Contraction-mediated mTOR, p70S6k, and ERK1/2 phosphorylation in aged skeletal muscle

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The age-related loss of skeletal muscle mass (sarcopenia) is associated with well-characterized functional limitations and physical disability (5). Although resistance training can improve muscle strength by either inducing muscle hypertrophy and/or neural adaptations, the capacity for hypertrophy in older humans and animals is impaired (6, 13, 35). The age-associated alterations in the cellular processes that mediate these processes in skeletal muscle and the extent to which they are affected by exercise are not well understood.

The phosphatidylinositol 3-kinase signaling substrate mammalian target of rapamycin (mTOR) has been implicated in skeletal muscle hypertrophy during overload (7). After an acute bout of contractile activity and during chronic periods of overload, phosphorylation of mTOR and its downstream target 70-kDa ribosomal protein S6 kinase (p70S6k) is increased (7, 8, 23, 24), and the degree of p70S6k phosphorylation after a single bout of contractile activity is strongly associated with the increase in muscle weight after 6 wk of chronic stimulation. Correspondingly, pharmacological inhibition of mTOR prevents overload-induced hypertrophy in both type I and type II fibers (7).

The impact of aging on the activation of mTOR has not been extensively explored. In vitro, the ability of amino acids to stimulate p70S6k activity and protein synthesis in aged muscle is impaired (10). In addition, skeletal muscles of aged animals display selective resistance to a variant of insulin-like growth factor-1 protein at the level of p70S6k phosphorylation (18). Although contractile activity is known to regulate mTOR in skeletal muscle, to our knowledge, the effect of aging on this signaling pathway has not been examined.

The p42/44 extracellular signal-regulated kinase (ERK) is emerging as an important signaling component in skeletal muscle. ERK regulates the activity of several nuclear transcription factors in response to both diverse systemic stimuli, including insulin and growth factors, and local stressors such as muscle contraction (29, 37, 39). There is compelling evidence that ERK controls muscle cell proliferation and differentiation and therefore may play an important role in the myogenic response to exercise (1, 2, 15, 17, 25). In addition, decreased ERK1/2 phosphorylation was recently observed in older humans after an acute bout of resistance exercise (38).

The purpose of the present study was to determine whether aging affects cellular pathways involved in skeletal muscle growth in response to a single bout of contractile activity. We specifically wished to examine the principal signaling pathway that controls the regulation of protein translation in skeletal muscle (Akt/mTOR) and the signaling kinase (ERK1/2) that is activated by a host of intrinsic and extrinsic stimuli and which may play a role in the myogenic response to exercise. Contraction-mediated activation of mTOR and ERK1/2 signaling was assessed in skeletal muscle from aged rats either immediately after or 6 h after a single bout of sciatic nerve stimulation. We hypothesized that aging would impair the ability of con-
traction to stimulate mTOR, p70S6K, and ERK1/2 phosphorylation.

MATERIALS AND METHODS

Materials. Primary antibodies [phospho mTOR (Ser2448), phospho p70S6K (Thr389), phospho ERK (Thr202/Tyr204), mTOR, ERK (p42)] were from Cell Signaling Technologies (Beverly, MA), and anti-p70S6K was from Santa Cruz Biotech (Santa Cruz, CA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary anti-
bodies were from Amersham Biosciences (Piscataway, NJ). All other chemicals were from Sigma Chemical (St. Louis, MO).

Animals. Protocols for animal use were approved by the Institutional Animal Care and Use Committee of Boston University. Adult (6 mo old, n = 15) and aged (30 mo old, n = 15) male Fischer 344 × Brown Norway rats were purchased from the National Institute on Aging. The Fischer 344 × Brown Norway rat was chosen because these animals exhibit a lower incidence of disease than other rat strains and demonstrate age-associated decrements in muscle mass and function that are similar to humans (6). On arrival, animals were acclimatized 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 high-frequency electrical stimulation (HFES) model has been previously described (23) and was chosen based on its efficacy in stimulating protein translation and muscle hypertrophy in vivo (3). The HFES model used in the present study produced 10 sets of 10 contractions with a protocol time of 30 min. This protocol results in concentric (shortening) contraction of the plantaris (Pla) and eccentric (lengthening) contraction of the tibialis anterior (TA). The TA and Pla were chosen because contractile activity has previously been shown to induce mTOR and ERK signaling in these muscles after HFES (20, 23). In addition, the HFES model allows a comparison of the effects of different types of signaling in these muscles after HFES (20, 23). In addition, the HFES model used in the present study was acclimatized for 3 days before experimentation and were given normal laboratory chow and water ad libitum. Animals were fasted overnight before the experimental protocol.

Preparation of skeletal muscle tissue lysates. TA and Pla muscles were rapidly dissected, trimmed of connective tissue, weighed, frozen in liquid nitrogen, and stored at −80°C. Samples for Western blotting analyses were homogenized in 10 volumes of buffer containing (in mM) 50 Tris–HCl, 100 NaF, 10 EDTA, 50 β-glycerophosphate, 1 Na3VO4, 3 benzamidine, 1 phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin. Homogenates were centrifuged at 10,000 g 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 a standard (Bio-Rad, Hercules, CA).

Western blotting. Equal amounts of protein (20 or 40 μg) were resolved by SDS-PAGE using either 10% p70S6K/ERK 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-base sodium (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 horseradish peroxidase-conjugated secondary antibodies (1:10,000 in blocking buffer) for 1 h at room temperature. Protein signals were detected with enhanced chemiluminescence Plus reagents (Amersham, Piscataway, NJ). Images were scanned, and band intensities were quantified by densitometry (Bioquant Image Analysis, Nashville, TN).

Statistical analyses. The change in kinase phosphorylation was calculated between experimental muscles and corresponding control muscles [change = (experimental − control)/control]. Data are presented as means ± SE of five observations per group. Differences in baseline parameters between age groups were determined by an unpaired t-test. Differences between control and experimental muscles in the aged animals for mTOR and p70S6K were also determined by a paired t-test. Age-related differences in ERK fold-change after contraction were determined by two-way ANOVA. Differences were considered significant at P < 0.05.

RESULTS

Muscle mass and aging. As expected, wet muscle weight of TA and Pla were 14 and 13% smaller, respectively, in aged animals (TA: 611 ± 16 vs. 710 ± 23 mg, P < 0.01; Pla: 377 ± 13 vs. 434 ± 11 mg, P < 0.01). Muscle protein per milligram of muscle tissue was not changed by age in either TA (adult: 0.21 ± 0.01 mg/mg muscle; aged: 0.20 ± 0.01 mg/mg muscle) or Pla (adult: 0.21 ± 0.01 mg/mg muscle; aged: 0.20 ± 0.01 mg/mg muscle).

Influence of age on mTOR, p70S6K, and ERK phosphorylation. At rest, there was no age-related difference in total protein expression for either mTOR, p70S6K, or ERK (Fig. 1). mTOR phosphorylation was 70% higher in the TA of aged animals (P < 0.05). However, there were no age-related differences in mTOR phosphorylation at baseline in the Pla. Similarly, basal phosphorylation of p70S6K was similar in Pla but was 75% higher in TA of aged compared with adult animals (P < 0.05). There were no age-related differences in ERK1/2 phosphorylation in either muscle.

mTOR phosphorylation after contractile activity in aged animals. Immediately after HFES in aged animals, mTOR phosphorylation was not increased compared with control in TA (Fig. 2). After 6 h of recovery, mTOR phosphorylation remained unchanged from control values in TA. mTOR phosphorylation was elevated in Pla (1.3 ± 0.3-fold, P < 0.05) immediately after HFES and to a lesser extent 6 h after HFES (0.6 ± 0.1-fold, P < 0.05). Total mTOR protein was not altered by contractile activity.

Fig. 1. Phosphorylation state and total protein content of mammalian target of rapamycin (mTOR), 70-kDa ribosomal protein S6 kinase (p70S6K), and extracellular signal-regulated kinase (ERK)1/2 in tibialis anterior (TA) and plantaris (Pla) muscles from adult and aged rats. Phosphorylations of mTOR and p70S6K were higher in TA of aged animals despite similar levels of total proteins (n = 5/group, P < 0.05).
Immediately after HFES, p70S6K phosphorylation increased 1.2 ± 0.3-fold in TA (P < 0.05, Fig. 3) but was not significantly different in Pla. After 6 h of recovery, p70S6K phosphorylation remained elevated in TA but appeared to be decreasing from the post-HFES timepoint (0.6 ± 0.2-fold, P < 0.05). p70S6K phosphorylation remained unaffected in Pla at 6 h. HFES did not alter total p70S6K protein across time points.

**ERK1/2 phosphorylation is impaired after contractile activity in aged animals.** ERK1/2 phosphorylation was increased significantly immediately after HFES in TA of adult animals (11.1 ± 2.9-fold) and to a lesser extent in aged animals compared with control (2.1 ± 0.5-fold, P < 0.05) (Fig. 4A). By 6 h after HFES, ERK1/2 phosphorylation was similar to control values in TA of both adult and aged animals. Similar results were observed in Pla. Immediately after HFES, ERK1/2 phosphorylation was significantly increased in Pla of both adult (6.5 ± 1.5-fold) and aged (1.8 ± 0.5-fold) animals, and the response was significantly greater in adult vs. aged animals (P < 0.05) (Fig. 4B). After 6 h of recovery, ERK1/2 phosphorylation in Pla had returned to control values in both age groups. Total ERK1/2 protein was not altered across time points by contractile activity in either age group.

**DISCUSSION**

In recent years, marked progress has been made in identifying the signaling substrates essential for muscle growth and development. In this report, we demonstrate that muscle contraction affects cellular pathways involved in skeletal muscle cell growth in aged animals. Our results suggest that the ability of HFES to stimulate mTOR signaling remains intact in aged animals, but the magnitude of this response is dramatically reduced. Older animals showed an equivalent response of...
mTOR and p70S6K immediately after muscle contraction, although the fold-change in kinase phosphorylation was smaller than previously reported in adult animals (3, 23). Also, the delayed activation of mTOR and p70S6K at 6 h was three to four times lower than previously observed in adult animals (3, 23). In addition, ERK signaling was induced briefly by contractile activity in aged animals, but the response was 3.5–5 times lower than that seen in adult animals.

The progressive skeletal muscle atrophy that occurs with age has been well documented (5, 11, 12). It is likely that many factors contribute to this phenomenon, including reduced physical activity, alterations in growth factor axes, protein and energy intakes, and increased proinflammatory cytokine production. Although skeletal muscle protein synthesis has been reported to decrease with age (4, 32–34, 41), this observation has not always been confirmed (30, 31, 40). Surprisingly, we found that basal phosphorylation of both mTOR and p70S6K are higher in TA, but not in Pla, of older animals, despite similar total protein expression of these kinases. Li et al. (18) reported that phosphorylation of p70S6K is ~40% higher in pooled hindlimb muscle from old mice, although these results were not statistically significant. Our findings suggest that aging increases mTOR signaling at rest but that this effect is muscle specific. Whether these changes are sufficient to increase protein synthesis in TA of older animals remains unclear.

Although previous studies have shown that mTOR signaling in skeletal muscle is impaired after supplementation with insulin-like growth factor-1 and amino acids (10, 18), to our knowledge, this is the first study to investigate mTOR signaling after acute muscle contraction in aged animals. Both mTOR and p70S6K play essential roles in regulating muscle growth during overload by stimulating protein translation (3, 7). The degree of acute p70S6K phosphorylation can predict the amount of wet muscle mass gained after repeated bouts of HFES (3). In adult rats, maximal activation of p70S6K after HFES occurs ~3–6 h after HFES (3). In the present study, we report that the muscle contraction is able to activate mTOR signaling and, presumably, muscle growth in aged animals. However, it is interesting to note that peak activations of mTOR and p70S6K occurred immediately after the exercise bout and were ~50% lower at 6 h, suggesting that aging may blunt the mTOR activation time course. In addition, the fold-changes in kinase phosphorylation reported in this study are lower than those reported in an identical study with adult rats of the same strain (23). In that study, peak fold-change in p70S6K was 2.4-fold compared with 0.6-fold in the present study. Similarly, peak mTOR phosphorylation was 4.6-fold compared with 1.3-fold here. This raises the question of whether protein synthesis is lower in aged animals after muscle contraction.

Phosphorylation of mTOR on Ser2448 has not been directly linked to downstream activation of p70S6K, although it has been suggested that this site plays a key role in regulating pathways involved in protein translation independent of Ser2448 phosphorylation (21). To date, Akt–protein kinase B (Akt/PKB) is the only kinase identified to phosphorylate Ser2448 of mTOR, and, therefore, increased phosphorylation at this site may reflect increased Akt/PKB activity. However, perhaps a better estimate of mTOR activity in the present study is from phosphorylation of p70S6K, because mTOR immunoprecipitates contain a kinase activity toward the p70S6K Thr389 site (9).

In fact, Sekulic et al. (26) have reported that mTOR, with a specific mutation at the Ser2448 phosphorylation site, can still activate p70S6K and 4E-BP1 on insulin stimulation of HEK293 cells. Thus the specific role of Ser2448 phosphorylation of mTOR observed after contraction in the present and previous studies and its subsequent effect on downstream kinase activity remain to be determined (8, 23).

Because the stimulus for increasing mTOR signaling after muscle contraction is unknown, the mechanism by which aging might affect its regulation remains elusive. Impaired activation of mTOR and p70S6K by age may be due to reduced availability of circulating growth factors, although evidence suggests that reduced expression of local growth factors or cytokines may also play a role (22, 28). Alternatively, this impairment may result from resistance to growth factors or amino acids at the receptor level or from alterations in protein levels of upstream signaling components.

Consistent with previous reports (2, 20, 27, 36), ERK phosphorylation was increased immediately after muscle contractile activity in adult and aged animals. Although the function of ERK1/2 signaling after muscle contractile activity is poorly defined, ERK1/2 is an important regulator of gene transcription via activation of nuclear transcription factors, such as myc and c-fos, and, therefore, may be involved in any number of adaptations that occur during contractile activity and/or exercise training in skeletal muscle. Although ERK1/2 activity does not appear to be sufficient for myogenesis in vitro or for muscle hypertrophy in vivo (15, 19), ERK1/2 signaling is required for proliferation of satellite cells and may play a permissive role in a network of signals required for regeneration and hypertrophy of skeletal muscle.

ERK1/2 phosphorylation in the present study was attenuated after muscle contractile activity in older animals. The age-related differences in ERK activation after contractile activity could not be explained by differences in ERK1/2 phosphorylation or total protein content at baseline. The finding that ERK1/2 phosphorylation is impaired after a single bout of contractile activity is in agreement with data from Williamson et al. (38). However, in this study, older individuals displayed a reduction in ERK activation after a single bout of resistance exercise, which was suggested to be the result of higher phosphorylation of ERK at baseline (38). In the present study, basal phosphorylation of ERK was not different between the adult and aged animals, and this apparent difference in basal phosphorylation between these studies may be related to possible species differences and the specific muscles examined. Together, these findings indicate diminished nuclear signaling after contractile activity in aged skeletal muscle that may be the result of impaired growth factor or cytokine signaling (14, 22, 28). Although the functional implications of diminished ERK signaling are unclear, these observations may help explain why aging skeletal muscle displays an altered gene expression profile in response to acute resistance exercise (16).

In conclusion, we report that phosphorylations of mTOR, p70S6K, and ERK1/2 are increased in skeletal muscle after a single bout of in situ muscle contractile activity in aged animals and that the responses are less than those seen in adult animals. These observations suggest that the anabolic response
to a single bout of contraction is attenuated with aging and may help explain the limited capacity for hypertrophy in aged animals. Future studies should determine whether these changes are sufficient to reduce protein synthesis and accumulation of muscle protein and also to seek appropriate interventions to ameliorate these effects.

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REFERENCES


