosphorylation and activity, we report that insulin, both *in vivo* and *in vitro*, activates p38 MAPK $\alpha$  and p38 MAPK $\beta$  in various rat muscles. Electrically stimulated contraction of the extensor digitorum longus (EDL) muscle also increased the activity of both p38 MAPK isoforms. Furthermore, an inhibitor of p38 MAPK inhibited stimulation of glucose uptake by either insulin or contraction.

## RESEARCH DESIGN AND METHODS

**Materials.** Activating transcription factor (ATF) 2 fusion protein and phosphospecific antibodies to p38 MAPK and cAMP response element binder (CREB) were purchased from New England Biolabs (Beverly, MA) and Calbiochem (San Diego, CA), respectively. Isoform-specific p38 MAPK antibodies ( $\alpha$  and  $\beta$ ) and anti-p38 MAPK antibody, which cross reacts with these two isoforms, were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody conjugated to agarose beads was from Upstate Biotechnology (Lake Placid, NY).

**Animals and treatments.** Animals were allowed access to standard rodent diet and water *ad libitum*. Food was withdrawn 12–14 h before experiments. For enzyme activity determinations after *in vivo* insulin injection, quadriceps and soleus muscles were obtained from male Wistar rats (250–300 g). The abdominal cavity was opened in anaesthetized (using ketamin/xylazin) rats, the portal vein was exposed, and 2 U insulin (or saline in control animals) was injected as a bolus. Quadriceps and soleus muscles were removed 3.5 min after insulin administration. Tissues were stored at  $-80^{\circ}\text{C}$ . For *in vitro* stimulation of p38 MAPK activity by insulin, intact EDL muscles isolated from young rats (40–60 g) were incubated with or without insulin for 4 min and immediately blotted at  $4^{\circ}\text{C}$ , clamp frozen, and stored at  $-80^{\circ}\text{C}$  until processed as described below. For glucose transport studies, EDL and soleus muscles (intact or strips) were obtained from young (60–80 g) or adult (200–250 g) Sprague-Dawley rats, respectively.

**Incubation protocols of isolated rat skeletal muscles and 2-deoxyglucose uptake.** Intact soleus and EDL muscles or muscle strips (20–30 mg) used for glucose uptake (insulin studies) were treated as previously described (16). Briefly, muscles or strips were preincubated with SB203580 (or DMSO) for 20 min at concentrations indicated in the figure legends. Muscles were then incubated for 30 min with insulin in the absence or continued presence of SB203580. For contraction studies, intact EDL muscles were dissected and prepared for electrically stimulated contraction *in vitro* as previously described (17) with minor modifications. In brief, muscles were first preincubated with SB203580 at the indicated concentrations for 20 min. Muscles were then stimulated with 25-V 0.2-ms pulses at a frequency of 70 Hz for 10 s, once per min for 20 min (in the absence or continued presence of SB203580). 2-Deoxyglucose uptake in insulin- or contraction-stimulated muscles was then determined as described (12). SB203580 treatment was continued during the glucose uptake experiments if it was present during the previous incubations.

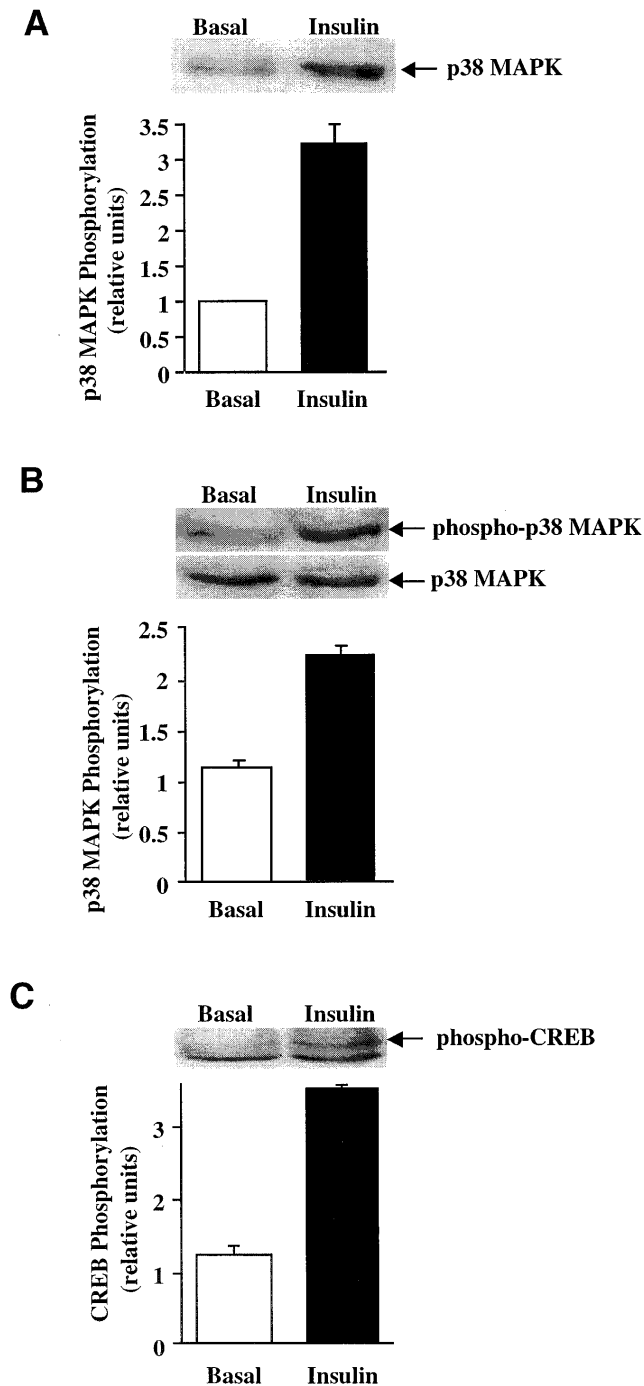
**Detection of p38 MAPK and CREB phosphorylation.** Tyrosine phosphorylation of p38 MAPK in soleus muscle was detected as described for muscle cells in culture with modifications (18). Soleus muscles (~500 mg) were homogenized as described (19) in cold homogenization buffer (250 mmol/l sucrose, 20 mmol/l  $\text{NaHCO}_3$ , and 5 mmol/l  $\text{NaN}_3$ ) supplemented with 1 mmol/l  $\text{Na}_3\text{VO}_4$ , 100 nmol/l okadaic acid, and protease inhibitors (1 mmol/l benzamide, 10  $\mu\text{mol/l}$  E-64, 1  $\mu\text{mol/l}$  leupeptin, 1  $\mu\text{mol/l}$  pepstatin A, and 0.2 mmol/l phenylmethylsulfonyl fluoride [PMSF]). Homogenate (500  $\mu\text{g}$ ) was diluted with an equal volume of 2 $\times$  cold lysis buffer (50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 10% glycerol [vol/vol], 1% Triton X-100 [vol/vol], 30 mmol/l sodium pyrophosphate, 10 mmol/l NaF, and 1 mmol/l EDTA) containing (added at time of experiment) 1 mmol/l dithiothreitol (DTT), 1 mmol/l  $\text{Na}_3\text{VO}_4$ , and 100 nmol/l okadaic acid and protease inhibitors, vortexed for 1 min, passed five times through a 25-gauge syringe, and then centrifuged for 10 min (1,000 rpm,  $4^{\circ}\text{C}$ ). Supernatant was subjected to immunoprecipitation for 2 h with anti-phosphotyrosine antibody conjugated to agarose beads (2  $\mu\text{g}$  per condition). Immunoprecipitates were washed four times with cold phosphate-buffered saline (PBS) supplemented with 0.1% NP-40 and 1 mmol/l  $\text{Na}_3\text{VO}_4$ , resuspended in 2 $\times$  Laemmli sample buffer, boiled for 5 min, and then immunoblotted for p38 MAPK. To detect phosphorylation of p38 MAPK

and CREB using phosphospecific antibodies, quadriceps (500 mg) were pulverized in liquid nitrogen then lysed with 1 ml 2 $\times$  Laemmli sample buffer containing (added at the time of experiment), 1 mmol/l benzamide, 1 mmol/l  $\text{Na}_3\text{VO}_4$ , 100 nmol/l okadaic acid, protease inhibitors, and 7.5%  $\beta$ -mercaptoethanol. Samples were vortexed for 1 min, passed five times through a 25-gauge syringe, and heated for 15 min at  $65^{\circ}\text{C}$ . Lysates were then centrifuged for 5 min at 1,000 rpm. To determine protein concentration, total proteins were precipitated from an aliquot of each sample with 10% trichloroacetic acid, washed twice with cold PBS, and samples were resuspended (in the lysis buffer used below to measure kinase activity). Bicinchoninic acid method was used according to the manufacturer's instruction (Pierce, Rockford, IL). Protein (150  $\mu\text{g}$ ) was resolved by 10% SDS-PAGE then immunoblotted for phospho-p38 MAPK or phospho-CREB (1:500 dilution of primary antibodies).

**In vitro p38 MAPK activity assay.** Protein kinase activity was measured as described (15) with modifications. Anti-p38 MAPK $\alpha$  or anti-p38 MAPK $\beta$  antibodies (2  $\mu\text{g}$  per condition) were adsorbed to protein A- or G-Sepharose beads, respectively, by incubating overnight at  $4^{\circ}\text{C}$  under constant rotation. Beads were washed twice with 1 ml cold PBS and once with 1 ml cold lysis buffer and were resuspended in lysis buffer. Muscles were pulverized in liquid nitrogen, lysed in 1 ml lysis buffer supplemented with protease inhibitors (1 mmol/l benzamide, 10  $\mu\text{mol/l}$  E-64, 1  $\mu\text{mol/l}$  leupeptin, 1  $\mu\text{mol/l}$  pepstatin A, 0.2 mmol/l PMSF), 1 mmol/l  $\text{Na}_3\text{VO}_4$ , 1 mmol/l DTT, and 100 nmol/l okadaic acid and were then vortexed for 1 min. Lysates were passed five times through a 25-gauge syringe then centrifuged for 5 min (1,000 rpm,  $4^{\circ}\text{C}$ ). Protein concentration of the supernatant was determined by the bicinchoninic acid method. p38 MAPK was immunoprecipitated from 250  $\mu\text{g}$  of total protein for 2–3 h with the preabsorbed Sepharose beads. Immunocomplexes were isolated and washed four times with 1 ml wash buffer (25 mmol/l HEPES, pH 7.8, 10% glycerol [vol/vol] 1% Triton X-100 [vol/vol], 0.1% bovine serum albumin, and 1 mol/l NaCl) supplemented with 1 mmol/l  $\text{Na}_3\text{VO}_4$ , 1 mmol/l DTT, 1 mmol/l PMSF, and 10 nmol/l okadaic acid and twice with 1 ml kinase buffer (50 mmol/l Tris/HCl, pH 7.5, and 10 mmol/l  $\text{MgCl}_2$ ) supplemented with 1 mmol/l  $\text{Na}_3\text{VO}_4$  and 10 nmol/l okadaic acid. Immunocomplexes were then incubated for 30 min at  $30^{\circ}\text{C}$  with 30  $\mu\text{l}$  reaction mixture (kinase buffer containing 5  $\mu\text{mol/l}$  ATP, 2  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP, and 5  $\mu\text{g}$  of myelin basic protein or 2  $\mu\text{g}$  ATF2 fusion protein per condition) on a platform shaker. Reaction was stopped by adding 30  $\mu\text{l}$  of 2 $\times$  Laemmli sample buffer and heating for 30 min at  $37^{\circ}\text{C}$ . Samples were centrifuged for 5 min (1,000 rpm) then 40  $\mu\text{l}$  of the supernatant was resolved by 13% SDS-PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Amount of radiolabeled phosphate transferred onto the substrate was determined by exposing the PVDF membrane to a phosphorimager cassette and quantitated using a Molecular Dynamics PhosphorImager System (Sunnyvale, CA).

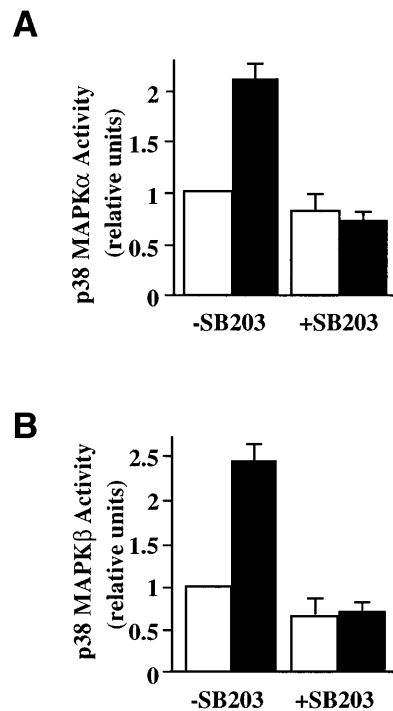
## RESULTS

**p38 MAPK phosphorylation and activity are enhanced by *in vivo* insulin treatment.** The mechanism of p38 MAPK activation requires prior phosphorylation on threonine and tyrosine in a TGY motif (20). Activation of p38 MAPK was assessed by three complementary approaches. First, soleus muscles from control (saline-injected) and insulin-injected (2 U/rat, 3.5 min) rats were solubilized and subjected to immunoprecipitation using anti-phosphotyrosine-directed antibodies. The immunoprecipitates were immunoblotted using an antibody that detects p38 MAPK $\alpha$  and p38 MAPK $\beta$  isoforms. Insulin injection resulted in a  $3.2 \pm 0.3$ -fold higher p38 MAPK signal in these immunoprecipitates (Fig. 1A). Second, an antibody that recognizes p38 MAPK only when activated by dual phosphorylation at Thr180 and Tyr182 (20) was used to probe muscle lysates. A  $2.2 \pm 0.1$ -fold increase in p38 MAPK phosphorylation caused by insulin was revealed by using this protocol (Fig. 1B). There was no difference in the total amount of p38 MAPK in the samples (Fig. 1B). Third, as an index of p38 MAPK activity *in vivo*, we measured phosphorylation of CREB, a downstream substrate of p38 MAPK (21,22). As observed for p38 MAPK, *in vivo* insulin treatment for 3.5 min increased CREB phosphorylation in muscle by  $3.50 \pm 0.05$ -fold (Fig. 1C). The phosphospecific CREB antibody also recognized a lower molecular weight protein, which presumably is ATF1.

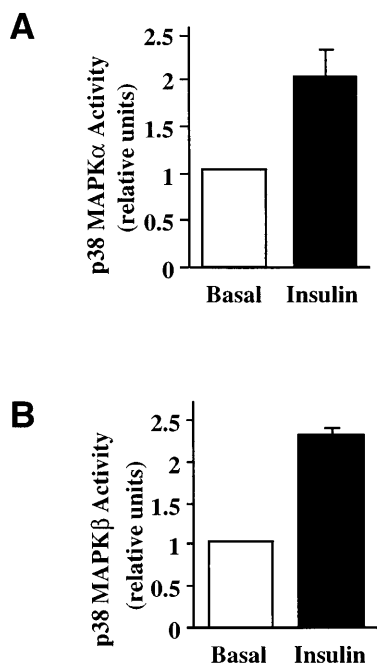


**FIG. 1.** Phosphorylation and activation of p38 MAPK by insulin. Soleus and quadriceps muscles were isolated from rats injected with insulin (portal vein, 3.5 min) or saline (basal). Anti-phosphotyrosine immunoprecipitates from soleus muscle lysates were immunoblotted with anti-p38 MAPK antibody (A). Quadriceps muscle lysates (150 µg) were immunoblotted with anti-phospho-p38 MAPK (B, upper immunoblot) or anti-phospho-CREB antibodies (C). Representative immunoblots are shown. The upper immunoblot shown in B was stripped of bound antibodies and reprobed with an anti-p38 MAPK antibody (B, lower immunoblot). The amount of phosphorylated protein was quantitated and is expressed in the graphs accompanying each immunoblot. Samples were resolved on the same gel, and the lowest basal was assigned a value of 1.0; all other values are expressed relative to this value. The graphs accompanying each immunoblot represent the mean ± SE of three experiments (A) or the mean ± SD of two experiments (B and C). Absence of error bars indicates a standard error that was too small to appear on this scale.

**Both p38 MAPK $\alpha$  and p38 MAPK $\beta$  isoforms are activated by insulin in vivo.** Figure 1 shows that insulin stimulated the phosphorylation of p38 MAPK and its activity toward CREB, one of its known endogenous substrates. However, the phosphospecific p38 MAPK antibody does not discriminate between isoforms of the enzyme. We used an in vitro kinase assay to determine which isoforms were activated by insulin in quadriceps muscles. Although skeletal muscle expresses three isoforms of the enzyme ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), immunoprecipitating antibodies are available only toward p38 MAPK $\alpha$  and p38 MAPK $\beta$ . Each of these proteins was immunoprecipitated using isoform-specific antibodies, and the ability of the immunisolated enzymes to phosphorylate myelin basic protein (MBP) was determined. Insulin treatment in vivo (3.5 min) increased the activity of p38 MAPK $\alpha$  by  $2.1 \pm 0.2$ -fold, as measured in vitro (Fig. 2A). Addition of 10 µmol/l SB203580 directly to the immunoprecipitates inhibited this activity (SB203580  $0.8 \pm 0.2$ -fold vs. basal, SB203580 + insulin  $0.7 \pm 0.1$ -fold vs. basal), confirming that the kinase activity measured was due to p38 MAPK. The in vitro kinase activity of p38 MAPK $\beta$  was stimulated by  $2.4 \pm 0.2$ -fold by in vivo insulin treatment (Fig. 2B). Addition of 10 µmol/l SB203580 to the immunoprecipitates also inhibited p38 MAPK $\beta$  (SB203580  $0.6 \pm 0.2$ -fold vs. basal, SB203580 + insulin  $0.70 \pm 0.05$ -fold vs. basal). In experiments in which ATF2 (a p38 MAPK substrate [20]) was used as the substrate in the kinase assays, insulin-stimulated p38 MAPK $\alpha$  activity was 2.2-fold above basal, whereas insulin-stimulated p38 MAPK $\beta$  activity was 2.3-fold above basal (data not shown).



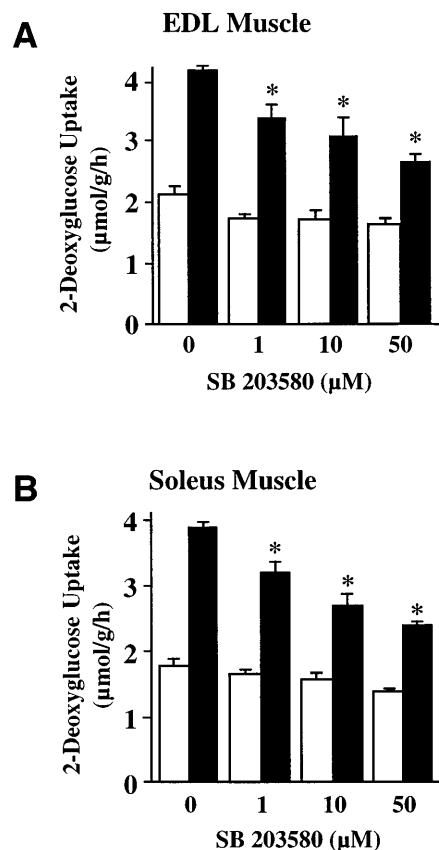
**FIG. 2.** Activation of p38 MAPK  $\alpha$  and  $\beta$  isoforms by insulin. p38 MAPK $\alpha$  (A) or p38 MAPK $\beta$  (B) was immunoprecipitated from quadriceps muscles, treated as described in Fig. 1. Kinase activities were determined by an in vitro kinase assay using MBP as substrate. Where indicated, 10 µmol/l SB203580 (SB203) was added directly to the kinase assay. Results are the mean ± SE of three experiments. Nonspecific activity was determined using an irrelevant IgG and was subtracted.



**FIG. 3.** Activation of p38 MAPK  $\alpha$  (A) and  $\beta$  (B) isoforms by insulin in isolated muscle. EDL muscles were isolated, then treated in vitro with 1 nmol/l insulin for 4 min. Muscles were then processed and kinase activity was determined as described in Fig. 2. Results are the mean  $\pm$  SE of three individual experiments. Basal kinase activity was assigned a value of 1.0, and insulin-stimulated activity is expressed relative to this value.

These results indicate that insulin activates at least two isoforms of p38 MAPK in rat skeletal muscle and that this activation is maintained in vitro.

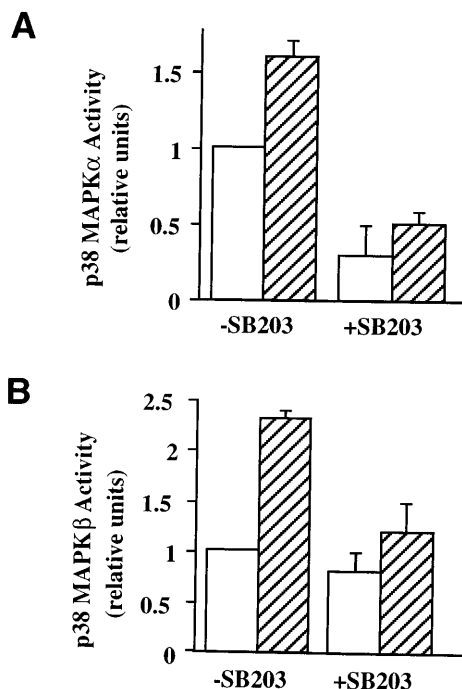
**Treatment of isolated muscles with insulin activates p38 MAPK $\alpha$  and p38 MAPK $\beta$ .** In Fig. 2, we demonstrated that administration of insulin in vivo resulted in p38 MAPK $\alpha$  and p38 MAPK $\beta$  activation. To determine if any systemic or humoral factors are necessary for this activation, isolated EDL muscles were either left untreated or treated with a submaximally effective concentration of insulin (1 nmol/l) for 4 min. The in vitro kinase activity of immunoisolated p38 MAPK $\alpha$  and p38 MAPK $\beta$  were determined. In vitro insulin treatment stimulated p38 MAPK $\alpha$  activity by  $2.0 \pm 0.3$ -fold (Fig. 3A) and p38 MAPK $\beta$  activity by  $2.3 \pm 0.1$ -fold (Fig. 3B). These results suggest that all of the necessary components for the activation of p38 MAPK by insulin are preserved in isolated skeletal muscle. **SB203580 lowers the insulin stimulation of 2-deoxyglucose uptake in isolated muscles.** The pyridinyl imidazole SB203580 inhibits the p38 MAPK $\alpha$  and p38 MAPK $\beta$  isoforms (23) (Fig. 2). In addition, this inhibitor was earlier shown to reduce the stimulation of glucose uptake by insulin in muscle and adipose cells in culture (15). To explore if a similar behavior was observed in skeletal muscle, isolated EDL muscle strips were treated with 1, 10, and 50  $\mu$ mol/l SB203580 for 20 min before treatment with a submaximal concentration of insulin (1 nmol/l) for 30 min (Fig. 4). Insulin increased 2-deoxyglucose uptake into EDL by 2.1-fold ( $2.1 \pm 0.1$  and  $4.2 \pm 0.01$   $\mu$ mol  $\cdot$  g $^{-1}$   $\cdot$  h $^{-1}$ , basal and insulin, respectively,  $P < 0.001$ ). Treatment with 1, 10, and 50  $\mu$ mol/l SB203580 before and during insulin action caused a statistically significant inhibition of insulin-stimulated 2-deoxyglucose uptake



**FIG. 4.** Reduction of insulin-induced 2-deoxyglucose uptake by SB203580. Isolated EDL (A) and soleus (B) muscle strips were incubated with or without the indicated concentrations of SB203580 for 20 min. Muscles were then incubated for 30 min with or without insulin in the absence ( $\square$ ) or continued presence ( $\blacksquare$ ) of SB203580. 2-Deoxyglucose uptake was then determined. Results represent the mean  $\pm$  SE of five different muscles in each group. \* $P < 0.01$  compared with insulin treatment in the absence of inhibitor.

of 20, 33, and 48%, respectively (Fig. 4A). Up to 100  $\mu$ mol/l, no further inhibition was observed at higher concentrations of the drug (data not shown). Similar inhibitory effects on insulin action in the EDL muscle were observed using another inhibitor of p38 MAPK, SB202190 (data not shown). In isolated soleus muscle strips, insulin increased 2-deoxyglucose uptake by 2.3-fold above basal ( $1.7 \pm 0.1$  and  $3.9 \pm 0.1$   $\mu$ mol  $\cdot$  g $^{-1}$   $\cdot$  h $^{-1}$ , basal and insulin, respectively,  $P < 0.0001$ ). Incubation with 1, 10, and 50  $\mu$ mol/l SB203580 caused a statistically significant inhibition of insulin-stimulated 2-deoxyglucose uptake by 35, 59, and 65%, respectively (Fig. 4B). No further inhibition was observed using higher concentrations of the inhibitor (data not shown). Basal uptake rates were affected only at high concentrations of the inhibitor (50  $\mu$ mol/l). From the above results, it is apparent that SB203580 reduces the insulin-stimulation of 2-deoxyglucose uptake by 50–60% in skeletal muscles, and that half of this effect is observed using  $\sim 1$   $\mu$ mol/l of the drug.

**p38 MAPK $\alpha$  and p38 MAPK $\beta$  are activated by electrically-induced contraction.** Contraction is a potent stimulator of glucose uptake in muscle (24), yet the signaling pathway linking contraction to glucose transporters is still unknown. Several enzymes have been shown to be activated and/or phosphorylated in exercising and/or contracting mus-



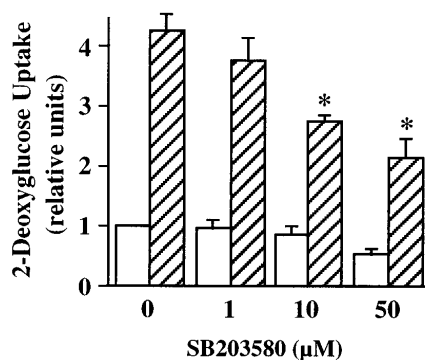
**FIG. 5.** Activation of p38 MAPK  $\alpha$  (A) and  $\beta$  (B) isoforms by contraction. EDL muscles were (▨) or were not (□) electrically stimulated in vitro for 20 min then processed for kinase activity, as described in Fig. 2. Where indicated, 10  $\mu\text{M}$  SB203580 (SB203) was added directly to the kinase assay. Results are the mean  $\pm$  SE of three individual experiments. Basal kinase activity was assigned a value of 1.0 and insulin-stimulated activity is expressed relative to this value.

cle, namely 5'-AMP kinase, extracellular regulated kinase, c-Jun NH<sub>2</sub>-terminal kinase, and p38 MAPK (25–27). The specific p38 MAPK isoforms activated in response to contraction have not been determined. Figure 5A shows that contraction (20 min) increased the activity of p38 MAPK $\alpha$  by  $1.6 \pm 0.1$ -fold above basal. Addition of 10  $\mu\text{M}$  SB203580 directly to the immunoprecipitates inhibited the enzyme. The p38 MAPK $\beta$  isoform was also activated by contraction ( $2.3 \pm 0.1$ -fold above basal [Fig. 5B]), and this activity was also inhibited by 10  $\mu\text{M}$  SB203580.

**Contraction-stimulated 2-deoxyglucose uptake is reduced by SB203580.** The results in Fig. 4 indicated that the insulin-dependent stimulation of glucose uptake was partially inhibited by SB203580. Therefore, it was of interest to determine if this compound would also alter the stimulation of glucose uptake elicited by contraction. Muscles were pretreated with increasing concentrations of SB203580 before and during electrical stimulation in vitro for 20 min, and 2-deoxyglucose uptake was subsequently determined. Contraction increased glucose uptake by  $\sim 4.1 \pm 0.3$ -fold above basal ( $P < 0.001$ ) (Fig. 6). Treatment with 1, 10, and 50  $\mu\text{M}$  SB203580 inhibited contraction-stimulated 2-deoxyglucose uptake by 13, 41, and 50%, respectively. Hence, like insulin, SB203580 reduced the contraction-induced stimulation of glucose uptake by 50%.

## DISCUSSION

Based on studies in cells in culture, we have recently proposed that the maximal stimulation of glucose transport by insulin may require an increase in the intrinsic activity of GLUT4, sub-



**FIG. 6.** Contraction-stimulated 2-deoxyglucose uptake is reduced by SB203580. Isolated EDL muscles were incubated with or without the indicated concentrations of SB203580 for 20 min. Muscles were then electrically-stimulated for 20 min in the absence (□) or continued presence (▨) of SB203580. 2-Deoxyglucose uptake was then determined. Results represent the mean  $\pm$  SE of at least three experiments. \* $P < 0.05$  compared with contraction in the absence of inhibitor.

sequent to its translocation to the plasma membrane (15). A survey of the literature suggests that there is a disparity between the extent of GLUT4 translocation and the stimulation of glucose uptake in mature muscle (14,28–35). In primary and cultured adipocytes, diverse conditions also dissociate stimulation of glucose uptake from GLUT4 translocation (8,36,37). Collectively, these studies support the notion that the intrinsic activity of glucose transporters may be subject to regulation and may determine the full stimulation of glucose uptake. Using cells in culture, we have shown that the inhibition of insulin stimulation of glucose uptake by SB203580 (15) and other chemically unrelated inhibitors of p38 MAPK (38) occurs without any interference of glucose transporter translocation to the cell surface. These results suggest that these compounds, by virtue of their inhibition of p38 MAPK, may negatively impact the intrinsic activity of the translocated glucose transporters, thereby reducing glucose uptake. Hence, it is imperative to examine in detail whether insulin activates p38 MAPK in mature muscle and to explore if inhibitors of the enzyme affect glucose uptake in this tissue.

**Activation of p38 MAPK by insulin.** Phosphorylation of p38 MAPK is often considered a measurement of enzyme activity (20). Using this approach, we demonstrate here that insulin administration in vivo rapidly stimulates p38 MAPK in quadriceps muscle of the rat. Consistent with this observation, insulin caused a rapid and transient phosphorylation of the enzyme in mouse gastrocnemius muscle (39). The phospho-specific p38 MAPK antibody recognizes all isoforms of the enzyme, and hence does not reveal selective activation of individual isoforms. Thus, we used isoform-specific antibodies in an in vitro kinase assay and observed that insulin activates p38 MAPK $\alpha$  and p38 MAPK $\beta$  in quadriceps and EDL muscles. These isoforms are the only ones inhibited by SB203580 (23). These results indicate that the enzymes maintain activity in vitro. To explore whether activation of p38 MAPK by insulin coincided with activation of downstream substrates of the enzyme, we measured the phosphorylation of CREB and observed an insulin-dependent phosphorylation of this substrate in rat quadriceps muscle. Another substrate of p38 MAPK, ATF-2, was shown to become phosphorylated in response to insulin in mouse hindlimb muscles (40). Activation of p38 MAPK by insulin has also been demonstrated in

L6 muscle cells (18) and 3T3-L1 adipocytes (15,41). In contrast to these observations, Goodyear et al. (25) were unable to detect an insulin-dependent effect on p38 MAPK activity in mixed hindlimb muscle preparations of rat using an in vitro kinase assay. The discrepancy between this study and those cited above may be due to differences in the time of insulin exposure. Goodyear et al. measured p38 MAPK activity 20 and 30 min into the insulin treatment (25), times at which the p38 MAPK phosphorylation observed by Guo et al. (39) had already subsided. In addition to insulin, several studies have explored the effect of muscle contraction on p38 MAPK activity. In vivo muscle contraction increased p38 MAPK activity/phosphorylation in human (42) and rat (25) skeletal muscle. In the present study, we extend these observations by showing that contraction leads to activation of the p38 MAPK  $\alpha$  and  $\beta$  isoforms in EDL muscle. Whether insulin and contraction activate the same or differently compartmentalized enzymes remains to be determined.

**Inhibition of the insulin- and contraction-dependent stimulation of glucose transport by SB203580.** The signaling mechanism by which contraction stimulates glucose uptake in skeletal muscle is undefined, but it appears to differ from that used by insulin. Thus, PI3K and Akt are not activated by contraction, and wortmannin has no effect on contraction-stimulated glucose uptake (3,43). In contrast, as shown here, insulin and contraction coincide in their ability to stimulate p38 MAPK $\alpha$  and p38 MAPK $\beta$ . The following observations suggest that there is a link between p38 MAPK activity and stimulation of muscle glucose. First, an inhibitor of p38 MAPK, SB203580, reduced the stimulation of glucose uptake elicited either by insulin or contraction; inhibition was already observed with a 1- $\mu$ mol/l concentration of the inhibitor. Although submicromolar concentrations of SB203580 inhibit recombinant p38 MAPK in vitro, the compound has only small effects on MAPK-activated protein kinase-2 activity and CREB phosphorylation when administered to HEK 293 cells, and full inhibition was only attained with a 10  $\mu$ mol/l dose (22). Moreover, the effect of insulin on glucose transport in the EDL muscle was also lowered by SB202190 (data not shown), another p38 MAPK inhibitor. Further evidence that p38 MAPK is the SB203580-sensitive pathway involved in glucose transport stimulation is provided by our recent observation of a significant reduction in insulin stimulation of glucose uptake in 3T3-L1 adipocytes expressing a dominant-negative p38 MAPK mutant (R.S., T.R., D.Y.K., P. Scherer, A.K., unpublished observations); the cells are described in the article by Engleman et al. (44).

We have previously shown that 10  $\mu$ mol/l SB203580 does not affect upstream insulin signals such as insulin receptor substrate (IRS)-1 phosphorylation, PI3K activity associated with IRS-1, or activation of Akt1, -2, and -3 in 3T3-L1 adipocytes (15). In muscle cells, up to 50  $\mu$ mol/l SB203580 did not alter insulin-stimulated Akt1, -2, and -3 activity, or T308 and S473 phosphorylation of Akt1, suggesting that the kinases upstream of Akt are not affected (D. Konrad, R.S., and A.K., unpublished observations). In contrast, a recent report demonstrated reduction of interleukin-2-stimulated Akt activation in T-cells by SB203580 (at concentrations >2  $\mu$ mol/l) (45). These results suggest that the effect of this compound on Akt activity may be more apparent in certain cell types and/or that its effect on this pathway is modulated by the stimulus (i.e., more specific for interleukin signaling).

It is unlikely that binding of SB203580 directly to the cell surface glucose transporters would be responsible for the inhibition of the insulin- or contraction-stimulated glucose uptake because of the following reasons. First, the maximal inhibition of insulin- or contraction-stimulated glucose uptake in muscle by SB203580 was ~50%. If SB203580 or other p38 MAPK inhibitors were to interact directly with cell surface glucose transporters, then full inhibition of glucose uptake would be expected at sufficiently higher doses of the drug. Second, the presence of 10  $\mu$ mol/l SB203580 during only the transport assay did not inhibit basal or insulin-stimulated glucose uptake in muscle and fat cells (15).

As mentioned above, the inhibition of contraction- or insulin-stimulated glucose uptake caused by SB203580 was only partial, leaving ~50% of the transport unabated. Our current hypothesis is that the translocated GLUT4 molecules are endowed with a low (basal) level of transport activity, which can be further enhanced by a mechanism sensitive to SB203580. Presently, it is difficult to determine if insulin- or contraction-induced GLUT4 translocation in muscle proceeds normally in the presence of SB203580. To estimate this would require the use of a method that would allow exofacial labeling of GLUT4, because subcellular fractionation cannot distinguish between transporters that are fully inserted into the plasma membrane and T-tubules and those that are docked but not fused. The exofacial label ATB-[<sup>3</sup>H]BMPA cannot be used to resolve this question, because it reacts with the active site of glucose transporters and, hence, may selectively label active transporters. Any reduction in ATB-[<sup>3</sup>H]BMPA labeling in the presence of SB203580 would not differentiate between fewer transporters and less active transporters at the cell surface. Our results in cells in culture suggest that p38 MAPK acts after GLUT4 insertion into the surface membranes. This conclusion is based on the observation that SB203580 inhibits insulin-induced glucose uptake but does not affect GLUT4 insertion into the plasma membrane (detected by exofacial exposure of a myc epitope on the transporter in intact cells) (15).

In conclusion, the present data are consistent with the hypothesis that a p38 MAPK-dependent activation step is a point of convergence of the signaling pathways used by insulin and contraction to increase glucose uptake in skeletal muscle. Future experiments using animal models lacking the enzymes responsible for p38 MAPK activation should allow us to further establish the role of this pathway in the regulation of muscle glucose uptake.

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