Activation of p38 MAP kinase enhances sensitivity of muscle glucose transport to insulin

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METHODS

EXERCISE SUCH AS RUNNING OR SWIMMING and electrical stimulation of muscles to contract induce an increase in glucose transport activity in skeletal muscle (20). This acute effect of muscle contractions on glucose transport is independent of the effect of insulin and reverses rapidly after cessation of contractile activity (20). As this increase in glucose transport wears off, it is replaced by an increase in insulin sensitivity. This phenomenon was discovered more than two decades ago by Ruderman’s group (9, 28) and has been further characterized (19), but the mechanism by which it is mediated has remained elusive, but in a study in which the 2-N-4-(1-azi-2,2,2-trifluoroethyl)-[2,3H]benzoyl-1,3-bis-(o-mannos-4-yloxy)-2-propylamine (ATB-[2,3H]BMPA) exofacial photolabeling technique was used to assess GLUT4 translocation, it was found that prior exercise increased GLUT4 labeling in response to a submaximal insulin stimulus that closely paralleled the increase in glucose transport (13). We interpreted this finding as indicating that the enhanced insulin sensitivity of glucose transport is mediated by increased GLUT4 translocation. However, evidence has accumulated suggesting that, in addition to causing GLUT4 translocation, insulin increases the intrinsic glucose-transporting activity of the glucose transporters (24, 31, 33). This raises the alternative possibilities that ATBBMPA binds only to activated GLUT4 or that activation of GLUT4 increases binding, so that what we interpreted as increased translocation could actually have been due to increased binding of the photolabel to GLUT4 at the cell surface.

The concept that GLUT4 is only partially active following translocation to the cell surface is based, at least in part, on the finding that inhibition of the p38 mitogen-activated protein kinase (MAPK, p38) with SB-203580, or expression of a dominant negative p38 mutant, reduces insulin-stimulated glucose transport ~50% without affecting GLUT4 translocation (24, 31, 33). Whether or not this concept is correct is currently the subject of controversy (8, 23). However, if it is correct, it would appear that stimulation of glucose transport by insulin or contractions is a two-step process, with the first being GLUT4 translocation and the second GLUT4 activation.

Muscle contractions have been shown to result in activation of p38 (10, 30, 35). It was found by Thong et al. (34) that the increase in p38 phosphorylation induced in skeletal muscle by exercise persists for at least 3 h after cessation of exercise, which is sufficiently long for the acute effect of exercise on glucose transport to wear off. This finding, together with the possible role of p38 in GLUT4 activation (31, 32) led Thong et al. to hypothesize that activation of p38 may be involved in mediating the increase in muscle insulin sensitivity after exercise (34). In this context, the purpose of this study was to evaluate the effect of activating p38 on insulin sensitivity of glucose transport in skeletal muscle and to determine whether activation of p38 mediates the increase in insulin sensitivity induced by exercise.

MATERIALS

2-Deoxy-[1,2-3H]glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). [14C]mannitol was obtained from ICN Radiochemicals (Irvine, CA). SB-203190 was purchased from Calbiochem (San Diego, CA). Anti-phospho-(Thr180/Tyr182)-p38 MAPK, total p38 MAPK, anti-phospho-cAMP response element-binding protein (CREB) (Ser133), anti-phospho-stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK) (Thr183/Tyr185), and anti-phospho-extracellular signal-related kinase (ERK)1/2 MAP kinase (Thr202/Tyr204) antibodies were purchased from Cell Signaling (Beverly, MA). The horseradish peroxidase

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M perchloric acid (27). Glycogen levels were measured for muscles
sured spectrophotometrically in muscle samples homogenized in 0.3
without anisomycin (10
1:5,000. Western blotting was performed as previously described (37).
(a concentration of 1:1,000. The appropriate HRP-conjugated second-
and SB-202190, followed by 30 min of
the absence of anisomycin and SB-202190, before exposure to anisomycin in the continued presence of
(17, 38). Soleus muscles were split longitudinally into strips before incubation, as described previously
(17), to allow adequate diffusion of oxygen and substrates. All
protocols were approved by the Animal Studies Committee of Wash-
ington University.

Muscle treatments. After dissection, muscles recovered for 60 min
in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB)
with 8 mM glucose, 32 mM mannitol, with a gas phase of 95%
O2-5% CO2, placed in a shaking incubator maintained at 35°C. 
Muscles were then transferred to flasks containing the medium with or
without anisomycin (10 μg/ml) for 30 min. In some experiments,
muscles were preincubated for 60 min in KHB with 10 μM SB-
202190 before exposure to anisomycin in the continued presence of
the compound. The muscles were then allowed to recover for 3 h in
the absence of anisomycin and SB-202190, followed by 30 min of
incubation with or without 60 μU/ml insulin before transport activity
measurement. SB-202190 is light sensitive; therefore, flasks contain-
ing this compound were wrapped in foil.

Measurement of glucose transport activity. To remove glucose
from the extracellular space, the muscles were rinsed for 10 min at
29°C in 2 ml of oxygenated KHB containing 40 mM mannitol and
with insulin if it was present during the previous incubation. After the
rinse step, muscles were incubated for 20 min at 29°C in flasks
containing 2 ml of KHB with 4 mM 2-deoxy-[1-14C]glucose (2-DG; 1.5 μCi/ml) and 36 mM [14C]mannitol (0.2 μCi/ml), with a gas phase of
95% O2-5% CO2, in a shaking incubator (12). The same additions
that were in the rinse were present during the determination of glucose
transport. The muscles were then blotted and clamp frozen and
processed for determination of intracellular 2-DG accumulation and
extracellular space, as described previously (38).

In vitro muscle contraction. Some muscles were electrically stimu-
lated to contract in vitro, as described previously (18). Ten tetanic
contractions were elicited by stimulation at 100 Hz for 10 s at a rate of
1 contraction/min for 10 min. Muscles were stimulated to contract
while incubated in rat serum with or without the p38 MAPK inhibitor
SB-202190. Muscles were then allowed to recover in KHB containing
8 mM glucose, 32 mM Mannitol, and 0.1% BSA for 3 h, followed by
30 min of incubation in the same medium with or without 60 μU/ml
insulin before 2-DG transport assays. Homogenates of clamp-frozen
epitrochlearis muscles were prepared as described previously (36).

Aliquots of muscle homogenate containing 30 μg of protein were
subjected to SDS-PAGE (10% resolving gel), and Western blot
analysis was used for measurement of phosphorylated p38 MAPK
(Thr180/Tyr182), CREB (Ser133), JNK (Thr183/Tyr185), and p44/42
MAP kinase (Thr202/Tyr204), and p38 MAPK antibodies were used at
a concentration of 1:1,000. The appropriate HRP-conjugated second-
ary antibodies (goat anti-mouse for-phospho-CREB and donkey anti-
rabbit for all other primary antibodies) were used at a concentration
of 1:5,000. Western blotting was performed as previously described (37).
Bands were visualized by ECL and quantified using densitometry.

Measurement of glycogen levels. Glycogen concentration was mea-
sured spectrophotometrically in muscle samples homogenized in 0.3
M perchloric acid (27). Glycogen levels were measured for muscles
incubated in the absence or presence of 10 μg/ml anisomycin in KHB.

Muscle samples were frozen after the full 3-h and 30-min incubation
period (30 min ± anisomycin, 3 h recovery).

Statistical analysis. Data are presented as means ± SE. Compari-
sions between the means of multiple groups were made using a
one-way analysis of variance (ANOVA) followed by a post hoc
comparison using Fishers least significant difference method.

RESULTS

Anisomycin increases p38 phosphorylation. Most of the
known activators of p38, such as the inflammatory cytokines
TNF-α and IL-6, have a variety of effects that could compli-
cate evaluation of the role of p38 activation in mediating an
increase in muscle insulin sensitivity. We, therefore, used
anisomycin, which is a well-characterized and relatively speci-
fic activator of p38 and JNK MAPKs (2, 15, 16, 39). Another
major effect of anisomycin is that it is a translational inhibi-
tor of protein synthesis; however, this effect does not appear to be a
problem in the present context, because we have shown that
inhibition of protein synthesis does not interfere with the
development of an increase in muscle insulin sensitivity after
contractile activity (7). To confirm that anisomycin activates
p38, soleus muscles were exposed to anisomycin, and phos-
phorylation of p38 on Thr180/Tyr182 was measured. As shown
in Fig. 1A, exposure of soleus muscle strips to anisomycin
increased the ratio of phospho-p38 to total p38 by ~2.5-fold. The
closely related pyridinyl imidazole compounds SB-203580 and
SB-202190 (SB compounds) are selective inhibitors of the
activation of p38 (5, 6). Pretreatment with SB-202190 comple-
tely blocked the increase in p38 phosphorylation induced by
anisomycin (Fig. 1A). As further evidence that p38 is activated
by anisomycin, we measured phosphorylation of CREB, which is
a downstream target of the p38 signaling pathway. As shown
in Fig. 1B, treatment of soleus muscles with anisomycin
induced an approximately twofold increase in CREB phos-
phorylation. The increase in CREB phosphorylation was com-
pletely blocked by pretreatment with SB-202190 (Fig. 1B).

Previous studies have shown that anisomycin also activates
the stress-activated protein kinase/JNK pathway, which is
closely related to the p38 MAPK pathway (16). As shown in
Fig. 1C, exposure to anisomycin increased JNK phosphoryla-
tion by ~60%. However, incubation with SB-202190 had no
effect on JNK phosphorylation (Fig. 1C). In addition, we
measured the effect of anisomycin treatment on phosphoryla-
tion of ERK1/2. There was no significant change in phosphor-
ylation of ERK in response to treatment with anisomycin or SB
(Fig. 1D).

Anisomycin induces an increase in 2-DG transport. Incuba-
tion of muscles with 10 μg/ml anisomycin induced a modest
increase in glucose transport activity in both the soleus (0.65 ±
0.07 μmol·ml⁻¹·10⁻¹ min⁻¹ basal vs. 1.33 ± 0.28
μmol·ml⁻¹·10⁻¹ min⁻¹, anisomycin, P < 0.05) and the
epitrochlearis (0.68 ± 0.07 μmol·ml⁻¹·10⁻¹ min⁻¹ basal vs.
1.21 ± 0.32 μmol·ml⁻¹·10⁻¹ min⁻¹, P < 0.05). This finding is
in keeping with earlier reports that anisomycin stimulates
glucose transport in 3T3-L1 adipocytes (1, 14). In those
studies, the anisomycin-induced increase in glucose transport
was attributed to activation of cell surface GLUT1 glucose trans-
porters (1, 14).

Anisomycin induces an increase in insulin sensitivity. The
acute effect of anisomycin on glucose transport activity did not
completely reverse during the 3.5-h recovery period. Never-
theless, it is clear that anisomycin markedly enhanced the effect of a submaximal insulin stimulus, 60 μU/ml, on 2-DG transport in both the soleus and epitrochlearis muscles (Fig. 2). This increase in insulin sensitivity is evident from a comparison of the increases in 2-DG transport induced by insulin in the control and anisomycin-treated muscles (Fig. 2).

Inhibition of p38 prevents the increase in insulin sensitivity induced by anisomycin. It has been shown that inhibition of p38 with SB-202190 causes an ~50% inhibition of the stimulation of glucose transport by insulin (31–33). To determine whether the inhibitory effect of SB-202190 on insulin action wears off sufficiently rapidly so as not to interfere with measurement of insulin sensitivity, we compared the effect of 60 μU/ml insulin on glucose transport in control muscles and muscles treated with SB-202190 3.5 h previously. As shown in Fig. 3, there was no difference in insulin-stimulated glucose transport between the control muscles and those that had been exposed to SB-202190. This finding made it possible to investigate the effect of inhibiting p38 on the anisomycin-induced increase in insulin sensitivity.

Soleus muscles strips were incubated with or without 10 μM SB-202190 for 60 min before and during treatment with anisomycin, allowed to recover for 3 h, and then incubated for 30 min with or without insulin. They were compared with muscles treated with insulin after the same incubation period but not exposed to SB-202190 or anisomycin. As shown in Fig. 4, pretreatment with SB-202190 completely inhibits the increase in insulin sensitivity of glucose transport induced by anisomycin.

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**Fig. 1.** Anisomycin (Aniso) increases p38 phosphorylation. Soleus muscle strips were incubated with or without 10 μM SB-202190 (SB) for 60 min followed by incubation with or without 10 μg/ml anisomycin for 30 min. Homogenates of clamp-frozen muscles were used for measurement of total p38 and phospho-p38 (P-p38; A); phospho-CAMP response element-binding protein (P-CREB; B); phospho-c-Jun NH2-terminal kinase (P-JNK; C), and phospho-extracellular signal-related kinase (P-ERK; D). Values are means ± SE for 6–8 muscles per group. *P < 0.001, anisomycin vs. basal and vs. anisomycin + SB; +P < 0.005, anisomycin and anisomycin + SB vs. basal.
Does anisomycin induce a change in muscle glycogen levels? A previous study by Coderre et al. (3) suggested that some GLUT4 vesicles in muscle are associated with glycogen, raising the possibility that the increase in insulin sensitivity after exercise is the result of a larger available pool of free GLUT4 vesicles due to glycogen depletion. In the present study, incubation of muscles with anisomycin had no significant effect on glycogen concentration (18.9 ± 1.1 μmol glucosyl units/g, control vs. 16.1 ± 1.9 μmol glucosyl units/g, anisomycin; n = 5 muscles/group).

Does activation of p38 mediate the contraction-induced increase in insulin sensitivity? Stimulation of soleus muscles to contract induced an approximately threefold increase in p38 phosphorylation that was completely prevented by preincubation with SB-202190 (Fig. 5). Viewed in the context of the evidence that activation of p38 by anisomycin increases insulin sensitivity of muscle glucose transport, this finding strongly suggested that contractions may increase insulin sensitivity by activation of p38. However, in both the soleus and epimysialar muscles, the same stimulation protocol that increased p38 phosphorylation induced increases in insulin sensitivity despite
preincubation with SB-202190 at the concentration that completely blocked p38 phosphorylation (Fig. 6).

**DISCUSSION**

The novel information provided by this study is that treatment of skeletal muscles with anisomycin is followed by a dramatic increase in insulin sensitivity of the glucose transport process. Anisomycin is a potent and well-characterized activator of p38 (25, 29, 39). Our results confirm that anisomycin activates p38 in skeletal muscle and show that the increase in insulin sensitivity of glucose transport induced by anisomycin is mediated by activation of p38. The evidence that p38 mediates the anisomycin-induced increase in insulin sensitivity is provided by the finding that pretreatment of muscles with SB-202190 completely prevented the increase in insulin sensitivity. The closely related pyridimyl imidazoles SB-203580 and SB-202190 are specific inhibitors of p38 activation (5, 6).

In the present study, SB-202190 blocked the increase in p38 activation by anisomycin, as evidenced by prevention of p38 phosphorylation on Thr180/Tyr182 and CREB phosphorylation on Ser133. CREB is a downstream substrate of the p38-signaling pathway (6). In addition to activating p38, anisomycin activates the JNK pathway (16), and treatment of muscles with anisomycin in the present study also resulted in increased phosphorylation of JNK. However, the anisomycin-induced increase in JNK phosphorylation was not inhibited by SB-202190.

Four isoforms of p38 MAPK have been identified in skeletal muscle α, β, δ, and γ (11, 21, 22, 26). The antibody used in the present study is not isoform specific; however, the SB compounds inhibit only the p38 MAPK α- and β-isoforms (4). Because the activation of p38 MAPK with anisomycin is completely inhibited by SB-202190, it appears that the α- and/or β-isoform(s) is/are involved in mediating the increase in insulin sensitivity seen with anisomycin.

A number of studies have provided evidence suggesting that activation of p38 mediates a step in the stimulation of glucose transport by insulin and contractions (31–33). It was found that inhibition of insulin- or contraction-stimulated activation of p38 reduced the increase in glucose transport induced by these agents by ~50% without affecting GLUT4 translocation to the cell surface (31–33). These findings were interpreted as evidence that the GLUT4 translocated to the cell surface is only partially active and that a second step that involves activation of p38 by insulin or contractions is necessary to fully activate the GLUT4 (31–33). Other studies have provided evidence that argues against this concept (8, 23).

The subsequent finding that the increase in p38 phosphorylation induced in skeletal muscle by exercise persists for at
least 3 h in vivo led Thong et al. (34) to suggest that p38 may be involved in mediating the increase in muscle insulin sensitivity after exercise. On the basis of our evidence that anisomycin increases insulin sensitivity by activating p38, it appeared highly probable that this hypothesis might be correct. However, inhibition of p38 activation with SB-202190 had absolutely no effect on the increase in insulin sensitivity of glucose transport induced by contractile activity. One possibility suggested by this finding is that contractile activity and anisomycin increase muscle insulin sensitivity by different mechanisms. Another is that contractions and anisomycin activate the same signaling pathway leading to increased insulin sensitivity but that, in addition to activating p38, contractions activate another step in the pathway that is downstream of p38, thus bypassing the inhibitory effect of SB-202190. Clearly this is a fertile area for further research.

In conclusion, the results of this study provide evidence that activation of p38 is followed by an increase in the sensitivity of glucose transport to insulin in skeletal muscle. However, activation of p38 is not necessary for the enhancement of muscle insulin sensitivity by contractile activity.

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