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Fujii, Nobuharu, Marni D. Boppart, Scott D. Dufresne, Patricia F. Crowley, Alison C. Jozsi, Kei Sakamoto, Haiyan Yu, Williams G. Aschenbach, Shokei Kim, Hitoshi Miyazaki, Liangyou Rui, Morris F. White, Michael F. Hirshman, and Laurie J. Goodyear. Overexpression or ablation of JNK in skeletal muscle has no effect on glycogen synthase activity. *Am J Physiol Cell Physiol* 287: C200–C208, 2004. First published March 10, 2004; 10.1152/ajpcell.00415. 2003.—c-Jun NH_2-terminal kinase (JNK) is highly expressed in skeletal muscle and is robustly activated in response to muscle contraction. Little is known about the biological functions of JNK signaling in terminally differentiated muscle cells, although this protein has been proposed to regulate insulin-stimulated glycogen synthase activity in mouse skeletal muscle. To determine whether JNK signaling regulates contraction-stimulated glycogen synthase activation, we applied an electroporation technique to induce JNK overexpression (O/E) in mouse skeletal muscle. Ten days after electroporation, in situ muscle contraction increased JNK activity 2.6-fold in control muscles and 15-fold in the JNK O/E muscles. Despite the enormous activation of JNK activity in JNK O/E muscles, contraction resulted in similar increases in glycogen synthase activity in control and JNK O/E muscles. Consistent with these findings, basal and contraction-induced glycogen synthase activity was normal in muscles of both JNK1- and JNK2-deficient mice. JNK overexpression in muscle resulted in significant alterations in the basal phosphorylation state of several signaling proteins, such as extracellular signal-regulated kinase 1/2, p90 S6 kinase, glycogen synthase kinase 3, protein kinase B/Akt, and p70 S6 kinase, in the absence of changes in the expression of these proteins. These data suggest that JNK signaling regulates the phosphorylation state of several kinases in skeletal muscle. JNK activation is unlikely to be the major mechanism by which contractile activity increases glycogen synthase activity in skeletal muscle.

electroporation; gene delivery; muscle contraction; exercise

THE c-JUN NH_2-TERMINAL KINASES (JNKs) are ubiquitously expressed intracellular signaling molecules that are activated by cytokines and exposure to environmental stresses such as osmotic stress, redox stress, and radiation (30). During the past few years, we (5, 6, 10–12, 22) and others (33, 49) have demonstrated that muscle contraction and physical exercise increase JNK activity in rat and human skeletal muscle. Muscle contractile activity robustly increases the activity of the JNK1 and JNK2 isoforms, and these increases are rapid and transient in nature (5, 11). The physiological function of contraction-stimulated JNK activation in skeletal muscle is not known.

In other cells and tissues, JNK signaling has been implicated in the regulation of numerous cellular processes, including apoptosis and survival signaling, T-cell maturation, brain development, cardiac hypertrophy, and ischemic or ischemia-reperfusion injury (17, 30). Depletion of JNK1 activity in knockout mice is protective against obesity and insulin resistance, probably because of chronic regulation of adipose cell size and distribution (26). Only one study has focused on the role of JNK in skeletal muscle, and the group that conducted it proposed that JNK is involved in the regulation of glycogen metabolism (36).

Glycogen content in skeletal muscle is controlled by the coordinated regulation of glycogen synthase and glycogen phosphorylase activities. It is well established that glycogen synthase activity is regulated by both allosteric and phosphorylation-dependent mechanisms. Insulin stimulation of glycogen synthesis may involve phosphorylation and activation of protein kinase B/Akt (Akt), serine phosphorylation, and deactivation of glycogen synthase kinase-3 (GSK-3), leading to dephosphorylation and activation of glycogen synthase (16). Moxham et al. (36) demonstrated that activation of JNK by anisomycin, a protein synthesis inhibitor and JNK activator, mimics insulin's action on glycogen synthesis in mouse skeletal muscle in vivo. This group concluded that insulin-induced JNK activation increases glycogen synthase activity through the activation of p90 ribosomal S6 kinase (RSK-3) and subsequent deactivation of GSK-3. Because muscle contraction can increase RSK activity and decrease GSK-3 activity in skeletal muscle (19, 31, 44), contraction-stimulated JNK activation may stimulate glycogen synthesis via a RSK-3-GSK-3-glycogen synthase signaling cascade.

Studies of signaling protein function in contracting skeletal muscle have been limited by the lack of a satisfactory model that allows the expression of foreign genes. However, direct intramuscular DNA injection in combination with electrical stimulation (in vivo electroporation) has recently received considerable attention as an effective gene delivery method in the field of gene therapy (4, 38). In vivo electroporation has several advantages for studying intracellular signaling in contracting skeletal muscle. With the use of this technique, gene

delivery and subsequent protein expression occur in adult animals without an immune response to the expression vector, making it less likely for an immunological response to dictate the observed physiological outcome (4, 34, 41). Other advantages include restricted localization and expression within a specific muscle, resulting in high reproducibility in a controlled experimental paradigm. Gene delivery by this method does not cause a disruption of genomic function by incorporation of the gene into the chromosome. In addition, time-consuming processes such as virus-based vector construction or transgenic animal generation can be eliminated.

In this study, we applied existing electroporation methodologies to overexpress JNK in mouse skeletal muscles. Moreover, JNK-deficient (JNK$^{-/-}$) mice were used as an ablation model of JNK. Using these approaches, we assessed the role of JNK signaling in the regulation of glycogen synthase activation in contracting skeletal muscle. Our data show that both JNK overexpression and ablation do not alter contraction-induced glycogen synthase activation in skeletal muscle in vivo. Moreover, JNK overexpression results in alterations in the phosphorylation state of several signaling proteins associated with extracellular signal-regulated kinase (ERK) and Akt signaling.

MATERIALS AND METHODS

Materials. Reagents for the protein assay and electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence reagents were obtained from PerkinElmer (Boston, MA), and all other standard chemicals were purchased from Sigma (Indianapolis, IN). Antibodies were obtained from the following sources: anti-JNK1 and anti-phospho-c-Jun, Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-JNK and anti-ERK1/2 antibodies, Promega (Madison, WI); anti-phospho GSK-3α/GSK-3β, anti-phospho-RSK-3, anti-phospho-Akt, anti-phospho-RSK (all isoforms, RSK-1–RSK-3), and anti-phospho-p70 S6 kinase (anti-p70^{S6K}) antibodies, Cell Signaling Technology (Beverly, MA); anti-phospho-ERK1/2, Quality Control Biochemistry (Hopkinton, MA); and anti-Akt and anti-GSK-3 antibodies, Upstate Biotechnology (Lake Placid, NY). Antibodies to insulin receptor substrate (IRS)-1 and Ser^{307} phospho-IRS-1 were generously provided by Dr. M. F. White (Joslin Diabetes Center, Boston, MA). Donkey anti-rabbit IgG horseradish peroxidase secondary antibody was purchased from Amersham Biosciences (Piscataway, NJ). The pCAGGS expression vector and lacZ/pCAGGS plasmid were kindly donated by Dr. J. Miyazaki (Osaka University, Osaka, Japan). The pcDNA3-HAN vector was kindly provided by Dr. K. Nakayama (Kyoto University, Kyoto, Japan). Endotoxin-free plasmid extraction kits were purchased from Qiagen (Valencia, CA). Protein A-agarose was purchased from Pierce (Rockford, IL), and [γ^{32}P]adenosine triphosphate (ATP) was obtained from PerkinElmer. A Grass S88 stimulator (Grass Instrument, Quincy, MA) was used to generate electrical pulses for both DNA delivery into skeletal muscle by in vivo electroporation and in situ muscle contraction via electrical nerve stimulation. Needle electrodes for in vivo electroporation were purchased from Cadwell Laboratories (Kennewick, WA), and subminiature electrodes for the in situ muscle contraction were obtained from Harvard Apparatus (South Natick, MA). Female ICR mice (8–10 wk old) purchased from Taconic (Germantown, NY) were used for JNK overexpression (O/E) by in vivo electroporation throughout this study. JNK1-deficient mice (JNK1$^{-/-}$) and JNK2-deficient mice (JNK2$^{-/-}$) (51) were donated by Dr. R. Davis (Howard Hughes Medical Institute, Chevy Chase, MD, and University of Massachusetts, Worcester, MA). All experimental mice used in this study were allowed free access to food and water until 1 h before the experiments.

Expression vector construction. Human JNK1 cDNA was cloned by performing PCR with a 5′-oligonucleotide (5′-cgggatccagcagaag-

caagcgtgacaacaatttttatagtg-3′) encoding recognition sequences for *Bam*HI and JNK1 5′-end coding sequence and with a 3′-oligonucleotide (5′-gcccaagtagtcatctacagcagcccagag-3′) containing a 3′-untranslated region of JNK1 (this region contains a *Xba*I site). The PCR product was subcloned into the *Bam*HI and *Xba*I sites of the pcDNA3-HAN vector, which has a Kozac sequence and a hemagglutinin (HA) epitope sequence upstream of the *Bam*HI site of the vector (45). HA-tagged JNK1 cDNA was excised with *Hin*dIII and *Xho*I and transferred to the *Xho*I site between the CAG promoter and a 3′-flanking region of a rabbit β-globin gene of pCAGGS expression vector after blunt end treatment (35). Plasmid DNA was prepared according to a standard procedure and dissolved in saline.

DNA injection into skeletal muscle and in vivo electroporation. DNA injection and in vivo electroporation were performed by using a modification of the method of Aihara and Miyazaki (4). Mice were anesthetized with pentobarbital sodium (90 mg/kg body wt ip), and 100 µg of HA-JNK/pCAGGS plasmid in 25 µl of saline were injected into the tibialis anterior muscle of one leg (JNK O/E) with an insulin syringe and a 29-gauge needle. For control, lacZ/pCAGGS was injected into the opposite leg. Square-wave electrical pulses (200 V/cm) were applied eight times with an electrical pulse generator at a rate of one pulse per second, with each pulse being 20 ms in duration. The electrodes were a pair of stainless steel needles inserted into the tibialis anterior muscles and fixed 5 mm apart. Ten days after gene delivery, the muscles were removed and prepared for analysis.

In situ muscle contraction. For the in situ muscle contraction experiments, mice were anesthetized with pentobarbital sodium (90 mg/kg body wt ip), the sciatic nerves of both hindlimbs were exposed, and subminiature electrodes were attached to the nerves (23, 42). Hindlimb muscles were electrically stimulated to contract for 15 min (train rate 1/s, train duration 500 ms, rate 100 pulses/s, duration 0.1 ms, 1–3 V). Immediately after contraction, the tibialis anterior muscle was rapidly dissected and frozen in liquid nitrogen for biochemical analysis or fixed with 4% paraformaldehyde for X-gal staining.

JNK1$^{-/-}$ and JNK2$^{-/-}$ mice. JNK1$^{-/-}$ and JNK2$^{-/-}$ mice were generated as reported by Dong et al. (18) and Yang et al. (51). Homozygous mutant (−/−) and wild-type mice were generated from intercrosses between heterozygous (+/−) mice, and treatment groups were derived from littermates. No experimental mice were backcrossed (i.e., all JNK$^{-/-}$ mice had mixed genetic backgrounds).

Analysis of β-galactosidase activity. Histochemical detection of β-galactosidase activity was performed according to standard procedures (28). Briefly, tibialis anterior muscles were dissected, rinsed with phosphate-buffered saline (PBS), and immediately fixed with 4% paraformaldehyde on a rocker for 2 h at 4°C. The muscles were washed twice with PBS for 30 min each time and incubated at 37°C with a solution of 1 mg/ml X-gal stain containing 5 mM K$_4$Fe(CN)$_6$, 2 mM MgCl$_2$, and 0.04% Igepal CA-630 for 12 h. X-gal-stained muscles were dissected, and portions that appeared blue were separated from those that appeared unstained. The fraction of tissue that expressed β-galactosidase activity was estimated by measuring weight.

Immunohistochemistry. Immunohistochemical analysis was performed according to standard procedures (29). Immediately after dissection, muscle was frozen in isopentane precooled by liquid nitrogen. Frozen sections (8 µm) were obtained, fixed in cold acetone (−20°C) for 10 min, and blocked with PBS (pH 7.4) containing 0.5% bovine serum albumin (BSA) and 0.5% Triton-X for 20 min. The sections were then incubated with FITC-conjugated hemagglutinin antibody (1:100) for 1 h at room temperature and rinsed in PBS. Muscle fiber nuclei also were stained with propidium iodide (10 µg/ml). After a final wash in PBS, and sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized with the use of a confocal microscope (Carl Zeiss, Thornwood, NY) at ×250 magnification.

Muscle processing. Tibialis anterior muscles were homogenized (Polytron; Brinkmann Instruments, Westbury, NY) in ice-cold lysis buffer containing 20 mM Tris·HCl, pH 7.5, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $NaVO_4$, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Homogenates were rotated for 1 h at 4°C and centrifuged at 14,000 *g* for 10 min at 4°C. Samples were quickly frozen and stored in liquid nitrogen. Supernatants were collected, and protein concentrations of the muscle lysates were measured according to the Bradford method (13).

JNK activity assay. JNK activity was assessed with the use of an in vitro immune complex assay as previously described in detail (6). Briefly, muscle lysates (250 μg) were immunoprecipitated with 1 μg of anti-JNK1 and 50 μl of prewashed protein A beads. After immunoprecipitation, the JNK immune complexes were washed and then suspended in 30 μl of kinase assay buffer containing 3 μg of inactive glutathione *S*-transferase (GST)-c-Jun(1–135) as substrate, 3.75 mM $MgCl_2$, 50 μM ATP, and 10 μCi [γ^{32}P]ATP. The kinase reaction was performed at 30°C for 30 min and terminated with Laemmli buffer. The reaction products were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the phosphorylated GST-c-Jun was visualized and quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics).

Immunoblotting. Muscle proteins (40 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked in Tris-buffered saline with either 5% milk or 5% BSA and immunoblotted using antibodies against HA, JNK1, phospho-JNK Thr^{183}/Tyr^{185}, phospho-c-Jun Ser^{63}, ERK1/2, phospho-ERK1/2 Thr^{202}/Tyr^{204}, RSK, phospho-RSK-1–RSK-3 Ser^{380}, RSK-3, phospho-RSK-3 Thr$^{353/356}$, GSK-3, phospho-GSK-3α Ser^{21}/β-Ser^9, Akt, phospho-Akt Ser^{473}, p70^{S6K}, and phospho-p70^{S6K} Thr^{389}. The blots were then incubated with secondary antibody for 1 h at room temperature followed by enhanced chemiluminescence. The intensity of the bands was quantified by densitometry.

Immunoprecipitation. Muscle lysates (500 μg) were incubated with 10 μl of IRS-1 antiserum for 2 h at 4°C. Immune complexes were precipitated with protein A beads during 1 h of incubation at 4°C. The beads were washed three times with washing buffer (50 mM Tris, pH 7.5, 1% NP-40, 150 mM NaCl, and 2 mM EGTA) and boiled for 5 min with Laemmli buffer. The proteins were separated by SDS-PAGE and immunoblotted to detect IRS-1 phosphotyrosine or IRS-1 phospho-Ser^{307}.

Glycogen synthase activity assay. Muscle was homogenized in 19 volumes of ice-cold glycogen synthase buffer (50 mM Tris, pH 7.8, 100 mM NaF, 5 mM EDTA), and glycogen synthase activity was measured as described previously (48). Glycogen synthase activity was expressed as %I form of total activity (i.e., enzyme activity in the absence of glucose-6-phosphate divided by activity in the presence of 6.7 mM glucose 6-phosphate).

Statistics. Data are expressed as means ± SE. Groups in Figs. 3–6 were compared by one-way ANOVA, and statistically significant differences were localized by the Bonferroni *t*-test. Statistical analysis of data in Fig. 7 was performed with a paired Student's *t*-test.

RESULTS

Delivery of foreign genes into skeletal muscle. To determine the distribution and efficiency of in vivo electroporation, we injected 100 μg of lacZ/pCAGGS vectors into tibialis anterior muscles, which were then electrically stimulated to facilitate gene transfer into muscle cells. Ten days later, the muscles were dissected and X-gal staining was performed to detect the activity of β-galactosidase. Figure 1*A* is a representative control muscle that was treated with saline injection followed by electroporation. Figure 1, *B–D*, shows representative muscles overexpressing LacZ. X-gal staining shows that β-galactosi-
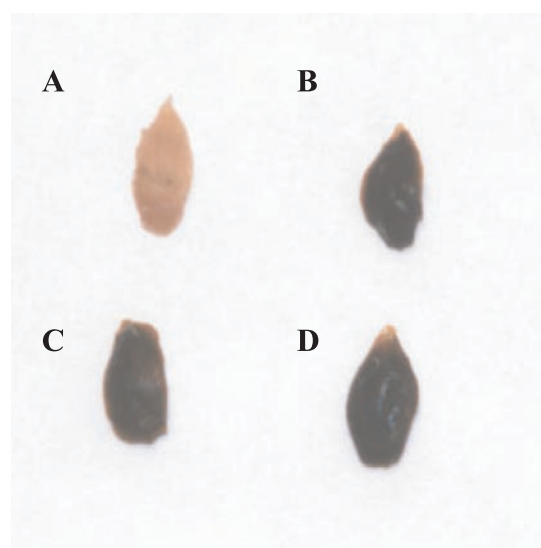

Fig. 1. Histochemical staining for β-galactosidase activity in the muscles after lacZ gene transfer by in vivo electroporation. LacZ/pCAGGS expression vector (100 μg) was injected into the tibialis anterior muscles of mice, followed by electroporation with the mice under anesthesia, as described in MATERIALS AND METHODS. Ten days after gene transfer, muscles were dissected and stained for β-galactosidase activity. Control muscles were electroporated after saline injection. A control (*A*) and three LacZ-expressing whole representative muscles (*B–D*) after staining for β-galactosidase are shown.

dase activity is detected in almost all areas of the tibialis anterior muscle. β-Galactosidase expression was observed not only on the surface but also deep within the tissue (data not shown). To estimate the fraction of tissue that expressed β-galactosidase activity, the X-gal-stained muscles were dissected, the portions that appeared blue were separated from those that appeared unstained, and each piece of the tissue was weighed. The percentage of fibers expressing β-galactosidase was determined to be 85.7 ± 2.3% (*n* = 6). Further increases in β-galactosidase expression were not observed when injections exceeded 100 μg (88.6 ± 1.4% with 200 μg of vector). A double-injection protocol (27) also did not further increase β-galactosidase activity (79.9 ± 12.0%). Plasmid injection without electroporation did not result in significant β-galactosidase expression in our system (data not shown). JNK expression reached near-maximum levels 7 days after transfection and remained elevated for at least 14 days (data not shown).

Overexpression of JNK by in vivo electroporation. Mice were injected with HA-JNK/pCAGGS in one leg and lacZ/pCAGGS in the opposite leg, followed by electroporation. Muscles injected with HA-JNK/pCAGGS expression vectors had a 25-fold increase in JNK protein expression compared with muscles injected with lacZ/pCAGGS, as determined by immunoblotting (Fig. 2, *A* and *B*). HA expression was detected only in JNK O/E muscles (Fig. 2*A*). The expression of HA-tagged JNK was confirmed under confocal microscopy after immunohistochemistry or immunofluorescence microscopy was performed on cross sections of the tibialis anterior muscles with an anti-HA antibody conjugated with fluorescein (Fig. 2*C*). No HA expression was detected by immunoblotting in extensor digitorum longus, a muscle adjacent to the tibialis anterior muscle, demonstrating the specificity of the injection procedure for the tibialis anterior muscle (data not shown).
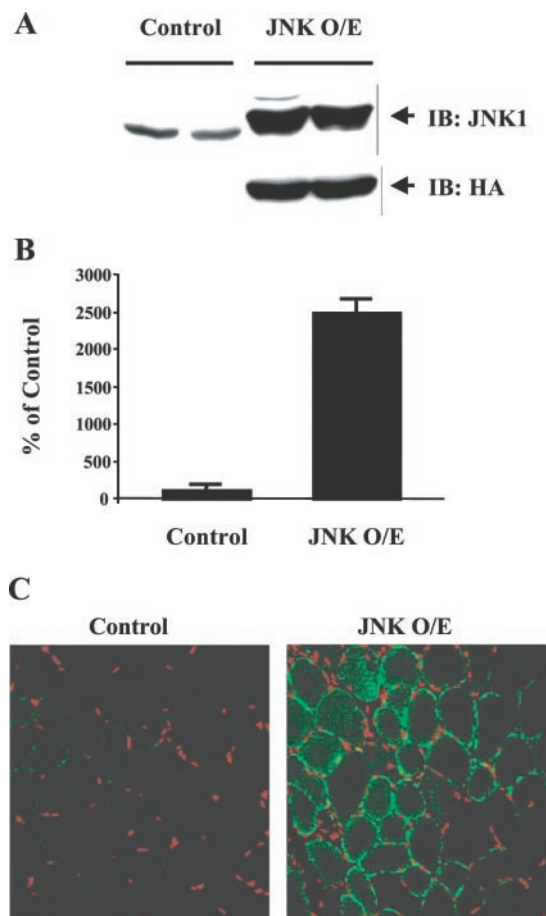
A

B

C

Fig. 2. Expression of c-Jun NH₂-terminal kinase (JNK) in tibialis anterior muscle. Hemagglutinin (HA)-tagged JNK1/pCAGGS expression vector (100 μg) was injected into the tibialis anterior muscle of one leg (JNK O/E) and lacZ/pCAGGS expression vector (100 μg) was injected into the opposite leg (control) of mice. Electroporation was applied as described in MATERIALS AND METHODS to enhance gene transfer into the muscle cells. Ten days after the gene transfer, muscles were dissected and processed for immunoblot (IB) analysis (*A* and *B*) or immunohistochemistry (*C*) to assess the expression level of JNK protein. A representative image (*A*) and average data (*B*) of the immunoblot analysis are shown. The muscle cells expressing HA-tagged JNK protein were detected by immunofluorescence analysis (*C*; original magnification, ×250). Values are means ± SE, *n* = 7–8/group.

Overexpressed JNK is functional. We next determined the effect of JNK overexpression on JNK activity by using an in vitro immune-complex assay developed for skeletal muscle (6, 22). Ten days after gene delivery by electroporation, mice were randomly divided into contraction or control groups and tibialis anterior muscles were contracted in situ for 15 min or served as sham-operated controls. Basal levels of JNK activity were significantly higher in JNK O/E muscles than in control muscles (Fig. 3). This increase in basal JNK activity did not correlate directly with the increase in total JNK protein expression, which we anticipated because we have found that in the rested, basal state, JNK activity is low. However, muscle contraction, a known activator of JNK in skeletal muscle, resulted in a much greater increase in JNK activity in the JNK O/E muscles than in control muscles (Fig. 3). Immunoblot analysis with the use of an anti-phosphospecific JNK antibody that recognizes the dual phosphorylation motif (Thr[183] and Tyr[185]) showed similar results with regard to JNK activity (Fig.

4). Consistent with JNK activity and phosphorylation, muscle contraction-induced phosphorylation of endogenous c-Jun, a downstream substrate of JNK, was greatly increased by JNK overexpression (data not shown). These results show that overexpressed JNK is functional in skeletal muscle in vivo and enhances JNK signaling evoked by muscle contraction.

Contraction-induced glycogen synthase activation in muscle is not coincident with an increase in JNK activity. Basal levels of glycogen synthase activity were not significantly altered in JNK O/E muscles (Fig. 5), despite threefold increases in JNK activity (Fig. 3). In the control muscles injected with lacZ, contraction significantly increased glycogen synthase activity 2.5-fold over basal level (Fig. 5). In the JNK O/E muscles, in which contraction-stimulated JNK activity was fivefold greater than in contraction-stimulated control muscles, contraction also increased glycogen synthase activity to the same level as in the control muscles. Thus the dramatically higher levels of JNK activity did not result in higher levels of glycogen synthase activity. Phosphorylation of c-Jun, a downstream substrate of JNK, corresponded to JNK activity and phosphorylation and was greatly increased by JNK expression (data not shown). The dissociation of JNK and glycogen synthase activities suggests that JNK is not a major regulator of glycogen synthase in skeletal muscle.

Muscle contraction-induced glycogen synthase activation is unchanged in mice lacking either JNK1 or JNK2. To further explore whether JNK participates in the regulation of glycogen synthase activity during muscle contraction, JNK1⁻/⁻ mice and JNK2⁻/⁻ mice underwent in situ muscle contraction as described above. As shown in Fig. 6, JNK1⁻/⁻ and JNK2⁻/⁻ mice had normal muscle contraction-induced glycogen syn-
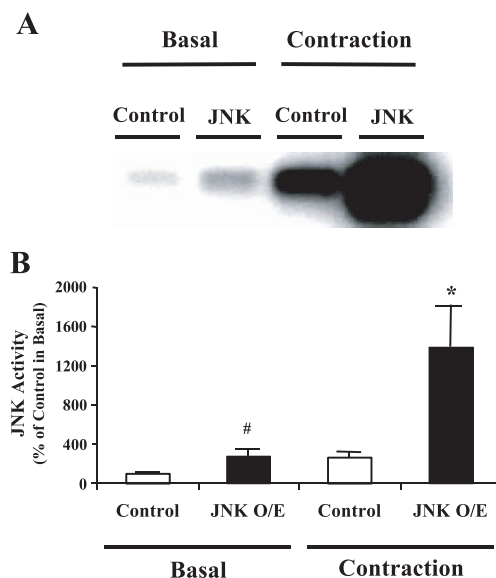
A

B

Fig. 3. JNK overexpression in muscle enhances contraction-induced JNK activation. Ten days after gene transfer, mice were separated into 2 groups. After mice underwent anesthesia, hindlimb muscle contraction was induced in 1 group for 15 min by electrical stimulation via the sciatic nerve (contraction), and the other group underwent sham operations but was kept at rest (basal). Mice were allowed free access to food and water until 1 h before experiments. JNK activity was assessed with the use of an in vitro immune complex assay. Control, LacZ/pCAGGS transfected leg; JNK O/E, HA-JNK/pCAGGS overexpressed leg. Values are means ± SE. #*P* < 0.01 vs. basal control; **P* < 0.01 vs. contraction control.
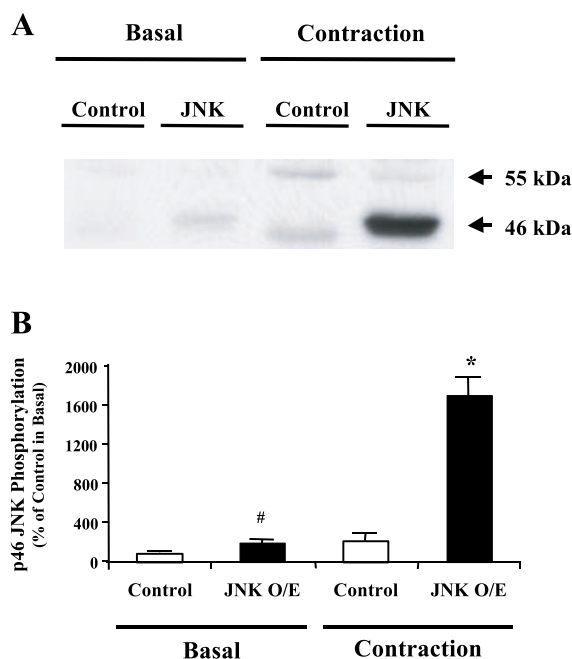
A

B

Fig. 4. Phosphorylation of overexpressed JNK protein. Mice with overexpressed JNK in the tibialis anterior muscle of one leg (JNK O/E) and transfected lacZ in the opposite leg (control) underwent in situ muscle contraction (contraction) or a sham operation (basal). Mice were allowed free access to food and water until 1 h before experiments. *A*: representative immunoblots for dually phosphorylated Thr^{183} and Tyr^{185} of JNK. *B*: quantified results of p46 JNK (major splicing isoform of JNK1) phosphorylation displayed as bar graphs. Values are means $\pm$ SE; $n = 6$–8/group. #$P < 0.05$ vs. basal control; *$P < 0.01$ vs. contraction control.

thase activation. Basal levels of glycogen synthase activity also were not altered in these $JNK^{-/-}$ mice. Immunoblotting of muscle lysates revealed that ablation of one isoform did not result in compensatory overexpression of the other isoform (data not shown). These results support the overexpression data and suggest that JNK is not involved in the signaling pathway leading to contraction-induced glycogen synthase activation in skeletal muscle.
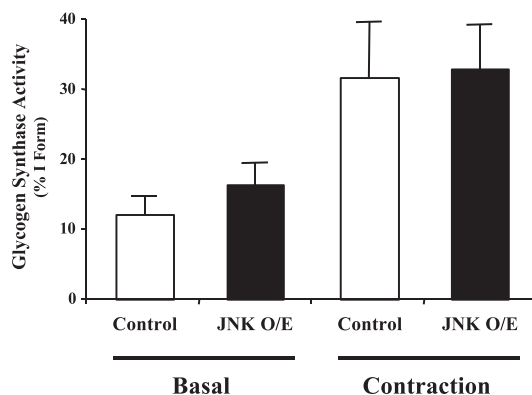

Fig. 5. JNK overexpression on muscle contraction-induced glycogen synthase activation had no effect. Mice overexpressing JNK in the tibialis anterior muscle of one leg (JNK O/E) and transfected lacZ in the opposite leg (control) underwent in situ muscle contraction (contraction) or sham operation (basal). Mice were allowed free access to food and water until 1 h before experiments. Glycogen synthase activity was measured in the absence (I form) or presence (total) of 6.7 mM glucose 6-phosphate, represented as %I form. Values are means $\pm$ SE; $n = 3$–4/group.
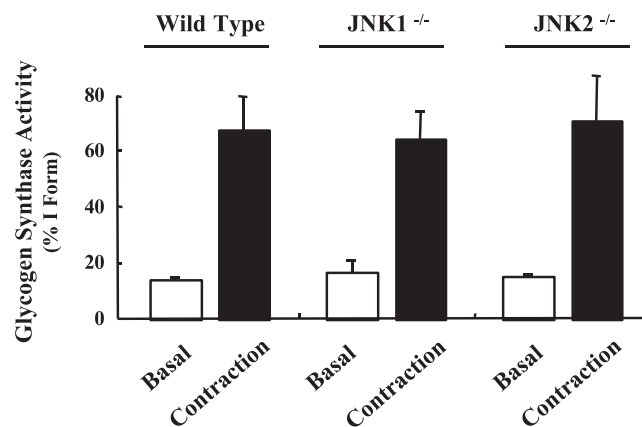

Fig. 6. Muscle contraction-induced glycogen synthase activation is intact in JNK1-deficient ($JNK1^{-/-}$) and JNK-deficient ($JNK2^{-/-}$) mice. Mice lacking either JNK1 or JNK2 were anesthetized, hindlimb muscles were contracted in situ by electrical stimulation via the sciatic nerve (contraction), and the opposite leg was sham operated and kept at rest (basal). Mice were allowed free access to food and water until 1 h before experiments. Glycogen synthase activity was measured in the absence (I form) or presence (total) of 6.7 mM glucose 6-phosphate and is represented as %I form. Values are means $\pm$ SE; $n = 4$/group.

Downregulation of protein phosphorylation with JNK overexpression. We next determined the effects of JNK overexpression on the phosphorylation state of several muscle signaling proteins on sites critical for the kinase activity of each protein. Interestingly, the threefold increase in JNK activity in the basal state was associated with significant decreases in the basal phosphorylation state of several proteins, including ERK1 (56% decrease from lacZ injected basal), ERK2 (58%), RSK-3 (51%), RSK-1–RSK-3 (50%), GSK-3α (34%), Akt (43%), and $p70^{S6K}$ (76%) (Fig. 7). The decreases in phosphorylation were not due to decreases in protein expression levels, because immunoblotting experiments revealed no differences in the detection of all proteins (data not shown). The effect of JNK overexpression in altering the basal phosphorylation state of cellular proteins was not indiscriminate, because increased basal JNK activity in JNK O/E muscles had no effect on the phosphorylation state of several other skeletal muscle proteins (e.g., GSK-3β, p38, AMP kinase; see Fig. 7). The lower levels of the RSKs and GSK-3α phosphorylation were unexpected because JNK has been proposed to activate RSK-3 and GSK-3 signaling in mouse skeletal muscle (36). Contractile activity, a potent activator of ERK and RSK signaling (24, 25, 50), resulted in normal activation of these proteins in JNK O/E muscles (data not shown). Thus muscle contractile activity can overcome the downregulation of the ERK signaling cascade induced by JNK overexpression.

IRS-1 Ser^{307} phosphorylation and tyrosine phosphorylation are not changed by JNK overexpression. Both the ERK and Akt signaling pathways can be regulated by IRS-1, via the Grb2-Sos-Ras complex and phosphatidylinositol 3-kinase (PI3-K), respectively. Ser^{307} is a major site of JNK phosphorylation on IRS-1, and phosphorylation of this site has been proposed to mediate the inhibitory effect of proinflammatory cytokines such as TNF-α on IRS-1 function in Chinese hamster ovary cells (2) and 3T3-L1 preadipocytes and adipocytes (43), as well as in rat, mouse, and human skeletal muscles (43). This raises the possibility that IRS-1 could be an upstream molecule
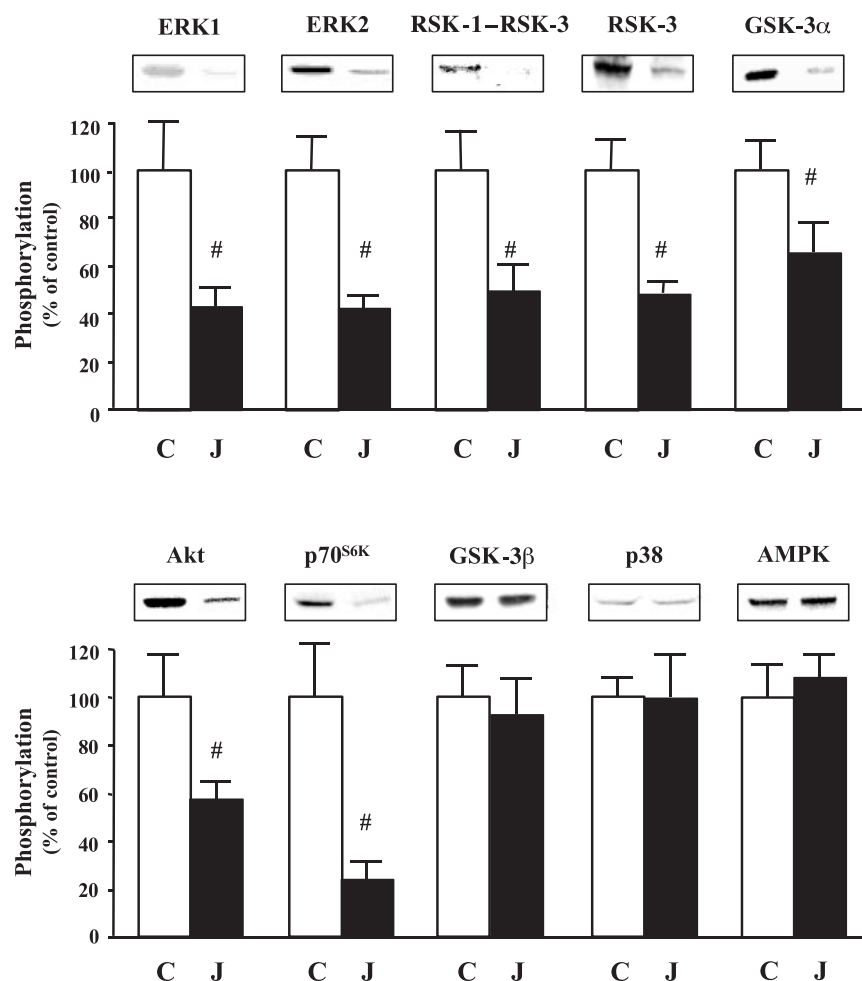
Fig. 7. JNK overexpression suppresses the phosphorylation of extracellular signal-regulated kinase (ERK), ribosomal S6 kinase (RSK), glycogen synthase kinase 3α (GSK-3α), protein kinase B/Akt (Akt), and p70 S6 kinase (p70S6K) but not GSK-3β, p38, and adenosine 5′-monophosphate-activated protein kinase (AMPK). Western blotting of muscle lysates from mice with JNK overexpression in the tibialis anterior muscle of one leg (J) and transfected with lacZ in the opposite control leg (C) was performed. Quantification of ERK1, ERK2, RSK-1–RSK-3, RSK-3, GSK-3α, Akt, p70S6K, GSK-3β, p38, and AMPK phosphorylation is displayed. Representative immunoblots of these proteins are shown above the bar graphs. Values are means ± SE. #$P < 0.05$, JNK O/E vs. respective control.

responsible for the suppression of basal phosphorylation levels of the ERK and Akt pathways by JNK overexpression. However, the levels of Ser[307] phosphorylation of IRS-1 and tyrosine phosphorylation were not changed by JNK overexpression (Fig. 8). Therefore, IRS-1 can be excluded from the list of candidates that may mediate the suppressed phosphorylation levels of molecules in the ERK and Akt pathways.

DISCUSSION

Studies of intracellular signal transduction in adult skeletal muscle have been relatively limited during the past decade because of the inability to adequately transfect a high percentage of muscle fibers with foreign genes. Cultured muscle cells such as the L6 and C_2C_{12} cell lines, which can be transfected readily with foreign genes, have been used widely in the analysis of signal transduction evoked by various stimuli. However, there are significant disadvantages to using these cells because they display a fetal phenotype and, importantly, cannot be used to study skeletal muscle contraction, which is the major function of this tissue. Another approach to gene delivery has been the use of adenovirus and adeno-associated virus vectors. Unfortunately, terminally differentiated muscle cells are resistant to adenovirus infection (1), and rejection of the virus-infected cells or the virus itself by multiple immunological responses makes this method difficult to use when efficient gene expression in skeletal muscle is attempted in

vivo (52). Our preliminary studies with recombinant adenovirus containing the cDNA encoding β-galactosidase delivered to newborn rats resulted in considerably lower (0–49%) and more diffuse (tibialis anterior, soleus, gastrocnemius, biceps femoris, and gracilis muscles) expression than did plasmid DNA injection followed by electroporation as described in the current study. Thus, although adenovirus-mediated gene transfer has great potential as a therapeutic strategy for the treatment of various diseases (28), this approach is not optimal for studying intracellular signaling in contracting muscle cells. Another approach would be to use adeno-associated viruses, because the lack of viral genes may make these vectors less immunogenic (32) and may increase the probability of infection of nondividing cells such as those in skeletal muscles (39). This approach also has limitations, however, including the DNA insertion capacity (transgene cassettes cannot be >4.6 kb), the lack of an efficient packaging cell line, and the random integration of the vectors into the host chromosomes (32). In our preliminary studies comparing lacZ-transfected muscles by electroporation with nontransfected muscles, we observed normal activation of JNK after in situ muscle contraction, which is activated in a tension-dependent manner (33), suggesting that in vivo electroporation does not impair muscle contraction. One limitation of our in vivo gene expression method is that some muscles that have relatively pure muscle fiber types, such as the soleus and red gastrocnemius muscles (oxidative fiber)
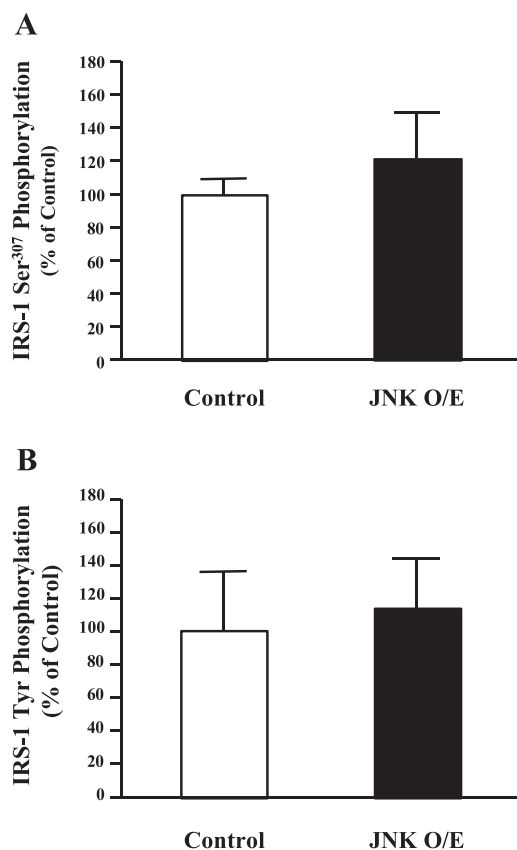
A

B

Fig. 8. Phosphorylation of insulin receptor substrate (IRS)-1 Ser[307] and tyrosine is not changed by JNK overexpression in skeletal muscle. Mice had JNK overexpression in the tibialis anterior muscle of one leg (JNK O/E) and lacZ transfected in the opposite leg (control). IRS-1 was immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-Ser[307] phospho-IRS-1 antibody or anti-phospho-tyrosine antibody. Quantification of IRS-1 Ser[307] phosphorylation (*A*) and IRS-1 tyrosine phosphorylation (*B*) is displayed. Values are means ± SE; *n* = 6/group.

and the white gastrocnemius muscle (glycolytic fiber), are not easily accessible for electroporation and therefore cannot be used. The tibialis anterior muscle, which was chosen for this study, contains both fiber types equally, and our observations in that muscle may not necessarily pertain to all muscles. However, our description of an in vivo electroporation technique that results in high levels of functional proteins derived from foreign genes is likely to be an important new strategy for defining the function of signaling molecules in skeletal muscle.

We used this gene transfer system to overexpress JNK1 in skeletal muscle, with the initial goal of determining whether JNK can regulate glycogen synthase in contracting skeletal muscle. During muscle contraction, glycogen breakdown is a major source of ATP regeneration for the working muscle. Simultaneously, glycogen resynthesis is activated by contractile stimuli (15, 40), providing a mechanism that protects the myofibers from declining energy stores under conditions of cellular stress. The finding that anisomycin treatment increases both JNK and glycogen synthase activities in skeletal muscle (36) raises the possibility that JNK is the stress signal that mediates glycogen synthase activation. Additional studies of L6 myotubes and rat adipocytes have suggested that the protein kinase C inhibitor Ro-31-8220 increases glycogen synthase activity and that this increase may be mediated by JNK

activation (46). With the use of several cell lines, JNK1 has also been shown to be activated by an increase in oxidant release from mitochondria caused by stimulated pyruvate metabolism, and redox-dependent JNK1 activation has been demonstrated to increase glycogen synthase activity through RSK-3 activation and the subsequent inactivation of GSK-3 (37). On the basis of these observations, we sought to determine whether JNK mediates glycogen synthase activity in both resting and contracting skeletal muscle in vivo. In contrast to the studies cited above, our data provide no support for the hypothesis that JNK activation is sufficient to activate glycogen synthase activity, because increased basal levels of JNK activity did not increase glycogen synthase activity. Furthermore, despite a large (15-fold over basal) increase in JNK activity associated with contraction in the JNK O/E mice, the increase in contraction-induced glycogen synthase activation was similar to that seen in control mice. In addition, muscle contraction-induced glycogen synthase activation was completely intact in both JNK1$^{-/-}$ and JNK2$^{-/-}$ mice. It may be possible that the disruption of one isoform could functionally compensate for the other isoform, although we have also demonstrated that the ablation of one isoform did not change the expression level of the other isoform. These findings are consistent with the JNK overexpression data and suggest that JNK signaling is unlikely to be involved in glycogen synthase regulation in contracting muscle.

RSK-3 has been proposed to be an intermediate in JNK-regulated glycogen synthase activity in mouse skeletal muscle in vivo (36) and in HeLa cells (37), and the mechanism by which RSK-3 regulates glycogen synthase may involve GSK-3 (36, 37). To determine whether JNK activation is sufficient to increase RSK-3 and GSK-3 phosphorylation in skeletal muscle, we examined the phosphorylation state of RSK-3 and GSK-3 in the JNK O/E muscles. Our results show that in resting muscle, JNK overexpression and the associated three-fold increase in JNK activity are associated with decreases in RSK-3 and GSK-3α phosphorylation. Muscle contraction, which increased JNK activity in both the control (3-fold) and JNK O/E muscles (15-fold), did not increase RSK-3 phosphorylation (data not shown). These results demonstrate that JNK activation is not sufficient for RSK-3 and GSK-3 phosphorylation in adult skeletal muscle tissue and suggest that subsequent phosphorylation and activation (or deactivation) of JNK, RSK-3, and GSK-3 are not mechanisms leading to increased glycogen synthase activity in contracting skeletal muscle. Instead, we think that other signaling molecules are involved, because we have shown that the regulatory subunit (R_{GL} or G_M) of the protein phosphatase 1G is necessary for muscle contraction- and exercise-stimulated glycogen synthase activity (7).

Our finding that JNK overexpression resulted in decreased RSK-3 and GSK-3 phosphorylation led us to examine whether JNK overexpression suppressed the phosphorylation state of other cellular protein kinases. Similarly to RSK-3 and GSK-3, JNK overexpression decreased ERK, RSK, Akt, and p70^{S6K} phosphorylation at sites that are important for the regulation of kinase activities. For the downregulation of the ERK signaling cascade, it is possible that JNK did not directly affect RSK-3 and GSK-3 phosphorylation but instead was a consequence of attenuated ERK phosphorylation. ERK1/2 can directly phosphorylate RSK isoforms (21), and, at least in vitro, RSK can

phosphorylate and inactivate GSK-3α and GSK-3β (47). In addition, GSK-3 phosphorylation and inactivation are mediated by the ERK/RSK signaling pathway in NIH/3T3 cells in response to EGF (20). Therefore, it is possible that decreased basal phosphorylation levels of ERK led to sequential decreases in basal phosphorylation levels of RSK and GSK-3α. Likewise, downregulation of p70[S6K] may be a consequence of decreased Akt phosphorylation.

One possible mechanism that could explain the suppressed phosphorylation of molecules in both the ERK and Akt pathways is the inhibition of IRS function by JNK overexpression. Phosphorylation of Ser[307] in IRS-1 is critical for the inhibitory effect of anisomycin and TNF-α on IRS-1-dependent signaling in Chinese hamster ovary cells, which is mediated by the association of activated JNK and IRS-1 (2, 43). Ser[307] phosphorylation triggers a decrease in the phosphorylation level of tyrosine residues on IRS-1 itself and promotes general inhibition of IRS-1 signaling, as revealed by reduced activation of both the PI3-K and ERK cascades (3). We thus hypothesized that IRS-1 might be an upstream regulator that inhibits the ERK and Akt cascades by JNK overexpression. As shown in Fig. 7, however, we saw no effect of JNK overexpression on IRS-1 Ser[307] phosphorylation or tyrosine phosphorylation in the muscles we studied. Therefore, IRS-1 Ser[307] phosphorylation and its subsequent decrease in IRS-1 signaling do not contribute to the inhibition of ERK and Akt signaling associated with JNK overexpression. This does not rule out the possibility that IRS-2 could initiate this downregulation, because IRS-2 is also phosphorylated by serine during anisomycin or TNF-α stimulation, which inhibits insulin-stimulated tyrosine phosphorylation. A residue analogous to Ser[307] of IRS-1 does not exist in IRS-2 (3), nor do phosphospecific antibodies to serine sites on IRS-2. Therefore, the possible role of IRS-2 in the inhibition of ERK and Akt signaling remains to be examined.

In NIH/3T3 fibroblast, JNK activation increases the expression of MAP kinase phosphatase-1 (MKP-1), a phosphatase specific for ERK. This results in decreased phosphorylation of ERK without a change in ERK protein expression in these cells (9). On the basis of these data, we recently measured MKP-1 protein expression by immunoblotting but saw no effect of JNK overexpression in the hindlimb muscles (Fujii N, Sakamoto K, and Goodyear LG, unpublished data). At this time, the mechanism for the downregulation of these multiple signaling molecules is unknown and is an important area of future investigation.

In summary, to explore the role of JNK signaling in skeletal muscle, we overexpressed wild-type JNK in mouse skeletal muscle with the use of an in vivo electroporation technique. JNK overexpression did not affect contraction-induced glycogen synthase activation in muscle, even though JNK activity and phosphorylation of c-Jun were dramatically increased. Moreover, contraction-induced glycogen synthase activation was normal in the muscles of JNK1$^{-/-}$ and JNK2$^{-/-}$ mice. These results suggest that JNK does not play a major role in the pathway leading to contraction-induced glycogen synthase activation in skeletal muscle. Overexpression-induced increases in basal JNK activity were associated with inhibition of the ERK and Akt signaling pathways. Elucidation of the mechanism whereby JNK downregulates ERK and Akt signaling in skeletal muscle is of great interest. Because JNK regu-

lates apoptotic events (17, 30) and ERK and Akt regulate cell growth events (8, 14) in various cell types, future studies should investigate the possibility that sustained JNK activation regulates apoptosis and/or atrophy cooperatively with suppression of the ERK and Akt signaling pathways in mature skeletal muscle.

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