Activation of p38 Mitogen-Activated Protein Kinase α and β 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 MAPKα and p38 MAPKβ 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 MAPKα and p38 MAPKβ. The activity of both isoforms was inhibited in vitro by 10 µmol/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 µmol/l SB203580. These results show that p38 MAPKα and -β 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

Translocation 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-λ, 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 MAPKα and p38 MAPKβ 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 (α and β) and anti-p38 MAPK antibody, which cross reacts with these two isoforms, were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho–p38 MAPK 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.

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 MAPKα and p38 MAPKβ isoforms. Insulin injection resulted in a 3.2 ± 0.3-fold higher p38 MAPK signal in these immunoprecipitates (Fig. 1A). There was no difference in the total amount of p38 MAPK in the samples (Fig. 1B).

As observed for p38 MAPK, in vivo insulin treatment for 3.5 min increased CREB phosphorylation in muscle by 3.5 ± 0.05-fold (Fig. 1C). The phosphospecific CREB antibody also recognized a lower molecular weight protein, which presumably is ATF1.
Both p38 MAPKα and p38 MAPKβ 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 (α, β, and γ), immunoprecipitating antibodies are available only toward p38 MAPKα and p38 MAPKβ. Each of these proteins was immunoprecipitated using isoform-specific antibodies, and the activity of the immunoprecipitated enzymes to phosphorylate myelin basic protein (MBP) was determined. Insulin treatment in vivo (3.5 min) increased the activity of p38 MAPKα by 2.1 ± 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 ± 0.2-fold vs. basal, SB203580 + insulin 0.7 ± 0.1-fold vs. basal), confirming that the kinase activity measured was due to p38 MAPK. The in vitro kinase activity of p38 MAPKβ was stimulated by 2.4 ± 0.2-fold by in vivo insulin treatment (Fig. 2B). Addition of 10 µmol/l SB203580 to the immunoprecipitates also inhibited p38 MAPKβ (SB203580 0.6 ± 0.2-fold vs. basal, SB203580 + insulin 0.70 ± 0.05-fold vs. basal). In experiments in which ATF2 (a p38 MAPK substrate [20]) was used as the substrate, insulin-stimulated p38 MAPKα activity was 2.2-fold above basal, whereas insulin-stimulated p38 MAPKβ activity was 2.3-fold above basal (data not shown).

**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.

**FIG. 2.** Activation of p38 MAPKα and β isoforms by insulin. p38 MAPKα (A) or p38 MAPKβ (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.
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 MAPKα and p38 MAPKβ.** In Fig. 2, we demonstrated that administration of insulin in vivo resulted in p38 MAPKα and p38 MAPKβ 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 immunosolated p38 MAPKα and p38 MAPKβ were determined. In vitro insulin treatment stimulated p38 MAPKα activity by 2.0 ± 0.3-fold (Fig. 3A) and p38 MAPKβ activity by 2.3 ± 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 MAPKα and p38 MAPKβ 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 µmol/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 2.3-fold above basal (1.7 ± 0.1 and 3.9 ± 0.1 µmol · g⁻¹ · h⁻¹, basal and insulin, respectively, P < 0.0001). Incubation with 1, 10, and 50 µmol/l SB203580 caused a statistically significant inhibition of insulin-stimulated 2-deoxyglucose uptake by 20, 33, and 48%, respectively (Fig. 4A). Up to 100 µmol/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 ± 0.1 and 3.9 ± 0.1 µmol · g⁻¹ · h⁻¹, basal and insulin, respectively, P < 0.0001). Incubation with 1, 10, and 50 µmol/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 µmol/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 ~1 µmol/l of the drug.

**p38 MAPKα and p38 MAPKβ 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-
**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, subsequent 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 (38). The p38 MAPKα and p38 MAPKβ isoforms 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 α and β 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, PEK 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 MAPKα and p38 MAPKβ. 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-µmol/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-µmol/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 µmol/l SB203580 does not affect upstream insulin signals such as insulin receptor substrate (IRS)-1 phosphorylation, PKB activity associated with IRS-1, or activation of Akt, -2, and -3 in 3T3-L1 adipocytes (15). In muscle cells, up to 50 µmol/l SB203580 did not alter insulin-stimulated Akt, -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 µmol/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 µmol/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-[^3H]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-[^3H]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 insulin 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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