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Acute resistance exercise activates rapamycin-sensitive and insensitive mechanisms that control translational activity and capacity in skeletal muscle

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Running title: Translational activity and capacity in skeletal muscle

Key points summary

- Ribosome biogenesis is the primary determinant of translational capacity, but its regulation in skeletal muscle following acute resistance exercise is poorly understood
- Resistance exercise increases muscle protein synthesis acutely, and muscle mass with training, but the role of translational capacity in these processes is unclear
- Here, we show that acute resistance exercise-activated pathways controlling translational activity and capacity through both rapamycin-sensitive and -insensitive mechanisms
- Transcription factor c-Myc and its downstream targets, which are known to regulate ribosome biogenesis in other cell types, were upregulated after resistance exercise in a rapamycin-independent manner and may play a role in determining translational capacity in skeletal muscle
- Local inhibition of myostatin was also not affected by rapamycin and may contribute to the rapamycin-independent effects of resistance exercise.

Abstract. This study aimed to determine i) the effect of acute resistance exercise on mechanisms of ribosome biogenesis, and ii) the impact of mammalian target of rapamycin on ribosome biogenesis, and muscle protein synthesis (MPS) and degradation. Female F344BN rats underwent unilateral electrical stimulation of the sciatic nerve to mimic resistance exercise in the tibialis anterior (TA) muscle. TA muscles were collected at intervals over the 36 h of exercise recovery (REx); separate groups of animals were administered rapamycin pre-exercise (REx+Rapamycin). Resistance exercise led to a prolonged (6 to 36 hours) elevation (30-50%) of MPS that was fully blocked by rapamycin at 6 hours but only partially at 18 h. REx also altered pathways that regulate protein homeostasis and mRNA translation in a manner that was both rapamycin-sensitive (proteasome activity; phosphorylation of S6K1 and rpS6) and insensitive (phosphorylation of eEF2, ERK1/2 and UBF; gene expression of the myostatin target Mighty as well as c-Myc and its targets involved in ribosome biogenesis). The role of c-Myc was tested *in vitro* using the inhibitor 10058-F4, which, over time, decreased basal RNA and MPS in a dose-dependent manner (correlation of RNA and MPS, $r^2 = 0.98$), even though it had no effect on the acute stimulation of protein synthesis. In conclusion, acute resistance exercise stimulated rapamycin-sensitive and -insensitive mechanisms that regulate translation activity and capacity.

Abbreviations:

AgNOR, argyrophilic proteins in nucleolar organizing regions; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ITS-1, internal transcribed spacer 1; MPB, muscle protein breakdown; MPS, muscle protein synthesis; mTORC1, mechanistic/mammalian target of rapamycin; TA, tibialis anterior; RRN3, RNA polymerase I transcription factor RRN3; TAF1B, TATA box binding protein-associated factor RNA Polymerase I B; UBF, upstream binding factor.

Introduction

Resistance exercise stimulates muscle protein synthesis and the initial increases in protein synthesis are accompanied by enhanced translational signaling. Further, there is evidence that alterations in translational signaling (e.g., through eIF2B ϵ (Mayhew *et al.*, 2011) and S6K1 (Baar & Esser, 1999)) underpin gains in muscle mass that are acquired through training. However, recently questions have been raised (Atherton & Smith, 2012; Phillips *et al.*, 2013) about the ability of relatively brief changes in individual translation signaling pathways to fully explain i) the prolonged nature of resistance exercise-induced enhancement of muscle protein synthesis (MPS) (Chesley *et al.*, 1992; Phillips *et al.*, 1997), and ii) gains in muscle mass achieved through training.

In 1967, it was reported (Hamosch *et al.*, 1967) that synergist ablation in the rat hindlimb enhanced *ex vivo* amino acid incorporation into protein using microsome fractions prepared from hypertrophied soleus muscles. Enhanced amino acid incorporation was accompanied by a robust increase in total RNA content. Given that ribosomal RNA accounts for approximately 85% of total RNA (Young, 1970), increased ribosomal mass, representing increased translational capacity, was hypothesized to be responsible for the increased rate of protein synthesis *ex vivo*. Collectively then, it follows that exercise-induced increases in MPS, and ultimately muscle mass, may be best explained by the changes in both translational activity ('ribosome activation') and translational capacity ('ribosome abundance').

Interestingly, the mechanistic/mammalian target of rapamycin complex 1 (mTORC1) is central to enhancing translation activity and also appears to be important in initiating a transcriptional program that supports ribosome biogenesis. Translationally, in response to anabolic stimuli (e.g., exercise and amino acids), the mTORC1 effectors S6K1 and 4E-BP1 are phosphorylated, resulting in the formation of a translation preinitiation complex and the initiation of protein synthesis (Holz *et al.*, 2005; Kubica *et al.*, 2005). mTORC1 also regulates ribosome mass through both a transcriptional program, upregulating ribosomal RNA synthesis and processing, and a translational program, increasing the translation of ribosomal proteins that contain a 5' terminal oligopyrimidine tract (Chauvin *et al.*, 2014). Thus, mTORC1 is a potent regulator of muscle hypertrophy (Bodine *et al.*, 2001), because of its dual role in influencing translational activity and capacity. Because S6K1 is a 'poor quality' substrate of mTORC1 (i.e., phosphorylation is more dependent on raptor (Kang *et al.*, 2013)), the drug rapamycin

completely blocks its phosphorylation at Thr389 by mTORC1; thus, rapamycin can be used as a tool to determine the impact of S6K1 in mediating responses to anabolic stimuli. Rapamycin abolishes the acute increase in muscle protein synthesis that occurs in the first few hours after resistance exercise (Drummond *et al.*, 2009), indicating that this early upregulation of MPS is mTORC1-dependent. However, the effects of rapamycin on anabolic signaling and MPS at later stages of recovery from acute resistance exercise are unclear. Therefore, an aim of the present study was to determine the impact of mTORC1 signaling on MPS in early and late recovery from acute resistance exercise. Secondly, in light of early (Hamosch *et al.*, 1967) and emerging (Chaillou *et al.*, 2014) evidence that ribosomal content plays an important role in directing anabolic responses, we aimed to determine mechanisms that regulate ribosome biogenesis after acute resistance exercise. Two targets that directly regulate ribosome biogenesis were brought to the forefront in our analysis: c-Myc and upstream binding factor (UBF). c-Myc enhances the expression of numerous genes that regulate ribosome biogenesis (Boon *et al.*, 2001), and UBF is a transcription factor that promotes ribosomal DNA transcription by RNA polymerase I (Kwon & Green, 1994; Voit & Grummt, 2001). Therefore, a secondary aim of the current work was to determine the regulation of UBF and c-Myc following resistance exercise. Lastly, we measured proteasome activity, the predominant protein degradation pathway, because net protein balance in recovery will be determined by both synthesis and breakdown, and breakdown after resistance exercise is understudied.

Materials and Methods

Animal use in acute exercise

Female Fisher 344-Brown Norway rats (10 months, $n = 5$ per group) were used according to a protocol approved by the University of California Davis Animal Care and Use Committee. Rats underwent a bout of acute unilateral resistance exercise using a protocol described previously (Baar & Esser, 1999; Hamilton *et al.*, 2010). Briefly, rats were anesthetized (2.5% isoflurane) and the sciatic nerve was stimulated (100 Hz, 3-6 volts, 1 ms pulse, 9 ms delay) using percutaneous wire electrodes to contract the hindlimb muscles for 10 sets of 6 repetitions (repetition length = 2 s). In this model, muscle fibers in the anterior (tibialis anterior (TA), extensor digitorum longus) and posterior (soleus, plantaris, gastrocnemius) compartments are fully recruited and contract simultaneously. As a result, the (smaller) anterior muscles perform high-force lengthening contractions. Following exercise, animals were given an analgesic (buprenorphine, 0.1 mg/kg) and returned to their cages for recovery. TA muscles were collected from animals under anesthesia at 1.5, 3, 6, 12, 18, and 36 h after exercise. For rapamycin treatments, rats were administered rapamycin (1.5 mg/kg body wt by i.p. injection) 15 min prior to the exercise protocol, and muscles were collected at 3, 6 and 18 h (note: a 12h+rapamycin group was added after the completion of the study for the primary purpose of determining the effect of rapamycin on mRNA targets (Fig. 3 B-D) that were upregulated at this time point). The non-exercised contralateral leg served as a within-animal control for every measure, and a group of animals that did not undergo exercise controlled for any artifacts of muscle collection order between legs (time = 0 in figures). TA muscles were surgically removed, dissected free of connective tissue, frozen in liquid nitrogen and powdered for further analysis.

Muscle protein synthesis

Changes in muscle protein synthesis were assessed by measuring the incorporation of exogenous puromycin into nascent peptides as described previously (Goodman *et al.*, 2011). Briefly, puromycin is a tyrosyl tRNA analog that results in premature release of translation products – puromycin conjugates – that can be subsequently measured by western blotting using an antibody against puromycin (see *Protein expression* below). Puromycin (EMD Millipore, cat.

540222) was dissolved in sterile saline and delivered (0.02 $\mu\text{mol/g}$ body wt by i.p. injection) 30 min prior to muscle collection.

Protein expression

Frozen muscle powder was homogenized in a sucrose lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 50 mM NaF, 1 mM NaVO_4 $\text{Na}_2(\text{PO}_4)_2$ and 0.1% DTT). Samples were centrifuged (10,000 $\text{g} \times 1$ min at 4°C) and the supernatant was removed for protein quantification by DC protein assay (Bio-Rad, cat. 500-0116). Protein concentrations were equalized, solubilized in Laemmli sample buffer and incubated at 95°C for 5 min. Samples were loaded on 4-20% gradient polyacrylamide gels with non-exercised and exercised samples in adjacent wells. Samples were separated by electrophoresis (200 V for 45 min), transferred to nitrocellulose membrane (wet transfer, 100 V for 1 h), blocked in 1% fish skin gelatin, and washed in Tris-buffered saline 0.1% tween (TBST) before overnight incubation in primary antibody at 4°C. Primary antibodies, diluted 1:1,000 in TBST, were from: Cell Signaling Technologies – phospho-ERK Thr202/Tyr204 (cat. 4370), phospho-S6K1 Thr389 (cat. 9205), phospho-rpS6 (cat. 2211), phospho-eEF2 Thr56 (cat. 2331), total S6K1 (cat. 2708), total S6 (cat. 2217), total eEF2 (cat. 2332), total ERK (cat. 4695); Santa Cruz Biotechnology – phospho-UBF Ser388 (cat. 21637) and Ser637 (cat. 21639); and Millipore – puromycin (cat. MABE343). Membranes were washed in TBST and incubated in secondary antibody (1:10,000) for 1 h at room temperature before washing in TBST and detection by chemiluminescence (Millipore, cat. WBKLS0500). Images for densitometry analysis were captured with a Bio-Rad ChemiDoc MP imaging system and quantified using Image Lab Software (Bio-Rad, v. 5).

RNA isolation and gene expression

Prior to RNA isolation, aliquots of frozen muscle powder were weighed in order to calculate RNA per milligram of wet muscle tissue. RNA was isolated using RNeasy RT reagent (Sigma, cat. R4533) according to the manufacturer's instructions and quantified by absorbance spectrophotometry (Epoch Microplate Spectrophotometer, BioTek Instruments Inc.). 1 μg of RNA was converted to cDNA using a reverse transcription kit (Life Technologies, cat. 4368814) with thermal cycling according to the manufacturer's instructions. cDNA was stored at -80°C

and diluted 1:10 in nuclease-free water prior to quantitative RT-PCR analysis (SYBR Green Supermix from Bio-Rad Laboratories, cat. 172-5121; Eppendorf Light Cycler PCR machine). Primer sequences are available on request. Gene expression was calculated using the delta delta threshold cycle method (Livak & Schmittgen, 2001) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. GAPDH expression did not change significantly at any time point after resistance exercise.

Proteasome and calpain activity

Proteasome and calpain activities were determined as described in detail elsewhere (Gomes *et al.*, 2012; Baehr *et al.*, 2014). Briefly, frozen muscle powder was dounce-homogenized in ice-cold buffer (50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT), centrifuged (12,000 g × 3 min at 4°C), protein concentrations were determined by the Bradford method, and samples were loaded onto black 96-well plates (26S proteasome, 14 µg protein as muscle lysate; 20S proteasome, 20 µg of protein as muscle lysate; calpain, 45 µg of protein as muscle lysate). Activity was determined by measurement of a fluorescent-tagged substrate (Leu-Leu-Val-Tyr—4-amino-7-methyl coumarin ; BACHEM, cat. I-1395) in the presence or absence of inhibitors (proteasome: Bortezomib, 2 mM; Calbiochem cat. 504314; calpain: 50 µM calpain inhibitor IV, Calbiochem cat. 208724). Kinetic reads were taken at 15 min intervals on a Fluoroskan Ascent 2.5 fluorometer for 2 h (excitation wavelength, 390 nm; emission wavelength, 460 nm), and all assays were linear up to 2 h and results were calculated from reads at 60 min.

Cell culture

C2C12 myoblasts were cultured in growth media (GM; high glucose DMEM, 10% FBS, 0.1% penicillin) until 95% confluence at which point they were differentiated in high glucose media containing 2% horse serum. Experiments were conducted on fully formed myotubes five days after the introduction of differentiation media. Long-term dose-response experiments were conducted using a c-Myc inhibitor (Sigma-Aldrich, cat. F3680) whereby myotubes were treated for 8 h with 25, 50 and 100 µM inhibitor, or a solvent control (dimethyl sulfoxide). Parallel dose-response experiments were run for analysis of total RNA and gene expression quantification. Three independent experiments were conducted for both MPS and RNA analysis. To determine

the role of c-Myc in the MPS response to an acute anabolic stimulus, a separate cohort of C2C12 myotubes were fasted for 30 minutes in phosphate buffered saline containing 2% horse serum. After 30 minutes, the cells were shifted to GM supplemented with 100 nM IGF-1 in the presence or absence of increasing amounts of the Myc inhibitor for a further 60 minutes before collection. For MPS analysis, myotubes were incubated with 1 μ M puromycin for 5 min before collection in a sucrose lysis buffer (see 'Protein expression' above). For total RNA analysis, myotubes were collected in RNazol RT reagent and RNA was isolated, quantified and analyzed for gene expression (see 'RNA isolation and mRNA abundance' above).

Staining of argyrophilic proteins in nucleolar organizing regions

Argyrophilic proteins in nucleolar organizing regions (AgNOR) can be stained as a morphological proxy of nucleolar activity and ribosomal DNA transcription (Morton *et al.*, 1983); importantly, nucleolar size is directly related to ribosome production (Hernandez-Verdun, 2006). C2C12 myotubes were grown on glass coverslips and stained according to a protocol that was adapted from previously described methods (Ploton *et al.*, 1986; Trere, 2000). Briefly, myotubes were fixed (95% ethanol, 5 min at room temp.), incubated in an absolute ethanol-glacial acetic acid mix (3:1 v/v), and hydrated using decreasing concentrations of ethanol, before incubation in a silver nitrate staining solution (33% silver nitrate, 0.33% gelatin, 0.33% formic acid). Next, myotubes were washed in distilled water and counterstained with 1% gold chloride. Myotubes were dehydrated using increasing concentrations of ethanol and coverslips were mounted. Images were obtained at 40X magnification using an Axio Imager M1 light microscope (Zeiss), and AgNORs were enumerated in a blinded fashion and according to methods previously described (Trere, 2000).

Statistical analysis

Effects for time after exercise were analyzed by one-way ANOVA, with Dunnett's post hoc for comparisons to the non-exercised control group. Effects of rapamycin were analyzed by two-way ANOVA (rapamycin \times time), with Tukey's post hoc. Western blot data was log-transformed to correct skewness and unequal variance before statistical analyses; data are geometric means \pm back-transformed SEM (Altman *et al.*, 1983; Olivier *et al.*, 2008). The remaining data are arithmetic means \pm SEM. Cell culture data was analyzed by one-way ANOVA with Tukey's post

hoc. Statistical analyses were performed using SigmaStat software (v.3.1, Systat Software, Point Richmond, CA, USA); statistical significance was set at $P < 0.05$.

Results

Muscle protein synthesis and total RNA

Resistance exercise stimulated a prolonged moderate (~45%) increase in MPS ($P = 0.042$ for effect of time after exercise; Fig. 1A). Rapamycin decreased MPS at 6 h ($P = 0.005$, RE_x vs RE_x+Rapamycin at 6 h) and at 18 h ($P = 0.027$, RE_x vs RE_x+Rapamycin at 18 h); however, the inhibitory effect appeared to be complete at 6 h and much less at 18 h (see bar graph inset in Fig. 1A showing the delta of MPS between the initial time point in RE_x and RE_x+Rapamycin groups and the 6 and 18 h time points). Total RNA ($\mu\text{g RNA/mg muscle}$) tended to rise gradually at 18 through 36 h after exercise ($P = 0.085$ for main effect of time after exercise; Fig. 1C).

Rapamycin administration reduced the effect of resistance exercise on total RNA ($P = 0.016$ for RE_x vs RE_x+Rapamycin main effect; Fig. 1C).

Translation signaling

Resistance exercise stimulated a rapid and sustained increase in S6K1 phosphorylation (1.5 through 18 h, all $P < 0.05$; Fig. 2A) that was completely inhibited at all time points by rapamycin. Interestingly, S6K1 phosphorylation had returned to basal levels by 18 and 36 hours even though protein synthesis was still elevated at these time points. Similarly, S6 phosphorylation was elevated from 1.5 through 18 h recovery (all $P < 0.05$ vs. non-exercised control group; Fig. 2B). S6 phosphorylation was completely blocked by rapamycin at 6 and 18 h as expected; however, S6 phosphorylation was increased on a relative basis (experimental/contralateral control) at 3 h due to a decrease in the contralateral control, and likely reflects ERK signaling that was elevated at this time point (Fig. 2C). eEF2 phosphorylation was decreased (indicative of activation) following resistance exercise, and interestingly, this occurred to similar levels in both the vehicle and rapamycin treated groups ($P = 0.41$ for RE_x vs RE_x+Rapamycin; Fig. 2D). Lastly, UBF phosphorylation at both Ser388 (Fig. 2E) and Ser637 (Fig. 2F) increased following resistance exercise and only the 3 h time point (Ser637 only) was sensitive to rapamycin.

Gene expression

ITS-1 expression, an estimate of ribosomal DNA transcription rate, tended to increase in post-exercise recovery ($P = 0.054$; Fig. 1B) in a manner that preceded changes in total RNA (Fig. 1C). The increase in ITS-1 was inhibited by rapamycin at 12 and 18 h (both $P < 0.05$, REx vs REx+Rapamycin at the same time point). c-Myc is a transcription factor for RNA polymerase I, as well as for ribosomal proteins and proteins controlling ribosomal RNA splicing; its expression was elevated at 3, 6 and 12 h (all $P < 0.05$; Fig. 3A) and was not affected by rapamycin ($P = 0.84$). The expression of nucleophosmin and nucleolin, which are targets of c-Myc that control pre-ribosomal RNA splicing, were elevated at 12 h (nucleophosmin and nucleolin, $P < 0.05$; Figs. 3B and 3C, respectively) and 36 h of recovery (nucleolin, $P < 0.05$). The elevation at 12 h of nucleolin, but not nucleophosmin, was rapamycin-sensitive (nucleolin, $P = 0.049$; nucleophosmin, $P = 0.43$; REx vs REx+Rapamycin at 12 h). Expression of TATA box binding protein-associated factor RNA Polymerase I B (TAF1B), which recruits RNA polymerase I to the ribosomal DNA promoter, was elevated at 12 h ($P < 0.05$; Fig 3D) and was not significantly affected by rapamycin ($P = 0.11$). Mighty gene expression, a readout of myostatin activity, was increased at 12 and 36 h post-exercise (both $P < 0.05$) suggesting a decrease in myostatin activity; Mighty expression was not affected by rapamycin ($P = 0.51$ for REx vs REx+Rapamycin; Fig. 4B). Further, as we had previously shown, increased Mighty expression corresponded with the cleavage and activation of Notch, which was not different between REx and REx+Rapamycin ($P = 0.69$; Fig. 4A).

Long-term c-Myc inhibition in myotubes

To determine whether c-Myc could control ribosome mass and protein synthesis in muscle cells, we treated C2C12 myotubes with the inhibitor 10058-F4. Long-term (8 h) treatment with the c-Myc inhibitor had a dose-dependent effect on both MPS (Fig. 5B) and total RNA (Fig. 5C). MPS and total RNA were strongly correlated ($r^2 = 0.98$, $P < 0.001$; Fig. 5D). c-Myc inhibitor treatment decreased nuclear UBF Ser637 phosphorylation ($P = 0.028$ at 100 μM), without significantly changing c-Myc or total UBF abundance in isolated nuclear and cytosolic fractions, respectively (Figs. 5E and 5F, respectively). Treatment with increasing concentrations of c-Myc inhibitor resulted in stepwise decreases in argyrophilic protein staining of nucleolar organizer regions, a morphological proxy of ribosome biogenesis (Fig. 5G). 10058-F4 blocks c-Myc activity by preventing its dimerization with Max and nuclear translocation (Huang *et al.*, 2006). This

inhibition/exclusion from the nucleus apparently resulted in a feedback signal that upregulated c-Myc expression in a stepwise manner at 50 and 100 μM ($P < 0.001$; Fig. 5H). Expression of the 5' external transcribed spacer, part of the pre-ribosomal RNA transcript, ITS-1 and RNA polymerase I-specific transcription initiation factor RRN3 were elevated at the 100 μM c-Myc inhibitor dose (Figs. 5I-K, respectively).

c-Myc and the Acute MPS Response

To determine whether c-Myc altered an acute protein synthesis in muscle cells, fasted cells were treated with growth media containing 100 nM IGF-1 for 1 h to stimulate protein synthesis. In this setting, low (25 μM) and moderate (50 μM) concentrations of the c-Myc inhibitor had no effect on either protein synthesis (Fig. 6A), or markers of mTORC1 activation (Fig. 6C-F). At the highest concentration (100 μM) the c-Myc inhibitor decreased protein synthesis and S6K1 and eEF2 signaling; interestingly though, 100 μM c-Myc inhibitor did not block 4E-BP1 or S6 phosphorylation.

Ubiquitin proteasome pathway and calpain activity

ATP-dependent (26S $\beta 5$) and ATP-independent (20S $\beta 5$) proteasome activities were not increased in early recovery from resistance exercise; 20S proteasome activity was elevated in late post-exercise recovery ($P < 0.05$ at 36 h vs. non-exercise control group; Fig. 7B). In the exercised leg, rapamycin increased the activity of the proteasomal subunits 26S $\beta 5$ at 6 h ($P < 0.001$ for REx vs REx+Rapamycin at 6 h; Fig. 7A) and 20S $\beta 5$ at 3 and 6 h of recovery ($P < 0.001$ for REx vs REx+Rapamycin at same time point, at 3 and 6 h of recovery; Fig. 7B). Calpain activity was not significantly altered by resistance exercise or by rapamycin (Fig. 7C).

Discussion

The effect of acute resistance exercise and the role of mTORC1 on the time course of translational signaling, protein synthesis, and mechanisms of ribosome biogenesis in skeletal muscle were examined in this study. The data provide a mechanistic basis for a hypothesis that skeletal muscle mass is regulated by changes in translational activity and capacity, and that these changes occur through both rapamycin-sensitive and -insensitive mechanisms. Our most important findings are that eEF2, UBF, c-Myc, TAF1B and nucleophosmin were activated, and that local myostatin activity was inhibited, through rapamycin-insensitive mechanisms, that rapamycin decreased precursor-ribosomal RNA, nucleolin and total RNA after resistance exercise, and that rapamycin has a limited effect on protein synthesis late in recovery.

Signaling and Protein Synthesis after Acute Resistance Exercise

We observed a prolonged elevation of protein synthesis after resistance exercise, as shown previously in humans (Chesley *et al.*, 1992; Phillips *et al.*, 1997). Increases in protein synthesis in early recovery after resistance exercise are underpinned by enhanced translational activity and this translational activity is mTORC1 dependent (Wong & Booth, 1990b; Baar & Esser, 1999; Kubica *et al.*, 2005; Glover *et al.*, 2008; Drummond *et al.*, 2009; Kimball & Jefferson, 2010). By comparison, mechanisms that underpin exercise-induced elevations in MPS in late recovery are poorly understood. Further, whether specific signaling events can predict the protein synthetic response has recently been challenged (Atherton & Smith, 2012; Phillips *et al.*, 2013). In the present study, mTORC1 signaling returned to near baseline levels by 18 h, a time when protein synthesis was at its peak, suggesting either that the high levels of protein synthesis late in the recovery period were the result of the earlier increase in mTORC1 activity or that mTORC1 was not required for the late rise in protein synthesis. To distinguish between these possibilities, we inhibited mTORC1 using rapamycin and observed that, as in humans (Drummond *et al.*, 2009), protein synthesis was completely blocked 6 h after exercise. However, the late (12-18 h) increase in protein synthesis appeared to be rapamycin insensitive, showing an induction from the fully suppressed state at 3-6 h postexercise (see inset of Figure 1A); this suggests that prolonged elevation of protein synthesis following resistance exercise involved mTORC1-independent mechanisms. This differs from the work of Kubica *et al.* (Kubica *et al.*, 2005), who showed that rapamycin completely blocked exercise-induced MPS at 16 h. The contrasting findings could be

due to differences in the models that were used. Kubica and colleagues (Kubica *et al.*, 2005) operantly trained rats to touch a bar 50 times and the “acute” protocol in fact consisted of four separate sessions with 1 day of rest between sessions and protein synthesis measured 16 h after the 4th session. By contrast, our protocol mimicked a single high intensity resistance exercise training session, involving maximal motor unit recruitment and high-force lengthening contractions. Since rapamycin blocked ribosome biogenesis, as determined by ITS-1 expression and total RNA content (Fig. 1B and C), the prolonged elevation of protein synthesis was not the result of an increase in translational capacity. Therefore, our data suggest that a rapamycin-insensitive process that controls translational activity is activated later in the recovery from acute resistance exercise.

To explore other potential mechanisms that could explain the prolonged increase in protein synthesis, we examined myostatin signaling, a major pathway controlling muscle mass (McPherron & Lee, 1997; Lee, 2004, 2010) that we have previously shown to be downregulated in the exercise model used in the present study (MacKenzie *et al.*, 2013). To our knowledge, the sensitivity of myostatin signaling to rapamycin after resistance exercise is not known. We assessed myostatin activity using one of its transcriptional targets (Mighty) as a readout (Marshall *et al.*, 2008; MacKenzie *et al.*, 2013). In the present study, resistance exercise resulted in a prolonged increase in Mighty expression that peaked in the late recovery period (12-18 h), when protein synthesis was also at its highest. The increase in Mighty expression is thought to occur through a mechanism whereby load across the muscle leads to cleavage and activation of notch (Akiho *et al.*, 2010), which can translocate to the nucleus and inhibit Smad2/3 activity (Carlson *et al.*, 2008). Here, we observed that resistance exercise increased notch activation and Mighty gene expression in a rapamycin-insensitive manner, thus suggesting local inhibition of myostatin in an mTORC1-independent manner. Thus the localized inhibition of myostatin could contribute to the rapamycin-independent rise in MPS at later (12-18 h) time points of recovery; however, this hypothesis requires further investigation.

Another rapamycin-insensitive effect that was surprising was the activation of eukaryotic elongation factor 2 (eEF2). This was unexpected since eEF2 is thought to be under the control of mTORC1 through S6K phosphorylation and inhibition of eEF2 kinase (Wang *et al.*, 2001). This finding of rapamycin-insensitive eEF2 dephosphorylation (activation) adds to data that distinguishes the unique ability of mechanical stimuli to stimulate anabolic signaling pathways

that are not only independent of phosphoinositide 3-kinase-activating hormones/growth factors (Hornberger *et al.*, 2004; Hornberger & Chien, 2006; West *et al.*, 2009; Hamilton *et al.*, 2010), but some of which are also independent of canonical mTORC1 activity (eEF2, UBF, Mighty and c-Myc in the present study). Interestingly, our data imply that peptide chain elongation may be controlled in an mTORC1-independent manner after resistance exercise and this might contribute to the prolonged increase in protein synthesis.

Ribosome Biogenesis after Acute Resistance Exercise

Another key finding from the current study was that markers of ribosome biogenesis were increased between 12 and 36 h after resistance exercise. We found that pre-ribosomal RNA, as indicated by ITS-1 levels, tended to increase gradually and in a sustained manner (plateau at 12-36 h) that preceded a trend toward increased total RNA. This finding is consistent with previous work showing that resistance exercise drives ribosome biogenesis, which is important in the regulation of muscle mass (Adams *et al.*, 2002; Goodman *et al.*, 2011; Chaillou *et al.*, 2014). We have expanded on this previous work to show that the exercise-induced increase in pre-ribosomal RNA, nucleolin a controller of pre-rRNA splicing, and total RNA were prevented by rapamycin. Collectively, this means that the initial rise in protein synthesis was the result of greater translational activity/efficiency (increased peptide synthesis per unit RNA), which is consistent with previous reports (Wong & Booth, 1990b, a; Fluckey *et al.*, 1996; Bickel *et al.*, 2005), but also suggests that an mTORC1-dependent increase in translational capacity may be important in the protein accretion that occurs as a result of training (Brook *et al.*, 2015). In general, the 12 and 18 h post-exercise time points are especially interesting since they appear to be a transition phase between diminishing mTORC1 signaling, sustained MPS, and increased ribosome biogenesis. However, it is important to note that even though rapamycin completely blocked ribosome biogenesis, it did not completely block MPS suggesting that ribosome biogenesis does not explain the prolonged increase in MPS after an acute bout of resistance exercise.

c-Myc was identified as an important target for investigating ribosome biogenesis since it regulates RNA polymerase I activity (Arabi *et al.*, 2005; Grandori *et al.*, 2005), ribosomal proteins, and proteins that control ribosomal RNA processing and ribosomal subunit intracellular localization (Boon *et al.*, 2001; Maggi *et al.*, 2008). In the present study, c-Myc gene expression was highly upregulated after resistance exercise (1.5 h through 12 h of recovery), which is in

agreement with earlier work (von Walden *et al.*, 2012), and the upregulation of c-Myc occurred in a rapamycin-insensitive manner. Peak c-Myc expression (3 h of recovery) preceded the upregulation of its targets TAF1B, which recruits RNA polymerase I to the ribosomal DNA promoter (Muth *et al.*, 2001), and nucleophosmin and nucleolin (12 h of recovery), which control c-Myc nucleolar localization and rDNA transcription (Li & Hann, 2013), splicing of the precursor-ribosomal RNA transcript (Boon *et al.*, 2001), and nuclear export of ribosomal subunits (Maggi *et al.*, 2008). To help clarify the relationship between c-Myc, ribosomal content and protein synthesis, we performed experiments *in vitro* in which we inhibited c-Myc activity in differentiated myotubes for 8 h. These experiments showed that increasing doses of c-Myc inhibitor resulted in stepwise decreases in both total RNA as well as protein synthesis, without causing cellular toxicity (see the ponceau image and the total UBF levels in Fig. 5). The fact that total RNA and protein synthesis rate were strongly correlated suggests that c-Myc affects the rate of protein synthesis in skeletal muscle by regulating translational capacity. In agreement with this hypothesis, inhibition of c-Myc had no effect on the acute activation of protein synthesis or mTORC1 activation in myotubes that had undergone a short fast followed by a strong anabolic stimulus. c-Myc regulates translational capacity by directly activating ribosomal DNA transcription as seen by the decreases in argyrophilic protein staining of nucleolar organizer regions, following treatment with the inhibitor (Fig. 5G). Our *in vivo* data showed that the increases in c-Myc, nucleophosmin and TAF1B gene expression are not rapamycin-sensitive and thus c-Myc may work together with mTORC1 to alter translational capacity after resistance exercise; however, further mechanistic research *in vivo* is required to test this hypothesis.

One mTORC1-regulated mechanism mediating ribosome biogenesis may be the activation of upstream binding factor (UBF), a nucleolar transcription factor that is integral in the formation of a preinitiation complex for the transcription of precursor rRNA (Sanij *et al.*, 2008). Phosphorylation of UBF is required for its mediation of ribosomal RNA synthesis (Voit *et al.*, 1995). A previous study (Hannan *et al.*, 2003) in NIH 3T3 cells showed that mTORC1 was required for UBF phosphorylation and subsequent serum-induced activation of ribosomal DNA transcription. In the present study however, resistance exercise increased UBF serine 388 and 637 phosphorylation in a largely mTORC1-independent manner. It was beyond the scope of the present study to determine the upstream kinase responsible for the rapamycin-insensitive phosphorylation of UBF; previous studies have shown that UBF can be phosphorylated by ERK

(Stefanovsky *et al.*, 2001) as well as cell cycle-associated kinases (Voit *et al.*, 1992; Voit & Grummt, 2001). A previous resistance exercise study (Figueiredo *et al.*, 2015) highlighted ERK-mediated activation of ribosome biogenesis via cyclin D1 and TIF-1A rather than UBF. Here, we report high UBF phosphorylation in the presence of rapamycin, which, combined with rapamycin-insensitive activation of the transcription of c-Myc, nucleophosmin and TAF1B, suggests that, even though mTORC1 is required for resistance exercise-dependent ribosome biogenesis (present data and (Hannan *et al.*, 2003; Nader *et al.*, 2005; Jastrzebski *et al.*, 2007; Thoreen *et al.*, 2012), mTORC1 is supported by mTORC1-independent mechanisms that remain to be fully elucidated.

The exact amount of time required after an acute bout (or number of bouts) to elicit a bona fide increase in translational capacity is unclear. Bickel and colleagues (Bickel *et al.*, 2005) reported increased total RNA concentration (μg RNA per mg muscle) after two bouts of exercise, but the plasticity of the ribosomal pool throughout resistance training is understudied (Figueiredo *et al.*, 2015). We hypothesize that the ribosomal pool is responsive to acute resistance exercise and that periodic induction of elements regulating ribosome biogenesis – including UBF phosphorylation as well as expression of c-Myc, nucleophosmin, nucleolin, and TAF1B – during the early stages of training may stimulate increases in translational capacity and facilitate subsequent adaptation (skeletal muscle remodelling and hypertrophy). According to such a model, increased translational capacity, achieved through training, would result in a smaller requirement for mTORC1 signaling to induce an anabolic response after an acute resistance exercise stimulus (Brook *et al.*, 2015). This could explain, at least in part, the paradoxical observation that individuals that exhibit the greatest hypertrophy after training express genes reflecting a downregulation of mTORC1 (Phillips *et al.*, 2013).

Protein Degradation after Acute Resistance Exercise

Compared with MPS, muscle protein breakdown (MPB) after resistance exercise is understudied. The ubiquitin proteasome pathway is the major protein degradation pathway in skeletal muscle (Rock *et al.*, 1994), and reduced proteasome activity contributes to pathological (Al-Khalili *et al.*, 2014) and aging (Hwee *et al.*, 2014) phenotypes in skeletal muscle. Conversely, proteasome activation accompanies compensatory hypertrophy induced by synergist ablation (Baehr *et al.*, 2014). In contrast to the chronic and supraphysiological stimulus imposed

by synergist ablation, acute resistance exercise in the present study did not elevate proteasome and/or calpain activity in early recovery; rather, it was not until the 36 h time point that ATP-independent chymotrypsin-like proteasome activity was significantly elevated. *A priori*, we hypothesized these pathways would be upregulated after exercise in response to contraction-induced damage, and to facilitate myofiber remodeling; however this was generally not the case and could be a reflection of the fact that the dynamic range of MPB is small compared to that of MPS after resistance exercise (Phillips *et al.*, 1997; Glynn *et al.*, 2010). Alternatively, a single bout of exercise may not have been sufficient to induce remodeling and/or activation of the ubiquitin proteasome system. While somewhat tangential to the main aims of the study, we noted that resistance exercise in the presence of rapamycin had a stimulatory effect on proteasome activity in early (3-6 h) recovery. The stimulatory effect of exercise plus rapamycin that we observed is in contrast to inhibitory effects reported *in vitro* (Osmulski & Gaczynska, 2013), but is consistent with more recent experiments *in vivo* showing that rapamycin activates the proteasome (Rodriguez *et al.*, 2014). Further research is required to explore potential mechanisms and implications of this finding toward therapies aimed at proteostasis and healthy aging of skeletal muscle. Taken together, the present work showed little evidence of proteasome or calpain activation (e.g., for remodelling or amino acid intracellular recycling) in early recovery from acute resistance exercise.

Summary

Overall, the present findings demonstrate that resistance exercise activates pathways controlling translational activity and capacity through both rapamycin-sensitive and -insensitive mechanisms. Rapamycin completely blocks protein synthesis 6 h after resistance exercise, but has a limited effect at 12 or 18 h. Rapamycin-insensitive pathways, including eEF2 and localized myostatin inhibition, may contribute to the prolonged rise in protein synthesis. c-Myc expression is upregulated after resistance exercise and may play a role in controlling translational capacity by stimulating ribosome biogenesis; however, further mechanistic work *in vivo* is clarify the role of Myc in load-induced muscle hypertrophy. In a broader context, given that muscle mass is an important clinical indicator of health, understanding how prolonged protein synthesis and ribosome biogenesis are controlled following exercise has the ability to identify potential targets to combat the loss of muscle with aging and disease.

Additional information

Competing interests

The authors declare no conflicts of interest.

Author contributions

Conception and design of the experiments: DW, SB, KB. Collection, analysis and interpretation of data: All authors. Drafting the article or revising it critically for important intellectual content: DW, SB, KB. All authors read and approved the final version of the manuscript and all authors listed qualify for authorship.

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Figure Legends

Figure 1. The effect of acute resistance exercise (REx, closed circles) on muscle protein synthesis (MPS; *A*; $P = 0.042$, for effect of time; representative blot at right), precursor ribosomal RNA (*B*; $P = 0.054$, for effect of time) and total RNA (*C*; $P = 0.085$, for effect of time). The '0' time point is a non-stimulated control group with its error term shaded to aid visual comparisons. Rapamycin was administered (pre-exercise, 1.5 mg/kg by i.p. injection) to groups shown in open circles. MPS was assessed by measuring the incorporation of puromycin into nascent peptides by western blot; inset shows change in MPS from the initial time point of each group to MPS at 6 and 18 h. Newly synthesized pre-rRNA was measuring using internal transcribed spacer 1 gene expression as a readout. †Difference between REx and REx+Rapamycin at the same time point, $P < 0.05$. Values are expressed as experimental/contralateral control muscles, means \pm SEM.

Figure 2. The effect of resistance exercise (REx, closed circles) on the phosphorylation of proteins that regulate translational activity (*A-D*) and capacity (*E & F*). The '0' time point is a non-stimulated control group with its error term shaded to aid visual comparisons. Rapamycin was administered (pre-exercise, 1.5 mg/kg by i.p. injection) to groups shown in open circles. Data are expressed as experimental/contralateral control muscles, with geometric means \pm SEM. *Difference between REx vs. non-stimulated control group, $P < 0.05$. †Difference between REx and REx+Rapamycin at the same time point, $P < 0.05$.

Figure 3. Expression of genes associated with ribosome biogenesis after resistance exercise (REx, closed circles). c-Myc (*A*), nucleophosmin (*B*), nucleolin (*C*), TATA box binding protein-associated factor RNA Pol I B (TAF1B; *D*). The '0' time point is a non-stimulated control group with its error term shaded to aid visual comparisons. Rapamycin was administered (pre-exercise,

1.5 mg/kg by i.p. injection) to groups shown in open circles. Values (means \pm SEM) are expressed relative to GAPDH and the contralateral control using the delta-delta Ct method. *Difference between REx vs. non-stimulated control group, $P < 0.05$. †Difference between REx and REx+Rapamycin at the same time point, $P < 0.05$.

Figure 4. Active Notch (Notch intracellular domain) protein expression (A) and Mighty gene expression (B) after resistance exercise (REx). Notch activation inhibits myostatin signalling, as reflected by an increase in Mighty gene expression. Time points: 0 (non-stimulated control group), 1.5, 3, 6, 12, 18, 36 h after REx. Rapamycin was administered (pre-exercise, 1.5 mg/kg by intraperitoneal injection) to groups shown in open circles. For active Notch, values are expressed as experimental/contralateral control muscles. For Mighty, values are expressed relative to GAPDH and the contralateral control using the delta-delta Ct method. Values are means \pm SEM. *Difference between REx vs. non-stimulated control group, $P < 0.05$.

Figure 5. Inhibition of c-Myc *in vitro* alters translational capacity. Differentiated C2C12 myotubes were treated with a myc inhibitor at the concentrations shown for 8 h (0 μ M = solvent control). For MPS analysis, myotubes were incubated with 1 μ M puromycin for 5 min before collection and detection of puromycin-conjugated nascent peptides by western blot. B) Muscle protein synthesis (MPS) and (C) total RNA in myotubes after c-Myc inhibitor treatment are (D) directly related. (E) c-Myc and (F) phospho UBF (Upstream Binding Factor) Ser637 protein, and (G) argyrophilic proteins in the nucleolar organizer region following treatment with the small molecule c-Myc inhibitor (0 μ M = vehicle control). The expression of (H) c-Myc, (I) 5' external transcribed spacer (5'ETS), (J) internal transcribed spacer 1 (ITS-1), and (K) RNA polymerase I transcription factor (RRN3) apparently demonstrates feedback upregulation at high inhibitor concentration. Data shown are representative of three independent experiments. Values

are means \pm SEM. Means with different letters are significantly different from each other, $P < 0.01$.

Figure 6. Acute muscle protein synthesis (MPS; A) and mTORC1 signaling (C-F) in myotubes treated with c-Myc inhibitor. Differentiated C2C12 myotubes were fasted for 30 min in PBS containing 2% horse serum prior to 60 min in growth media supplemented with 100 nM IGF-1 in the presence or absence of increasing doses of myc inhibitor. For MPS analysis, myotubes were incubated with 1 μ M puromycin for 5 min before collection and detection of puromycin-conjugated nascent peptides by western blot. Representative data shown are one of two independent experiments. Values are means \pm SEM. Means with * are significantly different than control, whereas † indicates different than the control refeed value, $P < 0.01$.

Figure 7. Ubiquitin proteasome pathway and calpain activities after resistance exercise (REx, closed circles). A) 26S β 5 and B) 20S β 5 reflect ATP- dependent and independent chymotrypsin-like activities of the β 5 proteasomal subunit (respectively), which is the most important subunit for proteasome function (Jager *et al.*, 1999). C) Calpain activity at each time point. The '0' time point is a non-stimulated control group with its error term shaded to aid visual comparisons. Rapamycin was administered (pre-exercise, 1.5 mg/kg by i.p. injection) to groups shown in open circles. Values are expressed as experimental/contralateral control muscles, means \pm SEM. *Difference between REx vs. non-stimulated control group, $P < 0.05$. †Difference between REx and REx+Rapamycin at the same time point, $P < 0.001$.