Effects of Chronic Overload on Muscle Hypertrophy and mTOR Signaling in Young Adult and Aged Rats

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We examined the effect of 28 days of overload on mammalian target of rapamycin (mTOR) and extracellular signal–regulated kinase (ERK) signaling in young adult (Y; 6-month old) and aged (O; 30-month old) Fischer 344 × Brown Norway rats subjected to bilateral synergist ablation (SA) of two thirds of the gastrocnemius muscle or sham surgery (CON). Although plantaris (PLA) muscle hypertrophy was attenuated by aging, mTOR phosphorylation was 44% and 35% greater in Y SA and O SA compared with CON (p = .038). Ribosomal protein S6 phosphorylation was 114% and 24% higher in Y SA and O SA compared with CON (p = .009). Eukaryotic initiation factor 2B phosphorylation was 33% and 9% higher in Y SA and O SA compared with CON (p = .04). Translational signaling in young adult and aged plantaris muscle is equally responsive to chronic overload.

Key Words: mTOR—Muscle hypertrophy—Muscle overload—Aging.

ACUMULATING evidence suggests that anabolic stimuli, such as skeletal muscle overload, growth factors, or nutritional supplementation, are capable of promoting skeletal muscle hypertrophy and attenuating the age-related decrement in muscle mass (1–3). Previous studies have demonstrated that aging skeletal muscle from both humans (4–6) and rodents (7,8) is highly adaptive and hypertrophies following overload, however, to a lesser extent than observed in the young (9–11).

The molecular mechanisms associated with the reduced plasticity in response to overload in aged compared with young skeletal muscle have yet to be fully elucidated. Nonetheless, it is evident that skeletal muscle hypertrophy is governed by competing molecular signals that activate muscle protein synthesis and/or muscle protein degradation. Studies in animals have demonstrated that contraction-induced activation of skeletal muscle protein synthesis is associated with cross talk between the molecular signaling pathways that underlie muscle anabolism (12,13) and those that promote muscle catabolism (14).

Central to the current investigation, the putative molecular candidates involved in skeletal muscle hypertrophy include mammalian target of rapamycin (mTOR) complex-1 and its downstream targets eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) and the 70-KDA ribosomal S6 kinase 1 (p70S6K1) and ribosomal protein S6 (rpS6) as well as the extracellular signal–regulated kinase (ERK) signaling pathway. mTOR, a central kinase in translation initiation, lies downstream of the PI3K/Akt signaling pathway. Phosphorylation of mTOR is induced by both contractile activity (15) and nutrients (16), which in turn causes the downstream phosphorylation of the translation regulators, 4E-BP1 (17) and p70S6K1 (18). The hyperphosphorylation of 4E-BP1 results in its dissociation from eIF4E and allows incorporation together with eIF4G in the eIF4F complex (17). This last step is critical to translation initiation of 5′-cap-dependent protein synthesis. Although these kinases are important to the activation of translation initiation, the role of rpS6 is less clear (19). Evidence suggests that it is important to the determination of cell size (20) and that its phosphorylation is partially achieved by p70S6K1 in a mitogen-activated protein kinase–dependent manner (21).

With advancing age, the molecular signaling pathways that are associated with the induction of muscle hypertrophy are attenuated following acute contractions, 7 and 14 days of muscle overload (9,11–13). Specifically, mTOR and p70S6K1 phosphorylation increase in aged rats but to a lesser extent than young adult rats following high-frequency electrical stimulation (HFES) (13). Conversely, 4E-BP1 phosphorylation and eIF4E–eIF4G association increase in young adult animals but no change was observed in aged animals after HFES (12). Decreased mTOR, p70S6K1, 4E-BP1, and rpS6 phosphorylation have also been reported following 7 days of muscle overload in aged compared with young adult animals (11). Following 14 days of muscle overload, eIF4E–eIF4G complex association increases in 6-month-old rats but not in 26-month-old rats (9).

Therefore, in the present study, we examined the activation of the mTOR and ERK signaling pathways and their downstream targets in young adult and aged rodents following 28 days of muscle overload. We hypothesized that mTOR signaling would be attenuated in aged versus young adult animals subjected to muscle overload for 28 days induced by bilateral ablation of two thirds of the gastrocnemius muscle. We further hypothesized that the examination of hypertrophy following 28 days of synergist ablation (SA) would be consistent with the response to resistance exercise training in humans and thus would be highly physiologically relevant.
Materials and Methods

Materials

Primary antibodies (phospho mTOR [Ser2448], phospho 4E-BP1 [Thr37/46], phospho p70S6K [Thr389], phospho rpS6 [Ser235/236], phospho GSK-3α/β [Ser21/9], eIF4E, phospho ERK [p42/44; Thr202/Tyr204], mTOR, 4E-BP1, p70S6K, rpS6, GSK-3α/β, eukaryotic initiation factor 2Be [eIF2Be], and ERK [p42/p44]) were obtained from Cell Signaling Technologies (Beverley, MA), and anti-eIF4G was obtained from Bethyl Laboratories (Montgomery, TX). Anti-rabbit and anti-mouse horseradish peroxidase–conjugated secondary antibodies were purchased from Cell Signaling Technologies. Anti-rabbit BioMag IgG beads were purchased from Qiagen (Valencia, CA). Total protein reagents were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). All other chemicals were obtained from Sigma Chemical (St Louis, MO) and Bio-Rad (Hercules, CA).

Animals

Young adult (Y; 6-month old, n = 16) and aged (O; 30-month old, n = 16) male Fischer 344 × Brown Norway rats were purchased from the National Institute on Aging. The animals were housed in an animal care facility at the Human Nutrition Research Center on Aging at Tufts University and were given ad libitum access to water and chow. The Fischer 344 × Brown Norway rat strain is less susceptible to disease (22) and show muscle atrophy that is characteristic in human aged skeletal muscle (23). The animals were housed in an animal care facility at the Human Nutrition Research Center on Aging at Tufts University on a 12-hour light–dark cycle and had free access to water and chow. Rats were acclimatized for 14 days and fasted overnight before initiation of experimental protocol. The study was approved by the Institutional Animal Use and Care Committee at Tufts University.

Synergist Ablation

Animals were weighed and anesthetized with 2%–3% isoflurane as an inhalant with 96%–99% oxygen as a vehicle. Under aseptic conditions, the animals were then subjected to either bilateral SA (Y, n = 8; O, n = 8) of two thirds of the gastrocnemius muscle or sham operated (CON; Y, n = 8; O, n = 8). SA was performed in order to induce overload for 28 days and promote compensatory hypertrophy in the plantaris (PLA) and soleus (SOL) muscles. Following surgery, each incision was closed using stainless steel wound clips (9 mm) with two sutures at the Achilles tendon for faster wound healing. The animals were also given a subcutaneous injection of 30–50 cc of warm Ringer’s solution and a subcutaneous injection of an analgesic (Buprenex—0.5 mg/kg body weight). Animals were monitored postsurgery for movement and checked twice daily for 7 days postsurgery. The animals received Buprenex everyday for 1–2 days postsurgery depending on postoperation stress levels and pain. After 14 days, the staples were removed, as the incisions were healed.

Preparation of Skeletal Muscle Tissue Lysates

All animals were sacrificed 28 days after SA or sham surgery. Animals were fasted 4 hours prior to sacrifice. Plantaris and SOL muscles were rapidly dissected, trimmed of connective tissue, weighed, and frozen in liquid nitrogen. Tissue samples were stored at −80°C. Samples of approximately 50 mg were prepared for Western blot analyses by homogenization in 20 volumes of muscle lysis buffer containing 50 mM Tris–HCl; 100 mM NaF; 10 mM ethylenediaminetetraacetic acid (EDTA); 50 mM β-glycerophosphate; 1 mM Na3VO4; 3 mM benzamidine; 1 mM phenylmethylsulfon; and 10 μg/mL each of aprotinin, leupeptin, and pepstatin. Homogenates were centrifuged at 10,000 g for 10 minutes at 4°C, the supernatants were collected, and aliquots were stored at −80°C. Protein concentration was determined using the Pierce Protein assay with bovine serum albumin (BSA) standards as a reference.

Immunoblotting

Equal amounts of protein (20 μg) were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis using 5% gel for mTOR, 7.5% for p70S6K, and 10% for 4E-BP1, rpS6, GSK-3α/β, eIF2Be, and p42/44 ERK. Proteins were transferred to nitrocellulose membranes (Bio-Rad). Equal protein loading was verified by Ponceau S staining. Membranes were blocked for 1 hour in tris-buffered saline and Tween-20 (TBS-T) containing 5% milk, washed with TBS-T, and then incubated overnight at 4°C with primary antibody (diluted 1:1000 in 5% BSA in TBS-T). Membranes were washed several times with TBS-T and then incubated for 1 hour at room temperature with anti-rabbit horseradish peroxidase–conjugated secondary antibody (diluted 1:2000 in 1% nonfat milk and TBS-T). Protein signals were detected with Pierce SuperSignal West Pico chemiluminescence substrate (Thermo Fisher Scientific). Images were scanned and band intensities quantified by optical density using standardized bandwidths (Alpha Innotech Corporation, San Leandro, CA).

Immunoprecipitation

The association of eIF4E with eIF4G and total eIF4E were quantified using a modified method by Kimball and colleagues (24). Briefly, eIF4E–eIF4G complex and unbound eIF4E were immunoprecipitated from 400 μg of muscle lysate by incubation overnight at 4°C with 3 μL anti-eIF4E antibody along with 200 μL of radioimmunoprecipitation assay buffer (50 mM Tris–HCl, 1% NP-40, 0.25%
Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, 1 mg/mL each aprotinin, pepstatin, and leupeptin). The antibody–antigen complex was collected by incubation for 2 hours at 4°C with 500 μL of goat anti-rabbit BioMag IgG beads. The beads were previously washed three times on a magnetic rack with low-salt buffer (LSB; 20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and 0.1% β-mercaptoethanol). Half of the initial volume was reconstituted with LSB-0.1% nonfat dry milk prior to incubation. Following bead incubation, remaining lysates were removed from the beads on a magnetic rack and the beads were washed twice with LSB and once with high-salt buffer (50 mM Tris–HCl [pH 7.4]; 500 mM NaCl; 5 mM EDTA; 1% Triton X-100; and 0.5% sodium deoxycholate, 0.1% SDS, and 0.04% β-mercaptoethanol). Beads were re-suspended in 100 μL of SDS sample buffer, and samples were boiled for 5 minutes at 100°C to release the immunoprecipitated proteins. Supernatants were collected using a magnetic rack, and 30-μL aliquots were used for immunoblotting analyses. Aliquots were run on a 7.5% polyacrylamide gel for quantification of total eIF4E-eIF4G association (eIF4G antibody diluted 1:1,000 in 5% BSA in TBS-T) or on a 15% polyacrylamide gel for quantification of total eIF4E (diluted 1:1,000 in 5% BSA and TBS-T).

Statistical Analyses

Data were analysed using SPSS statistical software package (version 16.0; Chicago, IL). Between-group comparisons were performed using a univariate general linear model with muscle mass or proteins of interest as dependent variables and age and group as fixed factors. Data are reported as means ± SEM. The criterion for statistical significance was \( p \leq .05 \).

RESULTS

Animal Characteristics

Whole-animal weights at Days 0 and 28 as well as specific muscle weights are presented in Table 1. At Day 0, O animals were significantly heavier than Y animals (49%, \( p = .000 \)). At Day 28, O animals remained significantly heavier than Y animals (24%, \( p = .000 \)) and the CON group were heavier than SA (11%, \( p = .01 \)). Y PLA and Y SOL weights were heavier than O PLA and O SOL weights (25%, \( p = .000 \); 14%, \( p = .02 \)). Y SA PLA and Y SA SOL weights were greater than Y CON PLA and Y CON SOL weights (17%, \( p = .000 \); 15%, \( p = .035 \), respectively). O SA PLA and O SA SOL weights were greater than O CON PLA and O CON SOL weights (14%, \( p = .000 \); 10%, \( p = .035 \), respectively). PLA and SOL weights were also normalized per grams of whole-animal weight (Figure 1). In PLA, the normalized muscle weights were 35% greater in Y SA and 20% greater in O SA compared with respective CON (\( p = .002 \), age by group interaction). Similarly, in SOL, the normalized muscle weights were 33% greater in Y SA and 16% greater in O SA compared with respective CON (\( p = .028 \), age by group interaction). For both PLA and SOL weights subjected to SA, the percentage increase was attenuated by aging.

mTOR Phosphorylation is Increased With SA in Plantaris but not Soleus Muscle

Total mTOR expression in PLA was unaffected by age or overload (Figure 2). mTOR phosphorylation was significantly greater in PLA muscle from SA compared with CON (\( p = .038 \); Figure 2). mTOR phosphorylation in PLA muscle was 44% and 35% higher in Y SA and O SA compared with respective CON. However, there was no effect of age on mTOR phosphorylation in PLA with SA (Figure 2). Total mTOR expression as well as mTOR phosphorylation in SOL muscle were unaffected by age or SA (Figure 3).

rpS6 Phosphorylation is Increased With SA in Plantaris but not Soleus Muscle

Similar to total mTOR expression, total rpS6 expression in PLA muscle was unaffected by age or overload (Figure 4). However, rpS6 phosphorylation in PLA muscle was significantly greater in SA compared with CON (\( p = .009 \); Figure 3). rpS6 phosphorylation in PLA muscle was 114% and 24% higher in Y SA and O SA compared with their respective

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Table 1. Animal Whole-Body Weights (grams) and Muscle Wet Weights (milligrams)

<table>
<thead>
<tr>
<th></th>
<th>Adult (6 mo)</th>
<th>Day 0*</th>
<th>Day 28**</th>
<th>Day 0*</th>
<th>Day 28**</th>
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<tbody>
<tr>
<td>SA</td>
<td></td>
<td>374.5 ± 16.8</td>
<td>374.5 ± 16.8</td>
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<td>CON</td>
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<td>387.4 ± 14.9</td>
<td>438.4 ± 24.9</td>
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<td>Muscle weight (mg)*, †</td>
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<tr>
<td>Plantaris</td>
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<tr>
<td>SA</td>
<td>506.5 ± 19.7</td>
<td>402.1 ± 14.4</td>
<td>196.1 ± 10.6</td>
<td>168.6 ± 10.4</td>
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<tr>
<td>CON</td>
<td>434.0 ± 13.8</td>
<td>352.8 ± 6.4</td>
<td>170.9 ± 8.1</td>
<td>152.6 ± 6.9</td>
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<table>
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<th>Adult (30 mo)</th>
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<tr>
<td>SA</td>
<td></td>
<td>567.3 ± 11.3</td>
<td>490.9 ± 9.7</td>
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<tr>
<td>CON</td>
<td></td>
<td>564.9 ± 11.1</td>
<td>518.6 ± 11.3</td>
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Notes: CON = control; SA = synergist ablation.
Data are represented as \( M ± SE \).
*Significant difference between young adult and aged (\( p < .05 \)).
†Significant difference between SA and CON (\( p < .05 \)).
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**eIF2B**

**Con**. There was no effect of age on rpS6 phosphorylation in PLA muscle with SA (Figure 4). In addition, total rpS6 expression as well as rpS6 phosphorylation in SOL muscle were unaffected by age or SA (Figure 3).

**eIF2Be Phosphorylation is Increased in Plantaris Muscle With SA**

Total eIF2Be expression was unaffected by age or overload (Figure 5). eIF2Be phosphorylation was significantly greater in SA compared with CON (p = .04; Figure 5). eIF2Be phosphorylation was 33% and 9% higher in Y SA and O SA, respectively, compared with their respective CON. There was no effect of age on eIF2Be phosphorylation with SA (Figure 5).

**GSKα/β, p70S6K1, 4E-BP1, eIF4E, and eIF4E–eIF4G Complex Association Are Unaffected by Age or SA in Plantaris Muscle**

Total GSKα/β, p70S6K1, and 4E-BP1 expressions were unaffected by age or overload (Figure 6). Similarly, GSKα/β, p70S6K1, and 4E-BP1 phosphorylation were unaffected by age or SA (Figure 6). Total eIF4E expression and eIF4E–eIF4G complex association were unaffected by age or SA (Figure 6).

**SOL mTOR and SOL rpS6, are unaffected by age or SA**

**Figure 1.** Plantaris (PLA) and soleus (SOL) weights normalized to body weight (milligrams muscle per grams body weight). Data are represented as mean ± SE. In PLA, the normalized muscle weights were greater in synergist ablation (SA) group compared with respective control (CON; p = .002, age by group interaction), irrespective of age. Similarly in SOL, the normalized muscle weights were greater in SA groups compared with respective CON (p = .028, age by group interaction), irrespective of age.

**Figure 2.** Response of rapamycin (mTOR) phosphorylation to synergist ablation (SA) in young adult and aged skeletal muscle compared with control (CON). Data are represented as mean ± SE. *Represents a significant difference (p = .038) between groups (SA vs CON). Representative gel bands are shown above.

**Figure 3.** Representative gel bands of soleus rapamycin (mTOR) and soleus rpS6. α/β tubulin included as a loading control. There was no measured effect of age or overload.
Total p44 ERK expression was significantly greater in O compared with Y \((p = .03;\) Figure 7). Total p44 ERK expression was 17% and 45% higher in O SA and O CON, respectively, compared with Y SA and Y CON. p44 ERK phosphorylation was unaffected by age or SA (Figure 7).

We also examined the effect of age and overload on total p42 ERK expression and p42 ERK phosphorylation. Total p42 ERK expression was unaffected by age or overload (data not shown). p42 ERK phosphorylation was also unaffected by age or SA (data not shown).

**Discussion**

The main findings from the present study were that chronic muscle overload for 28 days increased muscle mass in young adult and aged animals but there were no differences in mTOR or rpS6 phosphorylation between young adult and aged animals. Both plantaris and soleus muscles (normalized to body weight) from both young adult and aged animals underwent significant hypertrophy, although this effect was attenuated in aged compared with young adult animals. The hypertrophy of the plantaris but not soleus muscle was accompanied by an increase in the phosphorylation of mTOR and rpS6, but this increase was unaffected by age.

Previous reports have documented age-related deficits in muscle hypertrophy following muscle overload (10,11). After 7 days of muscle overload, the plantaris muscle weight increase in aged and young adult rats is 9.7% and 30%, respectively (11). However, the results from 7 days of muscle overload may be compromised by factors such as inflammation and edema, purportedly peaking 1–5 days postsurgery and normalizing by Day 16 (25). In a recent study that employed 14 days of compensatory hypertrophy, investigators reported that compared with control animals plantaris muscle in aged and young adult animals is 15% and 37% greater, respectively (9). In the present study, we observed a 14% (20% per grams body weight) and 17% (35% per grams body weight) increase in plantaris muscle weight in our aged and young adult animals, respectively, after 28 days. Longer duration overload consisting of 56 days results in a 44% reduction in plantaris muscle weight in 38-month-old rats compared with age-matched controls, whereas 8-month-old rats had a 53% increase (23).

Variations in the procedure used to induce muscle overload, such as unilateral versus bilateral ablation, as well as...
overload, mTOR phosphorylation increases 88% versus 292% in aged and young adult muscle, respectively (11). We observed a 35% and 44% increase in mTOR phosphorylation after a 28-day overload period in aged and young adult animals, respectively. The more prolonged overload paradigm employed in the present study, as well as other potential methodological differences, may explain the apparent discrepancies in these results. By 28 days of overload, any age-related differences in mTOR phosphorylation between young adults and aged animals may be attenuated.

Concurrent with an increase in mTOR phosphorylation, we found that rpS6 phosphorylation was significantly higher in overloaded muscle. It has been reported that 7 days of overload also initiates an increase in rpS6 phosphorylation in both young adult and aged muscle; although similar to mTOR phosphorylation, there was a significant impairment in aged versus young adult animals (11). We did not observe an effect of age probably because the aged control animals appeared to have higher levels of phosphorylated rpS6 than the young adult control. The concomitant increase in rpS6 phosphorylation we observed would suggest an mTOR-dependent mechanism. This is probable particularly because there was no measurable increase in p42/44 ERK phosphorylation, kinases that also phosphorylate rpS6 at Ser235/236 (21,27). However, we did not measure any changes in protein phosphorylation following our overload overload, mTOR phosphorylation increases 88% versus 292% in aged and young adult muscle, respectively (11). We observed a 35% and 44% increase in mTOR phosphorylation after a 28-day overload period in aged and young adult animals, respectively. The more prolonged overload paradigm employed in the present study, as well as other potential methodological differences, may explain the apparent discrepancies in these results. By 28 days of overload, any age-related differences in mTOR phosphorylation between young adults and aged animals may be attenuated.

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The significance of this association remains to be elucidated. It has been shown that although the 3-phosphorylation purportedly decreases with age (32), perhaps as an effect of eIF2B phosphorylation in either young adult or aged animals following 28 days of overload. GSK-3β phosphorylation would result in inhibition of its activity and, therefore, inhibition of GSK-3β-dependent phosphorylation of eIF2Be promoting protein synthesis. eIF2Be phosphorylation purportedly decreases with age (32), perhaps as an adaptive mechanism to allow for greater protein synthesis. Our results suggest the inhibition of translation initiation by negative feedback after a 28-day loading duration, an effect that at this time point does not extend to mTOR or rpS6. Of note, the increase in eIF2Be phosphorylation following muscle overload in both age groups may occur as early as 14 days after overload (9) signifying that the capacity for continued protein synthesis has been reached, an observation we confirm with our findings. Interestingly, activation of mTOR alone through leucine and/or insulin-like growth factor 1 repletion in rat fibroblasts has been demonstrated to increase eIF2Be messenger RNA and eIF2Be protein synthesis (33). The significance of this association remains to be elucidated. GSK-3α expression and phosphorylation were also unaffected by age or overload. It has been shown that although the α and β isoforms of GSK-3 are structurally similar, they have functionally distinct roles (34).

In both young adult and aged animals, eIF4E expression and eIF4E–eIF4G complex association were unaffected by our overload period. This may be due to the lack of 4E-BP1 phosphorylation, which would suggest that eIF4E is still complexed to its binding protein. 4E-BP1 phosphorylation would lead to its disassociation from eIF4E, a step necessary for eIF4E–eIF4G complex assembly and translation initiation. This is contrary to observations using a 14-day overload period where eIF4E–eIF4G association increased in young adult but not aged animals (9). It is likely that comparable to our observations on eIF2Be phosphorylation, the eIFs that are central to translation initiation are negatively responding to muscle overload of this duration, indicating the attainment of maximal protein synthesis. eIF2B may occupy a more central role in translation initiation than eIF4E (35).

Coupled to the effects of muscle overload on the mTOR signaling pathway, it has been shown that contractile activity directly activates p42/44 ERK (36,37). Moreover, p42/44 ERK phosphorylates rpS6 at Ser235/236 through p90 ribosomal S6 kinase (21,27). In the present investigation, p42/44 ERK phosphorylation was unaffected by age or muscle overload. Mylabathula and colleagues (38) observed a 20% higher basal level of total p44 ERK in aged compared with young adult rats. Reports of higher basal p44 ERK expression would conform to our observation of a 17%–45% increase of this protein in aged compared with young adult animals. Furthermore, the data suggest that while consideration must be given to different muscle groups under investigation, higher basal levels of total p44 ERK may be specific to aging. Neither muscle overloading nor aging had an effect on p42 ERK expression or phosphorylation.

In conclusion, chronic muscle overload for 28 days increased muscle mass in young adult and aged animals, and although hypertrophy was attenuated in the aged animals, there were no differences in mTOR or rpS6 phosphorylation between age groups. Both plantaris and soleus muscles (normalized to body weight) from both young adult and aged animals underwent significant hypertrophy, although this effect was attenuated in aged compared with young adult animals. The hypertrophy of the plantaris but not soleus muscle was accompanied by an increase in the phosphorylation of mTOR and rpS6, but this increase was unaffected by age. These data suggest that activation of anabolic signaling in plantaris muscle is similar between young adults and older animals following prolonged overload. Future studies should examine the detailed time course of anabolic signaling and muscle growth in response to overload using a standardized loading paradigm in both young adult and older animals.

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