Contractile Activity Stimulates the c-Jun NH$_2$-terminal Kinase Pathway in Rat Skeletal Muscle

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Doron Aronson, Scott D. Dufresne, and Laurie J. Goodyear

From the Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02215

Contractile activity plays a critical role in the regulation of gene transcription in skeletal muscle, which in turn determines muscle functional capabilities. However, little is known about the molecular signaling mechanisms that convert contractile activity into gene regulatory responses in skeletal muscle. In the current study we determined the effects of contractile activity in vivo on the c-Jun NH$_2$-terminal kinase (JNK) pathway, a signaling cascade that has been implicated in the regulation of transcription. Electrical stimulation of the sciatic nerve to produce contractions in anesthetized rats increased JNK activity by up to 7-fold above basal. Maximal enzyme activity occurred at 15 min of contraction and remained elevated at 60 min of contraction. The upstream activators of JNK, the mitogen-activated protein kinase kinase 4 and the mitogen-activated protein kinase kinase kinase 1 followed a similar time course of activation in response to contractile activity. In contrast, contraction induced a rapid and transient activation of the extracellular-regulated kinase pathway, indicating that the regulation of JNK signaling is distinct from that of extracellular-regulated kinase. The activation of the JNK signaling cascade was temporally associated with an increased expression of c-jun mRNA. These results demonstrate that contractile activity regulates JNK activity in skeletal muscle and suggest that activation of JNK may regulate contraction-induced gene expression in skeletal muscle.

Contractile activity in skeletal muscle causes a selective increase in gene expression, leading to the synthesis of specific muscle proteins (1, 2). This reprogramming of gene expression results in the induction of proteins involved in oxidative metabolism (3, 4) and an increase in the expression of the slow/white muscle fibers (21). The physiological consequences of these adaptations render the muscle resistant to fatigue during sustained contractile work. However, the molecular signaling mechanisms that convert skeletal muscle contractile activity into biochemical and gene regulatory responses are not known.

Exposure of cells to environmental stressors evokes a series of phosphorylation events leading to the modification of transcription factors and altered gene expression (6). One of the pathways mediating the response to cellular stressors relies on the c-Jun NH$_2$-terminal kinases (JNKs) for gene activation (6–9). JNKs are activated by the dual-specificity enzyme mitogen-activated protein kinase kinase 4 (MEK4) (8, 10). MEK4 is in turn activated by mitogen-activated protein kinase kinase 1 (MEKK1) (7, 11). JNKs (also referred to as stress-activated protein kinases) can be stimulated by a variety of cellular stresses such as UV radiation (9), osmotic (12) and heat shock (9, 13), protein synthesis inhibitors (9), and pro-inflammatory cytokines (9, 14). Once activated, JNKs can translocate to the nucleus (15), and their immediate downstream targets include a variety of transcription factors, notably c-Jun (9, 10), ATF-2 (16), and Elk-1 (15).

Although the JNK pathway has been extensively explored in the context of extreme cellular stresses (6), recent reports indicate that this signaling cascade may be involved in more physiological responses of transducing mechanical stimuli into transcriptional responses (17–19). We recently reported that exercise activates the extracellular-regulated kinase (ERK) as well as other upstream and downstream components of the ERK signaling cascade, including the p90 ribosomal S6 kinase, MEK1, and Raf-1 in human skeletal muscle (20). In the present study we determined the effects of contractile activity on JNK signaling in vivo using a model of electrically stimulated rat skeletal muscle. We show that contractile activity stimulates JNK activity as well as the activity of its upstream regulators MEK4 and MEKK1 and that the activation of JNK signaling is temporally related to a rapid up-regulation of c-jun mRNA expression. These results implicate the JNK pathway in the regulation of gene expression in response to contractile activity.

**EXPERIMENTAL PROCEDURES**

**Contraction of Skeletal Muscle—** Male Sprague-Dawley rats (200 ± 10 g) were anesthetized with sodium pentobarbital (75 mg/kg of body weight, intraperitoneal). The sciatic nerves of both hind limbs were exposed, and electrodes were attached. Hind limb muscles were stimulated with one 500-ms train of impulses (each impulse was of 0.1 ms duration) at a frequency of 100 Hz applied every 1 s for 15 or 5, 10, 15, 30, or 60 min. When the stimulation period exceeded 5 min, each additional 5-min period was separated by 2 min of rest. Immediately after stimulation, the gastrocnemius muscle was rapidly dissected and frozen in liquid nitrogen. This muscle mainly represents fast-twitch red and white muscle fibers (21).

For the signaling studies, the muscles were powdered and then Polytron-homogenized in ice-cold lysis buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na$_2$VO$_4$, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 10 μM leupeptin, 3 μM benazpectin, 5 μM pepstatin A, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Homogenates were rotated for 1 h at 4°C to ensure complete digestion of protein and the release of JNK from its inhibitor, SEK.

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§ To whom correspondence should be addressed: Joslin Diabetes Center, One Joslin Place, Boston MA 02215.

† The abbreviations used are: JNK, c-Jun NH$_2$-terminal kinase; MEK4, mitogen-activated protein kinase kinase 4; MEKK1, mitogen-activated protein kinase kinase kinase 1; ERK, extracellular-regulated kinase; GST, glutathione S-transferase; SEK, stress-activated protein kinase kinase; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
4 °C and centrifuged at 14,000 × g for 60 min at 4 °C to remove insoluble matter. Protein concentrations in the soluble extracts were estimated by the Bradford method (22). For Northern blotting, total RNA was extracted from muscle using TRI reagent according to the manufacturer’s protocol (Molecular Research Center, Inc.).

**Materials**—[γ-32P]ATP (3000 Ci/mmol) and [α-32P]dCTP were purchased from NEN Life Science Products. A PGEX vector designed to express a glutathione S-transferase (GST)-c-Jun fusion protein (amino-terminal residues 1–135) was provided by Dr. John Kyriakis, Massachusetts General Hospital. GST-SEK1, GST-MEK1, GST-ERK1 were from Upstate Biotechnology Inc. (Lake Placid, NY); anti-JNK1, anti-MEK4, and anti-MEKK1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-MEKK1 monoclonal antibody was from Transduction Laboratories (Lexington, KY); anti-phospho MEK4 was from New England Biolabs, Inc. (Beverly, MA); phospho-specific anti-ERK and anti-p55JNK were from Quality Controlled Biochemicals Inc. (Hopkinson, MA); and protein A-agarose and protein G-agarose were from Pierce. Protein concentrations were determined using a dye reagent from Bio-Rad; and all other chemicals were purchased from Sigma. Zeta-Probe blotting membranes were from Bio-Rad. A multiprime DNA labeling kit was from Amersham Life Science, Inc.

**Immunoblotting**—Muscle proteins (300 μg) were solubilized in Laemmli buffer and boiled for 5 min. Samples were then resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and incubated with phospho-specific anti-ERK, anti-p55JNK, or anti-MEK4. The filters were then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2500), and antibody binding was detected via the method of enhanced chemiluminescence.

**Kinase Assays**—JNK immune-complex kinase assays were carried out as described by Kyriakis et al. (9) with some modifications. Aliquots (250 μg) of precleared muscle extracts were immunoprecipitated with 1.0 μg of polyclonal anti-JNK1. The immunoprecipitates were washed twice with lysate buffer, twice with LiCl buffer, and twice with kinase buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiotheitol, 0.1% Triton X-100). The immunoprecipitates were resuspended in 50 μl of kinase buffer containing 1 μg of JNK-(1–135), 50 μM ATP, and 10 μCi of [γ-32P]ATP. The kinase reaction was performed at 30 °C for 30 min and terminated with Laemmli sample buffer. The reaction products were resolved using 10% SDS-PAGE, and the phosphorylated GST-c-Jun was quantitated by PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

MEK1 activity was assayed in an immune-complex kinase assay using GST-ERK as substrate, as described previously (20). The MEK4 assay was essentially similar to the MEK1 assay, except that muscle extracts were immunoprecipitated with 1.5 μg of anti-MEK4, and GST-JNK served as substrate. For the MEKK1 assay, 500 μg of muscle extracts were immunoprecipitated with 1.5 μg of polyclonal rabbit anti-MEKK1. The immunoprecipitates were washed three times in buffer A (150 mM NaCl, 20 mM Tris, pH 7.6, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 100 μM Na3VO4, 10 μg/ml aprotinin, 10% glycerol, 1% Nonidet P-40, 2 μg/ml leupeptin) and three times with MEK1 buffer (10 mM HEPES, pH 7.4, 10 mM MgCl2, 5 mM MnCl2, 5 μM DTT, 100 μM Na2VO3, 25 mM β-glycerophosphate, 1 mM dithiotheitol). The immune complexes were resuspended in 50 μl of kinase buffer containing 20 μM ATP, 10 μCi of [γ-32P]ATP, and 1 μl of recombinant kinase-inactive GST-MEK4 as substrate. The reactions were carried out for 30 min at 30 °C. Products were resolved on 10% SDS-PAGE, and the phosphorylated GST-MEK4 was quantified by PhosphorImager analysis of the dried gels.

**Northern Blot Analysis**—Aliquots (10 μg) of total RNA per sample were denatured and size-separated by electrophoresis in a 1% agarose, 6.7% formaldehyde gel and transferred to a nylon membrane in 10 × standard sodium citrate (1 × SSC = 0.15 M sodium chloride and 0.015 M sodium citrate). Equal loading of RNA was confirmed by ethidium bromide staining of the gels. RNA was UV cross-linked to the membrane, and the blots were hybridized with a 1.8-kilobase EcoRI fragment of the rat c-jun cDNA probe labeled with [α-32P]dCTP to ~107 dpm/μg using a multiprime DNA labeling kit. The membranes were washed stringently with the final wash in 0.5 SSC, 0.1% SDS at 55 °C for 30 min. The blots were then quantitated by PhosphorImager.

**RESULTS**

**Contractile Activity Stimulates JNK Dual Phosphorylation and Activity**—Activation of both JNK isoforms (46 and 55 kDa) requires dual phosphorylation of Thr-183 and Tyr-185 (23). To determine whether contraction increases JNK dual phosphorylation, we used a phospho-specific anti-p55JNK antibody that recognizes only the dual-phosphorylated form of the protein. Western blot analysis of muscle extracts showed that contractile activity increased the dual phosphorylation of the p55JNK (Fig. 1A). Densitometric analysis revealed that with 30 min of contraction, the dual phosphorylation of p55JNK increased by 2.2 ± 0.21-fold above basal (mean ± S.E.).

JNKs are the only known protein kinases that efficiently phosphorylate Ser-63 and Ser-73 on the NH2-terminal activation domain of c-Jun (9, 23, 24). To directly determine whether contractile activity increases JNK activity toward its physiologic substrate c-Jun, immune complex kinase assays were performed using c-Jun-(1–135) as substrate. Contractile activity increased c-Jun phosphorylation in anti-JNK immune complexes from skeletal muscle by 5.2 ± 0.6-fold (mean ± S.E.) at the 30-min time point (Fig. 1B). Taken together these results indicate that contractile activity increases the dual phosphorylation of JNK and that this event is associated with increased JNK activity.

**Differential Time Course for JNK and ERK Activation by Contraction**—We next examined the time course of contraction-induced JNK activation. Contraction resulted in a time-dependent increase in JNK activity (Fig. 2A). JNK activation required 15 min to reach a maximum where the increase in JNK activity was 6.9 ± 2.0-fold above basal (Fig. 2B). Thereafter, the activity of JNK decreased slightly but remained elevated with 30 and 60 min of contraction (Fig. 2B). In contrast, the time course of contraction-induced ERK activation, as determined by ERK1/2 dual phosphorylation, was transient (Fig. 3A). ERK1/2 phosphorylation reached maximal levels at 10 min and returned to near basal levels at 30 min (Fig. 3B).

**Contractile Activity Stimulates MEK4**—MEK4 (JNK kinase) is a dual-specificity kinase that specifically activates JNK and is highly expressed in skeletal muscle (8, 10). Although currently MEK4 is the only known upstream activator of JNK, studies in cells cultured from MEK4 knockout mice suggest that more than one pathway leading to JNK activation probably exists (25). Therefore, we determined if contractile activity stimulates MEK4 activity.

Activation of MEK4 requires phosphorylation of Ser-220 and Thr-224 (7). To determine the phosphorylation state of MEK4 in skeletal muscle, we used a phospho-specific antibody that recognizes MEK4 only when phosphorylated on Thr-224. As shown in Fig. 4A, this antibody recognizes a single 46-kDa
band, which corresponds to the reported molecular mass of MEK4 (8). The immunoblot in Fig. 4
shows that MEK4 phosphorylation increases with contractile activity in a similar
temporal profile to that of its downstream substrate, JNK. We
next determined if contraction increases MEK4 activity by an
immune complex kinase assay using GST-JNK as substrate.

Fig. 4. Regulation of MEK4 by contractile activity. A, muscle
proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with phospho-specific anti-MEK4 that
specifically recognizes the activated form of MEK4. B, phosphorylation. C, the intensity of each band on the
autoradiogram was quantified by densitometric scanning. Data are the
mean ± S.E. of three independent experiments expressed as fold in-
crease over basal.

Fig. 2. Time course of contraction-induced activation of JNK
in rat skeletal muscle. Hind limb muscles were contracted for the
indicated periods of time. A, muscle extracts were immunoprecipitated
with anti-JNK, and the immunoprecipitates were subjected to in vitro
kinase assay using GST-c-Jun(1–135) as substrate. A representative
autoradiogram is shown. Similar experiments were repeated three
times with nearly identical results. B, the intensity of each band on the
autoradiograms was quantified by PhosphorImager to determine rela-
tive JNK activity. Results are mean ± S.E.; each data point represents
three separate animal preparations.

Fig. 3. Time dependent effect of contractile activity on ERK
dual phosphorylation. A, muscle proteins (300 μg) were separated by
SDS-PAGE and transferred to nitrocellulose membranes. The phospho-
rylation (P) of the ERK1 and ERK2 isoforms was determined using phospho-specific antibodies that recognize only the dual-phosphoryl-
ated ERK1/2. B, the intensity of the combined ERK1/2 bands was
quantified by densitometric analysis. Relative ERK dual phosphoryla-
tion at the indicated time points is shown as mean ± S.E. Each data
point represents three separate animal preparations.

Contractile Activity Stimulates MEKK1 Activity—Although
originally identified as an activator of the ERK pathway (29), MEKK1 appears to function upstream of MEK4 in the regula-
tion of JNKs (7). We measured MEKK1 activity in an immune
complex kinase assay using GST-JNK as substrate. Fig. 4, B and C depict the time course of contraction-induced
MEK4 activation. Similar to JNK, MEK4 activity increased
gradually and was sustained through the 60-min time course.

MEK1 is a dual specificity kinase that parallels MEK4 in the
ERK signaling pathway (26–28). Similar to what was observed
with ERK and JNK, the time course of MEK1 activation dif-
fered markedly from that of MEK4 (Fig. 4 versus Fig. 5). Max-
imal activation of MEK1 (3.9 ± 1.2-fold above basal) was ob-
served at the earliest time point (15 s). This was followed by a
rapid decline in MEK1 activity to basal levels (Fig. 4). These
experiments demonstrate that the activation of the upstream
elements of the ERK signaling pathway is extremely rapid once
contractile activity commences.
MEK1 activities (Fig. 4 versus Fig. 5) suggest that MEKK1 is not the upstream activator of ERK signaling with contraction.

Effect of Contractile Activity on c-jun mRNA Expression—JNKs phosphorylate two serine residues in the transactivation domain of c-Jun leading to an increase in its transcriptional activity (9, 23, 24). Thus, we determined if JNK activation by contractile activity is associated with an increase in c-jun mRNA levels. Northern blot analysis revealed that resting skeletal muscle has a low expression of c-jun mRNA. However, as little as 15 min of contractile activity resulted in a 1.9 ± 0.1-fold increase in c-jun mRNA and remained at this level for the duration of the contraction protocol (Fig. 7). In addition, a 20-fold increase in c-fos mRNA occurred at the 15-min time point (data not shown). c-fos may be induced by either ERK (31) or JNK (15) phosphorylation of Elk-1, a known regulator of Fos transcription. These experiments demonstrate that contractile activity can induce a rapid increase in early response gene expression in skeletal muscle in a temporal pattern that corresponds to the activation of JNKs and ERKs.

DISCUSSION

Skeletal muscles from adult mammalian species exhibit a remarkable capacity for long-term adaptation to changing work demands (1, 2). The ability to respond to contractile activity through selective increases in gene expression and the synthesis of specific muscle proteins is critical for the functional adaptations of skeletal muscle cells. However, the mechanisms by which contractile activity is converted into gene regulatory events is not known.

Several cell types have been shown to respond to various mechanical stimuli such as stretch (17), shear stress (18), and increased load (32) by activating protein kinase phosphorylation cascades. Our previous study has shown that a similar response occurs in human skeletal muscle, as physical exercise results in the activation of the ERK signaling cascade (20). In the current study, we demonstrate that contractile activity in rat skeletal muscle potently stimulates JNK activity, as well as the upstream activators MEK4 and MEKK1.

The stimulation of the JNK pathway by contractile activity was sustained throughout the time course studied. In contrast, contraction induced a rapid and transient activation of the ERK pathway, indicating that the regulation of JNK signaling is distinct from that of ERK. Studies in cultured cells have indicated that the duration of the activation of the ERK (33) and JNK (34, 35) pathways is critical for determining transcriptional and cellular responses. Whether the different time courses of contraction-induced ERK and JNK activation are important for the physiological response of skeletal muscle
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remains to be established. Interestingly, studies in other mecha-
nically responsive cells have also shown differential time
courses of ERK and JNK activation. For example, shear-
induced ERK activation in endothelial cells is transient, reaching
a maximum by 5 min and decreasing to basal levels by 30 min
of shear exposure, whereas shear-stimulated JNK activation
remained elevated for many hours (18). These similarities im-
ply that the fundamental cellular transduction mechanisms of
mechanical stimuli may be conserved evolutionally and overlap
during different cell types.

The activation of JNK and ERK with contractile activity was
associated with the rapid induction of c-
-Jun and c-fos. This
temporal relationship suggests a role for JNK and ERK as
upstream regulators of early response genes in response to
contractile activity. The induction of early response genes
presumably represents transduction of early nuclear signals
to long term changes in gene expression that constitute the
cellular response to increased contractile activity. For example,
c-
-Jun and c-fos are involved in the induction of structural mus-
cle proteins including skeletal α-actin (36, 37) and myosin light
chain (38). Thus, activation of the JNK and ERK signaling
pathway with contraction may represent an important mecha-
nism for the conversion of mechanical stimuli into transcrip-
tional responses in skeletal muscle.

The JNK and ERK signaling pathways have been shown to
be involved in the adaptation of cardiac myocytes to increased
load (17, 19). Emerging data indicate that a similar response
occurs in arterial smooth muscle cells subjected to mechanical
load (39) or hypertension (32). In both cell types, the stimula-
tion of these pathways is associated with the induction of early
response gene expression (19, 32, 40). Although the nature of
the mechanical stimuli to which different muscle cell types are
normally exposed differs considerably, these studies, together
with our own data, suggest that the JNK and ERK cascades are
a signaling mechanism shared by different muscle cell types
to convert mechanical stimuli into early gene responses. In skel-
etal muscle, activation of these signaling pathways may be an
initial signaling event that results in long term adaptations to
increased muscle work.

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