Impaired overload-induced muscle growth is associated with diminished translational signalling in aged rat fast-twitch skeletal muscle

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Impaired overload-induced protein synthesis and growth in aged fast-twitch skeletal muscle may result from diminished responsiveness of signalling intermediates controlling protein translation. Yet, potential age-related signalling decrements have never been examined in direct parallel with impaired overload-induced muscle growth in any model. To this end, we used Western blotting to examine the contents and phosphorylation states of mammalian target of rapamycin (mTOR) and its downstream translational signalling intermediates, 70 kDa ribosomal protein S6 kinase (S6k), ribosomal protein S6 (rpS6), eukaryotic elongation factor 2 (eEF2), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), in conjunction with impaired growth in 1 week overloaded fast-twitch plantaris muscles (via unilateral gastrocnemius ablation) of old (O; 30 months) *versus* young adult (YA; 8 months) male Fischer344 × Brown Norway rats. The significantly ($P \leq 0.05$) diminished growth (assessed by total muscle protein content) in overloaded O muscles (5.6 ± 1.7 *versus* 19.3 ± 2.9% in YA) was accompanied by significant impairments in the phosphorylation states of mTOR (Ser^{2448}), S6k (impaired at the mTOR-specific Thr^{389} residue but not at Thr^{421}/Ser^{424}), rpS6 ($Ser^{235/236}$) and 4E-BP1 (gel shift), as well as deficits in total eEF2 accretion. Moreover, in overloaded muscles across both age groups, phospho-S6k at Thr^{389} (but not at Thr^{421}/Ser^{424}), 4E-BP1 phosphorylation status, and total eEF2 accretion were all positively correlated with percentage muscle hypertrophy, and negatively correlated with the phosphorylation (Thr^{172}) of 5′-AMP-activated protein kinase (AMPK; which inhibits translational signalling and protein synthesis in young muscle at rest). As previously published by ourselves, AMPK was hyperphosphorylated in O *versus* YA muscles used in the current investigation. The present results provide solid evidence that impaired overload-induced growth in aged fast-twitch muscle may partly result from multiple-level decrements in signalling pathway(s) controlling protein translation, and also provide an initial indication that AMPK hyperactivation with age may potentially lie upstream of these decrements.

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It is well known that ageing is accompanied by a loss of muscle mass (sarcopenia), particularly in fast-twitch fibres, a phenomenon which contributes to decreased muscular strength and power (Welle, 2002). This is an important problem for the elderly because it may lead to an increased risk of injury, a decrease in general mobility and loss of functional independence. While it is clear that older individuals are capable of some muscle growth with training, mechanical overload-induced muscle growth, particularly of fast-twitch fibres, is attenuated in old humans (Welle *et al.* 1996*b*; Häkkinen *et al.* 1998, 2001) and rodents (Blough & Linderman, 2000; Alway *et al.* 2002; Degens & Alway, 2003; Thomson & Gordon, 2005) when compared with the young. However, the mechanisms underlying this decreased capacity for fast-twitch skeletal muscle growth in the aged are still poorly understood.

For muscle growth to occur, the rate of protein synthesis must exceed that of protein breakdown. Protein synthesis is regulated in part by the activity of initiation and elongation factors that control the rate of mRNA translation. One critical signalling pathway controlling protein synthesis during mechanically induced skeletal muscle growth involves mammalian target of rapamycin (mTOR) (Bodine *et al.* 2001; Nader & Esser, 2001; Reynolds *et al.*

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2002). The mTOR protein lies downstream of Akt in the insulin signalling pathway, but can also be regulated through a variety of other hormonal, nutritional and metabolic signals. When active, mTOR promotes an increase in the efficiency and capacity for translation of mRNA to protein by increasing, either directly or indirectly, the activity of several proteins, including 70 kDa ribosomal protein S6 kinase (S6k), ribosomal protein S6 (rpS6), eIF4E-binding protein 1 (4E-BP1), and eukaryotic elongation factor 2 (eEF2) (Kimball *et al.* 1998; Nader *et al.* 2002). S6k promotes the translation of various ribosomal proteins and elongation factors (including eEF2), presumably via phosphorylation of rpS6 (Terada *et al.* 1994; Jefferies *et al.* 1997), and also promotes a general increase in protein elongation by an indirect activation of eEF2 (Wang *et al.* 2001). On the other hand, mTOR phosphorylation of 4E-BP1 promotes translation initiation of 5′-cap mRNAs, including most eukaryotic mRNAs (Gingras *et al.* 1999; Shah *et al.* 2000). With various forms of increased muscle loading, mTOR and its downstream targets are activated (Baar & Esser, 1999; Bodine *et al.* 2001; Nader & Esser, 2001; Reynolds *et al.* 2002; Bolster *et al.* 2003*b*; Parkington *et al.* 2003) and muscle protein synthesis is enhanced (Wong & Booth, 1990*a*,*b*; Hernandez *et al.* 2000; Pehme *et al.* 2004*a*).

Resting skeletal muscle protein synthesis declines with age in most cases (Yarasheski *et al.* 1993; Welle *et al.* 1995), in part due to decreased translational efficiency (Welle *et al.* 1996*a*). Mechanically induced increases in protein synthesis, especially in fast-twitch muscle, are also potentially attenuated with old age and may underlie the diminished growth (Welle *et al.* 1995; Tamaki *et al.* 2000; Pehme *et al.* 2004b). Potential signalling deficits underlying these age-related impairments are not well characterized. Despite equivocal mTOR and S6k data in resting muscle (Dardevet *et al.* 2000; Li *et al.* 2003; Guillet *et al.* 2004; Kimball *et al.* 2004; Cuthbertson *et al.* 2005), the responsiveness of S6k to various hormonal or nutritional stimuli is clearly impaired in aged skeletal muscle (Dardevet *et al.* 2000; Li *et al.* 2003; Guillet *et al.* 2004; Cuthbertson *et al.* 2005). Less is known about age-related decrements in mTOR, S6k and potential downstream signalling with respect to loading stimuli; however, S6k activation is diminished in association with failed regrowth in aged slow-twitch muscle that has been reloaded after disuse (Morris *et al.* 2004). Although there are reports that mTOR and S6k responsiveness may potentially be decreased with age after acute high-frequency electrical stimulation in fast-twitch muscle (Parkington *et al.* 2003, 2004), the responses between age groups were not directly compared in those studies. Furthermore, it is unknown whether such signalling alterations with age corresponded with impaired protein synthesis or impaired potential for longer-term muscle growth in that model. Thus, the potential connection between diminished mTOR and/or S6k activation in aged fast-twitch muscle under conditions known to demonstrate defective overload-induced protein synthesis and growth remains completely unexplored, especially with respect to downstream signalling intermediates (such as rpS6, eEF2 and 4E-BP1) controlling protein translation and accretion. We postulated that diminished mTOR, S6k, rpS6, eEF2 and 4E-BP1 signalling would exist in response to compensatory overload in aged fast-twitch muscle, a model demonstrating greatly impaired muscle protein synthesis (Pehme *et al.* 2004b) and growth (Thomson & Gordon, 2005) in aged animals.

Also unclear are the mechanism(s) of upstream regulation of mTOR and other translational signalling intermediates in aged and/or overloaded skeletal muscle. In young resting skeletal muscle (Bolster *et al.* 2002) and cardiac muscle (Horman *et al.* 2002; Chan *et al.* 2004), protein synthesis is negatively regulated by activation of the cellular energy sensor 5′-AMP-activated protein kinase (AMPK). This effect is likely to be due, in part, to AMPK's inhibition of mTOR, S6k, 4E-BP1 and/or eEF2 (Bolster *et al.* 2002; Horman *et al.* 2002; Chan *et al.* 2004) signalling. Importantly, we have demonstrated that AMPK phosphorylation is elevated with age in resting and overloaded fast-twitch skeletal muscle (Thomson & Gordon, 2005). Moreover, we found that the degree of hypertrophy is negatively correlated with the amount of AMPK phosphorylation in overloaded fast-twitch muscle after synergist ablation (Thomson & Gordon, 2005). Thus, we postulated that any potential impairments in mTOR, S6k, rpS6, eEF2 and 4E-BP1 signalling would correspond with the AMPK hyperphosphorylation and diminished overload-induced growth we observed in aged fast-twitch skeletal muscle (Thomson & Gordon, 2005). The purpose of this investigation was therefore to examine the contents and phosphorylation states of these proteins in response to overload (via gastrocnemius ablation) in young adult and old fast-twitch plantaris muscles.

Methods

Animals

Young adult (YA; 8 months; $n = 7$) and old (O; 30 months; $n = 7$) Fischer344 × Brown Norway F1 hybrid (FBN) male rats were housed in the animal care facility of East Carolina University Brody School of Medicine. Body weights, muscle weights and AMPK results have been previously reported for these animals (Thomson & Gordon, 2005). The FBN rat was chosen for use in this study because it is considered to be the preferred model for the study of age-related skeletal muscle dysfunction (Blough & Linderman, 2000; Degens & Alway, 2003). The animals were kept on a 12 h light–dark cycle and had free access to water and chow. The East Carolina University Animal Care and Use Committee approved all procedures prior to experimentation.

Synergist ablation procedure

Unilateral 1 week overload of the plantaris muscle (93% fast-twitch muscle by mass; Armstrong & Phelps, 1984) was achieved through the surgical ablation of the gastrocnemius muscle. The 1 week overload period was chosen because it is known that muscle protein synthesis in young rats is elevated at this time point of compensatory overload (Baillie & Garlick, 1991), and continues to be elevated at least 30 days after initiation of the stimulus (Pehme *et al.* 2004*a*,*b*). Furthermore, rat plantaris muscle protein synthesis in the compensatory overload model is greatly diminished with age (Pehme *et al.* 2004*b*), and we have shown that protein accretion is diminished by 71% in aged rats during 1 week of overload in plantaris muscles after gastrocnemius ablation (Thomson & Gordon, 2005). The advantage of unilateral ablation over bilateral ablation is that it allows within-animal comparisons to be made between sham-operated (control) and overloaded muscles, thus eliminating bias due to systemic differences between groups of animals and allowing a more precise measurement of signalling and muscle hypertrophy in each individual animal. Additionally, separate animals that underwent no prior surgery ($n = 3$ per group) were also killed to verify that the sham-operated control muscles truly reflected previously undisturbed resting muscle.

Rats were weighed and anaesthetized with 2–3% isoflurane and supplemental oxygen for gastrocnemius ablation. Under aseptic conditions, the distal two-thirds of the gastrocnemius muscle were surgically removed from the left hindlimb as previously described (Gordon *et al.* 2001*a*,*b*; Thomson & Gordon, 2005). A sham (control) operation was performed on the right hindlimb. This consisted of an incision through the skin and isolation of the Achilles tendon, but without disruption of the gastrocnemius muscle. The plantaris muscle from this limb served as the control. Animals were kept on a water-circulation heating pad during surgery. Following each procedure, the incision was closed using stainless steel surgical clips, and animals were subcutaneously injected with ∼10 ml of warm saline as well as a one-time subcutaneous injection of analgesic (Buprenex, 0.03 mg kg^{-1} body weight). Animals were active immediately after recovering from anaesthesia, and were checked twice daily thereafter during the 7 day post-operative period. No signs of postoperative complications (such as infection or undue distress) were observed. One week after surgery, the animals were weighed and anaesthetized with an intraperitoneal injection of ketamine and xylazine (90 and 10 mg kg^{-1} body weight, respectively). The animals were then decapitated, after which the plantaris muscles from both legs were quickly removed, trimmed of excess connective tissue, weighed on an analytical balance, frozen in liquid nitrogen, and stored at −80°C until further analysis.

Tissue homogenization and determination of protein concentration

Muscles were homogenized on ice as a 3.5% (w/v) solution in homogenization buffer (50 mM Hepes, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7.10H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg ml^{-1} leupeptin, 50 μg ml^{-1} pepstatin, 33 μg ml^{-1} aprotinin and 0.5 mM Na_3VO_4) in a ground glass homogenizer. An aliquot of homogenate was clarified by centrifugation for 10 min at 10 000 *g*, and protein concentration measurements were made on both the crude and clarified homogenates using a modified Lowry procedure (DC Protein Assay; Bio-Rad, Hercules, CA, USA). Protein assay results from the crude homogenate were used to calculate total protein per whole muscle (as previously reported by Thomson & Gordon, 2005), which allows a more precise estimation of muscle hypertrophy (protein accretion) than wet weight measurements because it accounts for potential differences in water weight due to oedema (Ianuzzo & Chen, 1979). Clarified homogenates were used for Western blotting (preliminary testing indicated no effect of clarification on Western blotting results).

SDS-PAGE, Western blotting and immunodetection

Clarified muscle homogenates were solubilized in sample loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% SDS, 2% β-mercaptoethanol, 0.1% bromophenol blue) at a concentration of 1 mg ml^{-1} and boiled for 5 min. Samples across conditions were equally represented on each SDS-polyacrylamide gel (7.5% for S6k, eEF2, mTOR and Akt, 15% for rpS6 and 4E-BP1). Proteins were separated by electrophoresis and then Western blotted for 1 h at 4°C onto a PVDF membrane (Millipore, Bedford, MA, USA) at 100 V in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol). Visual verification of transfer and equal protein loading amongst lanes was accomplished by Ponceau S staining of the membranes. For immunodetection, membranes were blocked for 1 h at room temperature in blocking buffer (5% non-fat dry milk in TBS-T (20 mM Tris-base, 150 mM NaCl, 0.1% Tween-20), pH 7.6), serially washed in TBS-T at room temperature, then probed for specific signalling proteins using antibodies (all from Cell Signalling Technology, Inc., Beverly, MA, USA) for the detection of Akt, phospho-Akt (Ser^{473}), mTOR, phospho-mTOR (Ser^{2448}), p70S6k, phospho-p70S6k (Thr^{389}), phospho-p70S6k (Thr^{421}/Ser^{424}), rpS6, phospho-rpS6 ($Ser^{235/236}$), 4E-BP1, eEF2 and phospho-eEF2 (Thr^{56}). Regulation at these residues is considered necessary for full or partial activation of these proteins (Kimball *et al.* 1998; Gingras *et al.* 1999; Shah *et al.* 2000; Browne & Proud, 2002).

Membranes were incubated overnight at 4°C in primary antibody buffer (5.0% BSA in TBS-T, pH 7.6, primary antibody diluted 1:1000, except for phospho-eEF2 which was diluted 1:2000), then serially washed in TBS-T, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in blocking buffer for 1 h, and again serially washed in TBS-T. The HRP activity was detected using enhanced chemiluminescence reagent (Femtoglow HRP Substrate Plus; Michigan Diagnostics, Troy, MI, USA) and exposure to autoradiographic film (Classic Blue Sensitive; Midwest Scientific, St Louis, MO, USA). The film was scanned and antigen concentration was then calculated by quantification of the integrated optical density (IOD) of the appropriate band(s) using Gel Pro Analyser software (Media Cybernetics, Silver Spring, MD, USA). For rpS6 and eEF2, blots were first probed with the phospho-specific antibody, stripped with 0.2 M NaOH at room temperature for 5 min, then reprobed with the antibody that detects total protein levels. For S6k, blots were probed with the total-S6k antibody, stripped, probed for phospho-Thr^{389} S6k, stripped again, and probed for phospho-Thr^{421}/Ser^{424} S6k. Because mTOR protein detection was not effective after stripping, phospho- and total mTOR values were obtained from separate blots using the exact same homogenate-loading buffer preparations and exact same lane loading. IODs were normalized to the sham-operated condition of the young adult animals. Relative phosphorylation for all proteins except 4E-BP1 was determined by normalizing the IOD for the phospho-protein to the IOD for the corresponding total protein. Phosphorylation of 4E-BP1 was expressed as the percentage of the total 4E-BP1 signal that was encompassed in the slower-migrating, hyperphosphorylated β and γ bands.

To verify that the unilateral ablation procedure did not alter signalling in the contralateral 'control' (sham-operated) muscles, we also compared the signalling status in the plantaris from the sham-operated leg of the YA animals with the plantaris muscles from a separate group of YA animals ($n = 3$) in which no ablation or sham surgery had been previously performed. No differences in protein concentration or phosphorylation were observed between the above groups for any of the measured proteins (data not shown). Furthermore, as previously reported (Thomson & Gordon, 2005), no AMPK protein concentration or phosphorylation differences existed in sham-operated plantaris muscles compared to plantaris muscles from animals experiencing no prior gastrocnemius ablation surgery. These results indicate that the unilateral ablation procedure did not alter the measured signalling proteins in contralateral sham-operated muscles.

Statistics

Group comparisons were made using analyses of variance, with repeated measures where appropriate, and Fisher's LSD *post hoc* comparisons when necessary. Where calculated, average responsiveness for YA and O animals to the overload stimulus (percentage change from sham muscle to overloaded muscle within each individual animal) was compared using Student's *t* test. All correlations were calculated as Pearson product–moment correlation coefficients. All values are expressed as means ± S.E.M., and statistical significance was set at a level of $P \leq 0.05$.

Results

Muscle wet weights and total protein contents

All of the muscle wet weight and total protein content results have been reported previously (Thomson & Gordon, 2005). Control plantaris muscles from O rats were significantly atrophied compared to their YA counterparts both in terms of wet weight (321.5 ± 6.7 *versus* 383.7 ± 12.8 mg) and of protein content (74.0 ± 5.5 *versus* 109.7 ± 6.5 mg). Overloaded plantaris muscles were also smaller in O than YA animals (wet weight, 352.9 ± 11.2 *versus* 497.3 ± 17.7 mg; protein content, 78.4 ± 6.6 *versus* 130.8 ± 8.7 mg). Although the increase in wet weight and protein content with overload was significant in both YA and O muscles, this hypertrophy (expressed as percentage increase) was significantly diminished in O compared with YA muscles (wet weight, 9.7 ± 2.3 *versus* 30.0 ± 4.3%; protein content, 5.6 ± 1.7 *versus* 19.3 ± 2.9).

Concentration and phosphorylation of Akt

Phosphorylation of Akt at Ser^{473} is one of two phosphorylation events involved in the activation of Akt. Akt phosphorylation status (phospho-Akt/total Akt) at Ser^{473} was significantly lower in O control muscles than in YA control muscles, and decreased with overload in YA muscles, while no such decrease relative to control was observed with overload in O muscles (Fig. 1*A*). The absolute concentration of phospho-Akt (in contrast to the Akt phosphorylation status relative to total Akt as reported above) was elevated with overload in YA and O muscles without any age-related differences (Fig. 1*B*). Total Akt protein concentration was elevated in O compared with YA muscles, regardless of loading status, and was higher in overloaded muscles regardless of age (Fig. 1*C*).

Concentration and phosphorylation of mTOR

While it is not clear what impact mTOR phosphorylation at Ser^{2448} has upon its actual activity (Sekulic *et al.*

2000), it is generally thought to be stimulatory (Nave *et al.* 1999). The phosphorylation status of mTOR (phospho-mTOR/total mTOR) at Ser^{2448} was significantly elevated after overload in YA muscles, while no such increase relative to control was observed with overload in O muscles (Fig. 2*A*). Furthermore, although the absolute concentrations of phospho-mTOR and total mTOR were elevated after overload in both age groups (Fig. 2*B* and *C*), the average percentage increase in phospho-mTOR concentration from control to overloaded plantaris muscles was significantly impaired in O *versus* YA rats (88 ± 51 *versus* $292 \pm 74\%$, respectively; $P = 0.042$).

Concentration and phosphorylation of S6k

Activation of S6k requires phosphorylation at several sites. It is generally thought that phosphorylation at Thr^{421}/Ser^{424} (among other sites in a C-terminal autoinhibitory domain) through poorly understood mechanisms occurs early in the activation process and contributes to the 'opening up' of the protein and exposure of other sites, including Thr^{389}, for subsequent phosphorylation (Pullen & Thomas, 1997). Of the various phosphorylation sites, Thr^{389} is the main site regulated downstream of mTOR activity (Pearson *et al.* 1995) and is most indicative of *in vivo* S6k activity (Weng *et al.* 1998). Phosphorylation status of S6k (phospho-S6k/total S6k) at Thr^{389} (Fig. 3*A*) and Thr^{421}/Ser^{424} (Fig. 3*B*) increased with overload in YA and O muscles compared with control muscles. While no age-related differences were observed for S6k phosphorylation status at Thr^{421}/Ser^{424}, S6k phosphorylation status at Thr^{389} was lower in overloaded O muscles than in overloaded YA muscles. Differences in absolute concentrations of phospho-S6k phosphorylated at Thr^{421}/Ser^{424} and Thr^{389} were similar to those reported above for S6k phosphorylation status, with O rats demonstrating an impaired phosphorylation response to overload at Thr^{389} but not at Thr^{421}/Ser^{424} (Fig. 3*C* and *D*). Furthermore, absolute concentrations

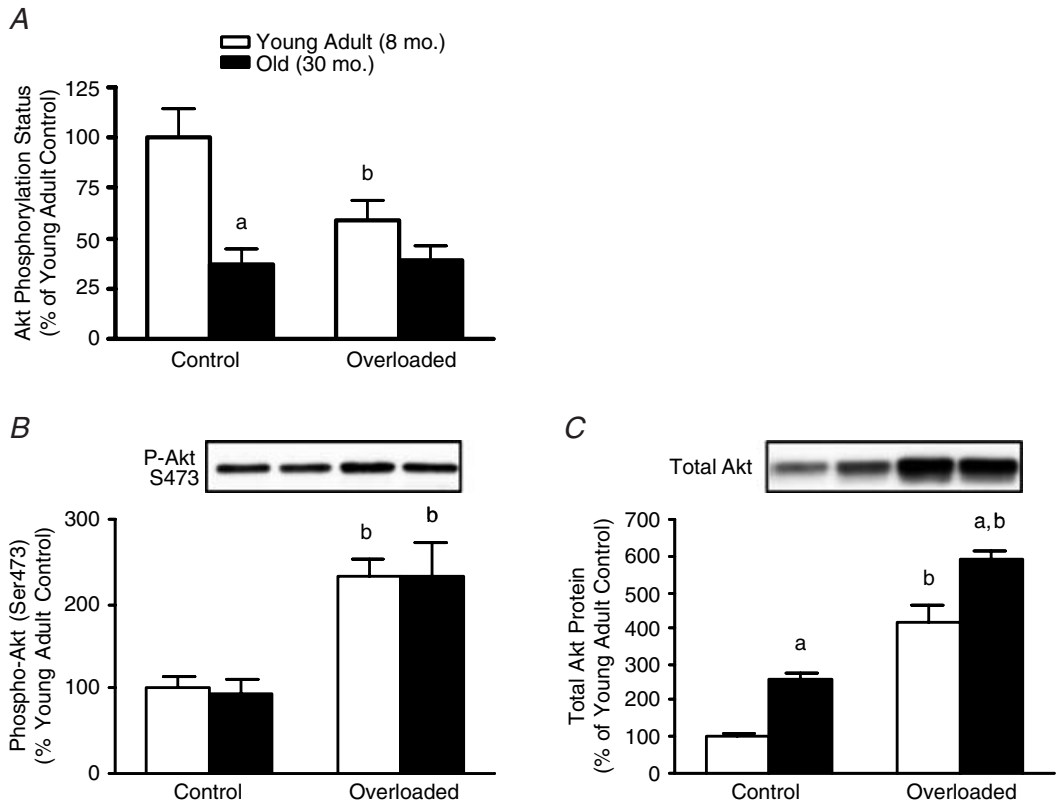

Figure 1. Total and phosphorylated Akt response to overload in young adult and old skeletal muscle
A, plantaris Akt phosphorylation status (phospho-Akt integrated optical density (IOD)/total Akt IOD) at Ser^{473} is lower in old (O; 30 months; $n = 7$) than young adult (YA; 8 months; $n = 7$) control muscles, and is lower after 1 week of functional overload (unilateral gastrocnemius ablation) in YA, but not in O, rats. *B*, plantaris phospho-Akt concentration is elevated after overload regardless of age. *C*, plantaris total Akt protein concentration is greater in O compared with YA rats regardless of overload status and is elevated after overload regardless of age. Data (means ± S.E.M.) are expressed as percentages of YA control (sham-operated) values. [a]Significant ($P \leq 0.05$) effect of age, [b]significant effect of overload.

of phospho-S6k phosphorylated at Thr^{389} in the overloaded plantaris were significantly correlated with the degree of hypertrophy (as measured by total muscle protein content) elicited by overload across all animals ($r = 0.59$, $P = 0.03$; Fig. 7*A*), and negatively correlated with the phosphorylation status of AMPK (Thr^{172}, AMPK data previously reported Thomson & Gordon, 2005) in the same muscle ($r = -0.59$, $P = 0.03$; Fig. 7*B*). Neither the degree of hypertrophy nor AMPK phosphorylation status, was correlated with S6k phosphorylated at Thr^{421}/Ser^{424} ($r = 0.13$ and -0.18, respectively). Total S6k concentration increased in the overloaded muscles of both YA and O rats compared with control muscles (Fig. 3*E*).

Concentration and phosphorylation of ribosomal protein S6

Activation of rpS6 is thought to consist of at least five phosphorylation events, in a stepwise fashion beginning with the phosphorylation of Ser^{236} and then Ser^{235} by S6k (Martin-Perez & Thomas, 1983). Phosphorylation status of rpS6 (phospho-rpS6/total rpS6) at Ser$^{235/236}$ was elevated with overload, regardless of age, and was significantly lower in O muscles than in YA muscles independent of overload status (Fig. 4*A*). Both the absolute concentration of phospho-rpS6 and total rpS6 protein concentration were elevated with overload independent of age (Fig. 4*B* and *C*).

Concentration and phosphorylation of eEF2

Phosphorylation of eEF2 at Thr^{56} is inhibitory on its activity (Browne & Proud, 2002). No significant effects of age or overload were observed on eEF2 phosphorylation status (phospho-eEF2/total eEF2) at Thr^{56} (Fig. 5*A*), although there was a non-significant trend for eEF2 phosphorylation status to increase with overload in O muscles ($P = 0.13$). Absolute concentrations

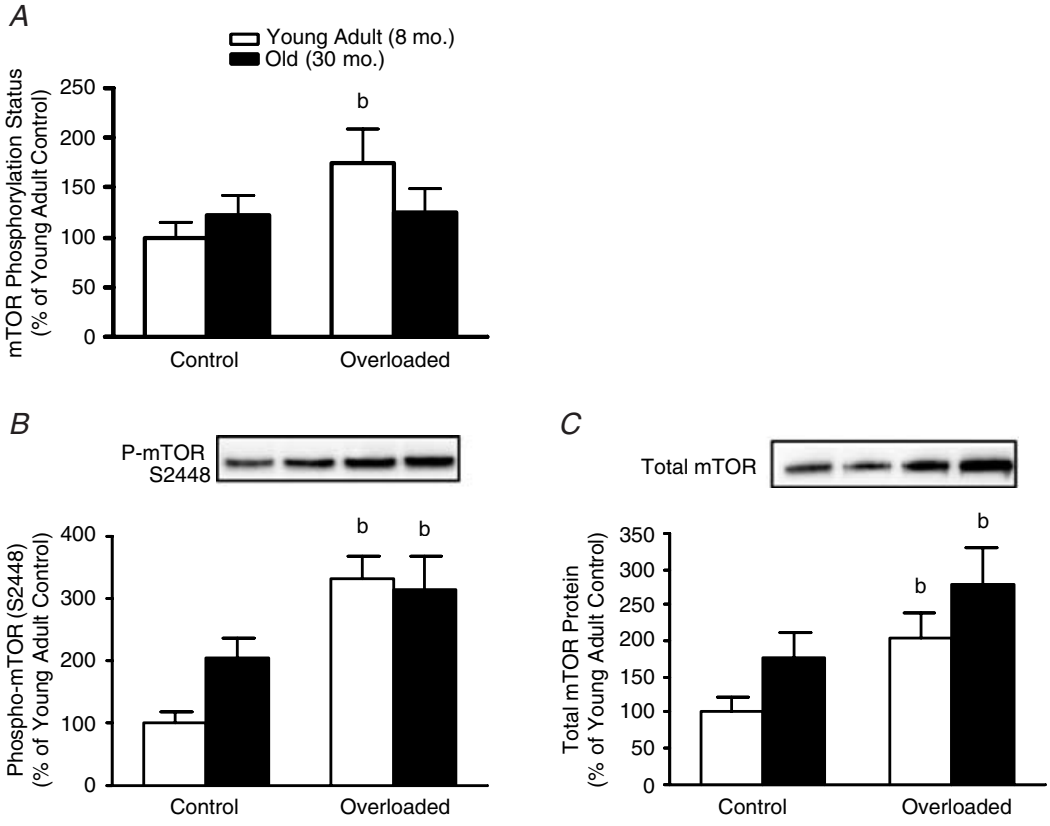

Figure 2. Total and phosphorylated mTOR response to overload in young adult and old skeletal muscle
A, plantaris mammalian target of rapamycin (mTOR) phosphorylation status (phospho-mTOR IOD/total mTOR IOD) at Ser^{2448} is elevated after 1 week of functional overload compared to control (sham-operated) conditions in YA ($n = 7$), but not O ($n = 7$) muscles. *B*, plantaris phospho-mTOR concentration is elevated after overload regardless of age (but note that the average percentage increase in phospho-mTOR concentration from control to overloaded plantaris muscles was significantly less O *versus* YA rats, 88 ± 51 *versus* $292 \pm 74\%$, respectively; $P = 0.042$). *C*, plantaris mTOR protein concentration is elevated after overload regardless of age. Data (means ± S.E.M.) are expressed as percentages of YA control (sham-operated) values. [b]Significant ($P \leq 0.05$) effect of overload.

of phospho-eEF2 increased in the overloaded muscles in both age groups, and were also higher in O *versus* YA in both control and overloaded muscles (Fig. 5*B*). Interestingly, total eEF2 concentration increased with overload in YA, but not O muscles (Fig. 5*C*). The average intra-animal increase of total eEF2 concentration in the overloaded plantaris relative to that of the control leg was also significantly greater for YA rats than for O rats (38.9 ± 9.8 *versus* $5.6 \pm 5.5\%$, respectively; $P = 0.01$). Additionally, this percentage increase in total eEF2 protein concentration was strongly correlated across ages with the percent hypertrophy observed in the plantaris ($r = 0.84$, $P = 0.0002$; Fig. 7*C*), and negatively correlated with the phosphorylation status (Thr^{172}) of AMPK in these

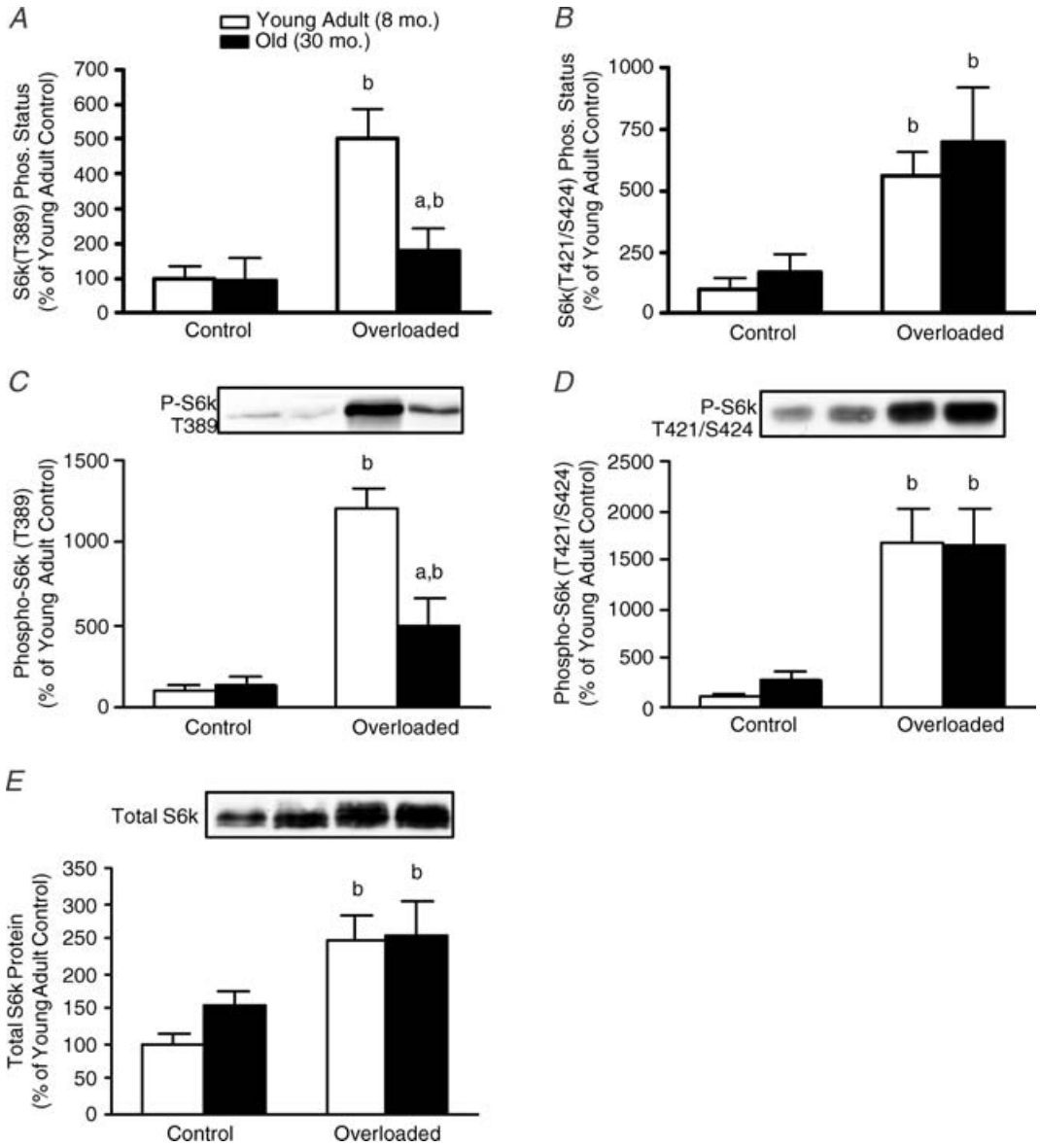

Figure 3. Total and phosphorylated S6k response to overload in young adult and old skeletal muscle
A, plantaris 70 kDa ribosomal protein S6 kinase (S6k) phosphorylation status (phospho-S6k IOD/total S6k IOD) at Thr^{389} is elevated after 1 week of functional overload compared with control (sham-operated) conditions in YA ($n = 7$) and O ($n = 7$) muscles, but is lower after overload in O than in YA muscles. *B*, plantaris S6k phosphorylation status at Thr^{421}/Ser^{424} is elevated after overload compared with control (sham-operated) conditions regardless of age. *C*, plantaris phospho-S6k (Thr^{389}) concentration is elevated after overload in YA and O muscles, but is lower after overload in O than in YA muscles. *D*, plantaris phospho-S6k (Thr^{421}/Ser^{424}) concentration is elevated after overload regardless of age. *E*, plantaris total S6k protein is elevated after overload regardless of age. Data (means ± S.E.M.) are expressed as percentages of YA control (sham-operated) values. [a]Significant ($P \leq 0.05$) effect of age, [b]significant effect of overload.

overloaded plantaris muscles ($r = -0.63$, $P = 0.02$; Fig. 7*D*) (AMPK data previously reported by Thomson & Gordon, 2005).

Concentration and phosphorylation of 4E-BP1

4E-BP1 phosphorylation status (phospho-4E-BP1/total 4E-BP1) was increased in the overloaded muscles of both YA and O rats, but was significantly lower with overload in the O than in the YA muscles (Fig. 6*A*). Total 4E-BP1 protein concentration was increased with overload but was not different between YA and O muscles (Fig. 6*B*). Phosphorylation of 4E-BP1 occurs on at least seven sites (Wang *et al.* 2005) which leads to its dissociation from the translation initiation factor eIF4E and therefore promotes enhanced cap-dependent protein translation. Though additional kinases are necessary for full phosphorylation and activation of 4E-BP1, phosphorylation of the protein by mTOR is thought to be crucial in the initial events that 'prime' 4E-BP1 for phosphorylation by other proteins (Gingras *et al.* 1999), and may regulate later phosphorylation events as well (Wang *et al.* 2005). In this investigation, the phosphorylation status of 4E-BP1 was calculated as the percentage of the total 4E-BP1 that was encompassed in the slower-migrating, hyperphosphorylated β and γ bands (Fig. 6*B*). Although the true relationship between each specific hyperphosphorylated 4E-BP1 band and resultant 4E-BP1 activity has not been definitively established to our knowledge (e.g. it is not known which bands are representative of increased eIF4E activity), it is generally believed that 4E-BP1 hyperphosphorylation results in increased dissociation from eIF4E and thus increased eIF4E activation and translation initiation (Gingras *et al.* 1999; Shah *et al.* 2000). Accordingly, we observed that the phosphorylation of 4E-BP1 (percentage of 4E-BP1 signal encompassed in the β and γ bands) in the overloaded plantaris muscles was positively correlated with percentage plantaris hypertrophy across all animals ($r = 0.66$, $P = 0.01$; Fig. 7*E*), and negatively associated with the phosphorylation status of AMPK (Thr^{172}, AMPK data previously reported Thomson & Gordon, 2005) in the same muscle (strong but non-significant trend: $r = -0.53$, $P = 0.051$; Fig. 7*F*).

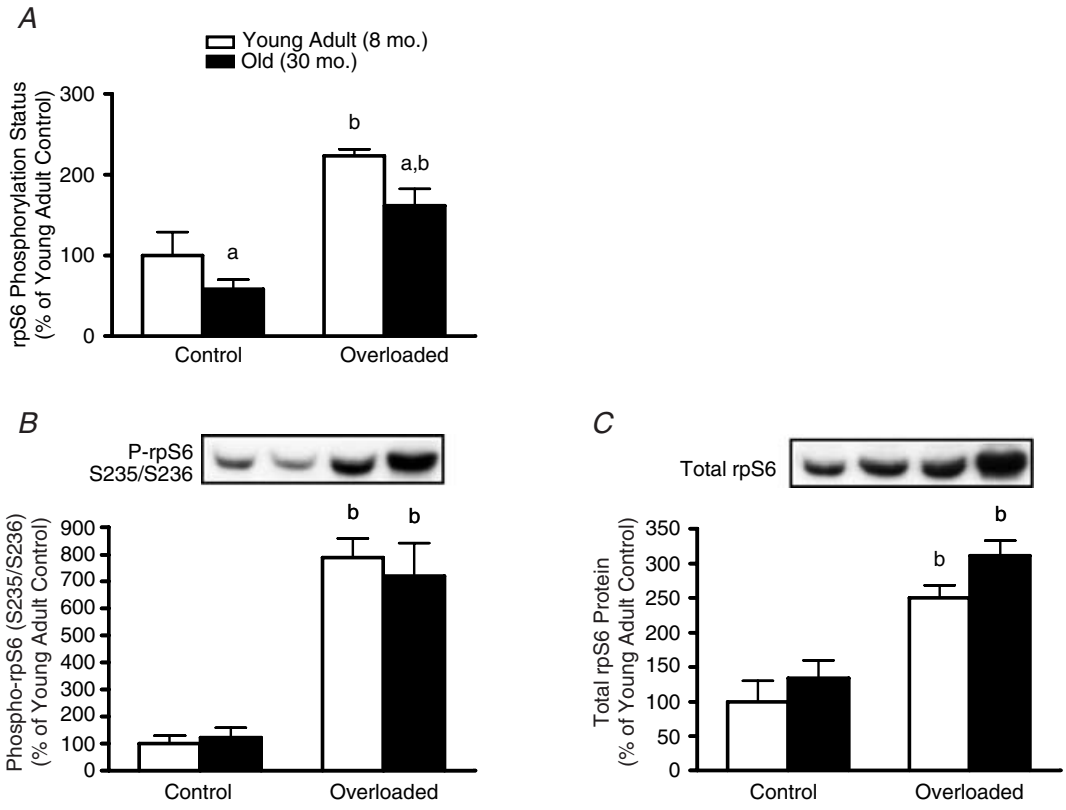

Figure 4. Total and phosphorylated rpS6 response to overload in young adult and old skeletal muscle
A, plantaris ribosomal protein S6 (rpS6) phosphorylation status (phospho-rpS6 IOD/total rpS6 IOD) at $Ser^{235/236}$ is lower in O ($n = 7$) than YA ($n = 7$) rats and is elevated after 1 week of functional overload regardless of age. *B*, plantaris phospho-rpS6 concentration is elevated after overload regardless of age. *C*, plantaris total rpS6 concentration is elevated after overload regardless of age. Data (means ± S.E.M.) are expressed as percentages of YA control (sham-operated) values. [a]Significant ($P \leq 0.05$) effect of age, [b]significant effect of overload.

Discussion

We and others have previously shown that fast-twitch muscle protein synthesis (Pehme *et al.* 2004*b*) and growth (Blough & Linderman, 2000; Alway *et al.* 2002; Degens & Alway, 2003; Pehme *et al.* 2004*b*; Thomson & Gordon, 2005) are reduced in old rats compared with young rats after ablation, tenotomy or denervation of a synergistic muscle. It is doubtful that these results solely reflect a delayed response with age, as some have observed highly diminished growth in aged muscle (despite robust growth in young muscle) by using the same model as that used in the current investigation over a much longer overload period (8 weeks *versus* 1 week) (Blough & Linderman, 2000). Because of their importance in the hypertrophic response of skeletal muscle to chronic overload (Baar & Esser, 1999; Bodine *et al.* 2001), we compared the contents and phosphorylation states of mTOR and several of its downstream targets in YA and O rat fast-twitch plantaris muscle after 1 week of an overload-induced growth stimulus. Ours is the first report of deficits in the phosphorylation states of mTOR, S6k, rpS6 and 4E-BP1, as well as deficits in the accretion of eEF2 total protein, in conjunction with impaired overload-induced growth in aged fast-twitch skeletal muscle. Moreover, when compared with previously reported AMPK data from the same animals (Thomson & Gordon, 2005), our results also indicate that this age-related suppression of fast-twitch muscle translational signalling under chronic overload conditions may be at least partly related to AMPK hyperactivation.

Our current results confirm those of others (Bodine *et al.* 2001; Reynolds *et al.* 2002), who demonstrated the importance of mTOR-S6k signalling in the hypertrophic response of chronically overloaded young muscle. However, we now extend upon those results to show site-specific increases in S6k phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} as well as an increase in rpS6 phosphorylation at $Ser^{235/236}$ with overload. We also show that S6k phosphorylation at Thr^{389} (an mTOR-specific residue; Pearson *et al.* 1995; Burnett *et al.* 1998) was significantly correlated with degree of muscle hypertrophy in overloaded muscles, while phosphorylation at Thr^{421}/Ser^{424} was not. Our data further elucidate findings that increased

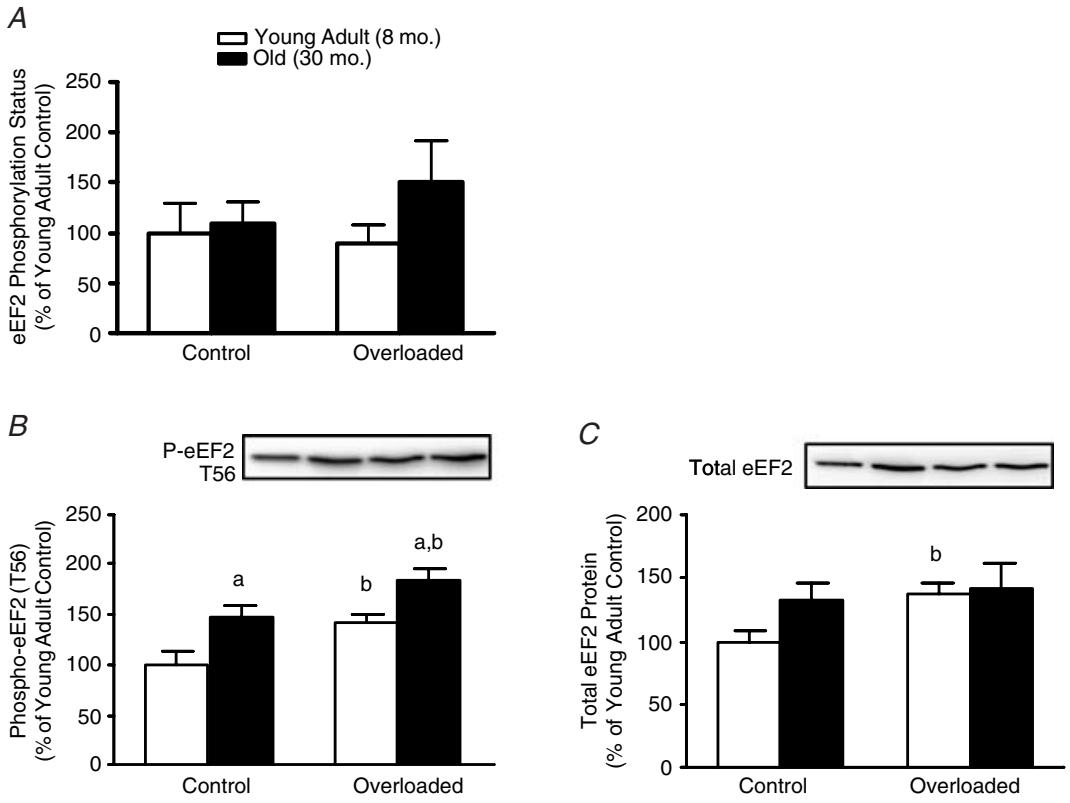

Figure 5. Total and phosphorylated eEF2 response to overload in young adult and old skeletal muscle
A, plantaris eukaryotic elongation factor 2 (eEF2) phosphorylation status (phospho-eEF2 IOD/total eEF2 IOD) at Thr^{56} is not statistically different between O ($n = 7$) and YA ($n = 7$) rats or changed with 1 week of functional overload. *B*, plantaris phospho-eEF2 concentration is greater in O compared with YA rats regardless of overload status and is elevated after overload regardless of age. *C*, plantaris eEF2 protein concentration is elevated after overload in YA, but not O rats. Data (means ± S.E.M.) are expressed as percentages of YA control (sham-operated) values. [a]Significant ($P \leq 0.05$) effect of age, [b]significant effect of overload.

S6k phosphorylation at unspecified residues (multiple bands assessed by gel shifts) is significantly correlated with muscle hypertrophy elicited by chronic electrical stimulation (Baar & Esser, 1999). The fact that the mTOR (Parkington *et al.* 2003), S6k (Baar & Esser, 1999), and hypertrophy (Baar & Esser, 1999) responses to electrical stimulation are all greater in fast-twitch (*versus* slow-twitch) muscle further confirms the importance of mTOR-S6k signalling in mechanically induced skeletal muscle growth. Not surprisingly, mTOR and/or S6k phosphorylations are decreased during unloading- or immobilization-induced atrophy in young adult (Bodine *et al.* 2001; Reynolds *et al.* 2002) and old (Morris *et al.* 2004) muscle, although others have reported no effect of immobilization on S6k phosphorylation in young adult muscle (Childs *et al.* 2003).

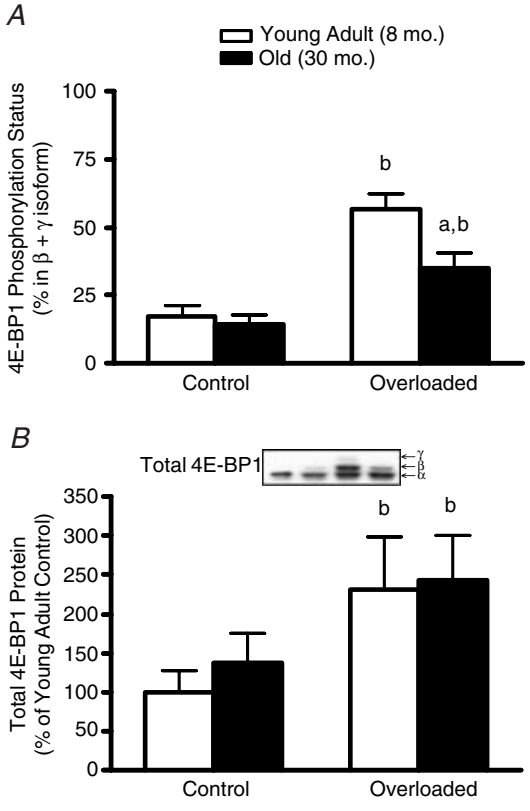

Figure 6. Total and phosphorylated 4E-BP1 response to overload in young adult and old skeletal muscle
A, plantaris eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) phosphorylation status (expressed as a percentage of total 4E-BP1 signal encompassed in the slower-migrating, hyperphosphorylated β and γ bands) is elevated after 1 week of functional overload compared with control (sham-operated) conditions in YA ($n = 7$) and O ($n = 7$) muscles, but is lower after overload in O than in YA muscles. *B*, plantaris 4E-BP1 total protein concentration is elevated after overload regardless of age. Data (means ± S.E.M.) are expressed as percentages of YA, control (sham-operated) values. [a]Significant ($P \leq 0.05$) effect of age, [b]significant effect of overload.

In stark contrast to the anabolic response of mTOR, S6k and rpS6 to overload in young adult muscle, the phosphorylation states of mTOR, S6k (at Thr^{389} only), and rpS6 were significantly lower in the overloaded muscles of O compared with YA rats (although S6k and rpS6 phosphorylation states were still elevated above O control muscle). The attenuated phosphorylation responses of mTOR and S6k (Thr^{389}) to chronic overload in the present investigation agree with the finding that S6k phosphorylation at Thr^{389} is attenuated in old slow-twitch muscle when stimulated with reloading after immobilization (Morris *et al.* 2004), as well as reports that mTOR/S6k responsiveness may potentially be reduced after acute contractions in old fast-twitch muscle (Parkington *et al.* 2003, 2004). The present investigation further advances the developing paradigm that mTOR dysregulation (as opposed to other signalling pathways) may play a central role in impaired translational signalling in ageing skeletal muscle, because the diminished responsiveness to muscle overload of S6k phosphorylation at Thr^{389} (but not at Thr^{421}/Ser^{424}) agrees with the fact that mTOR phosphorylation in response to overload was also diminished with age. Of the various sites, Thr^{389} is most indicative of *in vivo* S6k activity (Weng *et al.* 1998; Isotani *et al.* 1999) and is the primary site phosphorylated by mTOR (Pearson *et al.* 1995; Burnett *et al.* 1998). This is further supported by our finding that S6k phosphorylation at Thr^{389} (but not at Thr^{421}/Ser^{424}) was correlated to the hypertrophic response in overloaded muscles. Finally, the potential for diminished rpS6 signalling with age in overloaded skeletal muscle has not been previously examined to our knowledge. Interestingly, the fact that rpS6 phosphorylation at $Ser^{235/236}$ was reduced with age in both overloaded and control muscles (while upstream S6k Thr^{389} phosphorylation was only reduced in overloaded muscles) indicates that resting muscle rpS6 phosphorylation at $Ser^{235/236}$ may also be regulated by mechanisms other than S6k, similar to other tissue types (Martin-Perez & Thomas, 1983; Pende *et al.* 2004). Nevertheless, our results collectively indicate that impaired S6k activation by mTOR (but perhaps not S6k activation by other pathways) may be a major factor underlying the diminished protein synthesis and growth with age in overloaded fast-twitch skeletal muscle (Pehme *et al.* 2004*b*).

In the young adult animals, the overload-induced increase in total eEF2 protein concentration in the absence of a concomitant increase in eEF2 relative phosphorylation status (per total eEF2 protein) at Thr^{56} (which is inhibitory upon eEF2 activity; Browne & Proud, 2002) appears to indicate an increased drive for protein elongation because of the presumed resultant increase in unphosphorylated (active) eEF2. On the contrary, old muscles demonstrated no such anabolic increase in total eEF2 content. Further, old animals demonstrated a

higher phospho-eEF2 content *versus* young adult animals regardless of overload status as well as a slight (albeit non-significant; $P = 0.13$) overload-induced increase in eEF2 phosphorylation status (relative to total eEF2). The higher eEF2 phosphorylation in aged fast-twitch muscle is interesting, because S6k normally acts to inhibit eEF2 phosphorylation (and thus increase its activity) through inhibition of eEF2 kinase (eEF2k) (Wang *et al.* 2001; Browne & Proud, 2004). Others have recently reported an acute increase in eEF2 phosphorylation at

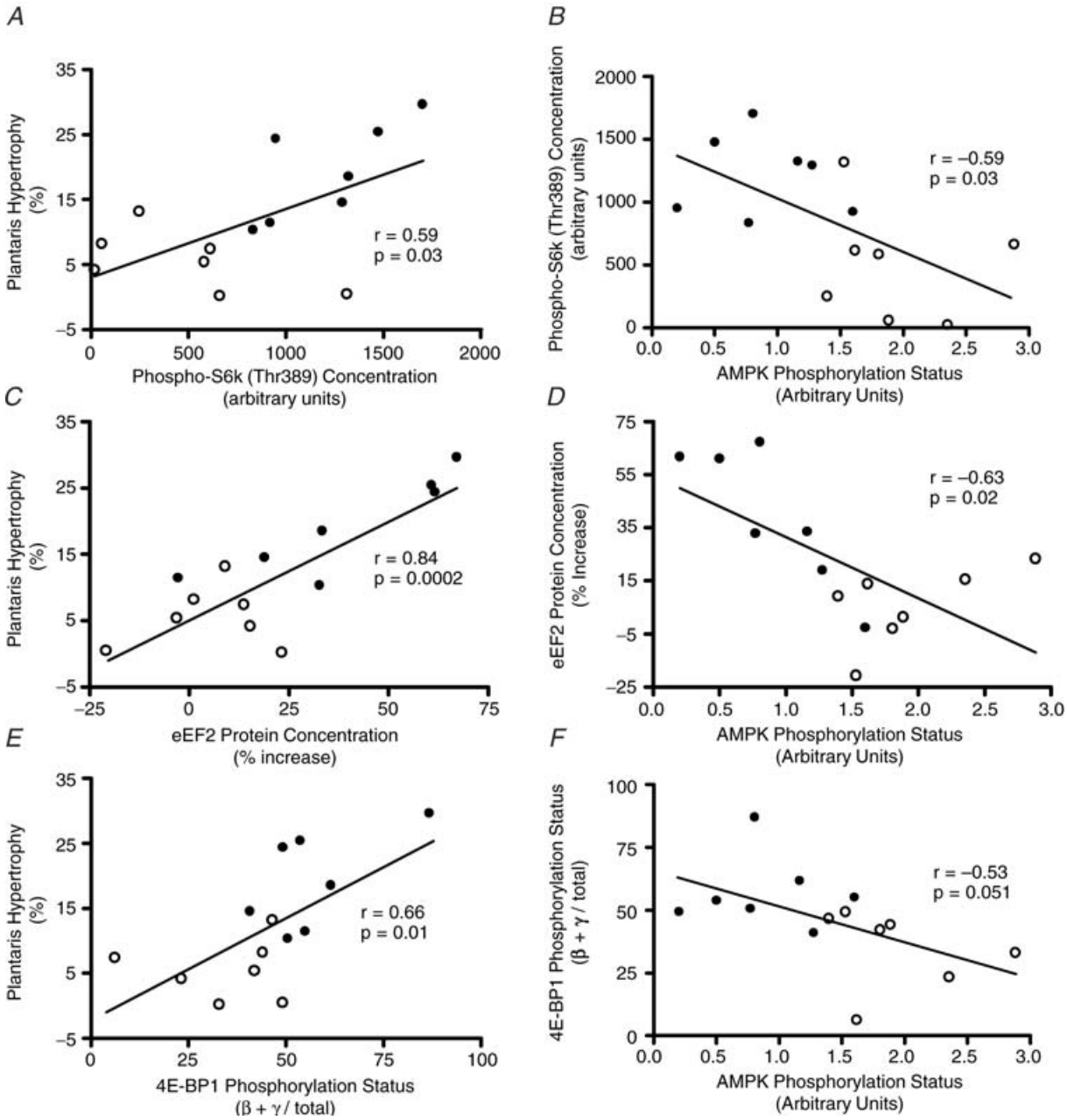

Figure 7. Relationships of selected variables to muscle hypertrophy and AMPK phosphorylation status
A and *B*, phospho-S6k (Thr^{389}) content is positively correlated with percentage hypertrophy, and negatively correlated with 5′-AMP-activated protein kinase (AMPK) phosphorylation status (Thr^{172}) in overloaded fast-twitch plantaris muscles of YA (●) and O (○) rats ($n = 7$ per group). Note that no such correlations existed between phospho-S6k at Thr^{421}/Ser^{424} and either AMPK phosphorylation status or percentage hypertrophy. *C* and *D*, accretion of eEF2 protein content is positively correlated with percentage hypertrophy, and negatively correlated with AMPK phosphorylation status in overloaded fast-twitch plantaris muscles of young adult and old rats. *E* and *F*, phosphorylation of 4E-BP1 (percentage of 4E-BP1 signal encompassed in the β and γ bands) is positively correlated with percentage hypertrophy, and negatively associated ($P = 0.051$) with AMPK phosphorylation status in overloaded fast-twitch plantaris muscles of YA and O rats.

Thr^{56} in response to aerobic exercise (Rose *et al.* 2005), which does not elicit hypertrophy; however, the current investigation is the first to examine the response of eEF2 to hypertrophic skeletal muscle overload. The increased total eEF2 content in young but not aged fast-twitch muscles in the current investigation suggests that total eEF2 may play an important role in the anabolic response of skeletal muscle to overload. Accordingly, we found a strong significant correlation between the increase in eEF2 total protein concentration and the degree of hypertrophy in the overloaded muscles. Notably, some (Terada *et al.* 1994; Jefferies *et al.* 1997), but not all (Stolovich *et al.* 2002; Pende *et al.* 2004), studies have also linked S6k and rpS6 to the selective translation of mRNAs with a 5′-terminal polypyrimidine (5′TOP) tract, which encode ribosomal proteins and elongation factors including eEF2. Thus, the loss of an overload-induced increase in eEF2 total protein concentration in old muscles could be attributable to their diminished mTOR/S6k/rpS6 signalling. More research is warranted to examine the possibility that eEF2 content is upregulated by mTOR/S6k/rpS6 signalling in overloaded young fast-twitch muscle, as well as the intriguing possibility that this response is lost in old muscle due to diminished mTOR/S6k/rpS6 signalling.

Similar to mTOR and S6k (Thr^{389}) phosphorylation, the 4E-BP1 phosphorylation response to overload, though still significant, was greatly reduced in our aged muscles compared to those of young adult animals. These findings further suggest that mTOR signalling is deficient in old fast-twitch muscles under overload conditions, as both S6k (Thr^{389}) and 4E-BP1 lie independently downstream of mTOR and are considered reliable indicators of *in vivo* mTOR activity (Hay & Sonenberg, 2004). Because it binds to eIF4E and prevents it from forming the eIF4F initiation complex with eIF4A and eIF4G, unphosphorylated 4E-BP1 is an important regulatory protein inhibiting translation initiation (Nader *et al.* 2002; Bolster *et al.* 2003*a*). The fact that overload elicited an increased 4E-BP1 phosphorylation status in our young adult muscles (resulting from a greater increase in phospho-4E-BP1 relative to total 4E-BP1) indicates a potential freeing of eIF4E to participate in eIF4F formation and initiation of protein translation in 7 day overloaded plantaris muscles of young adult rats. These results agree with observations of decreased 4E–BP1–eIF4E association and increased eIF4E–eIF4G association in overloaded plantaris muscles of young adult rats 14 days after synergist ablation, effects that were both blocked by rapamycin inhibition of mTOR (Bodine *et al.* 2001). Thus, increased 4E-BP1 phosphorylation in overloaded young adult fast-twitch muscle appears to be mTOR-dependent, which may explain the reduced 4E-BP1 phosphorylation (concomitant with diminished mTOR phosphorylation) in response to overload in our old muscles. Our current data also further show that 4E-BP1 phosphorylation in overloaded muscles is significantly correlated with hypertrophy in those muscles, and implicate the impaired 4E-BP1 phosphorylation response to muscle overload in old animals as another potential mechanism underlying the diminished growth observed in aged fast-twitch muscle.

In our overloaded muscles, the age-related impairments in the phosphorylation states of mTOR and downstream translational signalling intermediates (S6k, rpS6 and 4E-BP1), along with deficits in eEF2 protein accretion, indicate that mTOR dysregulation may play a primary role on several levels in the diminished protein synthesis and growth response to mechanical loading in aged fast-twitch muscle (Tamaki *et al.* 2000; Pehme *et al.* 2004*b*; Thomson & Gordon, 2005). However, the upstream mediator(s) in aged fast-twitch muscle that may lead to impaired mTOR signalling under such conditions remain to be identified. To this end, we chose to examine the potential relationship of this diminished translational signalling to AMPK, which is known to inhibit S6k, 4E-BP1 and eEF2 signalling and protein synthesis in resting young skeletal and/or cardiac muscle (Bolster *et al.* 2002; Horman *et al.* 2002; Chan *et al.* 2004), probably via its inhibition of mTOR (Cheng *et al.* 2004) as well as its mTOR-independent inhibition of eEF2 via eEF2 kinase (Browne *et al.* 2004). AMPK is activated by cellular energy depletion, which is exacerbated in old *versus* young skeletal muscle at rest and with exercise (Bastien & Sanchez, 1984; Marcinek *et al.* 2005). Accordingly, we recently reported (using the same animals and muscles as in the current study) that AMPK phosphorylation (Thr^{172}) is significantly elevated with age in resting and overloaded plantaris muscles (Thomson & Gordon, 2005). Moreover, there was a strong negative correlation between the degree of AMPK phosphorylation and the degree of hypertrophy in the overloaded muscles, implicating AMPK as a potentially important negative regulator of overload-induced skeletal muscle hypertrophy (Thomson & Gordon, 2005).

Correlational results in the current investigation raise the possibility that AMPK hyperphosphorylation may be a potential upstream mechanism by which translational signalling and overload-induced growth is suppressed in aged fast-twitch muscles. Using AMPK data previously obtained from the same muscles (Thomson & Gordon, 2005), we found that AMPK phosphorylation state was negatively correlated with the amount of S6k phosphorylated at the mTOR-specific Thr^{389} residue, but not at Thr^{421}/Ser^{424}. This fits with previously reported data showing that AMPK activation attenuates amino-acid-stimulated phosphorylation of S6k at Thr^{389}, but not at Thr^{421}/Ser^{424} (Krause *et al.* 2002; Moller *et al.* 2004). Similar to S6k, a negative relationship ($P = 0.051$) was also observed between the phosphorylation statuses of AMPK and 4E-BP1. Because S6k (Thr^{389}) and 4E-BP1 are

both independent targets of mTOR (Hay & Sonenberg, 2004), it is possible that AMPK inhibited S6k (Thr^{389}) and 4E-BP1 phosphorylation in these muscles via its reported suppression of mTOR phosphorylation (Bolster *et al.* 2002). Additionally, the negative correlation between AMPK phosphorylation status and the percentage increase in eEF2 content in the overloaded muscles points to the possibility that AMPK may also somehow suppress total eEF2 protein accretion, possibly by suppressing S6k and its specific targeting of 5′TOP mRNA translation (Terada *et al.* 1994; Jefferies *et al.* 1997), which includes eEF2. Overall, such correlative evidence is intriguing when taken in concert with the fact that AMPK is hyperphosphorylated in aged fast-twitch muscle (Thomson & Gordon, 2005) and is also known to negatively regulate translational signalling and protein synthesis in resting skeletal muscle (Bolster *et al.* 2002). Nevertheless, more research is warranted to establish any potential causal relationship between AMPK hyperactivation and suppressed translational signalling and growth with overload in aged fast-twitch muscles.

In conclusion, here we have reported evidence of impaired phosphorylation states of mTOR and its downstream translational signalling intermediates S6k (specifically at the mTOR-specific Thr^{389} residue but not at Thr^{421}/Ser^{424}), rpS6, and 4E-BP1 in overloaded fast-twitch skeletal muscles of old rats, as well as deficits in the accretion of eEF2 total protein in the same muscles (further indicating impaired S6k and rpS6 signalling). These findings are novel, as they are the first to demonstrate impaired translational signalling in direct conjunction with diminished overload-induced growth in aged skeletal muscle. Moreover, our data also raise the interesting possibility that AMPK hyperactivation may lie upstream of this suppressed translational signalling with age in overloaded fast-twitch muscle, perhaps acting via an mTOR-dependent mechanism. Further work is necessary to establish the causality of AMPK in this respect. Regardless of the upstream mechanism(s), the results of this investigation provide solid evidence that the impaired overload-induced growth observed in old fast-twitch muscle may be closely related to underlying decrements in signalling pathways controlling protein translation.

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