Differential activation of mTOR signaling by contractile activity in skeletal muscle

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Differential activation of mTOR signaling by contractile activity in skeletal muscle. Am J Physiol Regul Integr Comp Physiol 285: R1086–R1090, 2003. First published July 24, 2003; 10.1152/ajpregu.00324.2003.—The cellular mechanisms by which contractile activity stimulates skeletal muscle hypertrophy are beginning to be elucidated and appear to include activation of the phosphatidylinositol 3-kinase (PI3-kinase) signaling substrate mammalian target of rapamycin (mTOR). We examined the time course and location of mTOR phosphorylation in response to an acute bout of contractile activity. Rat hindlimb muscle contractile activity was elicited by high-frequency electrical stimulation (HFES) of the sciatic nerve. Plantaris (Pla), tibialis anterior (TA), and soleus (Sol) muscles from stimulated and control limbs were collected immediately or 6 h after stimulation. HFES resulted in mTOR phosphorylation immediately after (3.4 ± 0.9-fold, P < 0.01) contractile activity in Pla, whereas TA was unchanged compared with controls. mTOR phosphorylation remained elevated in Pla (3.6 ± 0.6-fold) and increased in TA (4.6 ± 0.9-fold, P < 0.05) 6 h after HFES. Interestingly, mTOR activation occurred predominantly in fibers expressing type Ila but not type I myosin heavy chain isoformal. Furthermore, HFES induced modest ribosomal protein S6 kinase phosphorylation immediately after exercise in Pla (0.4 ± 0.1-fold, P < 0.05) but not TA and more markedly 6 h after both Pla and TA (1.4 ± 0.4-fold vs. 2.4 ± 0.3-fold, respectively, P < 0.01). Akt/PKB phosphorylation was similar to controls at both time points. These results suggest that mTOR signaling is increased after a single bout of muscle contractile activity. Despite reports that mTOR is activated downstream of Akt/PKB, in this study, HFES induced mTOR signaling independent of Akt/PKB phosphorylation. Type fiber-dependent mTOR phosphorylation may be a molecular basis by which some fiber types are more susceptible to contraction-induced hypertrophy.

The maintenance of skeletal muscle mass is regulated by a balance between protein synthesis and protein degradation. Contractile activity and mechanical overload in skeletal muscle stimulate protein synthesis, leading to enhanced fiber size and strength, and alterations in metabolic properties (4, 21). Although the biochemical mechanisms responsible for these adaptations are not fully understood, recent evidence suggests that the phosphatidylinositol 3-kinase (PI3-kinase) signaling cascade plays an essential role.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates muscle cell growth and myogenesis (3, 8). Control of cell growth by mTOR appears to be dependent on its ability to enhance translation initiation, whereas the mechanism by which it regulates myogenesis is unknown. mTOR is activated by a variety of stimuli, including insulin, growth factors, and amino acids. In response to insulin and growth factors, mTOR activation is believed to occur through sequential activation of PI3-kinase, phosphoinositide-dependent kinase (PDK)-1, and Akt/PKB, the latter of which phosphorylates Ser2448 of mTOR in vitro (10, 16). In contrast, there is evidence that activation of mTOR by amino acids does not require Akt/PKB, suggesting that mTOR is a point of convergence for nutrient and growth factor signaling pathways (2, 14, 16).

When activated, mTOR influences translation initiation by at least two distinct mechanisms. The first involves 5′ cap binding via phosphorylation of the eukaryotic initiation factor (eIF) binding protein (eIF4E BP1). On hyperphosphorylation, this protein releases from a complex with eIF4E and allows eIF4G to bind eIF4E, thereby increasing the affinity of eIF4E for the 5′ cap (11). In addition, mTOR phosphorylates the ribosomal protein S6 kinase (p70 S6K) which, in turn, phosphorylates the S6 ribosomal protein and allows the upregulation of a subclass of mRNAs encoding the translational apparatus (11).

p70 S6K appears to play an important role in regulating contraction-induced muscle hypertrophy. A single bout of high-frequency electrical stimulation (HFES) results in prolonged phosphorylation of p70 S6K for up to 36 h (1, 9). The degree of p70 S6K phosphorylation after a single bout of HFES is closely associated with the increase in muscle weight after 6 wk of chronic exercise; hypertrophy; ribosomal protein S6 kinase; Akt

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stabilization (1). Therefore, the acute pattern of \( p70^{S6K} \) activation is likely preserved during training and is essential for muscle growth. This is supported by the finding that when mTOR is pharmacologically blocked loading fails to activate \( p70^{S6K} \) and hypertrophy is prevented (3).

The role of Akt/PKB in regulating contraction-induced muscle hypertrophy is less clear. Although Akt/PKB is an important regulator of muscle size and can prevent muscle atrophy in vivo (3, 17), the downstream targets of Akt/PKB after muscle contractile activity are unknown. Akt/PKB phosphorylation does not follow a time course similar to that of \( p70^{S6K} \) after a single bout of muscle contractions (15). Rather, Akt/PKB activation appears after 1 min and returns to baseline within 15–30 min after the onset of contraction (19). Although the time course of mTOR phosphorylation is not known, this information may prove useful in identifying the putative signaling pathways involved in stimulating protein synthesis after muscle contractile activity.

The purpose of this study was to determine whether mTOR is phosphorylated in response to a single bout of muscle contractile activity and to compare the pattern of activation to both upstream (Akt/PKB) and downstream (\( p70^{S6K} \)) kinases. In light of evidence that \( p70^{S6K} \) is not activated in contracting soleus muscle (1, 15), we were further interested in determining whether contraction-induced mTOR phosphorylation is fiber type dependent. We hypothesized that mTOR phosphorylation would be increased primarily 6 h after rather than immediately after muscle contractile activity and would be associated with \( p70^{S6K} \) phosphorylation in both the plantaris (Pla) and tibialis anterior (TA) muscles. We further hypothesized that an early induction of Akt/PKB phosphorylation could be dissociated from that of mTOR.

**MATERIALS AND METHODS**

**Materials.** Primary antibodies (anti-phospho Akt (Ser308), anti-phospho \( p70^{S6K} \) (Thr389), anti-phospho mTOR (Ser2448), anti-Akt, anti-mTOR) were from Cell Signaling Technologies (Beverly, MA), and anti-\( p70^{S6K} \) was from Santa Cruz Biotechnology (Santa Cruz, CA). Type I and IIa myosin heavy chain (MHC) antibodies were grown from hybridoma cell lines A4.951 (American Type Culture Collection, Manassas, VA) and SC-71 (DSMZ, Braunschweig, Germany). Anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham Biosciences (Piscataway, NJ). Biotinylated secondary antibodies for immunofluorescence experiments were from Vector Laboratories (Burlingame, CA). All other chemicals were from Sigma (St. Louis, MO).

**Animals.** Protocols for animal use were approved by the Institutional Animal Care and Use Committee of Boston University. Six-month-old male Fischer 344 × Brown Norway rats (383 ± 5 g) were purchased from the National Institute on Aging. On arrival, animals were aclimatized for 3 days before experimentation and were given normal laboratory chow and water ad libitum. Animals were fasted overnight before the experimental protocol.

**Electrical stimulation.** The HFES model was chosen on the basis of its efficacy in stimulating protein translation and muscle hypertrophy in vivo (1). This protocol results in concentric (shortening) contraction of the Pla and soleus (Sol) and eccentric (lengthening) contraction of the TA. In rats, the fiber type composition of these muscles is as follows: Pla 7% type I, 52% type IIa, 41% type IIb; TA 3% type I, 61% type IIa, 36% type IIb; Sol 83% type I, 17% type IIa, 0% type IIb (12). Animals (n = 10) were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine plus 5 mg/kg xylazine, and the sciatic nerves of both legs were exposed. Platinum wire electrodes were attached to the sciatic nerve of the experimental hindlimb immediately superior to the point of trifurcation. Animals were secured in a fashion to allow the experimental limb to freely move. Each contraction was elicited by stimulating the experimental nerve with a 3-s train of pulses (frequency 100 Hz, duration 1 ms at 10–12 V) by a Grass S48 stimulator (Grass Telefactor, Quincy, MA). Animals performed a total of 100 contractions grouped into 10 sets of 10 contractions. Each contraction was followed by a 10-s rest, and a 60-s rest interval separated each set, resulting in a protocol time of 30 min. Animals were killed by a lethal dose of pentobarbital sodium either immediately after or 6 h after HFES.

**Preparation of skeletal muscle tissue lysates.** TA, Pla, and Sol muscles were rapidly dissected, trimmed of connective tissue, weighed, frozen in liquid nitrogen, and stored at −80°C. Muscle pieces for Western blotting analyses were homogenized in 10 vols of buffer containing (in mM) 50 Tris-HCl, 100 NaF, 10 EDTA, 50 β-glycerophosphate, 1 Na3VO4, 3 benzamidine, and 1 PMSF, with 10 µg/ml each of aprotinin, leupeptin, and pepstatin. Homogenates were centrifuged at 10,000 g for 10 min at 4°C, and aliquots were stored at −80°C. The protein concentration of the supernatant was determined by the Bradford assay with BSA as standard (Bio-Rad, Hercules, CA). Muscle pieces for immunohistochemical analyses were embedded in Tissue-Tek mounting medium and frozen in isopentane cooled to the temperature of liquid nitrogen.

**Western blotting.** Equal amounts of protein (20 or 40 µg) were resolved by SDS-PAGE with either 10% (\( p70^{S6K} \)/Akt) or 7.5% (mTOR) gels. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and equal protein loading was verified by Ponceau S staining. Membranes were blocked for 1 h in Tween-20 Tris-buffered saline (TTBS) containing 5% milk followed by incubation with the appropriate primary antibody (diluted 1:1,000 in 2% BSA in TTBS) overnight at 4°C. After several washes in TTBS, membranes were incubated with either anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (1:10,000 in blocking buffer) for 1 h at room temperature. Protein signals were detected with ECL Plus reagents (Amersham Biosciences). Images were scanned, and band intensities were quantified by densitometry (Bioquant Image Analysis, Nashville, TN).

**Double immunofluorescence.** Ten-micrometer cross sections of TA muscles were cut at −20°C, placed on slides, and dried for 30 min at room temperature. Sections were fixed in acetone for 10 min at −20°C and blocked in PBS containing 5% normal goat serum (Jackson Immunoresearch, West Grove, PA) for 30 min. Endogenous biotin binding sites were blocked with an avidin/biotin blocking kit (Vector Laboratories) according to the manufacturer’s instructions. Slides were then incubated with anti-phospho mTOR antibody (1:100) overnight at 4°C. Slides were rinsed and then incubated for 30 min with biotinylated anti-rabbit IgG secondary antibody diluted 1:400 in Tris-buffered saline containing 2% normal goat serum. Slides were then incubated with fluorescein avidin DCS (Vector Laboratories) diluted 1:200 in 10 mM HEPES pH 7.4–150 mM NaCl for 20 min. After the
blocking steps were repeated, sections were incubated with either anti-MHC I or anti-MHC IIa antibodies for 1 h at room temperature. The MHC signal was visualized as described for phospho-mTOR with a biotinylated anti-mouse IgG secondary antibody and Texas red avidin DCS (Vector Laboratories). Control slides were performed with either the second or both primary antibodies omitted. Slides were coverslipped with Vectashield mounting medium (Vector Laboratories) and examined with a Nikon Eclipse E400 microscope (Melville, NY). Images were captured at ×10 magnification with the Bioquant Nova Image analysis system (Bioquant Image Analysis). Overlay images were created in Adobe Photoshop.

Statistical analyses. The fold change in kinase phosphorylation was calculated between experimental muscles and corresponding control muscles [fold change = (experimental – control)/control]. Data are presented as means ± SE of five observations per group. Differences between the experimental and control muscles were determined with a paired Student’s t-test. Differences were considered significant at \( P < 0.05 \).

RESULTS

Contractile activity did not alter Akt/PKB phosphorylation. Immediately after and 6 h after an acute bout of HFES, we failed to detect any change in Akt/PKB phosphorylation by the Western blotting strategy in either TA or Pla muscle (Fig. 1). Total Akt/PKB protein in response to HFES did not differ between experimental and control muscles at either time point.

Contractile activity increases mTOR phosphorylation in type IIa muscle fibers. Compared with controls, mTOR phosphorylation was increased 3.4 ± 0.9-fold immediately after HFES in Pla (\( P < 0.01 \)) whereas there was no change in TA muscle (Fig. 2). By 6 h of recovery, mTOR phosphorylation remained elevated in Pla (3.6 ± 0.6-fold, \( P < 0.05 \)) and increased in TA (4.6 ± 0.9-fold, \( P < 0.05 \)). mTOR phosphorylation was not altered in Sol at either time point. Furthermore, immunofluorescence experiments in TA showed that mTOR phosphorylation after HFES was localized to both the membrane and intracellular region of the muscle fiber (Fig. 3). Strikingly, 6 h after stimulation, an increase in mTOR phosphorylation was observed in a subset of fiber types. With the use of antibodies for the MHC isoforms, mTOR phosphorylation was determined to be specific to fibers predominantly expressing the MHC IIa isoform, whereas the increase in phosphorylation in type I fibers was negligible.

\( p70^{S6K} \) activation with contractile activity. The increase in mTOR phosphorylation was associated with an increase in the phosphorylation state of \( p70^{S6K} \) (Fig. 4). Immediately after contractile activity, \( p70^{S6K} \) phosphorylation was modestly increased \((0.4 ± 0.1\text{-fold, } P < 0.05)\) in Pla but was not significantly different in TA. After 6 h of recovery, \( p70^{S6K} \) phosphorylation increased \(1.4 ± 0.4\text{-fold and } 2.4 ± 0.3\text{-fold in Pla and TA, respectively (} P < 0.01\)) \( p70^{S6K} \) phosphorylation was not altered in Sol at either time point. Total \( p70^{S6K} \) and mTOR proteins did not change in response to HFES or differ between muscles examined.

DISCUSSION

The present study is the first to describe mTOR phosphorylation after a single bout of contractile activity in adult skeletal muscle. HFES resulted in mTOR phosphorylation that was fiber type dependent, and this response was delayed in the TA. \( p70^{S6K} \) followed a pattern of activation similar to that of mTOR, whereas Akt/PKB phosphorylation remained unchanged. The finding that contractile activity regulates mTOR signaling is in agreement with Reynolds et al. (18), who reported that 2 wk of synergist ablation increases mTOR phosphorylation approximately twofold in rat Pla. Furthermore, treatment with a specific inhibitor of mTOR during synergist ablation abolishes 95% of muscle fiber growth, suggesting that mTOR signaling is required for loading-induced hypertrophy (3). The specific mechanism by which mTOR regulates loading-induced muscle growth is unclear but likely involves stimulation of translation initiation through its ability to phosphorylate \( p70^{S6K} \) and eIF4E BP1.

A further novel finding of this study is that contraction-mediated mTOR phosphorylation is localized primarily to type IIa muscle fibers. Because the HFES protocol recruits all motor units of the contracting

Fig. 1. Akt/PKB phosphorylation after in situ muscle contractile activity. Rat tibialis anterior (TA; A and C) and plantaris (Pla; B and D) muscles were contracted via sciatic nerve stimulation and collected either immediately after (0 h) or 6 h after stimulation. Akt/PKB phosphorylation (Thr308) in stimulated muscle (Stim) and sham-operated controls (Con) was determined by Western blotting with an anti-phospho Akt/PKB antibody. There were no differences in Akt/PKB phosphorylation or total protein at either time point.

Fig. 2. Increased mammalian target of rapamycin (mTOR) phosphorylation after in situ muscle contractile activity. Rat TA (A and D), Pla (B and E), and soleus (Sol; C and F) muscles were assayed for mTOR phosphorylation with an anti-phospho mTOR (Ser2448) antibody. Data are means ± SE for \( n = 5\)/group. *Significantly different from control (\( P < 0.05 \)).
muscle, this finding appears not to be the result of differential recruitment of specific motor units during HFES. Another possibility is that muscle damage induced by the eccentric contractions performed by the TA activates mTOR in "injury-sensitive" type II fibers. However, this is unlikely because we observed a degree of total mTOR phosphorylation in the concentrically contracting Pla similar to that we observed in the eccentrically lengthening TA. Furthermore, the injury hypothesis fails to explain the lack of mTOR phosphorylation in type IIb fibers (by process of elimination), which also incur damage during eccentric contractions (13). It is important to note that fiber type-dependent activation of mTOR may be unique to the acute nature of the stimulus or the stimulation patterns used in the present study. For example, hypertrophy of both type I and type II fibers during chronic loading is blocked by treatment with the mTOR inhibitor rapamycin (3). However, fiber type-dependent activation of mTOR signaling in the present study is supported by the observation that mTOR and p70s6k phosphorylation is unchanged in Sol after contractile activity.

Both mTOR and p70s6k followed similar time courses in TA and Pla after contractile activity, suggesting that mTOR may activate p70s6k after muscle contractile activity in vivo. The finding that p70s6k phosphorylation is increased after muscle contractile activity is in agreement with the findings of others (1, 15). The degree of p70s6k phosphorylation is strongly correlated with the increase in wet muscle mass after chronic HFES, whereas rapamycin treatment inhibits p70s6k and muscle fiber hypertrophy (1, 3). Together, these data suggest that mTOR-dependent activation of p70s6k is required for contraction-induced muscle hypertrophy.

Contrary to our hypothesis, we failed to detect a change in Akt/PKB phosphorylation in response to contractile activity at either time point. Sherwood et al. (20) did not show any change in Akt/PKB activity with 15 s to 60 min of HFES. However, previous studies reported that Akt/PKB phosphorylation is induced briefly (1–30 min) by acute muscle contractile activity in vivo (15, 19) and during compensatory hypertrophy (3). The discrepancy in the literature may reflect the influence of different modes of contractile activity (acute vs. chronic), stimulation patterns, duration/number of contractions, and/or length of stimulation protocols on Akt/PKB activity. However, the observa-
tion that Akt/PKB phosphorylation is unchanged after 6 h of recovery from a single bout of HFES is consistent with previous reports (15).

Although Akt/PKB phosphorylates mTOR in vitro (10, 16), its role in regulating mTOR activity after contractile activity in vivo remains unclear. Multiple Akt isoforms (Akt1, Akt2, Akt3) are present in skeletal muscle and appear to have distinct physiological roles (5–7). Although Akt1 knockout mice display growth retardation and normal glucose homeostasis (5, 7), Akt2-deficient mice have impaired insulin-stimulated glucose uptake in skeletal muscle (6). Although all three isoforms are briefly activated by muscle contraction (19), the downstream signaling molecules by which Akt/PKB exerts its effects in contracting skeletal muscle are unknown. Overexpression of active Akt/PKB induces muscle growth that is inhibited by rapamycin (17), suggesting that Akt/PKB control of muscle growth is mediated through mTOR. However, the observation that increased mTOR and p70S6K phosphorylation are not associated with Akt/PKB phosphorylation after muscle contractile activity suggests that these events may occur independent of Akt/PKB kinase activity. In addition, the dissociation between Akt/PKB and mTOR phosphorylation may reflect that cellular localization of Akt/PKB, rather than total kinase activity, may be critical for mTOR activation after muscle contractile activity.

In summary, we report that mTOR phosphorylation is elevated after muscle contractile activity and is associated with p70S6K but not Akt/PKB. Furthermore, mTOR phosphorylation in TA is localized to intracellular and membrane regions of type IIa fibers, suggesting that this subpopulation of fibers may be more responsive to hypertrophy. Presently, the stimulus responsible for increasing mTOR and p70S6K phosphorylation in contracting skeletal muscle is unknown.

DISCLOSURES

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