Insulin-independent pathways mediating glucose uptake in hindlimb-suspended skeletal muscle

Thomas L. Hilder,1 Lisa A. Baer,2 Patrick M. Fuller,3 Charles A. Fuller, Richard E. Grindeland,2 Charles E. Wade,2 and Lee M. Graves1

1Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina; 2Life Sciences Division, National Aeronautics and Space Administration Ames Research Center, Moffett Field; and 3Section of Neurobiology, Physiology and Behavior, University of California, Davis, California

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Insulin-independent pathways mediating glucose uptake in hindlimb-suspended skeletal muscle. J Appl Physiol 99: 2181–2188, 2005. First published August 11, 2005; doi:10.1152/japplphysiol.00743.2005.—Insulin resistance accompanies atrophy in slow-twitch skeletal muscles such as the soleus. Using a rat hindlimb suspension model of atrophy, we have previously shown that an upregulation of JNK occurs in atrophic muscles and correlates with the degradation of insulin receptor substrate-1 (IRS-1) (Hilder TL, Tou JC, Grindeland RF, Wade CE, and Graves LM, FERS Lett 553: 63–67, 2003), suggesting that insulin-dependent glucose uptake may be impaired. However, during atrophy, these muscles preferentially use carbohydrates as a fuel source. To investigate this apparent dichotomy, we examined insulin-independent pathways involved in glucose uptake following a 2- to 13-wk hindlimb suspension regimen. JNK activity was elevated throughout the time course, and IRS-1 was degraded as early as 2 wk. AMP-activated protein kinase (AMPK) activity was significantly higher in atrophic soleus muscle, as were the activities of the ERK1/2 and p38 MAPKs. As a comparison, we examined the kinase activity in solei of rats exposed to hypergravity conditions (2 G). IRS-1 phosphorylation, protein, and AMPK activity were not affected by 2 G, demonstrating that these changes were only observed in soleus muscle from hindlimb-suspended animals. To further examine the effect of AMPK activation on glucose uptake, C2C12 myotubes were treated with the AMPK activator metformin and then challenged with the JNK activator anisomycin. While anisomycin reduced insulin-stimulated glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels, metformin significantly increased glucose uptake to control levels. By using a rat HLS model of atrophy, we show that AMPK activity is responsible for inhibiting ATP-consuming pathways, such as fatty acid and cholesterol synthesis, while stimulating ATP-generating pathways (reviewed in Ref. 16). AMPK is activated during muscle contraction by cellular stresses, such as hypoxia and ischemia, and exogenously by the AMP analog aminoimidazole-4-carboxamide-1-β-d-riboside (AICAR) (14). AMPK is responsible for inhibiting ATP-consuming pathways, such as fatty acid and cholesterol synthesis, while stimulating ATP-generating pathways (reviewed in Ref. 16). Further interest in AMPK has stemmed from its ability to stimulate insulin-independent glucose uptake in muscle (19, 37) and by the observation that the antidiabetic effects of the drug metformin may be mediated by AMPK (38, 48). The activation of skeletal muscle ERK1/2 and p38 MAPKs is thought to mediate, in part, the effect of AMPK on glucose uptake. ERK1/2 activation occurs in an AMPK- and proline-rich tyrosine kinase 2 (Pyk2)–dependent manner in response to glucose, exercise, or AICAR treatment, resulting in elevated phospholipase D and atypical protein kinase C (PKC) activities and enhanced GLUT-4 translocation to the membrane (7, 8). AICAR treatment or expression of a constitutively active AMPK has also been shown to activate p38 and MAPK kinase 3 (MKK3), a p38 activating kinase (46). In turn, p38 has recently been demonstrated to be a GLUT-4 “activator.” Pharmacological inhibitors of p38, such as SB-203580, reduce glucose uptake but do not block GLUT-4 membrane translocation (40). AMPK and p38 have also been reported to enhance the expression of glucose transporters GLUT-1 and GLUT-4 (10, 11, 24). Because atrophic soleus muscle displays insulin resistance (13, 21, 34, 43), we hypothesized that the activation of AMPK may provide an alternate mechanism to maintain glucose uptake. By using a rat HLS model of atrophy, we show that AMPK activity is elevated through a 13-wk time course of atrophy in the slow-twitch soleus. ERK1/2 and p38 MAPK activities are also elevated, possibly as a compensatory mechanism to mediate insulin-independent glucose uptake. Compar-

Skeletal muscle atrophy occurs in response to a number of stimuli, including prolonged bed rest, exposure to microgravity, severe human immunodeficiency virus infection, cancer cachexia, and others (reviewed in Refs. 25, 27). Muscle unloading primarily affects slow-twitch muscles such as the soleus, resulting in an increased expression of fast-type myosin heavy chain (3, 28, 32, 33, 41), a loss in the ability to oxidize fatty acids (4), a dependence on carbohydrates as a fuel source (4, 12), and increased insulin resistance (13, 21, 34, 43). Using the rat hindlimb-suspension (HLS) model of muscle atrophy, we recently demonstrated that c-Jun NH2-terminal kinase (JNK) activity was elevated in the soleus muscle during atrophy and correlated with the degradation of insulin receptor substrate-1 (IRS-1), suggesting that this may be one mechanism involved in the development of insulin resistance (20). The AMP-activated protein kinase (AMPK) responds to changes in energy levels in skeletal muscle as the ratio of AMP to ATP increases and is stimulated both allosterically by AMP and covalently by the AMPK kinase phosphorylation of the catalytic α-subunit (reviewed in Refs. 14–16). AMPK is activated during muscle contraction by cellular stresses, such as hypoxia and ischemia, and exogenously by the AMP analog aminoimidazole-4-carboxamide-1-β-d-riboside (AICAR) (14). AMPK is responsible for inhibiting ATP-consuming pathways, such as fatty acid and cholesterol synthesis, while stimulating ATP-generating pathways (reviewed in Ref. 16). Further interest in AMPK has stemmed from its ability to stimulate insulin-independent glucose uptake in muscle (19, 37) and by the observation that the antidiabetic effects of the drug metformin may be mediated by AMPK (38, 48). The activation of skeletal muscle ERK1/2 and p38 MAPKs is thought to mediate, in part, the effect of AMPK on glucose uptake. ERK1/2 activation occurs in an AMPK- and proline-rich tyrosine kinase 2 (Pyk2)-dependent manner in response to glucose, exercise, or AICAR treatment, resulting in elevated phospholipase D and atypical protein kinase C (PKC) activities and enhanced GLUT-4 translocation to the membrane (7, 8). AICAR treatment or expression of a constitutively active AMPK has also been shown to activate p38 and MAPK kinase 3 (MKK3), a p38 activating kinase (46). In turn, p38 has recently been demonstrated to be a GLUT-4 “activator.” Pharmacological inhibitors of p38, such as SB-203580, reduce glucose uptake but do not block GLUT-4 membrane translocation (40). AMPK and p38 have also been reported to enhance the expression of glucose transporters GLUT-1 and GLUT-4 (10, 11, 24). Because atrophic soleus muscle displays insulin resistance (13, 21, 34, 43), we hypothesized that the activation of AMPK may provide an alternate mechanism to maintain glucose uptake. By using a rat HLS model of atrophy, we show that AMPK activity is elevated through a 13-wk time course of atrophy in the slow-twitch soleus. ERK1/2 and p38 MAPK activities are also elevated, possibly as a compensatory mechanism to mediate insulin-independent glucose uptake. Compar-

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Address for reprint requests and other correspondence: L. M. Graves, Dept. of Pharmacology, Univ. of North Carolina, CB #7365, Chapel Hill, NC 27599-7365 (e-mail: lmg@med.unc.edu).

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ing these results to a time course of animals subjected to twice gravity suggested that the loss of IRS-1 protein, the development of insulin resistance, and the activation of AMPK are important events associated with muscle atrophy.

MATERIALS AND METHODS

Reagents. The IRS-1 antibodies were from Upstate Biotechnology (Lake Placid, NY). Antibodies directed against phospho-AMPK-α (Thr172), AMPK-α, phospho-MKK3/6 (Ser189/Ser207), MKK3, phospho-PKC-ζ/λ (Thr165), phospho-p38 (Thr180/Tyr185), and p38 were from Cell Signaling Technology (Beverly, MA). The PKC-α antibody was from Biosource (Camarillo, CA). The phospho-Pyk2 antibody was from Pharmingen (San Diego, CA), while the total Pyk2 monoclonal antibody was a gift from Dr. H. Shelton Earp (University of North Carolina). Antibodies against phospho-ERK (Tyr204) and ERK1/2, protein A-agarose beads, and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A-horseradish peroxidase was from Amersham Biosciences (Piscataway, NJ). [1,2-3H]-2-deoxy-D-glucose was from MP Biomedicals (Irvine, CA). All RT-PCR reagents were from Invitrogen (Carlsbad, CA), and all other chemicals were purchased from Sigma (St. Louis, MO).

Animal subjects. Animal experimentation was conducted in accordance with the guidelines of the National Aeronautics and Space Administration Institutional Animal Care and Use Committee, University of California at Davis Animal Care and Use Committee, and the National Research Council Guide for the Care and Use of Laboratory Animals.

HLS experiments. One hundred twenty male Sprague-Dawley rats (Simonsen Farms, Gilroy, CA), weighing between 180 and 200 g, were shipped to Ames Research Center. Forty rats were specifically dedicated to the experiments described in this study. On arrival, rats were housed in standard vivarium cages (47 × 26 × 21 cm) lined with corncob bedding and maintained under the following conditions: light cycle, 12 h light, beginning at 0600, and 12 h dark, 23 ± 1°C, and 30–50% humidity. Rat chow (powdered diet, Purina 5102, Richmond, IN) and water were available ad libitum. Animals were weighed daily after arrival to monitor body weight for group assignments. The rats were randomly assigned to HLS (17, 36, 45) or control ambulatory (Amb) groups at one of five times: baseline, and 2, 4, 8, and 13 wk. Except for the baseline group (N = 6), an untreated control group, all time groups had 12 rats within each treatment group. Seven days before the initiation of HLS, animals (HLS and Amb) assigned to the 13-wk group were surgically implanted with a Data Sciences biotelemetry according to the manufacturer’s instructions (Data Sciences, St. Paul, MN; #TA-FA40), for continuous recording of temperature and activity. Animals were housed in specialized suspension/metabolic cages for the duration of the study (17). Food and water consumption and body mass data were collected twice weekly for the duration of the study. All animals used in the study were anesthetized with isoflurane (2.5% in O2), bled by cardiac puncture, and decapitated. Muscles were immediately excised and frozen in liquid nitrogen.

Hypergravity experiments. A second group of rats, essentially identical to the group used in the suspension study, was shipped to the Chronic Acceleration Research Unit at the University of California, Davis. Forty rats in this group were specifically dedicated to the experiments described in this study. With few exceptions (gravitational load and housing within metabolic cages), conditions and animal procedures were identical to those employed in the HLS study described above. Thirteen-week animals [hypergravity (2 G) and Amb] were implanted with Data Sciences biotelemetry (#TA-FA40), as described above. Animals were exposed to continuous 2-G centrifugation (centrifuge diameter, 4.5 m). For the duration of the study, the centrifuge was stopped for no more than 1 h, twice weekly, for animal maintenance (food and water consumption and body mass data collection and cage changes). Seven days before death, without alteration of the centrifugation conditions, all animals [2 G and normal gravity (1 G)] were transferred to specialized metabolic cages (17) and placed on a powder diet ad libitum (Purina 5102).

In vitro JNK assay. Two soleus muscles from Amb and HLS rats at each time point were snap-frozen in liquid nitrogen following excision, crushed into a powder with a mortar and pestle, and lysed in modified RIPA buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 0.5% deoxycholate, 1.0% Nonidet P-40, 10 mM EDTA, 10 mM EGTA, 250 μM PMSF, 200 μM sodium vanadate, 5 μg/ml leupeptin, and 0.01 μM microcystin). Assays were conducted as described (20).

Immunoprecipitation. From each of the lysesates generated above, 1 mg was tumbled at 4°C for 1 h with 5 μl of Pyk-2 antibody in a total of 500 μl of modified RIPA buffer. Twenty microliters of protein A-agarose beads were added and tumbled for an additional hour. Bead complexes were collected by centrifugation and washed three times in modified RIPA, and Pyk-2 was eluted in 2× sample buffer with boiling for 3 min.

Immunoblotting. Blots were carried out as described (20) with the lysesates generated above. Phospho-blots were performed first; the membranes were then stripped and reprobed for total protein. Protein A-horseradish peroxidase was used as a secondary antibody for the phospho-Pyk-2 blot; other secondary antibodies were used as per manufacturer’s instructions. While every effort was made to ensure equal protein loading, discrepancies in total protein levels were seen when proteins were heavily phosphorylated (e.g., see Fig. 5A; compare Amb with HLS total protein). This can be attributed to the fact that phospho- and total protein antibodies are often derived from the same epitopes, differing only by the phosphate, which then interferes with the ability of the total antibody to bind the epitope. Densitometry was performed with the NIH ImageJ program. Results are expressed as the average of the level of phospho-AMPK to total AMPK for two animals (SD).

Glucose uptake assay. C2C12 mouse muscle myoblasts (ATCC, Manassas, VA) were grown in 12-well plates to confluence in DMEM with 10% fetal bovine serum, 10 U/ml penicillin, and 10 μg/ml streptomycin sulfate. Cells were differentiated to myotubes for 3 days in DMEM with 2% horse serum, penicillin, and streptomycin. On the 4th day, the cells were treated with 2 mM metformin or vehicle for 24 h. For the last 4 h, the cells were incubated in serum-free DMEM with DMSO or 5 μg/ml anisomycin, and insulin was added at 5 μg/ml for the last 15 min. Cells were then rinsed twice in KRPH buffer (5 mM Na2HPO4, 20 mM HEPES, pH 7.4, 1 mM MgSO4, 1 mM CaCl2, 137 mM NaCl, and 4.7 mM KCl), and glucose uptake was assessed with 50 μM 2-deoxy-β-glucose (0.2 μCi/ml [1,2-3H]-2-deoxy-β-glucose) with or without 10 μM cyclohexatin B in KRPH for 10 min at room temperature. Cells were then washed three times with PBS and lysed in 1 ml of 0.05 N NaOH per well and [3H] counted. Results are expressed as the average picomole of 2-deoxy-β-glucose taken up into the cells in 10 min ± the SE of the mean for four assays performed in triplicate.

RT-PCR. Differentiated C2C12 were treated for 24 h, with or without 2 mM metformin, rinsed twice in PBS, and lysed in TRIZol reagent. RNA was isolated per manufacturer’s instructions (Invitrogen). Four micrograms of RNA were reverse transcribed using the SuperScript First-Strand Synthesis System. PCR was performed with the following primers: GLUT-1, 5′-ATCATCCTTACGGGTGTC-CC-3′ and 5′-TCAACACTTGGAATGTCGCC-3′; GLUT-4, 5′-AAAG-TGCCGTGAAAC-3′ and 5′-CCCTAAGTTCAGTCTG-3′; and β-actin, 5′-GTCGTCACCCAGGTGATGTTG-3′ and 5′-GCAATTGCTGGGGTGATGG-3′. Reaction steps were as follows: 94°C, 2 min; 94°C, 30 s, 50°C, 30 s, 72°C, 45 s, 30 cycles; 72°C, 10 min.
RESULTS

Increased JNK activity correlates with IRS-1 degradation. The serine phosphorylation of IRS-1 blocks insulin-dependent signaling (1) and targets IRS-1 for degradation (18, 42, 47). The degradation of IRS-1 in response to certain stimuli occurs in a JNK-dependent manner (1, 22, 29, 31). In our laboratory’s previous study (20), we showed that a 38-day HLS of female rats significantly activated JNK and correlated with IRS-1 degradation only in slow-twitch muscles like the soleus. To determine whether these are early or late responses to muscle atrophy, we examined soleus muscles following a 0- to 13-wk time course of HLS. Muscle masses at each point are summarized in Table 1; significant atrophy occurred after 2 wk and was sustained throughout the time course. In comparison, soleus muscle masses from 1-G and 2-G animals increased similarly to those from the Amb group. JNK assays of lysates from these muscles demonstrated that JNK activity was elevated after only 2 wk of HLS and was higher in atrophic muscle at every time point (Fig. 1A). By contrast, in muscles obtained from rats exposed to 2-G conditions, the specific activity of JNK was significantly lower than during HLS-induced atrophy, and no significant differences in JNK activity were seen between solei from animals at 1 G and 2 G (Fig. 1B). Although some sample variation was observed, which is believed to reflect normal animal variability, prolonged JNK activity was only observed in atrophic soleus muscle.

Serine 307 phosphorylation of IRS-1 results in an inhibition of its tyrosine phosphorylation (1), and serine phosphorylation of IRS-1 leads to its ubiquitin-mediated degradation (18, 42, 47). Examination of IRS-1 protein from Amb and HLS soleus muscles showed that IRS-1 Ser307 phosphorylation and degradation occurred in atrophic muscles, consistent with an increase in JNK activity in these samples (Fig. 2A). This change occurred early (2 wk), and both Ser307 phosphorylation and IRS-1 degradation increased over the entire time course of atrophy. By contrast, neither IRS-1 Ser307 phosphorylation nor degradation were affected by exposure to 2-G conditions (Fig. 2B), indicating that IRS-1 degradation and the development of insulin resistance selectively occurred in atrophic conditions. Although the animals in the 2-G study did show an unexpected increase in Ser307 phosphorylation and IRS-1 degradation at the 60-day time point for unknown reasons, the magnitude of these parameters did not differ between the 1-G and 2-G animals, suggesting that this was an artifact of the samples examined at this time point. The levels of IRS-1 returned to normal levels in both sets of animals at the 90-day time point.

AMPK activity is increased in atrophic muscle. Muscle wasting conditions are associated with increased fatigue (12) and are believed to be induced through decreases in creatine phosphate and ATP and the concomitant increases in ADP, AMP, free phosphate, intracellular lactate, and/or decreased pH (9, 12). This potential change in the AMP-to-ATP ratio led us to examine the activity of AMPK during the development of muscle atrophy. Immunoblotting with a phospho-specific antibody against the activated form of AMPK revealed that AMPK was significantly activated in the atrophic soleus muscle (Fig. 3A). This activity was sustained throughout the time course of muscle atrophy, while total AMPK protein levels declined slightly. Comparison of the amount of phospho- and total AMPK by densitometry showed that this activation was

### Table 1. Soleus muscle weights following atrophy or 2-G time courses

<table>
<thead>
<tr>
<th>Time</th>
<th>Amb</th>
<th>HLS</th>
<th>Time</th>
<th>Amb</th>
<th>2 G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>127.6 (SD 12.5)</td>
<td>127.6 (SD 12.5)</td>
<td>Baseline</td>
<td>131.3 (SD 5.7)</td>
<td>131.3 (SD 5.7)</td>
</tr>
<tr>
<td>2 wk</td>
<td>155.6 (SD 13.2)</td>
<td>77.4 (SD 6.6)</td>
<td>15 days</td>
<td>146.0 (SD 12.1)</td>
<td>152.0 (SD 9.0)</td>
</tr>
<tr>
<td>4 wk</td>
<td>176.5 (SD 15.8)</td>
<td>51.2 (SD 8.0)</td>
<td>30 days</td>
<td>176.8 (SD 23.5)</td>
<td>151.7 (SD 7.2)</td>
</tr>
<tr>
<td>8 wk</td>
<td>190.7 (SD 21.6)</td>
<td>44.8 (SD 5.0)</td>
<td>60 days</td>
<td>199.6 (SD 7.8)</td>
<td>170.3 (SD 3.9)</td>
</tr>
<tr>
<td>13 wk</td>
<td>183.5 (SD 11.9)</td>
<td>48.8 (SD 6.1)</td>
<td>90 days</td>
<td>196.8 (SD 19.2)</td>
<td>182.7 (SD 7.5)</td>
</tr>
</tbody>
</table>

Masses are means (SD) for 5–6 muscles in mg. Data from ambulatory (Amb), hindlimb suspended (HLS), and hypergravity (2 G) are shown.
AMPK AND MAP KINASE ACTIVITY IN ATROPHIC SOLEUS MUSCLE

Fig. 2. Insulin receptor substrate-1 (IRS-1) degradation is prevalent only in the atrophic soleus muscle. Lysates from atrophic (A) and 2-G (B) soleus muscle were immunoblotted for pSer307 (top) or total IRS-1 (bottom). Each lane represents a lysate from 1 animal, and the blot is representative of 2 independent experiments.

~20-fold higher in HLS vs. Amb controls at 4 and 8 wk of atrophy. As observed with IRS-1 degradation, AMPK activity was not altered by exposure to 2 G (Fig. 3B), suggesting that activation of AMPK may be an atrophy-specific event.

**ERK1/2 and PKC-ζ/λ activity do not correlate with Pyk-2 in atrophic muscle.** Previous studies have demonstrated that AMPK can activate a pathway dependent on Pyk2, ERK1/2, phospholipase D, and atypical PKC to stimulate glucose transporter translocation and glucose uptake in fast-twitch muscles like the extensor digitorum longus (7, 8). Therefore, we examined the activity of these enzymes in the soleus from HLS time course lysates. Immunoblotting for the activated form of Pyk2 (pTyr402) or total Pyk2 protein showed that Pyk2 activity and expression dramatically decreased during atrophy throughout the 13-wk period (Fig. 4A). In comparison, both ERK1/2 and PKC-ζ/λ activities increased modestly in the HLS animals, whereas no significant change in the total amount of these enzymes was observed (Fig. 4, B and C). Taken together, these results suggested that activation of Pyk2 was not required for activation of ERK1/2 or the atypical PKCs in HLS muscle. Comparison of muscle lysates from control and 2-G animals showed no significant differences in ERK activity, with the exception of the 60-day samples, which showed a loss of ERK activity (Fig. 4D).

**p38 Activity is elevated during atrophy.** Activation of AMPK with AICAR has also been shown to activate a pathway dependent on MKK3/6 and p38 MAPK to increase glucose transporter transcription and enhance transporter activation (10, 11, 24, 46). In our previous study, p38 activity was only activated in atrophic soleus muscle while the fast-twitch gas-

Fig. 3. AMP-activated protein kinase (AMPK) activity is elevated only in the HLS soleus muscle. Lysates from atrophic (A) and 2-G (B) soleus muscles were immunoblotted with a phospho-specific antibody against the AMPK-α subunit (top) and for total AMPK (bottom). Results are representative of 2 independent experiments. Densitometry shown in A is expressed as the ratio of phospho- to total AMPK.
trocnemius showed no change in activity following a 38-day HLS (20). Immunoblot analysis of the time course of HLS lysates revealed that both MKK3/6 (pSer189/207) and p38 (pThr180/Tyr182) were significantly activated throughout the time course of atrophy (Fig. 5, A and B). Although some reduction in immunoreactive MKK3/6 protein (MKK3) was observed after 2 wk of HLS, this may be an artifact of the significant increase in MKK3/6 phosphorylation during HLS (see note in MATERIALS AND METHODS). Again, comparison of the control (1-G) and 2-G muscle samples showed little sustained difference in p38 activity (Fig. 5C). Similar results were observed with MKK3/6 (data not shown).

![Image](53x376) to 412x721

**Fig. 4.** ERK1/2 and atypical protein kinase C (PKC) activation appear to be independent of proline-rich tyrosine kinase 2 (Pyk2). A: Pyk2 was immunoprecipitated from control and atrophic soleus lysates and immunoblotted for the active, phospho-protein (top) and for total protein (bottom). Immunoblots are shown for ERK1/2 (B) and atypical PKC (C) from control and atrophic soleus muscle lysates, with phospho blots (top) and total protein blots (bottom). D: immunoblot for active (top) and total (bottom) ERK1/2 from control and 2-G soleus lysates. Results are representative of 2 independent experiments.

![Image](53x55 to 411x272)

**Fig. 5.** The activities of p38 and its activating enzyme MAPK kinase (MKK) 3/6 are elevated in atrophic soleus muscle. Immunoblots for MKK3/6 (A) and p38 (B and C) are shown, with phospho-specific blots (top) and total protein blots (bottom). Results are representative of 2 independent experiments.
Activation of AMPK by metformin enhances glucose uptake during stress. We sought to determine the relationship between activated JNK and AMPK on glucose uptake in skeletal muscle. Because we could not perform these studies in animals, differentiated C2C12 mouse muscle myotubes were treated with metformin in the presence or absence of the JNK activator anisomycin. Metformin is a potent activator of AMPK, and activation of AMPK has been demonstrated to increase the expression of glucose transporters and promote glucose uptake (10, 24). Therefore, we investigated whether metformin-dependent AMPK activation promoted glucose uptake in the presence of activated JNK. The rationale for these experiments was that insulin-stimulated glucose uptake would be inhibited in the presence of activated JNK, whereas AMPK-dependent uptake would not.

The ability of these stimuli to increase the tyrosine phosphorylation of IRS-1 and promote glucose uptake in response to a 15-min dose of insulin was measured. As shown in Fig. 6A, insulin reproducibly increased glucose uptake 1.5-fold in these cells and robustly led to the tyrosine phosphorylation of IRS-1 and activation of Akt. Interestingly, anisomycin treatment potently activated JNK and inhibited insulin-stimulated glucose uptake, tyrosine phosphorylation of IRS-1, and activation of Akt (Fig. 6A and data not shown). Metformin treatment alone led to a threefold increase in glucose uptake that was not further increased in response to insulin. While anisomycin partially suppressed glucose uptake in the metformin-treated cells, glucose uptake remained at a level higher than vehicle- and insulin-treated cells. Additionally, metformin treatment did not alleviate the loss of tyrosine phosphorylation of IRS-1 in response to anisomycin.

To address how glucose uptake was maintained during a suppression of insulin signaling, RT-PCR was performed to examine the expression of GLUT-1 and GLUT-4 in response to metformin. A 24-h metformin treatment resulted in a roughly 2.5- and 1.5-fold increase in GLUT-1 and GLUT-4 mRNA, respectively, whereas no change in actin expression was observed by these treatments (Fig. 6B). Thus these results suggested that metformin-dependent AMPK activation may increase both GLUT-1 and GLUT-4 transporter expression, independently of insulin or IRS-1 signaling, and provide a mechanism for maintaining glucose uptake in atrophic skeletal muscle.

DISCUSSION

In this study, we sought to investigate how atrophic slow-twitch muscles like the soleus become insulin resistant, yet maintain glucose uptake independently of insulin-dependent regulation of IRS-1. The role of JNK in insulin resistance and IRS-1 serine phosphorylation and degradation has been clearly established through the targeted disruption of JNK in mice; obese mice lacking JNK1 have lower circulating plasma insulin and glucose and no detectable Ser307 phosphorylation compared with obese JNK1+/+ mice (22). Similarly, obese mice deficient in the JNK-interacting protein, a JNK scaffold required for its activation, have no detectable Ser307 phosphorylation in muscle and adipose tissue compared with the obese wild-type controls (26). Further studies have demonstrated a direct interaction of JNK with IRS-1 and shown that serine phosphorylation of IRS-1 prevents its binding to, and tyrosine phosphorylation by, the insulin receptor (1). While a number of kinases are capable of serine phosphorylation of IRS-1, the phosphorylation of Ser302 and Ser307 was shown to be solely JNK dependent, and phosphorylation of those residues is prominent in animal models of obesity and insulin resistance (44).

A secondary objective of these studies was to investigate whether 2-G conditions elicited IRS-1 phosphorylation changes similar to those observed during HLS. In the HLS time course that we investigated, increased IRS-1 Ser307 phosphorylation and degradation correlated with elevated JNK activity in the atrophic soleus muscle similar to that observed in our initial study. The results of the present study demonstrated that this was an early event that was sustained throughout a lengthy time course of atrophy. Interestingly, exposure of animals to 2-G conditions did not result in similar alteration in JNK and IRS-1 signaling or ERK, p38, AMPK, Pyk2, or PKC-δ. These results are consistent with the modest change in soleus muscle mass after 2-G treatment and suggest that simply altering muscle load is insufficient to activate JNK and associated signaling events. The source of JNK activation in atrophic muscle, whether an external factor such as a change in cytokine signaling or an internal signal resulting from cytoskeletal alterations or muscle tension, remains unknown.
The results of this study suggest that the loss of IRS-1 protein would reduce the capacity of slow-twitch muscle to respond to insulin and would impair insulin-stimulated glucose uptake. AMPK is a possible mediator of insulin-independent glucose uptake in skeletal muscle, and activation of AMPK occurs in response to a number of diverse stimuli, such as hyperosmotic stress, hypoxia, and exercise, that induce the depletion of ATP (reviewed in Ref. 14). Activation of AMPK has been shown to enhance glucose uptake, and stimulation of AMPK with the AMP analog AICAR sensitizes insulin-resistant muscle to glucose uptake (39). Similarly, the antidiabetic agent metformin activates AMPK and results in greater glucose disposal in skeletal muscle (38).

Therefore, activation of AMPK may promote glucose uptake in insulin-resistant atrophic muscle. Consistent with this proposal, we observed a significant increase in AMPK activity that was only found in the atrophic muscle samples. Previous studies showed that administration of AICAR resulted in an acute activation of AMPK and increased expression of hexokinase-II, citrate synthase, and 3-hydroxyacyl-CoA-dehydrogenase, only in fast-twitch muscles like the extensor digitorum longus; however, AICAR was without effect in the soleus (5, 8). Thus activation of AMPK in the atrophic soleus (Fig. 3A) may be a unique event that reflects the change to more of a fast-twitch phenotype and/or the conversion from an oxidative to glycolytic muscle.

Long-term expression of a constitutively activated AMPK construct in cultured muscle myotubes increased the expression of both GLUT-1 and GLUT-4 (10), and prolonged administration of AICAR to rats elevated GLUT-4 expression in skeletal muscle (24). In the C2C12 myotubes, we observed that GLUT-1 and GLUT-4 mRNA were increased in response to metformin. In addition, metformin treatment allowed glucose uptake to proceed independently of insulin (Fig. 6). By contrast, studies in 3T3-L1 adipocytes showed that expression of constitutively activated MKK3/6 upregulated GLUT-1 but downregulated GLUT-4 expression (11). Thus while these data suggest cell-type-specific differences, prolonged activation of AMPK, either independent of, or in cooperation with, ERK and p38, can promote changes in GLUT expression. Moreover, activation of AMPK by contraction, stress, AICAR, or creatine feeding resulted in increased activity of the transcription factors myocyte enhancer factor-2 and GLUT-4 enhancing factor (2, 23, 30), which have been shown to increase GLUT-4 expression. The MAPks ERK1/2 and p38 are known activators of these transcription factors (35), and, in response to osmotic or oxidative stress, the incubation of muscle cells with inhibitors of the p38 and ERK1/2 pathways completely blocked myocyte enhancer factor-2 DNA binding (2). Therefore, AMPK-dependent activation of these MAPks may contribute to increased GLUT-4 expression and glucose uptake.

Additionally, the atypical PKCs are responsible for translocation of GLUT-4 from intracellular membranes to the muscle surface; expression of constitutively active PKC-λ increases GLUT-4 translocation independently of insulin, whereas dominant-negative PKC-λ inhibits insulin-stimulated uptake (6). In the atrophic soleus, atypical PKC activity was found to be significantly elevated compared with control muscle (Fig. 4C). This, combined with the loss of IRS-1 protein, suggests that the atypical PKCs are activated independently of insulin to stimulate glucose uptake in the atrophic soleus.

In summary, our data point to a model in which JNK activation during atrophy results in phosphorylation and loss of IRS-1 and, therefore, a decreased ability to respond to insulin. The corresponding activation of AMPK, a unique event in slow-twitch muscle, may accommodate the increased demand for carbohydrates in the atrophic muscle through activation of signals leading to increased GLUT-1 and GLUT-4 expression. The activation of the MAPks p38 and ERK1/2, either in response to AMPK activation or other mechanisms, may contribute to increased expression of glucose transporters and the enhanced translocation of GLUT-4 to the membrane through the insulin-independent atypical PKC activation. Further studies using transgenic animals expressing constitutively active or dominant-negative versions of these kinases may clarify the role of these enzymes in glucose uptake during atrophy of slow-twitch muscles.

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REFERENCES

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