p38γ MAPK regulation of glucose transporter expression and glucose uptake in L6 myotubes and mouse skeletal muscle

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SKELETAL MUSCLE represents a significant site for whole body insulin-stimulated glucose disposal (13) and insulin resistance in this tissue is a characteristic feature in the development of type 2 diabetes mellitus (12, 20, 43). In addition to diabetes, skeletal muscle insulin resistance is common to other clinical and subclinical conditions of obesity, stress, cachexia, pregnancy, starvation, sepsis, and trauma. These conditions are often associated with systemic factors that activate cellular signals, many of which are transmitted via mitogen-activated protein kinases (MAPK). Of the four main MAPK subgroups, the extracellular-signal-regulated kinases (ERK) appear to be primarily involved in cellular growth and transformation (40), and the c-Jun NH2-terminal kinases (JNK) and p38 MAPK are regulated by cytotoxic and environmental stress. JNK has recently been implicated in obesity- and TNF-α-mediated insulin resistance by negative regulation of insulin-stimulated phosphoinositide-3 kinase (PI3K) activity (2, 24). For p38 MAPK, numerous activators of these enzymes are associated with insulin resistance; however, data regarding the potential role of p38 MAPK in the regulation of glucose transport in skeletal muscle have been controversial (17, 49).

Four p38 MAPK isoforms have been reported: p38α (34), p38β (27, 48), p38γ (33, 35), and p38β (18). Activation of p38 MAPK occurs via dual phosphorylation of conserved TGY motifs by the MAPK kinases, MKK6 and MKK3 (41). Functional differences between the isoforms are related in part to their differential expression, activation, and substrate specificity. p38α and p38β are ubiquitously expressed, while p38γ is predominantly expressed in glandular tissues, lung, and kidney (28, 51). Interestingly, p38γ (ERK6, SAPK3) and MKK6 are primarily expressed in skeletal muscle (10, 21, 33).

In studies using the pyridinyl imidazole class of inhibitors, p38α and p38β activity has been shown to be required for insulin-stimulated glucose uptake through a mechanism that involves increases in the intrinsic activity of GLUT4 (45, 46, 49). In contrast to these studies, activation of p38 by overexpression of a constitutively active MKK6 decreased insulin signaling, GLUT4 expression, and insulin-stimulated glucose uptake in 3T3-L1 adipocytes and L6 cells (16, 17). In addition, overexpression of dominant negative p38α or MKK6 leads to increased insulin-stimulated glucose uptake in 3T3-L1 adipocytes (17). Thus there are data suggesting that p38 is necessary for insulin effects on glucose transport and other studies suggesting that p38 is a negative regulator of insulin-stimulated glucose uptake (16, 17).

Like insulin, exercise is known to increase skeletal muscle glucose transport. In adult skeletal muscle, exercise and contractile activity increase the phosphorylation and activity of multiple p38 isoforms via a mechanism that likely involves activation of MKK6/3 (4, 19, 23). One study has suggested that the well-known effects of contractile activity to stimulate glucose transport in skeletal muscle are impaired when muscles are treated with a p38 inhibitor (47). Interestingly, unlike p38α and p38β, p38γ is resistant to the effects by known p38 inhibitors (11). Compared with the α- and β-isoforms of p38, we have evidence that p38γ is very highly regulated by muscle contraction (6). Thus the γ-isofom is almost exclusively expressed in skeletal muscle and is highly regulated by skeletal muscle contraction, yet nothing is known about the role of

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p38γ in the regulation of basal and contraction-mediated glucose uptake.

To elucidate the role of p38γ in the regulation of basal and contraction-stimulated glucose uptake in L6 myotubes and skeletal muscle, we transfected and overexpressed wild-type p38γ or MKK6 into L6 myotubes and/or adult mouse skeletal muscle in vivo. We also utilized vector-based small interfering RNA (siRNA) technology to knock down p38γ in L6 myotubes. Our findings suggest that p38γ is involved in the regulation of GLUT1 expression and basal glucose uptake in L6 myotubes. Our findings also suggest that p38γ negatively regulates GLUT4 expression and contraction-mediated glucose uptake in adult skeletal muscle in vivo.

EXPERIMENTAL PROCEDURES

Materials. Antibodies used were purchased from the following: phospho-p38, MKK6, phospho-ATF-2 (Cell Signaling Technology), p38γ (Upstate Biotechnology), FLAG (Sigma), GLUT1 (Chemicon), and GLUT4 (gift from Robert Smith). Anti-mouse and anti-sheep IgG- horseradish peroxidase (HRP) were from Upstate Biotechnology. The anti-rabbit IgG-HRP and ECL-Plus Western blotting detection kit were purchased from Amersham Pharmacia Biotech. 2-Deoxy-d-[3H]glucose was from Perkin Elmer.

Plasmid construction. Currently, there are no p38γ inhibitors available, so to study p38γ function in L6 cells we expressed p38γ WT and constitutively active MKK6 (MKK6CA). The human FLAG-p38γWT and MKK6CA mutants (donated by Dr. Jiahuai Han) were subcloned from a pcDNA3 vector into a pCAGGS vector (gift from Dr. J. Miyazaki of Osaka University) (38). The MKK6 mutant was created by introducing a constitutive negative charge by replacing a glutamate residue in MKK6 with a glutamine residue (38). The MKK6 mutant was efficiently activated both endogenous and recombinant p38 (42). FLAG-p38 and MKK6 cDNA constructs were excised with HindIII and XhoI and transferred into the pCAGGS vector. The CAG promoter has extremely high activity in muscle, as demonstrated in the immediate-early enhancer-chicken β-globin gene of pCAGGS (38). The CAG promoter and a 3′-flanking region of a rabbit β-globin gene of pCAGGS expression vector after blunt-end treatment (37). This expression vector drives a target gene under the control of the CAG promoter, allowing for high activity in muscle, as demonstrated in both transgenic mice (39) and intramuscular DNA injection (50).

Plasmid DNA was grown in E. coli TOP10 cells, extracted using the Endo-free Plasmid Mega Kit (Qiagen), and the DNA was suspended in saline.

RNA interference. pSilencer vectors harboring the U6 promoter (Ambion, TX) were used to drive the expression of four different pairs of p38γ-specific oligonucleotides. The siRNA constructs contain a U6 promoter followed by a p38γ-specific, 21-mer sense oligonucleotide, a spacer, the 21-mer antisense oligonucleotide, and a U6 termination sequence consisting of five thyminides (Fig. 1A). Four pairs of oligonucleotides were generated and cotransfected with the FLAG-p38γWT plasmid in L6 cells. All four siRNAs oligos were successful in blocking FLAG-p38γWT expression by >85%, and one set, which had >94% knockdown efficiency, was used for subsequent experiments. While FLAG-p38γWT expression was dramatically decreased, p38γ-specific siRNA did not affect expression of FLAG-p38β or endogenous expression of p38α and p38β (Fig. 1B). Levels of endogenous p38γ were below the limits of detection by immunoblotting; however, it is known that p38γ is expressed in differentiating muscle cells as determined by Northern blotting (33).

Cells and transfections. L6 cells were seeded into 12-well plates and maintained in growth medium consisting of αMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2-95% air at 37°C. Myoblasts were grown in monolayers and allowed to reach confluence. Myoblasts were induced to differentiate by exposure to 2% fetal bovine serum for 6 days. Transient transfections were performed using a total of 2 μg of DNA per well, using lipofection (Lipofectamine 2000, Invitrogen). Recombinant protein expression was allowed for 48 h, at which time cells were used for the determination of glucose transport and immunoblotting.

Glucose uptake in L6 myotubes. L6 myotubes were serum-starved for 5 h in αMEM before any treatment. Cells were washed twice in PBS and incubated in 2,4-dinitrophenol (DNP). 500 μM; 10 min). After stimulation, 2-deoxy-d-[3H]glucose uptake was measured by incubating cells at room temperature for 5 min in transport solution (140 mM NaCl, 20 mM HEPES-Na, pH 7.4, 5 mM KCl, 0.5 μM/L 2-deoxy-d-[3H]glucose, 2.5 mM MgSO4, 1.0 mM CaCl2). Nonfacilitated glucose uptake was determined in the presence of 10 μM cytochalasin B. Net accumulation of 2-deoxy-d-[3H]glucose was determined, and rates of uptake were calculated.

Sensitivity to stimulated glucose uptake of L6 cells increases, but transfection efficiency decreases with differentiation. Therefore, we determined the optimal stage at which myotubes could be both effectively transfected and stimulated by DNP. By the eighth day of differentiation, p38γ was transected on day 6 of differentiation, and subsequent experiments were performed 48 h later.

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Fig. 1. Overexpression of p38γ in L6 myotubes. L6 myoblasts were seeded and differentiated in 2% FBS. Myotubes were transfected on day 6 of differentiation, and subsequent experiments were performed 48 h later. A: four pairs of p38γ-specific oligonucleotides were cloned into the pSilencer vector. FLAG-p38γ was reduced by 89.6, 97.2, 94.5, and 94.7% response to the 4 different p38γ-specific siRNAs. Pair 2 exhibited the maximum silencing effect and was used for all subsequent experiments. B: representative immunoblot with a total p38 antibody showing the specificity of p38γ-specific siRNA. Expression of wild-type p38γ (p38γWT) was reduced over 90% in response to p38γ-specific siRNA, while expression of endogenous p38αβ and overexpression of FLAG-p38γWT was unaffected.
...were used (H11021/H18528 samples were homogenized in lysis buffer (20 mM Tris–HCl, 500 mM EDTA, 10 mM Na3P2O7, 100 mM NaF, 2 mM NaVO4, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine, 1 mM PMSF), and total protein concentrations were determined by the Bradford method (Bio-Rad). Samples were resolved by 8% SDS-PAGE, transferred to nitrocellulose membranes, blocked in 5% milk, and probed using the respective antibodies. Immunocomplexes were detected using chemiluminescence.

Statistical analysis. Results are expressed as means ± SE or transformed and expressed relative as a percentage of the mock-transfected control. ANOVA and Student’s paired and unpaired t-tests were used (P < 0.05).

RESULTS

Overexpression of constitutively active MKK6 increases basal glucose uptake in L6 myotubes. To study the regulation of basal glucose uptake by p38γ, we transfected L6 myotubes with MKK6CA alone or with p38γ-specific siRNA. Expression of the activating MKK6 mutant resulted in an increase in basal rates of glucose uptake in the myotubes (Fig. 2A). These changes in basal glucose uptake were likely due to alterations in GLUT1 protein expression, as there was a tendency for GLUT1 expression to be higher in cells overexpressing MKK6CA compared with pCAGGS transfected cells. (Fig. 2B). Because MKK6 activates all p38 isoforms, we also designed vector-based siRNA constructs...
to selectively knockdown p38γ expression in L6 cells. The increases in basal glucose uptake in response to MKK6CA overexpression were reversed when cells were cotransfected with the p38γ-specific siRNA (Fig. 2A).

Overexpression of p38 in injected muscles. In adult skeletal muscle, the expression of FLAG-p38γ was 10-fold higher compared with the endogenous expression of p38γ (Fig. 3A). The high degree of overexpression did not affect endogenous p38γ expression (Fig. 3A). Using a pan-p38 antibody that recognizes all p38 isoforms, Fig. 3B shows that overexpression of FLAG-p38γ did not affect total expression of endogenous p38 isoforms. Recombinant FLAG-p38γ is functional, as evidenced by the finding that it is phosphorylated in response to in situ muscle contractions (Fig. 3C). Total (endogenous + recombinant) p38 phosphorylation under both the basal and contraction-stimulated conditions was significantly increased in FLAG-p38WT muscles compared with pCAGGS controls (Fig. 3C). The changes in p38 phosphorylation in muscles overexpressing p38γ-WT were associated with increases in phosphorylation of the p38 downstream substrate ATF-2 (Fig. 3D).

p38γ positively regulates basal glucose uptake in adult skeletal muscle. We next used this gene transfer method to overexpress p38γ-WT in skeletal muscle of free-living animals to determine whether basal glucose uptake and GLUT1 expression are regulated by p38γ. Skeletal muscles overexpressing p38γ-WT exhibited significantly higher basal rates of glucose uptake compared with pCAGGS-transfected muscles (Fig. 4A). This increase was not due to detectable changes in GLUT1 expression in the muscle (Fig. 4B, 85.8 ± 13.5 vs. 100.0 ± 17.9%, P = 0.54), nor was it due to increases in GLUT4 (described below). Collectively, the data from L6 cells and adult skeletal muscle suggest that p38γ positively regulates basal glucose uptake.

Overexpression of constitutively active MKK6 decreases DNP-stimulated glucose uptake in L6 myotubes. We next investigated the role of p38γ in stimulated glucose uptake. L6 myotubes were transfected with MKK6CA and stimulated with DNP, an uncoupler of oxidative phosphorylation, to determine whether p38γ regulates increases in glucose uptake caused by cellular energy demand. DNP-stimulated glucose uptake was significantly lower in cells overexpressing MKK6CA compared with pCAGGS-transfected cells (Fig. 5). Interestingly, the effect of MKK6CA on DNP-stimulated glucose uptake was reversed when cells were cotransfected with p38γ-specific siRNA (Fig. 5). Thus knockdown of p38γ completely restores the impairment in glucose uptake caused by overexpression of MKK6CA. While the effects of MKK6 and p38γ-siRNA on basal glucose uptake are paralleled by changes in GLUT1...
expression, the effects on DNP-stimulated glucose uptake were not associated with detectable changes in GLUT4 protein levels (data not shown).

**DISCUSSION**

Mitogen-activated protein kinases, particularly JNK and p38 MAPK, are activated under conditions of environmental and cellular stress. These physiological and cellular perturbations are commonly associated with skeletal muscle insulin resistance, and recent studies suggest that JNK is a negative regulator of insulin signaling in 3T3-L1 adipocytes (24). How-

*p38γ negatively regulates contraction-mediated glucose uptake.* Contractile activity also increases glucose uptake through a mechanism that may also involve cellular energy demand. Because p38γ is highly activated by exercise, we determined whether p38γ regulates glucose uptake in contracting skeletal muscle. Mice injected with empty vector or p38γWT into the tibialis anterior muscle were anesthetized, and in situ contraction-stimulated glucose uptake was determined. The mean contraction-mediated glucose uptake was 16% lower in skeletal muscle overexpressing p38γWT compared with pCAGGS-transfected muscles, and this effect was close to reaching statistical significance ($P = 0.08$, Fig. 6A). Total GLUT4 expression was significantly lower in skeletal muscle overexpressing FLAG-p38γWT (Fig. 6B). Thus these data suggest that p38γ is a negative regulator of GLUT4 expression and, subsequently, attenuates the increases in glucose uptake in response to changes in cellular energy demand (DNP stimulation and contractile activity in L6 cells and adult skeletal muscle, respectively).
ever, previous data regarding the role of p38 in the regulation of glucose uptake in skeletal muscle are controversial. The majority of these studies have focused exclusively on the ubiquitously expressed p38α and p38β isoforms using p38 inhibitors. The workhorse among p38 inhibitors has been the pyridinyl imidazole derivatives (SB compounds), which are only effective against p38α and p38β activity. Unfortunately, these compounds have also been shown to inhibit Akt (32), JNK (9), ERK (36, 44), and nucleoside transporters (25) in a variety of cell lines. Skeletal muscle p38γ is unique in that it is insensitive to inhibition by known p38 inhibitors (18).

In the current study, we suggest that p38γ positively regulates basal glucose uptake in L6 myotubes. Overexpression of constitutively active MKK6, which activates all p38 isoforms, resulted in increases in rates of basal glucose uptake. This increase was associated with a trend for higher cellular GLUT1 expression and increased basal glucose uptake in 3T3-L1 adipocytes and L6 myotubes (17). Increases in GLUT1 expression and increased basal glucose uptake in 3T3-L1 adipocytes and L6 myotubes resulted in increases in rates of basal glucose uptake. This constitutively active MKK6, which activates all p38 isoforms, resulted in increases in basal glucose uptake in L6 myotubes. Overexpression of these compounds have also been shown to inhibit Akt (32), JNK (9), ERK (36, 44), and nucleoside transporters (25) in a variety of cell lines. Skeletal muscle p38γ is unique in that it is insensitive to inhibition by known p38 inhibitors (18).

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In agreement with our data from L6 cells, we also found that p38γ increases basal glucose uptake in adult skeletal muscle. Overexpression of p38γ-WT resulted in significantly elevated rates of basal glucose uptake; however, this was not accompanied by detectable changes in levels of total GLUT1 expression compared with pCAGGS-transfected muscles. Our data are consistent with previous reports suggesting that p38 is involved in the regulation of basal glucose uptake and demonstrate that the γ isoform is pivotal in mediating this effect in skeletal muscle (14, 17, 25).

We also show that overexpression of constitutively active MKK6 significantly attenuated DNP-stimulated glucose uptake in L6 myotubes. These data suggest that the p38γ isoform is primarily responsible for this effect since full recovery of DNP-stimulated glucose uptake resulted when L6 cells were cotransfected with p38γ-specific siRNA oligos. We did not observe significant changes in GLUT4 expression in response to overexpression of MKK6CA or p38γ-siRNA; however, L6 cells are known to exhibit relatively low levels of GLUT4, and changes in protein expression may be below the limits of detection by immunoblotting.

We have previously reported that compared with the p38α and p38β isoforms, p38γ was highly activated after prolonged exercise in humans (6); however, the physiological role of exercise-stimulated p38γ signaling in skeletal muscle has remained obscure. Here, we demonstrate that total GLUT4 expression is significantly lower in skeletal muscle overexpressing p38γ-WT in vivo. This was associated with decreases in in situ contraction-mediated glucose uptake, suggesting that p38γ negatively influences contraction-mediated glucose uptake in skeletal muscle in vivo. The effect of p38γ activation on contraction-stimulated glucose uptake is at least partially due to the negative regulation of GLUT4 protein expression in skeletal muscle. Our data agree with a previous report that showed that constitutively active MKK6/3 mutants downregulated GLUT4 expression in 3T3-L1 adipocytes (17). Recent studies have shown an upregulation of MAPK (including p38) in adipose tissue from individuals with type 2 diabetes and have suggested that the p38 pathway, in particular, might contribute to the loss of GLUT4 expression observed in adipose tissue from type 2 diabetic patients (8, 31). However, these studies did not determine isoform-specific effects of p38 signaling in the regulation of GLUT4 expression. Our data indicate that p38γ is involved in the regulation of GLUT4 expression in skeletal muscle in vivo and, therefore, may represent a novel target for the regulation of glucose homeostasis in this tissue.

While the present data are the first to suggest that p38γ is a negative regulator of GLUT4 expression in adult skeletal muscle in vivo, the effect of p38α and p38β on stimulated glucose transport has yet to be resolved. Klip and colleagues (49) have reported that insulin-activated p38, and the stimulation of glucose uptake, was reduced by preincubation of 3T3-L1 adipocytes and L6 myotubes with p38 inhibitors. These authors concluded that insulin activates GLUT4 intrinsic activity, most likely by utilizing a p38-dependent signal in L6 cells. The same group has shown that contraction-stimulated 2-deoxyglucose glucose transport was reduced by up to 50% when isolated muscles were pretreated with SB-203580 (47). On the other hand, studies have indicated that increases in glucose uptake resulting from hyperosmolarity, insulin, or osmotic shock are not impaired by treatment with p38 inhibitors (5, 30). Another study reported that activation of p38 by treatment of cells with anisomycin did not stimulate glucose transport (30). Interestingly, both ERK and JNK are activated by exercise (19), and, like p38γ described here, ERK and JNK have been shown to positively regulate GLUT1 expression and negatively regulate insulin-stimulated glucose uptake in cell systems (2, 7, 16, 17). While our data indicate that p38γ negatively regulates DNP-stimulated glucose uptake in L6 cells and contraction-mediated glucose uptake in skeletal muscle, the possibility remains that p38α and p38β may have the opposite effect. This is plausible considering different isoform-specific tissue expression, cellular localization, activation, and downstream signaling. For example, p38γ is the only known MAPK to exhibit a PI3K discs-large ZO-1 (PDZ) binding domain (22), which confers the ability for specific downstream signaling to proteins such as syntrophin (22), aquaporin-4 (1), and potentially nitric oxide synthase (29).

In conclusion, our results demonstrate that p38γ MAPK signaling positively regulates GLUT1 expression and basal glucose uptake in L6 myotubes and adult skeletal muscle. Our results also identify p38γ as a negative regulator of GLUT4 expression and DNP- and contraction-stimulated glucose uptake in L6 myotubes and adult skeletal muscle, respectively. MAPKs are involved in the regulation of numerous cytokine signaling networks, which have commonly been associated with peripheral insulin resistance. Here, we demonstrate the isoform-specific involvement of p38γ in the regulation of glucose uptake in L6 myotubes and skeletal muscle in vivo.
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