Phosphorylation of insulin receptor substrate-1 serine 307 correlates with JNK activity in atrophic skeletal muscle

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Abstract c-Jun NH_2-terminal kinase (JNK) has been shown to negatively regulate insulin signaling through serine phosphorylation of residue 307 within the insulin receptor substrate-1 (IRS-1) in adipose and liver tissue. Using a rat hindlimb suspension model for muscle disuse atrophy, we found that JNK activity was significantly elevated in atrophic soleus muscle and that IRS-1 was phosphorylated on Ser^{307} prior to the degradation of the IRS-1 protein. Moreover, we observed a corresponding reduction in Akt activity, providing biochemical evidence for the development of insulin resistance in atrophic skeletal muscle.

Key words: Skeletal muscle atrophy; Hindlimb suspension; Insulin resistance; Insulin receptor substrate; c-Jun NH_2-terminal kinase

1. Introduction

Skeletal muscle atrophy is a consequence of prolonged muscle disuse from limb immobilization, extended bed rest, space flight or other factors [1]. Muscle unloading has been shown to primarily affect slow-twitch, anti-gravity muscles such as the soleus and adductor longus, resulting in muscle wasting, increased fatigue, and decreased contractile function (reviewed in [2]). Decreased protein synthesis [3] and increased protein degradation [4,5] are believed to contribute to the overall loss in skeletal muscle protein. Numerous metabolic alterations are associated with the development of atrophy, including an increased expression of fast-type myosin heavy chain (MHC) with the selective loss of the slow-type MHC [6–10], and a diminished ability to oxidize fatty acids [11] with an enhanced dependence upon carbohydrates as a fuel source [11,12]. Finally, the development of atrophy is accompanied by an increase in insulin resistance in the slow-twitch muscles [13–16] by a mechanism that is poorly understood.

The c-jun NH_2-terminal kinase (JNK) members of the MAP kinase family are part of a phosphorylation cascade that phosphorylate both nuclear and non-nuclear proteins in response to cellular stress signals [17]. In skeletal muscle, JNK is activated during stretch, exercise, and contraction [18–20], although the physiological significance of JNK activation in muscle function is unknown. Recent studies have implicated JNK-dependent serine phosphorylation of the insulin receptor substrate-1 (IRS-1) in negative regulation of the insulin receptor. The phosphorylation of IRS-1 Ser^{307} occurs in response to cytokines, metabolites, cell stress, or chronic insulin stimulation [21–29], in both JNK-dependent and -independent manners. Ser^{307} phosphorylation of IRS-1 prevents association of its protein tyrosine binding domain with the insulin receptor β-subunit, thereby preventing IRS-1 binding to the receptor and the insulin-dependent activation of PI 3-kinase [23]. Additionally, Ser^{307} phosphorylation of IRS-1 following chronic insulin stimulation has been shown to lead to its ubiquitin-proteasome-mediated degradation [28,30,31]. Finally, obese mice display elevated JNK levels in adipose, liver, and muscle tissue; similar mice in a $JNK1^{-/-}$ background (but not $JNK2^{-/-}$) have lower Ser^{307} IRS-1 and circulating insulin and plasma glucose [26], indicating that JNK1 may be a novel therapeutic target for obesity and type 2 diabetes.

In this study, we used a rat hindlimb suspension model for skeletal muscle disuse atrophy, and examined the role of JNK with respect to insulin resistance. We observed an atrophy-dependent increase in JNK activity and IRS-1 Ser^{307} phosphorylation. Therefore, similar to obesity-induced insulin resistance, JNK activation during atrophy may be a negative regulator of insulin signaling and provide a target for anti-atrophy therapeutics.

2. Materials and methods

2.1. Materials

Antibodies directed against IRS-1, IRS-2, and phospho-IRS-1 (Ser^{307}) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies against Akt, phospho-Akt (Ser^{473}), phospho-p38 (Thr^{183}/Tyr^{185}), JNK1/2, and phospho-JNK (Thr^{183}/Tyr^{185}), glycogen synthase kinase-3β (GSK-3β), and phospho-GSK-3β (Ser^{9}) were purchased from Cell Signaling Technology (Beverly, MA, USA). The glucose transporter-4 (GLUT4) antibody was from Chemicon (Temecula, CA, USA). The insulin receptor β-subunit antibody was from PharMingen (San Diego, CA, USA). Erk 1/2, phospho-Erk

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Abbreviations: AMB, ambulatory; Gastroc, gastrocnemius; GLUT4, glucose transporter-4; GSK-3β, glycogen synthase kinase-3β; HLS, hindlimb suspended; IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase

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(Tyr^{204}), p38, and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Rat hindlimb suspension

Female, virgin Sprague–Dawley derived albino rats were received from Simonsen Laboratories (Gilroy, CA, USA). Upon receipt, animals were individually housed in National Aeronautics and Space Administration (NASA) designed HLS/metabolic cages. Animals were kept in a room maintained at 22 ± 2°C with a 12:12 h light/dark cycle. The rats were fed certified rodent chow (TD 8728C, Harlan Teklad, Madison, WI, USA) in powder form to prevent contamination of urine during the study's metabolic collection periods. Chow and water were provided ad libitum throughout the study.

Prior to the study, female rats were acclimated for 7 days. Female rats, age 77 days, were given a number, then randomly assigned ($n = 10$/group) so that initial body mass between the different treatment groups did not differ. Animals were kept as ambulatory (AMB) controls or subjected to hindlimb suspension (HLS) according to the Morey–Holton procedure [32]. For this procedure, the tails of rats are lightly attached to a swivel on the top of the cage, providing a 30° head-down tilt. The swivel moves in a 360° angle and is attached to a set a bearings on an x–y plane, allowing for full access to food and water. At no point during the suspension are the hind limbs allowed to contact the surface of the cage. An experimental period of 38 days was selected to examine the effect of chronic exposure to HLS; at the end of the study, the rats were anesthetized with isoflurane, bled by cardiac puncture then decapitated. Following excision and weighing, the gastrocnemius (gastroc) and soleus were then frozen in liquid nitrogen. All procedures used in this study conformed to the National Research Council guide for use and care of laboratory animals. The animal protocol for this study was approved by the IACUC.

2.3. In vitro JNK assay

Three gastroc and soleus muscles from AMB and HLS rats were snap-frozen in liquid nitrogen, and crushed into a powder with a mortar and pestle. The powder was lysed on ice in modified RIPA buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 0.5% deoxycholate, 1.0% NP-40, 10 mM EDTA, 10 mM EGTA, 250 μM PMSF, 5 μg/ml leupeptin, 200 μM sodium vanadate, 0.01 μM microcystin). From each lysate, 0.375 mg of protein was tumbled with GST-c-jun coupled to glutathione Sepharose 4B beads for 1 h at 4°C. The bead complexes were washed twice in modified RIPA and once in MOPS buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1% Triton X-100). Kinase assays were performed at 30°C for 20 min in 50 μl assay buffer (16 mM MOPS, pH 7.2, 1.6 mM EGTA, 0.8 mM DTT, 0.08% Triton X-100, 10 mM $MgCl_2$, 0.5 mM ATP, 0.4 mCi/ml [γ-^{32}P]ATP). Reactions were terminated in sodium dodecyl sulfate (SDS) sample buffer with boiling and separated on a 12% SDS–PAGE gel. Following drying, the gel was exposed to film for 2 h and developed. GST-c-jun bands were then excised from the dried gel and counted. Results are reported as the average of three experiments performed in duplicate ± standard error of the mean. Significance was determined by two-factor ANOVA with replication.

2.4. Immunoblotting

For the lysates generated above, 100 μg was separated on an 8% SDS–PAGE gel, then transferred to PVDF (2.5 h, 500 mA). Blocking of membranes and antibody dilutions were performed according to the manufacturer's directions. Phospho blots were performed first; membranes were then stripped (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol; 55°C, 30 min), and reprobed for total protein. Proteins were detected using an enhanced chemiluminescent reagent. Exposure time of the membrane to film for gastroc and soleus immunoblots was identical so that direct comparisons of phospho and total protein could be made between the muscles. Protein levels were quantified with the NIH ImageJ densitometry program; results are shown as the average density ± standard deviation for three animals, and significance was determined with a two-sample t-test.

Table 1
Average body weight and muscle masses following a 38-day hindlimb suspension

	Ambulatory	Hindlimb suspended
Body mass (g)	254.6 ± 6.4	251.3 ± 7.2
Gastrocnemius (g)	1.789 ± 0.0543	1.383 ± 0.0230
Soleus (g)	0.123 ± 0.0035	0.050 ± 0.0018

Data are expressed as the mean ± standard deviation for 10 animals.

3. Results

3.1. MAP kinase family member activation during skeletal muscle atrophy

While JNK activity has been described during conditions of hypertrophy [18–20], we hypothesized that loss of muscle mass following extended disuse may also activate this protein

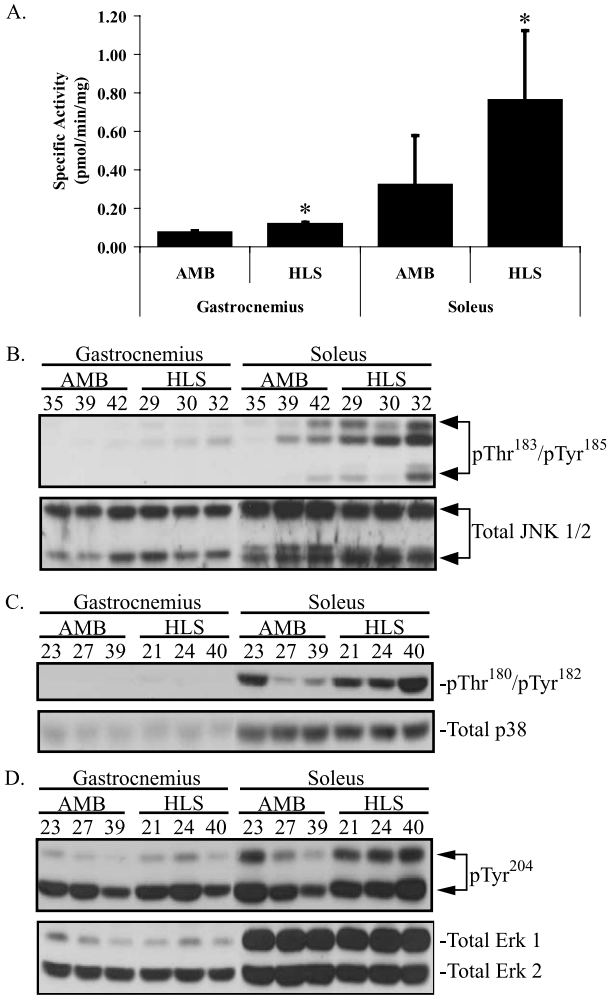

Fig. 1. The activity MAP kinase family members are elevated in atrophic muscle. A: GST-c-jun coupled to glutathione Sepharose beads was used to pulldown JNK from lysates from AMB or HLS gastroc or soleus muscle. JNK-dependent phosphorylation of GST-c-jun was assayed from muscle lysates from three different animals in duplicate. *$P < 0.05$ for the difference between HLS and AMB for gastroc or soleus using two-factor ANOVA with replication. B–D: Muscle lysates were separated by SDS–PAGE and immunoblotted for JNK (panel B), p38 (panel C), and Erk 1/2 (panel D). In each case, membranes were probed for the activating phosphorylation sites (top), then reprobed for total protein (bottom). Proteins were visualized by enhanced chemiluminescence. Arrows indicate the alignment of phospho-protein to total protein. Above each lane is the number used to identify each animal upon the random distribution into AMB or HLS at the beginning of the study.

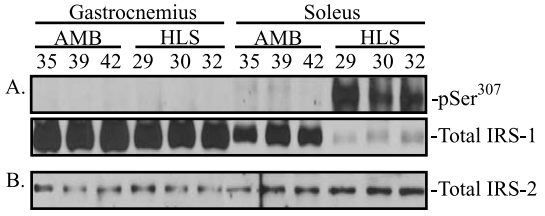

Fig. 2. Ser[307] phosphorylation of IRS-1 correlates with degradation of IRS-1 protein. Lysates were immunoblotted for IRS-1 phosphoserine 307 (pSer[307]; panel A, top), then stripped and reprobed for total IRS-1 (panel A, bottom); IRS-2 levels were also examined by immunoblotting (panel B).

kinase. Exposure of rats to a 38-day hindlimb suspension regiment resulted in a slight loss in gastroc mass and a more pronounced loss of soleus mass as summarized in Table 1. Assaying JNK from AMB or HLS rat soleus demonstrated elevated JNK activity (2.5-fold higher versus control) in the atrophic soleus (Fig. 1A). To ensure this was specific to atrophic, slow-twitch muscle and not a general phenomenon of atrophied skeletal muscle, JNK activity from gastroc (fast-twitch) also was measured. Comparison of JNK from AMB and HLS gastroc muscles showed a much smaller, yet significant increase in activity. Immunoblotting with antibodies directed against the JNK activating phosphorylation sites (Thr[183]/Tyr[185]) also demonstrated that the increase in activity was much more pronounced in atrophic soleus (Fig. 1B). The change in activity was not due to expression differences, as no change was seen in either the 54- or 46-kDa JNK1 and JNK2 protein (Fig. 1B). Comparable results were obtained with male rats exposed to shorter (21-day) hindlimb suspension (data not shown).

Similar to JNK, p38 and Erk are activated during short stints of hypertrophy [33], and p38 activity is elevated following a 10-day cast immobilization [34]. In our 38-day hindlimb suspension, the activities of p38 (Fig. 1C) and Erk 1/2 (Fig. 1D) were both greater in the atrophic soleus as compared to the control, with the differences not being attributed to altered expression.

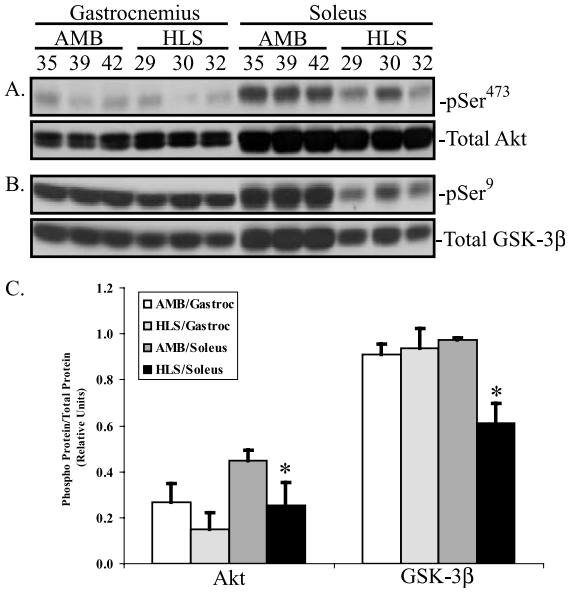

Fig. 3. Components of the insulin signaling pathway are reduced during atrophy. Lysates generated as described in Fig. 1 were immunoblotted for Akt (panel A) or GSK-3β (panel B), either for the activating phosphoserine (top) or for total protein (bottom). Densitometry analysis of phospho-protein relative to total protein is shown in panel C; results are reported as the average of three muscles ± standard deviation, with $*P < 0.05$ for the difference between HLS and AMB for the soleus.

3.2. Serine 307 phosphorylation of IRS-1

JNK has been shown to negatively regulate insulin signaling through the phosphorylation IRS-1 on Ser[307], while preventing this phosphorylation restores insulin responsiveness in mice [26]. Using a phospho-specific antibody to Ser[307], we examined the phosphorylation of IRS-1 in AMB and HLS skeletal muscle. As shown in Fig. 2A, Ser[307] phosphorylation was only detected in HLS soleus; IRS-1 was not phosphorylated in the fast-twitch gastroc although JNK activity was slightly higher in the HLS gastroc versus AMB (Fig. 1). The increased phosphorylation on Ser[307] appears to have caused a loss in total IRS-1 protein in HLS soleus as compared to the AMB control (Fig. 2A), whereas IRS-2, shown to be degraded under some conditions [35], was unaffected by atrophy (Fig. 2B). Therefore, JNK activity correlated with the Ser[307] phosphorylation and the degradation of IRS-1 in atrophic soleus muscle.

3.3. Ser[307] phosphorylation of IRS-1 reduces Akt and GSK-3β activities

Insulin binding to its membrane receptor leads to receptor tyrosine autophosphorylation, association of IRS-1 with the receptor, and the recruitment of PI 3-kinase. Since phosphorylation of IRS-1 on Ser[307] prevents association with the insulin receptor [23], and IRS-1 protein is lost in atrophic soleus, we hypothesized that the coupling of PI 3-kinase to the insulin receptor would be affected. Using phospho-specific antibodies to active forms of the kinase, the activities of Akt (Fig. 3A) and GSK-3β (Fig. 3B), an Akt substrate, were analyzed. Consistent with the loss of IRS-1 protein, a specific loss of both Akt and GSK-3β was observed in the atrophic soleus. While total GSK-3β protein also declined,

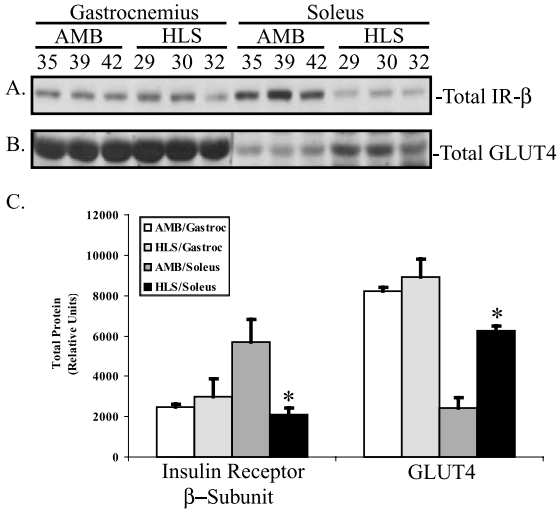

Fig. 4. Insulin receptor expression, but not GLUT4, is reduced in atrophic soleus muscle. Lysates were immunoblotted for total insulin receptor β-subunit (panel A) or GLUT4 (panel B). Densitometry analysis of total protein (panel C) is as described in Fig. 3.

the loss of phospho-GSK-3β was still significantly lower in atrophic soleus when compared to AMB muscle (Fig. 3C).

3.4. Insulin receptor-β and GLUT4 alterations in atrophic muscle

Insulin resistance in skeletal muscle tissue may result from a loss of the insulin receptor or decreased expression of the glucose transporters. Therefore, the expression of these proteins was examined from AMB and HLS muscle lysates. Interestingly, the β-subunit of the insulin receptor was significantly reduced in atrophic soleus muscle compared to AMB muscle (Fig. 4A,C). Conversely, the expression of GLUT4, the insulin-responsive member of the glucose transporter family, increased significantly in atrophic soleus as compared to control (Fig. 4B,C). Again, these changes were specific to the slow-twitch soleus, as the atrophic gastroc demonstrated no significant change compared to control muscle.

4. Discussion

Serine phosphorylation and the subsequent degradation of IRS-1 provide one mechanism for insulin resistance. Using a 38-day hindlimb suspension of rats as a model for skeletal muscle disuse atrophy, we demonstrate increased JNK activity during atrophy, which corresponds with [34] and contradicts [36] previous reports. The JNK activity correlates with increased Ser^{307} phosphorylation of IRS-1, and the corresponding degradation of the protein. Additionally, the activities of Akt and GSK-3β, enzymes normally activated in response to insulin, are suppressed in atrophic soleus muscle. Reduced insulin receptor levels were also found during atrophy, in agreement with previous studies [37,38], although not to the same extent as the loss of IRS-1. Interestingly, these findings were specific to atrophic slow-twitch muscle, the primary muscle group affected by disuse and weightlessness [39]. Thus, we propose that the insulin resistance observed during muscle atrophy is not due to loss of insulin-mediated glucose transport, as the levels of GLUT4 increase in atrophic muscle. Although reductions in IRS-1 protein levels were demonstrated in obese insulin-resistant human skeletal muscle [40] and chronically insulin-stimulated cultured L6 muscle cells [41], to our knowledge this study is the first demonstration of Ser^{307} phosphorylation of IRS-1 in atrophic skeletal muscle tissue.

Current evidence suggests two possible methods for IRS-1 phosphorylation and degradation. JNK has been shown to associate with and directly phosphorylate IRS-1 in vitro [23], and cell-permeable JNK inhibitory compounds and peptides, as well as mutations in the IRS-1 JNK-binding domain, reduce Ser^{307} phosphorylation [25,27]. Additional evidence from obese JNK1 knockout mice has shown that these mice have lower circulating insulin and glucose and Ser^{307} phosphorylation when compared to obese $JNK1^{+/+}$ mice [26]. However, JNK-independent phosphorylation of IRS-1 has also been described. Chronic insulin stimulation of a number of cell types in culture has shown that the phosphorylation and degradation of IRS-1 can be prevented by pretreatment of cells with wortmannin or rapamycin, suggesting that the PI 3-kinase/Akt/mTOR pathway is responsible [30,42–44]. Taken together, these JNK-dependent and -independent results suggest that chronic insulin stimulation of PI 3-kinase/Akt/mTOR and cell stress-induced JNK activation may be distinct signals that result in IRS-1 serine phosphorylation, degradation, and insulin resistance.

Our results favor the JNK-dependent phosphorylation of IRS-1 for a number of reasons. First, decreased protein synthesis is prominent in muscle atrophy, partially due to reduced p70 S6-kinase activity [3]. The activities of mTOR and p70 S6-kinase are intimately linked, suggesting the mTOR-dependent activity is not responsible for IRS-1 serine phosphorylation. Second, the loss of insulin receptor numbers during atrophy (Fig. 4A and [37,38]), combined with the reduction in Akt activity (Fig. 3A), suggests the overstimulation of the PI 3-kinase/Akt/mTOR pathway is not significantly contributing to IRS-1 serine phosphorylation and degradation. Third, human muscle atrophy studies with the head-down bed rest model have demonstrated that there are no changes in circulating insulin-like growth factor-I (IGF-I) or insulin [45]. Together, these data suggest that chronic receptor stimulation does not contribute to IRS-1 serine phosphorylation and insulin resistance. Rather, our data are consistent with a model of cell stress-induced JNK activity contributing to the development of insulin resistance in muscle atrophy.

Moreover, Erk and p38 both contribute to glucose uptake. High extracellular glucose and exercise have been shown to activate Erk, which in turn, through the activation of atypical PKCs, stimulates glucose uptake independent of insulin [46]. p38 is hypothesized to be an activator of GLUT4, as inhibitors of p38 do not prevent the translocation of GLUT4 to the membrane but block insulin-stimulated glucose transport [47,48]. The fact that Erk and p38 activities are elevated in atrophic skeletal muscle is intriguing in that they provide a mechanism to explain how glucose uptake can occur in the absence of insulin signaling through IRS-1 in atrophic muscle. While atrophic, slow-twitch muscle prefers carbohydrates as a fuel source, it appears that the inherent activation of the stress kinases during atrophy – JNK to degrade IRS-1 and Erk/p38 to enhance glucose uptake – may be two competing signals that maintain glucose uptake. Further studies will be needed to clarify the role of these MAP kinases throughout the progression of skeletal muscle atrophy.

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