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ORIGINAL ARTICLE

Comparative effects of whey protein versus L-leucine on skeletal muscle protein synthesis and markers of ribosome biogenesis following resistance exercise

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Abstract We compared immediate post-exercise whey protein (WP, 500 mg) versus L-leucine (LEU, 54 mg) feedings on skeletal muscle protein synthesis (MPS) mechanisms and ribosome biogenesis markers 3 h following unilateral plantarflexor resistance exercise in male, Wistar rats (~250 g). Additionally, in vitro experiments were performed on differentiated C_2C_{12} myotubes to compare nutrient (i.e., WP, LEU) and 'exercise-like' treatments (i.e., caffeine, hydrogen peroxide, and AICAR) on ribosome biogenesis markers. LEU and WP significantly increased phosphorylated-rpS6 (Ser235/236) in the exercised (EX) leg 2.4-fold ($P < 0.01$) and 2.7-fold ($P < 0.001$) compared to the non-EX leg, respectively, whereas vehicle-fed control (CTL) did not (+65 %, $P > 0.05$). Compared to the non-EX leg, MPS levels increased 32 % and 52 % in the EX leg of CTL ($P < 0.01$) and WP rats ($P < 0.001$), respectively, but not in LEU rats (+15 %, $P > 0.05$). Several genes associated with ribosome biogenesis robustly increased in the EX versus non-EX legs of all treatments; specifically, c-Myc mRNA, Nop56 mRNA, Bop1 mRNA, Ncl mRNA, Npm1 mRNA, Fb1 mRNA, and Xpo-5 mRNA. However, only LEU significantly increased 45S pre-rRNA levels in the EX leg (63 %, $P < 0.001$). *In vitro* findings confirmed that 'exercise-like' treatments similarly altered markers of ribosome biogenesis, but only LEU increased 47S pre-rRNA levels ($P < 0.01$). Collectively, our data suggests that resistance exercise, as well as 'exercise-like' signals in vitro, acutely increase the expression of genes associated with ribosome biogenesis independent of nutrient provision.

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Moreover, while EX with or without WP appears superior for enhancing translational efficiency (i.e., increasing MPS per unit of RNA), LEU administration (or co-administration) may further enhance ribosome biogenesis over prolonged periods with resistance exercise.

Keywords Whey protein · Leucine · Muscle protein synthesis · Ribosome biogenesis

Background

L-leucine (LEU) and whey protein (WP) are well-known stimulators of muscle protein synthesis (MPS). Mechanistically, LEU (Anthony et al. 2000; Bolster et al. 2004; Dardevet et al. 2000; Dreyer et al. 2008; Wang and Proud 2006) and WP (Anthony et al. 2007; Ha and Zemel 2003; Hulmi et al. 2009, 2010; Koopman et al. 2007) have been shown to activate key mediators in the protein kinase B-mammalian target of rapamycin (Akt-mTOR) signaling cascade which, in turn, up-regulates MPS. Many scientists speculate that the anabolic effects of WP are mitigated through LEU and that LEU is the anabolic 'trigger' that initiates MPS in skeletal muscle (Norton et al. 2009, 2012; Wilkinson et al. 2013). Indeed, several studies have demonstrated that WP or LEU-rich branch chained amino acids (BCAAs) can synergistically enhance post-resistance exercise MPS mechanisms (Dreyer et al. 2008; Karlsson et al. 2004; Tang et al. 2009; Tipton et al. 2007). Limited evidence has also shown that adding LEU to WP provides benefit in enhancing post-resistance exercise anabolism markers (Churchward-Venne et al. 2012; Koopman et al. 2005, 2008). However, no data to our knowledge have directly compared LEU to WP with regards to how these agents independently alter post-exercise Akt-mTOR signaling and/or MPS.

Ribosomes are ribonucleoprotein complexes that catalyze MPS by converting messenger RNA (mRNA) into protein, and these organelles play an integral role in regulating skeletal muscle mass (Chaillou et al. 2014). As mentioned above, numerous studies have shown that WP and/or WP with added LEU can increase MPS through an enhanced translational efficiency (or increase MPS per unit of RNA). However, aside from increases in translational efficiency, it has been hypothesized that MPS can also be heightened through longer-term increases in ribosome biogenesis (Chaillou et al. 2014). Simply stated, ribosome biogenesis is the synthesis of new ribosomes through the coordinated up-regulation in genes encoding ribosomal proteins, rRNA processing proteins, ribosome assembly proteins, and nuclear export proteins (Thomson et al. 2013). Likewise, the synthesis of new ribosomes requires the RNA polymerase I (Pol I) and RNA Pol III-mediated up-regulation of ribosomal DNA transcription yielding increases in 47S pre-ribosomal RNA (47S pre-rRNA) and 5S rRNA, respectively (Chaillou et al. 2014). 47S pre-RNA is further processed to 18S, 5.8S and 28S rRNAs, and the latter rRNAs are integrated into the 60S ribosomal subunit while the former rRNA is integrated into the 40S ribosomal subunit (Thomson et al. 2013). While an in-depth description of ribosome biogenesis is beyond the scope of this paper, it should be noted that 'master regulators' of ribosome biogenesis are posited to exist. Beyond being able to increase translational efficiency, mTOR signaling in yeast and mammals has been shown to be coupled to increases ribosome biogenesis; this occurs through the mTOR-mediated phosphorylation of upstream binding factor 1 (Ubf1) which, in turn, up-regulates RNA Pol I activity (Mayer and Grummt 2006). In support of this, rapamycin treatment in HeLa cells has been reported to decrease the expression of genes related to ribosome biogenesis (Iadevaia et al. 2012) as well as 45S pre-rRNA (Mayer et al. 2004). c-Myc is an immediate early response gene that: (1) is up-regulated in response to a variety of cellular stress stimuli, and (2) encodes a transcription factor that increases the Pol II-mediated expression of numerous genes related to growth, proliferation, and metabolism (Guo et al. 2000; Nie et al. 2012). However, as with mTOR, c-Myc has pleiotropic roles and has also been shown to be a master regulator of ribosome biogenesis by controlling Pol I and Pol III-mediated rDNA transcription (Grandori et al. 2005).

Recent data have demonstrated that resistance exercise can increase markers of skeletal muscle ribosome biogenesis. For instance, Nader et al. (2014) reported that markers of ribosome biogenesis, chiefly 45S pre-rRNA and c-Myc mRNA, were significantly elevated 4 h following acute resistance exercise in humans. Likewise, Stec et al. (2015) reported that younger subjects presented increases in ribosomal proteins 24 h following a resistance exercise bout. Notwithstanding, and in spite of the aforementioned studies that have clearly demonstrated exercise with or without nutritional provision acutely enhances MPS through translational efficiency, sparse literature to our knowledge has examined if resistance exercise with or without nutrient provision acutely enhances the expression of genes involved with ribosome biogenesis.

Given that chronic provision with supplemental amino acids (chiefly LEU) (Bird et al. 2006) and WP (Burke et al. 2001; Cribb et al. 2006; Volek et al. 2013) with resistance exercise have been shown to increase muscle mass compared to resistance exercise alone, it stands to reason that the consumption of these nutrients following exercise may enhance the expression of genes associated with ribosome biogenesis. Therefore, the purpose of this study was to compare the post-exercise feeding effects of WP and LEU on markers of translational efficiency (i.e., MPS and mTOR pathway activity) and ribosome biogenesis (i.e., nuclear/cytoplasmic/total

RNA as well as the expression of 45S pre-rRNA and mRNAs related to ribosome processing, assembly, and nuclear export) 3 h following resistance exercise in rats. Additionally, in vitro experiments were performed on differentiated C_2C_{12} myotubes comparing the effects of WP versus LEU markers of ribosome biogenesis, and additional 'exercise-like' treatments [i.e., caffeine (CAFF), hydrogen peroxide (H_2O_2), and 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR)] were examined to further ascertain how resistance exercise may mechanistically affect the expression of the ribosome biogenesis markers assayed herein.

Methods

Animal experimental procedures

All experimental procedures described herein were approved by Auburn University's Institutional Animal Care and Use Committee. Male, Wistar rats (~250 g) purchased from Harlan Laboratories, Indianapolis, IN, USA) were allowed to acclimate in the animal housing facility 5 days prior to experimentation. Animal quarters were maintained on a constant 12 h light: 12 h dark cycle, at ambient room temperature, and water and standard rodent chow (24 % protein, 58 % carbohydrate, 18 % fat; Teklad Global #2018 Diet, Harlan Laboratories) was provided to animals ad libitum.

The day prior to the experiment, food was removed from home cages resulting in an 18 h overnight fast. The morning of experimentation, animals were removed from their quarters between 0700 and 0800, transported to the Molecular and Applied Sciences Laboratory in the School of Kinesiology building and were allowed to acclimate for approximately 3–4 h. Thereafter, rats were placed under isoflurane anesthesia and resistance trained via electrical stimulation resulting in dynamic plantar flexions. Briefly, animals were fastened to an apparatus to allow the two hind limbs to move freely. Two subcutaneous electrodes connected to a Grass S48 Stimulator (Grass Medical Instruments, Quincy, MS, USA) were placed parallel to the gastrocnemius in each rat's right leg. Four sets of 8 stimulations were delivered with the following settings: 70 mV, 100 Hz, 2000 ms train duration, 0.2 TPS train rat, and 0.2 ms duration. Between sets rats were allowed 2 min of recovery. Immediately following the final training set, rats were administered either 500 mg of WP [COMBAT (MusclePharm, Denver, CO, USA) which is a proprietary blend prominently comprised of WP concentrate; $n = 10$ rats], 54 mg of LEU (EMD Chemicals, Inc., San Diego, CA, USA; $n = 8$ rats), or 1 ml of water (CTL; $n = 10$ rats). The WP dose was determined per the species conversion calculations of Reagan-Shaw et al. (Reagan-Shaw et al. 2008), whereby the human body mass for an average male was expected to be 80 kg. Per this calculation, rats received approximately an 18.8 g human equivalent dose of WP. The LEU dose was based upon the concept that WP contains roughly 12–14 % LEU. Per the species conversion calculations provided above, LEU-treated rats received approximately a 2.8 g human equivalent dose. Each test ingredient was suspended in 1 ml of water and administered via gavage feeding. The gavage feeding procedure involved keeping the animals under light isoflurane anesthesia post-exercise for approximately 30 s while gavage feeding occurred. Rats were then allowed to recover 3 h prior to being euthanized under CO_2 gas in a 2 L induction chamber (VetEquip, Inc., Pleasanton, CA, USA). 30 min prior to euthanasia, animals were injected with puromycin dihydrochloride (5.44 mg in 1 ml of diluted in phosphate buffered saline provided intraperitoneally; Ameresco, Solon, OH, USA) in order to determine relative MPS levels via the SUnSET method (Goodman and Hornberger 2013). Of note, a 3 h post-exercise time point was picked to sample the physiological responses given that Phillips et al. (Burd et al. 2009; Phillips et al. 1997) have shown a 3–4 h post-exercise MPS nadir to occur over a 48 h post-exercise period.

Tissue preparation

For protein analyses, approximately 50 mg of gastrocnemius muscle was extracted from the exercised (EX) leg and the non-exercised (non-EX) leg using standard dissection techniques and placed in 500 µl of ice-cold cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1 % Triton, 20 mM sodium Pyrophosphate, 25 mM Sodium Fluoride, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin]. Samples were then homogenized via micropestle manipulation, insoluble proteins from RIPA homogenates were removed with centrifugation at $500 \times g$ for 5 min, and supernatants were assayed for total protein content using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) prior to immunoblotting and phosphoproteomic analyses described below. An additional 50 mg of gastrocnemius muscle was extracted from the EX leg and the non-EX leg and placed in 500 µl of Ribozol (Ameresco). Samples were then homogenized via micropestle manipulation and RNA isolation was performed per manufacturer's recommendations. Unprocessed gastrocnemius muscle removed during dissections was flash-frozen in liquid nitrogen and stored at −80 °C for later potential analyses.

Directed Akt-mTOR phosphoproteomics for in vivo experimentation

PathScan® Akt Signaling Antibody Array Kits (chemiluminescent readout; Cell Signaling, Danvers, MA, USA)

were used to identify phosphorylated proteins primarily belonging to the Akt-mTOR pathway signaling cascade as previously described (Mobley et al. 2015). Briefly, RIPA homogenates were diluted to 1.0 µg/µl using cell lysis buffer included in the kit and assayed according to manufacturer's instructions. Glass chips containing the Akt signaling markers were developed using an enhanced chemiluminescent reagent provided by the kit, and chip-spot densitometry was performed through the use of a digitized gel documentation system and associated densitometry software (UVP, Upland, CA, USA). Of note, each sample well of the assay was accompanied by a positive and negative control which was used to normalize between-sample values for each phosphorylated target. The calculation of each phosphorylated target was as follows:

(Density value of the target−density of the negative control)/
summation of densities for all phospho targets

It should be noted that this high throughput antibody chip array for muscle phosphorylation markers was used rather than single antibodies due to resource constraints. Moreover, others in the literature have used chemiluminescent-based phosphoarray chips for screening purposes just as we have utilized them herein (Olszewski et al. 2012; Victoni et al. 2014). Finally, prior publications from our laboratory using this method has shown that different doses of whey protein feedings in rats increases p-rps6, p-mTOR, and p-p70sk6 in a dose-dependent manner [supplementary Fig. 1 in (Mobley et al. 2015)], and these arrays are sensitive at detecting differences in phospho-signaling events that occur in rodents in response to cardiac ischemia–reperfusion (McGinnis et al. 2015).

Western blotting for in vivo experimentation

The SUnSET method was employed in order to examine if WP or LEU differentially affected post-exercise MPS. Gastrocnemius homogenates were prepared for Western blotting using 4x Laemmli buffer at 2 µg/µl. Thereafter, 20 µl of prepped samples were loaded onto hand-casted 12 % SDS–polyacrylamide gels (C.B.S. Scientific Company, San Diego, CA, USA) and subjected to electrophoresis (200 V @ 75 min) using pre-made 1x SDS-PAGE running buffer (C.B.S. Scientific Company). Proteins were then transferred to polyvinylidene difluoride membranes (Whatman™, Westran® Clear Signal), and membranes were blocked for 1 h at room temperature with 5 % nonfat milk powder. Mouse anti-puromycin IgG (1:5000; Millipore, Billerica, MA, USA) was incubated with membranes overnight at 4° C in 5 % bovine serum albumin (BSA), and the following day membranes were incubated with anti-mouse IgG secondary antibodies (1:2000, Cell Signaling) at room temperature for 1 h prior to membrane development described below. Given that mixed gastrocnemius muscle fibers can potentially yield varying normalizing protein amounts, membranes were stained with Coomassie following puromycin densitometry in order to ensure equal loading of samples between lanes. Membrane development was performed using an enhanced chemiluminescent reagent (Amersham, Pittsburgh, PA, USA), and band densitometry was performed through the use of a digitized gel documentation system and associated densitometry software (UVP).

Nuclear and Cytoplasmic RNA isolation for in vivo experimentation

Nuclear, cytoplasmic and total RNA fraction concentrations were extracted using a Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada). Briefly, 6–12 mg of frozen gastrocnemius muscle was homogenized via micropestle manipulation using provided lysis buffer. Once homogenized, samples underwent centrifugation at 14,000×*g* for 3 min. Supernatants were retained for cytoplasmic RNA fractions while pellets were retained for nuclear RNA fractions. Once isolated, nuclear and cytoplasmic RNA fractions were immediately subjected to further processes described in the manufacturer's instructions. Once nuclear and cytoplasmic RNA fractions were isolated, concentrations were immediately quantified in duplicate using a Nanodrop Lite spectrophotometer (Thermo Scientific). Prior studies have used this same technique to localize nuclear and cytoplasmic RNA fractions in various target tissues (Jaffe et al. 2015; Zaghlool et al. 2013). Furthermore, we confirmed on pilot samples that nuclear RNA isolates presented 4.8-fold greater 45S pre-rRNA levels versus the cytoplasmic fraction (*data not shown*) demonstrating relatively pure subcellular RNA fractionation.

Real-time PCR for in vivo experimentation

Following RNA isolation, total RNA concentrations were analyzed using a Nanodrop Lite spectrophotometer (Thermo Scientific) and 2 µg of gastrocnemius RNA was reverse transcribed into cDNA for real-time PCR (RT-PCR) analyses using a commercial qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). RT-PCR was performed using gene-specific primers (Table 1) and SYBR green-based methods in a RT-PCR thermal cycler (3 min DNA polymerase activation at 95 °C followed by 40 cycles of 95 °C at 15 s and 60 °C at 15 s) (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed using primer designer software (Primer3Plus, Cambridge, MA, USA), and melt curve analyses demonstrated that one PCR product was amplified per reaction. While primer efficiency curves were only run on a subset of primer sets, past primer

Table 1 Gene-specific primers used for RT-PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Rat primers		
c-Myc	CCCTCAGTGGTCTTCCCCTA	GCGAGTCCGAGGAAGAAGAG
Raptor	CACACAGGCACACACACATG	GGAAGACCAGGACACCCATG
45S pre-rRNA	TGGGGCAGCTTTATGACAAC	TAGCACCAAACGGGAAAACC
Nop56	GCATCCACAGTGCAGATCCT	CCCTTGTCTTCAGGGCTCTG
Bop1	CCCTTGATTGCCACTGTCCT	ACATAAGGTCAAGGCTGGGC
Ncl	GAGGGCAGAACAATCAGGCT	GCATTAGGTGATCCCCTGGG
Rpl3	ATGGTCGCTTCCAGACCATG	TGCGATCTTTCTTGAGCGGT
Npm1	TTCCGGATGACTGACCAGGA	ACTTCCTCCACTGCCAGAGA
Fb1	TGAAGCCTCAGGAGCAGTTG	GGTGGCCTGTACACACCTAC
Xpo-5	GGACAAGAGCTGGGCAGAAT	CCGTCAGAAGGGCAAGATGT
Nxt1	ATAGCGAGTGACTGCTTCCG	CCTTTGTCACCCCCACTAGC
Rps16 (HKG)	TCGCTGCGAATCCAAGAAGT	CCCTGATCCTTGAGACTGGC
Mouse primers		
c-Myc	TCTCTCCTTCCTCGGACTCG	GTGTCTCCTCATGCAGCACT
Raptor	GTGGGTGGTAAAGGCCTACC	AGCGCACATCTCCATTGACA
47S pre-rRNA	GCCTGTCACTTTCCTCCCTG	GCCGAAATAAGGTGGCCCTC
Nop56	AGTGAACCAGAAGAGGCAGC	CAGCAACTTCAGGCTCCTCA
Bop1	TGTGCCTCCACACAGAATCC	TTGCTGGAGGCTGAATCAGG
Ncl	AAGCCATGAAATCTGGGGCA	GATGCAAACACAGCCTCTGC
Rpl3	CATTGGCGTGACAAAAGGCA	CAACTTTGCGCAAACCTCGA
Npm1	AGGCGGTTCTCTTCCCAAAG	TCCTGGTCAGTCATCCGGAA
Fb1	CTGCGGAATGGAGGACACTT	GATGCAAACACAGCCTCTGC
Gusb (HKG)	TCAGCTCTGTGACCGATACG	GCCACAGACCACATCACAAC
Rer1 (HKG)	GCCTTGGGAATTTACCACCT	CTTCGAATGAAGGGACGAAA
Rpl-7l1 (HKG)	ACGGTGGAGCCTTATGTGAC	TCCGTCAGAGGGACTGTCTT

HKG housekeeping gene. Other abbreviations for each gene are provided in the body of the text

efficiency curves generated from our laboratory using this method of primer design and PCR protocol have yielded primer efficiencies within 90–110 % (McGinnis et al. 2015; Mobley et al. 2015). Fold-change values from the CTL non-EX condition were performed using the Livak method (i.e., $2^{-\Delta\Delta CT}$ assuming 100 % primer binding efficiency), where $2^{-\Delta CT} = [\text{housekeeping gene (HKG) CT} - \text{gene of interest CT}]$ and $2^{-\Delta\Delta CT}$(or fold - change)=[$2^{-\Delta CT}$value/$2^{-\Delta CT}$ average of CTL non - EX condition]. Of note, for in vivo analyses ribosomal protein S16 (Rps16) was used as a HKG given that it remained stable across all treatments and EX conditions.

Procedures for in vitro experimentation

C_2C_{12} myoblasts were grown in growth medium (GM; DMEM, 10 % FBS, 1 % penicillin/streptomycin, 0.1 % gentamycin) on 60 mm plates (Griener Bio-One GmbH, Maybachstr, Frickenhausen, GER) at a seeding density of 3.5×10^5 under standard culture conditions (37 °C in a 5 % CO_2 atmosphere). Differentiation was induced 48 h after myoblast growth reached 80–90 % confluency by removing GM and replacing it with differentiation medium [DM; DMEM, 2 % (vol/vol) horse serum, 1 % penicillin/streptomycin, 0.1 % gentamycin]. DM was then replaced every 24 h for 7d to allow for myotube growth.

After 7 days of DM treatments, cells were treated once for 6 h with: (1) DMEM only (CTL, $n = 6$), (2) 10 mM (1.3 µg/ml) L-leucine (LEU, $n = 6$) (EMD Chemicals, Inc.), (3) 100 mM (13 µg/ml) whey protein hydrolysate (WP, $n = 6$) (MusclePharm), (4) 5 mM (1.0 µg/ml) CAFF ($n = 6$) (Ameresco), (5) 1 mM (0.012 µl/ml) H_2O_2 ($n = 6$) (Ameresco), and (6) 2 mM (0.5 µg/ml) AICAR ($n = 6$) (Ameresco). Of note, DMEM was used as the vehicle for each respective treatment. The LEU dosage was based upon a prior study showing that mM concentrations amplified Akt phosphorylation (p) in C_2C_{12} myotubes (DeLong et al. 2011). The WP dosage was based upon the amino acid profile of WP with LEU making up approximately 12–14 % of the amino acid content (Hulmi et al. 2010).

Increases in intramuscular calcium (Beaton et al. 2002), reactive oxygen species (Reid and Durham 2002), and AMPK (Dreyer et al. 2006) activation have all been shown to be increased in skeletal muscle during resistance exercise;

thus, beyond the WP and LEU effects, we were also interested in examining if these 'exercise-like' signals promoted an increase in ribosome biogenesis markers. The CAFF dosage was based upon a previous study showing that 5 mM increased intracellular calcium levels in L6 myotubes (Barres et al. 2012). The AICAR dosage was based upon prior studies reporting that 2 mM increases AMPK activation in C_2C_{12} myotubes (Egawa et al. 2014), and we have performed preliminary experiments whereby 2 h 1 mM AICAR treatments increases AMPKα (Thr172) phosphorylation in C_2C_{12} myotubes by approximately 25-fold (*data not shown*). The H_2O_2 dosage was supported by a previous study that demonstrated this dosage of H_2O_2 increased atrophy-related mechanisms (McClung et al. 2009).

Following treatments, myotubes were lysed using 500 µl of Ribozol (Ameresco) per the manufacturer's recommendations. Total RNA concentrations were analyzed using a Nanodrop Lite spectrophotometer (Thermo Scientific), and 2 µg of cDNA was synthesized using a commercial qScript™ cDNA SuperMix (Quanta) per the manufacturer's recommendations. Real-time PCR was performed using gene-specific primers (Table 1) and SYBR green chemistry as discussed above. Fold-change values from the CTL treatment were performed using the Livak method (i.e., $2^{-\Delta\Delta CT}$ assuming 100 % primer binding efficiency), where $2^{-\Delta CT}$=[housekeeping gene (HKG) CT − gene of interest CT] and $2^{-\Delta\Delta CT}$(or fold - change)=[$2^{-\Delta CT}$value/$2^{-\Delta CT}$ average of CTL treatment]. For in vitro analyses beta-glucoronidase (Gusb) was used as a HKG for all 'nutrient signal' comparisons given that it remained stable across all treatments. Moreover, the geometric mean of retention in endoplasmic reticulum sorting receptor 1 (Rer1) and ribosomal protein 7 like 1 (Rpl7l1) was used as the HKG values for all 'exercise-like' treatment comparisons given that it remained stable across all treatments.

Statistics

Statistical comparisons of in vivo-dependent variables were run using two-way [treatment (WP versus LEU versus CTL) x exercise (EX versus non-EX legs)] mixed-factorial ANOVAs using SPSS v 22.0 (IBM, Armonk, NY, USA). When main effects for exercise were observed, within-treatment Student's paired t tests (EX versus non-EX leg) were performed. When a significant treatment or interaction was observed, between-treatment differences were examined using protected LSD post hocs. Statistical comparisons of in vitro dependent variables were performed using one-way ANOVAs, and protected LSD post hoc analyses were performed to determine where significant between-treatment effects occurred. All data herein are presented in figures and tables as mean ± standard error values and statistical significance was set at $p < 0.05$.

Results

Effects of WP versus LEU on in vivo post-exercise Akt-mTOR signaling markers

A significant treatment effect ($P = 0.030$) was observed with regards to p-Akt (Ser473) levels, as this marker was significantly greater in the LEU versus CTL treatment ($P < 0.01$, Fig. 1a). No significant effects for exercise ($P = 0.32$), treatment ($P = 0.25$) or an EX*treatment interaction ($P = 0.32$) were observed with regards to p-Akt (Thr308) levels (Fig. 1b). A significant exercise effect ($P < 0.001$) was observed with regards to p-mTOR (Ser2481) levels, as this marker was significantly greater in the CTL and WP non-EX legs versus the corresponding EX legs ($P < 0.05$, $P < 0.01$; respectively; Fig. 1c). No significant effects for exercise ($P = 0.20$), treatment ($P = 0.65$) or EX*treatment interaction ($P = 0.15$) were observed with regards to p-ERK1/2 (Thr202/Tyr204) levels (Fig. 1d). A significant exercise effect ($P = 0.003$) was observed with regards to p-70s6 k (Thr389) levels, as this marker was significantly higher in the LEU EX leg versus non-EX legs ($P < 0.01$; Fig. 1e). No significant effects for exercise ($P = 0.59$), treatment ($P = 0.76$) or EX*treatment interaction ($P = 0.50$) were observed with regards to p-RSK1 (Thr421) levels (Fig. 1f). A significant exercise effect ($P < 0.001$) was observed with regards to p-rpS6 (Ser235/236) levels, and this marker was significantly higher in the LEU and WP EX legs versus non-EX legs ($p < 0.001$; Fig. 1g). A significant exercise effect ($P = 0.010$) was observed with regards to p-4E-BP1 (Thr37/46) levels, as this marker was increased in the CTL EX versus non-EX legs only ($P < 0.05$; Fig. 1h).

Other phosphorylated markers of the Akt-mTOR signaling pathway that were altered between treatments and/or exercise are presented in Fig. 2. A significant exercise effect ($P < 0.001$) and treatment effect ($P < 0.001$) was observed with regards to p-GSK-3α (Ser21) levels. This marker was significantly greater in the LEU and WP treatments versus the CTL treatment ($P < 0.001$, $P < 0.001$, respectively), and was also significantly decreased in the CTL and LEU EX legs versus the corresponding non-EX legs ($P < 0.001$, $P < 0.001$, respectively; Fig. 2a). A significant exercise effect ($P = 0.009$) and EX*treatment interaction ($P = 0.009$) was observed with regards to p-GSK-3β (Ser9) levels. This marker was significantly decreased in the CTL and LEU EX legs versus the corresponding non-EX legs ($P < 0.001$, $P < 0.001$, respectively), and was also significantly elevated in the LEU and WP EX legs versus CTL EX legs ($P = 0.011$, $P < 0.001$, respectively; Fig. 2a). A significant treatment effect ($P < 0.001$) was observed with regards to p-AMPK (Thr172) levels. This marker

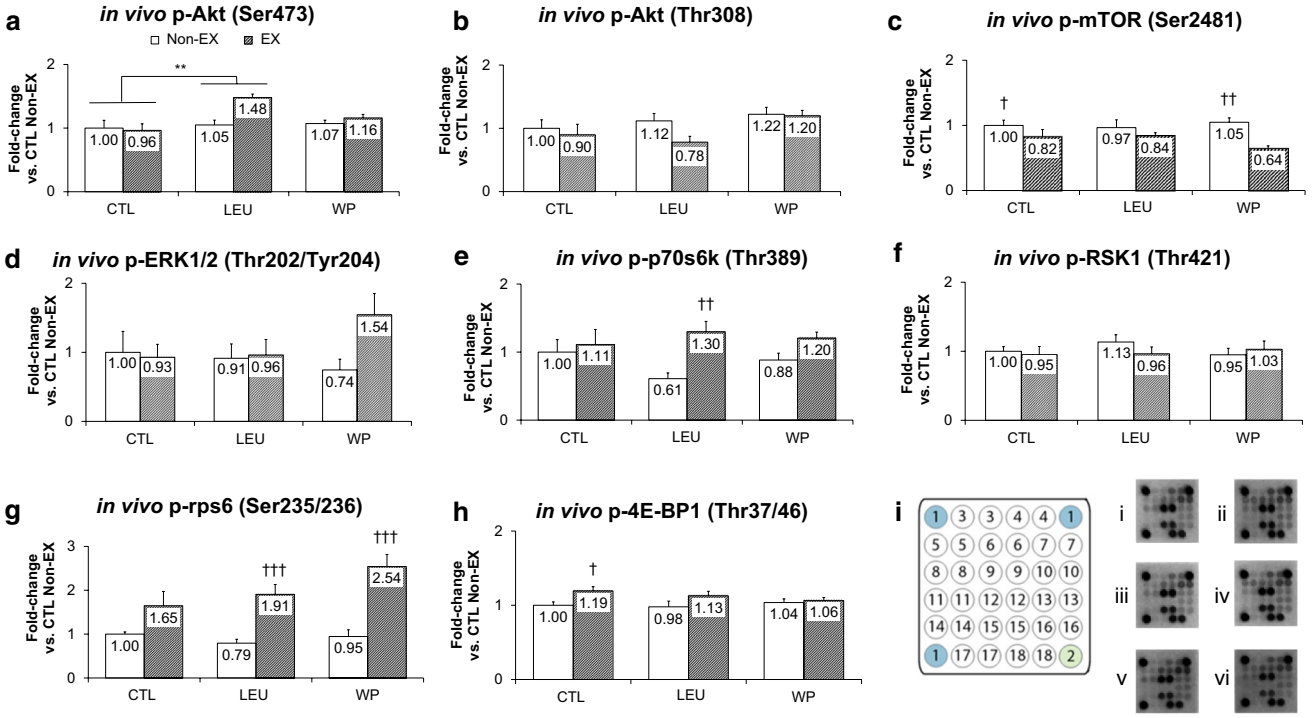

Fig. 1 Effects of WP versus LEU on post-exercise Akt-mTOR substrates in vivo. Effects of whey protein (WP) versus leucine (LEU) on gastrocnemius p-Akt (Ser473) (**panel a**), p-Akt (Thr308) (**panel b**), p-mTOR (Ser2481) (**panel c**), p-ERK1/2 (Thr202/Tyr204) (**panel d**), p-p70s6k1 (Thr389) (**panel e**), p-RSK1 (Thr421) (**panel f**), p-rps6 (Ser235/236) (**panel g**) and p-4E-BP1 (Thr37/46) (**panel h**). Data are numerically standardized to control (CTL) non-exercised (non-EX) values and presented as mean ± standard error [CTL, n = 8–10 per bar; LEU, n = 7–8 per bar; WP, n = 10 per bar]. Significant between-treatment differences are represented with *superscript symbols* (**$P < 0.01$) whereas within-treatment differences are represented with a *separate set of symbols* ($^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$; $^{\dagger}P < 0.05$). Schematic of Akt-mTOR array (**panel i** , *left inset*): *1*, positive control; *2*, negative control; *3*, p-Akt (Thr308); *4*, p-Akt (Ser473); *5*, p-rps6 (Ser235/236); *6*, p-AMPK-α (Thr172); *7*, p-PRAS40 (Thr246); *8*, p-mTOR (Ser2481); *9*, p-GSK-3α (Ser21); *10*, p-GSK-3β (Ser9); *11*, p-p70s6 k (Thr389); *12&14*, p-p70s6 k (Thr421/Ser424); *13*, p-BAD (Ser112); *15*, p-PTEN (Ser380); *16*, p-PDK1 (Ser241); *17*, p-ERK1/2 (Thr202/Tyr204); *18*, p-4E-BP1 (Thr37/46). Representative digitized array images (**panel i** , *right inset*) for CTL non-EX (**i**), CTL EX (**ii**), LEU non-EX (**iii**), LEU EX (**iv**), WP non-EX (**v**) and WP EX (**vi**)

was significantly decreased in the WP and LEU treatments versus CTL treatment ($P < 0.001$, $P < 0.001$, respectively; Fig. 2c). A significant exercise effect ($P = 0.033$) was observed with regards to p-PRAS40 (Thr246) levels. This marker was significantly higher within the LEU non-EX leg versus the EX legs ($P < 0.05$; Fig. 2d). No exercise effect ($P = 0.464$), treatment effect ($P = 0.940$) or EX*treatment interaction ($P = 0.382$) was observed with regards to p-PTEN (Ser380) (Fig. 2e). A significant exercise effect ($P < 0.001$) was observed with regards to p-BAD (Ser112) levels. This marker was significantly increased within the LEU and WP EX legs versus corresponding non-EX legs ($P < 0.001$, $p < 0.01$, respectively; Fig. 2f).

Effects of WP versus LEU on post-exercise MPS levels in vivo

A significant exercise effect ($P < 0.001$) was observed with regards to MPS responses. WP treatment elicited the greatest increase in an MPS response in the EX versus non-EX legs ($P < 0.001$; Fig. 3a), and a significant increase in MPS occurred in the EX versus non-EX legs within the CTL treatment ($P < 0.01$). Interestingly, LEU did not statistically increase MPS in the EX versus non-EX legs ($P > 0.05$).

Effects of WP versus LEU on post-exercise markers of ribosome biogenesis in vivo

A significant exercise effect ($P < 0.001$) was observed with regards to c-Myc mRNA expression. CTL, LEU and WP-fed rats experienced a robust increase in c-Myc mRNA in the EX versus non-EX legs ($P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively; Fig. 4a). No exercise effect ($P = 0.128$), treatment effect ($P = 0.089$) or EX*treatment interaction ($P = 0.156$) was observed with regards to Raptor mRNA expression (Fig. 4b). A significant exercise effect ($P = 0.013$) was observed with regards to 45S pre-rRNA expression, as this marker increased in the EX versus non-EX legs of LEU-fed rats ($P < 0.001$) though there was

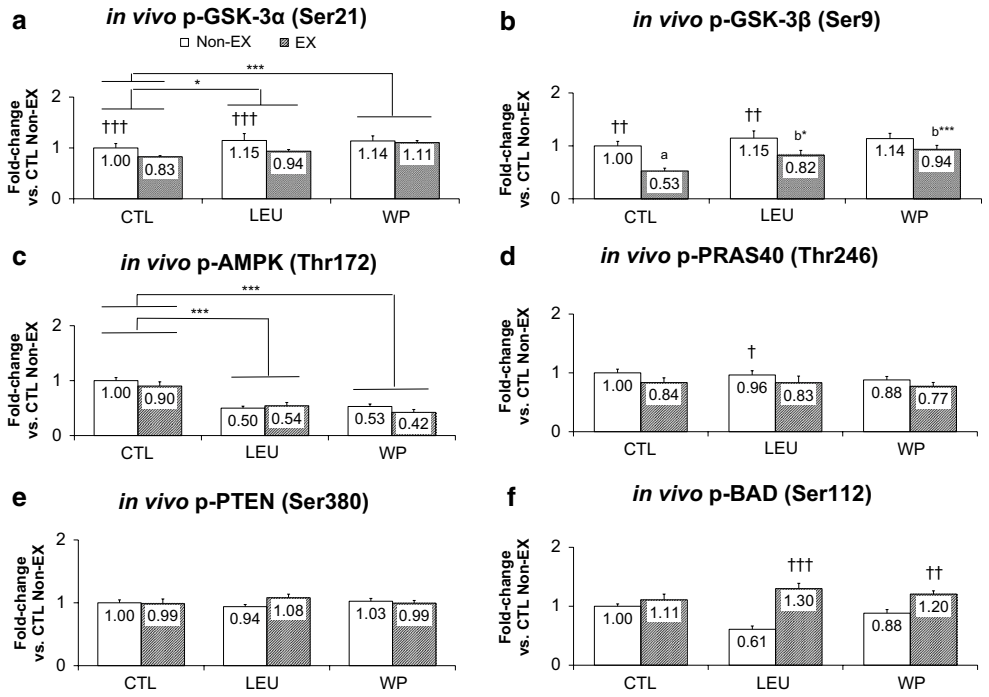

Fig. 2 Effects of WP versus LEU on additional post-exercise Akt-mTOR substrates in vivo. Effects of whey protein (WP) versus leucine (LEU) on gastrocnemius p-GSK-3α (Ser21) (**panel a**), p-GSK-3β (Ser9) (**panel b**), p-AMPK (Thr172) (**panel c**), p-PRAS40 (Thr246) (**panel d**) and p-PTEN (Ser380) (**panel e**) and p-BAD (Ser112) (**panel f**). Data are numerically standardized to control (CTL) non-exercised (non-EX) values and presented as mean ± standard error [CTL, $n = 8$–10 per bar; LEU, $n = 7$–8 per bar; WP, $n = 10$ per bar]. Significant between-treatment differences are represented with *superscript symbols* ($^{*}P < 0.05$; $^{***}P < 0.001$), within-treatment *differences* are represented with a *separate set of symbols* ($^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$; $^{\dagger}P < 0.05$), and between-treatment EX leg differences (EX*treatment interaction $P < 0.05$) are represented by *different superscript letters* whereby the degree of significance is further denoted by accompanying *asterisks* (e.g., **a** versus **b***, $P < 0.05$; **a** versus **b*****, $P < 0.001$)

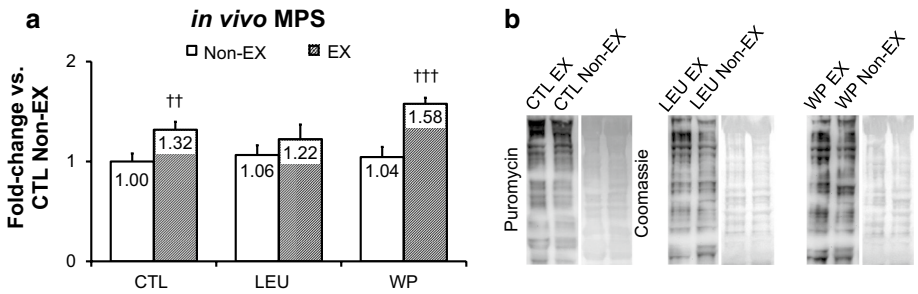

Fig. 3 Effects of WP versus LEU on post-exercise skeletal muscle protein synthesis in vivo. Effects of whey protein (WP) versus leucine (LEU) on gastrocnemius muscle protein synthesis (MPS; **panel a**). Data are numerically standardized to control (CTL) non-exercised (non-EX) values and presented as mean ± standard error [CTL, $n = 8$–10 per bar; LEU, $n = 7$–8 per bar; WP, $n = 10$ per bar]. Significant within-treatment differences are represented with *superscript symbols* ($^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$). Representative digital images of puromycin integration into skeletal muscle protein (SUnSET determination of muscle protein synthesis) with representative digital images of Coomassie *Brilliant Blue* staining to visually ensure equal loading of proteins (**panel b**)

no significant increase within the WP or CTL treatments (Fig. 4c).

Given that some of the select aforementioned markers of ribosome biogenesis were altered by exercise we were next interested in determining subcellular total RNA concentrations; these being surrogates for ribosome content. A significant EX*treatment interaction ($P = 0.002$) was observed with regards to nuclear RNA content, as this marker was significantly elevated in the EX legs of CTL versus LEU and WP rats ($P = 0.009$, $P < 0.007$, respectively; Fig. 4d). No significant exercise effect ($P = 0.722$), treatment effect ($P = 0.171$) or EX*treatment interaction

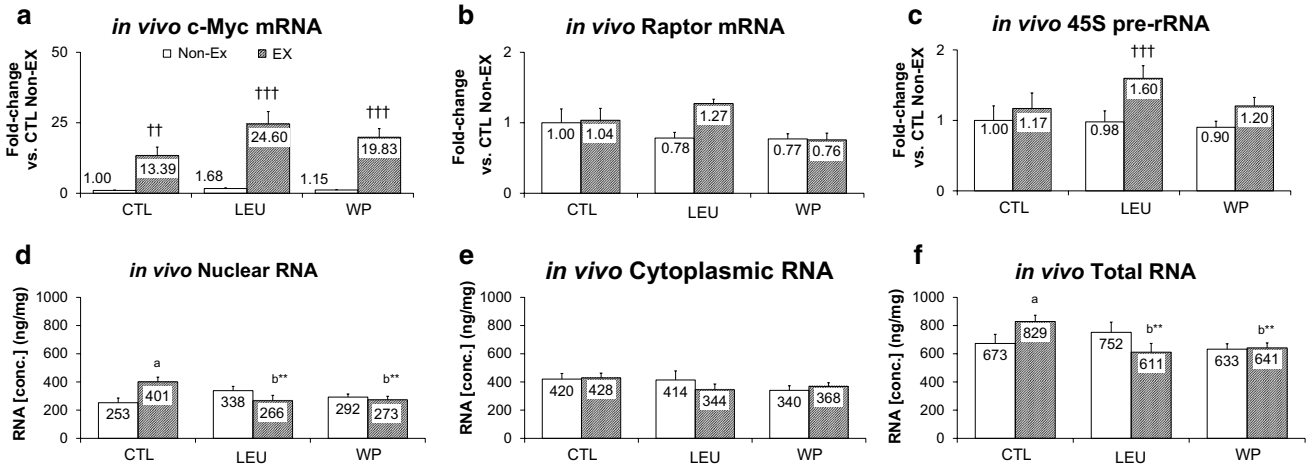

Fig. 4 Effects of WP versus LEU on post-exercise markers of ribosome biogenesis in vivo. Effects of whey protein (WP) versus leucine (LEU) on gastrocnemius c-Myc mRNA (**panel a**), Raptor mRNA (**panel b**), 45S pre-rRNA (**panel c**), nuclear RNA (**panel d**), cytoplasmic RNA (**panel e**) and total tissue RNA (**panel f**). mRNA data are numerically standardized to control (CTL) non-exercised (non-EX) values, and sub-cellular RNA are presented as concentration values. All data are presented as mean ± standard error [CTL, $n = 8$–10 per bar; LEU, $n = 7$–8 per bar; WP, $n = 10$ per bar]. Significant within-treatment differences are represented with a *separate set of symbols* ($^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$; $^{\dagger}P < 0.05$), and between-treatment EX leg differences (EX*treatment interaction $P < 0.05$) are represented by *different superscript letters* whereby the degree of significance is further denoted by accompanying *asterisks* (e.g., **a** versus **b****, $P < 0.01$)

($P = 0.476$) was observed with regards to cytoplasmic RNA content (Fig. 4e). A significant EX*treatment interaction ($P = 0.032$) was observed with regards to total RNA content, as this marker was significantly elevated in the EX legs of CTL versus LEU and WP ($P = 0.004$, $P < 0.006$, respectively; Fig. 4f), though these effects were likely driven by nuclear RNA content differences between treatments described above.

Of note, the assayed muscle weights for RNA content between each treatment group were not different ($P > 0.05$) as were as follows: CTL Non-EX = 9.4 ± 0.7 mg, CTL EX = 9.1 ± 0.7 mg, LEU Non-EX = 10.1 ± 1.3 mg, LEU EX = 9.0 ± 0.7 mg, WP Non-EX = 10.3 ± 0.8 mg, WP EX = 9.7 ± 0.4 mg.

Resistance exercise robustly increases markers of rRNA processing, ribosome assembly and nuclear ribosome export independent of post-exercise nutrient provision in vivo

A significant exercise effect ($P < 0.001$) was observed with regards to Nop56 mRNA expression, as this marker was significantly higher in the EX versus non-EX legs in the CTL, LEU and WP treatments ($P < 0.001$, $P < 0.001$, $P < 0.01$, respectively; Fig. 5a). A significant exercise effect ($P < 0.001$) was observed with regards to block of proliferation 1 (Bop1) mRNA expression, as this marker was significantly higher in the EX versus non-EX legs in the CTL, LEU and WP treatments ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively; Fig. 5b). A significant exercise effect ($P < 0.001$) was observed with regards to nucleolin (Ncl) mRNA expression, as this marker was significantly increased in the EX versus non-EX legs in the CTL, LEU and WP treatments ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively; Fig. 5c). A significant exercise effect ($P < 0.001$) was observed with regards to ribosomal protein like 3 (Rpl3) mRNA expression, as this marker was significantly increased in the EX versus non-EX legs in the CTL and LEU treatments only ($P < 0.01$, $P < 0.01$, respectively; Fig. 5d). A significant exercise effect ($P < 0.001$) was observed with regards to nucleophosmin (Npm1) mRNA expression, as this marker was significantly higher in the EX versus non-EX legs in the CTL, LEU and WP treatments ($P < 0.001$, $P < 0.001$, $P < 0.05$, respectively; Fig. 5e). A significant exercise effect ($P < 0.001$) and treatment effect ($P = 0.006$) was observed with regards to fibrillarin (Fb1) mRNA expression. This marker was significantly higher in the EX versus non-EX legs in the CTL, LEU and WP treatments ($P < 0.001$, $P < 0.01$, $P < 0.001$, respectively; Fig. 5f). Moreover, Fb1 mRNA expression was greater in the CTL versus LEU treatment ($P = 0.002$). A significant exercise effect ($P < 0.001$) was observed with regards to exportin-5 (Xpo-5) mRNA expression, as this marker was significantly higher in the EX versus non-EX legs in the CTL, LEU and WP treatments ($P < 0.05$, $P < 0.001$, $P < 0.05$, respectively; Fig. 5g). Finally, no significant exercise effect ($P = 0.112$), treatment effect ($P = 0.602$) or EX*treatment interaction ($P = 0.758$) was

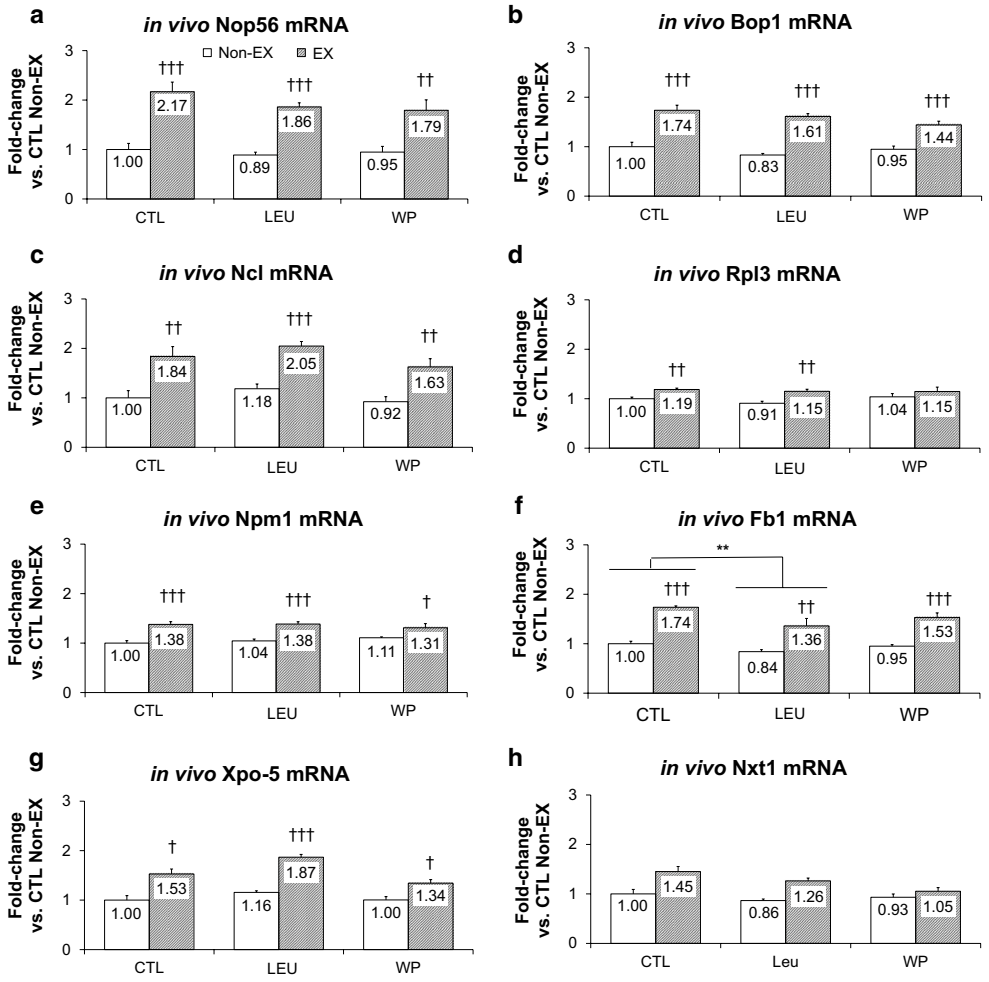

Fig. 5 Effects of WP versus LEU on post-exercise markers of rRNA processing, ribosome assembly and nuclear ribosome export in vivo. Effects of whey protein (WP) versus leucine (LEU) on gastrocnemius Nop56 (**panel a**) and Bop 1 (**panel b**), Ncl (**panel c**) and Rpl3 (**panel d**), Npm1 mRNA (**panel e**), Fbl mRNA (**panel f**), Xpo-5 mRNA (**panel g**), and Nxt1 mRNA (**panel h**). Data are numerically standardized to control (CTL) non-exercised (non-EX) values and presented as mean ± standard error [CTL, n = 8–10 per bar; LEU, n = 7–8 per bar; WP, n = 10 per bar]. Significant between-treatment differences are represented with *superscript symbols* (**$P < 0.01$), within-treatment differences are represented with a *separate set of symbols* (†††$P < 0.001$; ††$P < 0.01$; †$P < 0.05$)

observed with regards to NTF2-like export factor 1 (Nxt1) mRNA expression (Fig. 5h).

Mechanistic investigation in vitro reveals that various nutrient and 'exercise-like' signals drive the genetic expression of ribosome biogenesis markers

Regarding regulators of ribosome biogenesis, c-Myc mRNA significantly increased in the WP versus the CTL and LEU treatments ($P < 0.001$; Fig. 6a). H_2O_2 significantly increased c-Myc mRNA versus the CTL and CAFF treatments ($P < 0.05$), and AICAR significantly increased the expression of this gene over all of the select 'exercise-like' treatments ($P < 0.01$; Fig. 6a). Raptor mRNA was downregulated in the WP versus the CTL and LEU treatments ($P < 0.01$; Fig. 6b). Raptor mRNA in the CTL, CAFF and AICAR treatments were significantly greater than the H_2O_2 treatment ($P < 0.01$; Fig. 6b). LEU increased 47S pre-rRNA over CTL ($P < 0.01$) and WP treatments ($P < 0.01$; Fig. 6c). Interestingly, CAFF elicited a robust increase in 47S pre-rRNA expression over all other 'exercise-like' treatments ($P < 0.001$), and H_2O_2 increased 47S pre-rRNA expression versus the CTL ($P < 0.05$) and AICAR treatments ($P < 0.001$; Fig. 6c).

Regarding regulators of rRNA processing, LEU and WP significantly increased Nop56 mRNA versus the CTL treatment ($P = 0.001$, $P < 0.001$, respectively; Fig. 6d), though when comparing both nutrients, WP significantly increased the expression of this gene versus the LEU treatment ($P < 0.05$; Fig. 6d). AICAR significantly increased the expression of Nop56 mRNA versus the CAFF and CTL treatments ($P < 0.001$), and H_2O_2 increased the expression

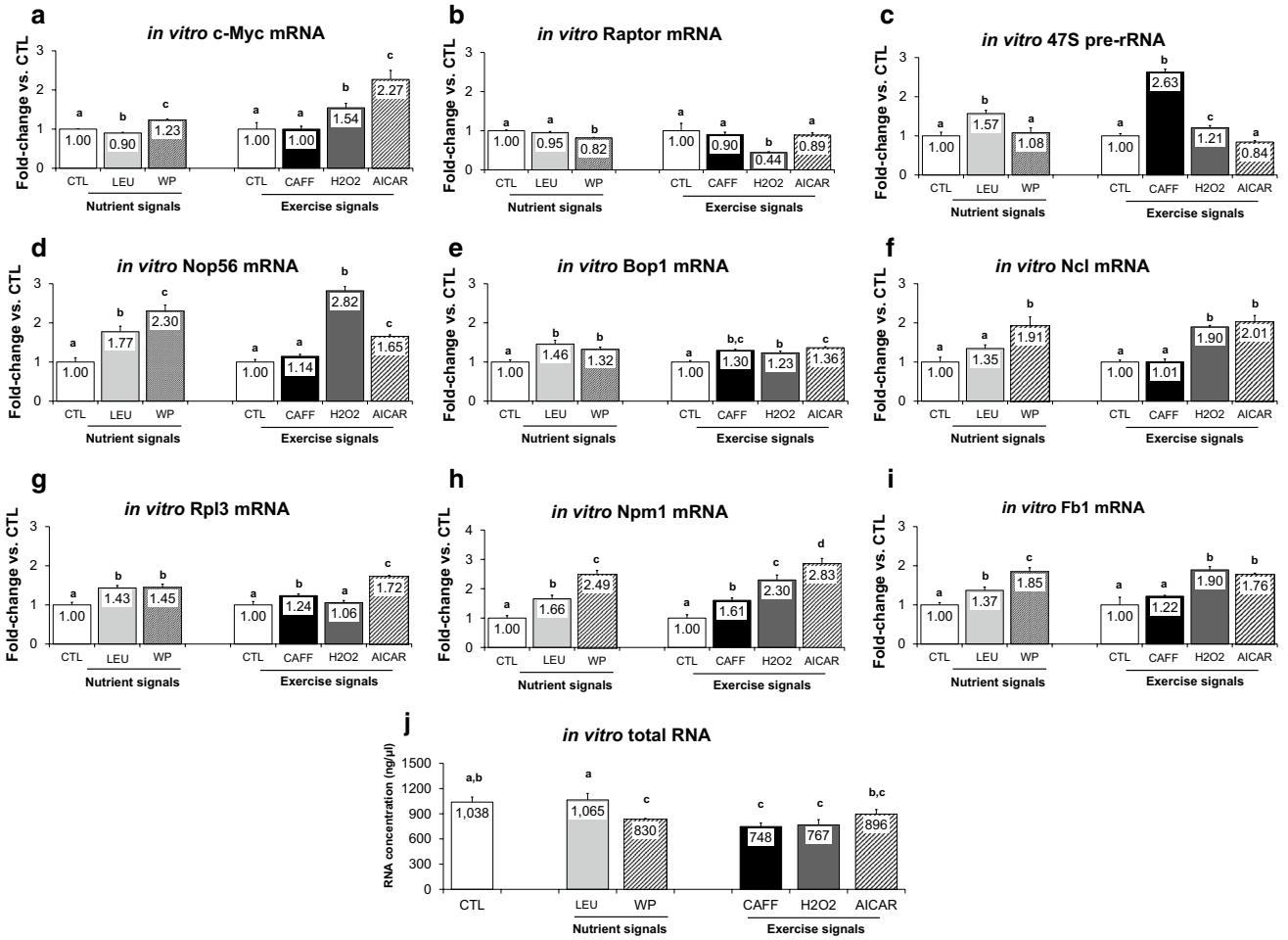

Fig. 6 Effects of nutrient or exercise signals on various in vitro markers of ribosome biogenesis, rRNA processing, rRNA assembly and nuclear ribosome export. Effects of nutrient or exercise signals on various myotube markers of ribosome biogenesis: c-Myc (**panel a**), Raptor (**panel b**), 47S pre-rRNA (**panel c**), Nop56 mRNA (**panel d**), Bop1 mRNA (**panel e**), Ncl mRNA (**panel f**), Rpl-3 mRNA (**panel g**), Npm1 mRNA (**panel h**), Fb1 mRNA (**panel i**), and total RNA (**panel j**). PCR data are numerically standardized to control (CTL) treatments are presented as mean ± standard error ($n = 6$ per treatment). One-way ANOVAs with a Fisher's LSD post hoc test were performed and significant between-treatment differences are represented with *different superscript letters* ($p < 0.05$)

of this gene over all other 'exercise-like' treatments ($P < 0.001$; Fig. 6d). LEU and WP significantly increased Bop1 mRNA versus the CTL treatment ($P < 0.01$, $P < 0.05$, respectively; Fig. 6e). CAFF, H_2O_2 and AICAR treatments elicited modest but significant increases in Bop1 mRNA versus the CTL treatment ($P < 0.01$; Fig. 6e).

Regarding regulators of ribosome assembly, Ncl mRNA was significantly greater in the WP versus the LEU and CTL treatments ($P < 0.05$, $P = 0.001$, respectively; Fig. 6f). H_2O_2 and AICAR increased Ncl mRNA versus the CTL and CAFF treatments ($P < 0.001$; Fig. 6f). LEU and WP increased Rpl3 mRNA versus the CTL treatment ($P = 0.001$; Fig. 6g). CAFF significantly increased Rpl3 mRNA versus the CTL and H_2O_2 treatments ($P < 0.05$), and AICAR significantly increased Rpl3 mRNA versus the CTL and H_2O_2 treatments ($P < 0.001$; Fig. 6g).

Regarding regulators of nuclear ribosome export, LEU and WP increased Npm1 mRNA versus the CTL treatment ($P < 0.001$; Fig. 6h), and WP was significantly greater than the LEU treatment ($P < 0.001$). CAFF, H_2O_2 and AICAR increased Npm1 mRNA versus the CTL treatment ($P < 0.001$), with AICAR exhibiting a greater up-regulation of this gene versus other 'exercise-like' treatments ($P < 0.001$; Fig. 6h). LEU and WP significantly increased Npm1 mRNA versus the CTL treatment ($P < 0.001$, $P < 0.001$, respectively), and WP elicited a greater increase in the expression of this gene versus the LEU treatment ($P < 0.001$; Fig. 6i). CAFF, H_2O_2, and AICAR increased Fb1 mRNA versus the CTL treatment ($P < 0.01$), while H_2O_2 and AICAR elicited greater increase in the expression of this gene over CAFF ($P < 0.01$, $P < 0.001$, respectively; Fig. 6i).

Finally, compared to the CTL treatment, total RNA was not altered in the LEU treatment ($P = 0.74$), although this marker was lower in the WP treatment ($P = 0.013$; Fig. 6j). Likewise, compared to the CTL treatment, total RNA was lower in the CAFF ($P = 0.001$) and H_2O_2 ($P = 0.002$) treatments and trended lower in the AICAR treatment ($P = 0.082$).

Discussion

We report that WP and LEU both stimulate select markers of Akt-mTOR substrate phosphorylation 3 h following resistance exercise in vivo, though a superior post-exercise MPS response exists when WP versus LEU is fed immediately following exercise. While our study is limited in that it is acute and only examined a single post-exercise time point, several hypotheses can be generated. Specifically, given that the employed exercise model as well as 'exercise-like' signals in vitro acutely increase mRNAs related to different processes of ribosome biogenesis, longer-term resistance exercise may enhance various facets of ribosome biogenesis independent of nutrient provision. Furthermore, LEU was more effective than WP in increasing rDNA transcription, and WP was more effective than LEU in enhancing MPS. These findings are discussed in further detail in the following paragraphs.

Human relevancy of the acute rat exercise and feeding model

It is first important to discuss that the acute exercise rat model employed herein has limitations; specifically, the potential species differences that exist between rats and humans as well as the 'human relevancy' of the leg-kicking model. Indeed, rats and humans differ with regards to whole body metabolism (Davies and Morris 1993). However, acute rodent exercise and feeding models have been employed in prior literature, and rat hind limb skeletal muscle tissue has been analyzed in order to make 'human-relevant' conclusions. For instance, a hallmark study by Booth and Wong (Wong and Booth 1990) utilized an unloaded concentric plantarflexor stimulation (192 repetitions) bout using subdermal electrodes, and the authors reported that gastrocnemius MPS increased by 47 % 12–17 h post-exercise. Herein, we report that gastrocnemius MPS in exercised, un-fed rats increased by 32 % at 3 h post-exercise after 32 repetitions using the same stimulation set-up. A hallmark human study by Phillips et al. (Phillips et al. 1997) demonstrated that acute resistance exercise in the fasted state increases mixed muscle MPS in the vastus lateralis by ~100 % 3 h post-exercise. Thus, while acute leg-kicking rat studies show a lessened MPS response relative to human data, both models do show a significant directional increase in MPS. With regards to the phospho-signaling response to the model, we report that non-fed rats experienced a 65 % (non-significant) increase in p-rps6 levels and this is similar to a human study by Rasmussen's group who reported that younger humans experience a ~75 % (non-significant) increase in this marker 3 h following resistance exercise as well (Drummond et al. 2011). However, our findings differ from human literature in that we detected a modest 11 % (non-significant) increase in p-p70s6 k (Thr389) and a significant 19 % increase p-4E-BP1 (Th37/46) in our model 3 h post-exercise in non-fed rats, whereas Dreyer et al. (Dreyer et al. 2006) reported significant increases in p-p70s6 k up to 2 h post exercise but no change in p-4E-BP1. Collectively, when comparing our current data to past rodent and human models, it is apparent that subtle differences do exist although we posit that this acute exercise model is relatively translatable to humans.

With regards to whey protein feeding studies in rats, these studies have been previously performed by our group (Mobley et al. 2015) as well as others (Norton et al. 2012), and it is apparent that rodent studies have reported findings that parallel human findings (Tang et al. 2009) with regards to whey protein-induced increases in post-prandial MPS levels in non-exercised limbs.

It should finally be noted as to why we chose to analyze the gastrocnemius muscle rats versus other muscle groups. The rat gastrocnemius is made up of a heterogeneous pool of slow-twitch and fast-twitch fibers that is more similar to human locomotor muscles (Armstrong and Phelps 1984). Alternatively, the plantaris and soleus muscles are predominantly fast-twitch and slow-twitch muscles (Armstrong and Phelps 1984), respectively. Thus, we chose not to analyze the latter two muscle groups given that their proportions are less 'human relevant' relative to larger human muscle groups which are heterogeneous with regards to their fiber type make-up.

Effects of WP versus LEU on post-exercise Akt-mTOR signaling and MPS

Farnfield et al. (Farnfield et al. 2012) reported that the exercise-induced phosphorylation of rpS6, p70s6 k, and 4E-BP1 are synergistically enhanced with WP feeding in untrained young and/or older participants 2 h following resistance exercise, and others (Apro and Blomstrand 2010) have reported that resistance exercise and a LEU-containing BCAA drink elicit both separate and combined effects on p70S6 k (Thr389) phosphorylation. Herein, we report that LEU- and WP-fed rats similarly increased p-rps6 (Ser235/236) in the EX leg, but this response did not differ between groups. Likewise, beyond modest

LEU-induced increases in p-p70s6 k in the EX versus non-EX leg, the feeding conditions did not amplify exercise-induced increases in p-4E-BP1 and there was a main effect for exercise regarding a decrease in p-mTOR (Ser2481). These phenomena may have been due to one of the several factors which include: (1) the employed exercise bout, while increasing MPS as discussed above, may have been a relatively 'mild' anabolic stimulus due to only employing only concentric contractions for 32 repetitions, and (2) the phosphorylation status of upstream Akt-mTOR signaling substrates (i.e., Akt, ERK, RSK1, etc.) likely peaked at an earlier post-exercise time point (1–2 h) and returned to basal levels by 3 h post-exercise, as this has been shown to occur in humans with and without whey protein ingestion following resistance exercise (Farnfield et al. 2009). Likewise, resistance exercise-induced decreases in p-mTOR (Ser2448) have been reported in humans despite increases in p-p70s6 k and p-rps6 (Gonzalez et al. 2015), and this may be due to negative feedback on mTOR that occurs within hours after exercise in order to prevent uncontrolled increases in MPS [i.e., a refractory period posited by Wilson and colleagues (Wilson et al. 2011)].

Notwithstanding, we are the first to show that WP elicits a greater post-exercise MPS response in exercised skeletal muscle compared to a dose of LEU that was similar to what was contained within the WP dose. While this finding is unique, our findings are tempered by the fact that CTL (non-fed) animals experienced a significant increase in MPS levels in the EX leg as well. However, post hoc MPS analyses between the EX legs of all treatments ($P = 0.063$, not reported in the results due to the lack of an interaction), revealed that the 58 % increase in MPS in WP rats approached statistical significance ($P = 0.082$) when compared to the 32 % increase in MPS in the CTL rats. When comparing the WP versus LEU MPS responses, it is also important to note that LEU may have led to earlier (90–120 min) increases in MPS which, in turn, may have led to a 3 h downward shift in MPS. Of note, this hypothesis is supported by Wilson et al. (Wilson et al. 2011) who reported that LEU-induced increases in MPS peak at 90 min and this can lead a subsequent post-prandial refractory period whereby MPS is diminished. Notwithstanding, there is ample evidence to suggest that WP provides a greater anabolic stimulus versus LEU alone. For instance, chronic WP supplementation has been consistently shown to lead to significant gains in lean body mass (Katsanos et al. 2008; Phillips 2014; Tipton and Phillips 2013), whereas chronic LEU supplementation has not proven to be efficacious (Balage and Dardevet 2010). Moreover, WP has been shown to promote a greater muscle mass recovery after immobilization-induced atrophy in aged rats compared to LEU feeding alone (Magne et al. 2012). Hence, given that WP increased post-exercise MPS in the current study whereas LEU did not, we hypothesize that other 'bioactive compounds' (i.e., microRNAs and/or di- or oligopeptides beyond LEU alone) may be present in WP which drive this superior increases in MPS. This has been posited in prior literature (Izumi et al. 2013; Madureira et al. 2010) and should be further explored.

Differential effects of WP versus LEU on other Akt-mTOR pathway markers following exercise

Glycogen synthase kinase 3 (GSK-3) is a serine or threonine kinase that elicits inhibitory effects on downstream kinases (Ali et al. 2001; Woodgett 1994, 2001). Akt and p70s6 k can phosphorylate and inhibit GSK-3 (Zhang et al. 2006). However, when GSK-3 is not phosphorylated, it inhibits eukaryotic elongation factors which results in decreased MPS (Bodine et al. 2001; Rommel et al. 2001); Vyas et al. (2002) confirmed this by demonstrating that an increased GSK-3β phosphorylation results in a decrease in GSK activity and subsequent myotube hypertrophy in vitro.

In the present study, LEU and WP increased GSK-3α phosphorylation compared to CTL rats independent of exercise. Moreover, LEU and WP increased GSK-3β phosphorylation compared to CTL rats in the exercised limb. Thus, our data suggest that some of the anabolic effects of LEU and/or WP with exercise may operate through GSK-3 inhibition. However, we report that, regardless of nutritional provision, GSK-3 phosphorylation is decreased with exercise; a finding which parallels our findings with exercise-induced decreases in mTOR phosphorylation. This finding is difficult to reconcile given that muscle contraction in situ has been shown to increase the phosphorylation of GSK-3α and GSK-3β immediately after contraction (Sakamoto et al. 2002). However, others have reported that resistance exercise and BCAA provision do not affect GSK-3β phosphorylation immediately, 1 h or 2 h after exercise (Blomstrand et al. 2006). Likewise, it remains possible in the current study that, as with decreases in mTOR phosphorylation 3 h following exercise, the employed muscle contraction may have led to rapid rises in the phosphorylation of GSK-3α and GSK-3β (0–1 h following exercise) which subsequently led to an exercise-induced refractory period in these markers by 3 h post-exercise. Thus, more research is needed to better elucidate if resistance exercise and/or nutrient provision around exercise affects GSK-3 phosphorylation and activity.

As described by others (Anthony and Anthony 2008), AMP-activated protein kinase (AMPK) inhibits mTOR signaling and downstream anabolic signaling. We observed that LEU and WP decreased AMPK phosphorylation compared to CTL rats independent of exercise suggesting that AMPK activity is inhibited with these two treatments. Other studies (Jakubowicz and Froy 2013; Li et al. 2011)

agree with our findings, and the authors of these studies attribute the reduction in AMPK activity to a high protein diet or WP, respectively, with an emphasis on LEU content being the primary contributing component to AMPK inhibition. Ropelle et al. (Ropelle et al. 2008) similarly demonstrated that rats fed a high-protein diet present a decrease in AMPK phosphorylation and an increase in mTOR phosphorylation, although this study used casein protein instead of WP. Saha et al. (Saha et al. 2010) confirmed that p-AMPK (Thr172) decreased in rat extensor digitorum longus muscle incubated for 1 h in the presence of a higher concentration of LEU versus other BCAAs while concurrently increasing protein synthesis. A more recent study (Wilson et al. 2011) demonstrated that AMPK (Thr172) phosphorylation is further decreased with LEU administration after the consumption of a protein-containing meal, thus prolonging MPS. Thus, our findings support recent findings in the literature suggesting that LEU and WP can suppress AMPK phosphorylation which, in turn, may suppress AMPK activity and allow downstream mTOR signaling to occur to a greater degree or for a longer duration after exercise.

Effects of exercise-like signals on aspects of ribosome biogenesis

Herein, we report that c-Myc mRNA, a gene which encodes a transcription factor that activates a sundry of genes related to ribosome biogenesis (Chaillou et al. 2014; Guo et al. 2000), robustly increased 3 h following resistance exercise independent of nutrient provision. Our data are in agreement with Nader et al. (Nader et al. 2014) who similarly demonstrated that c-Myc mRNA expression robustly increases (>50-fold) in human skeletal muscle 4 h following an acute resistance exercise bout. Beyond being a putative regulator of ribosome biogenesis, and as mentioned earlier, the c-Myc transcription factor acts as an enhancer of RNA polymerase II activity to increase the expression of numerous genes related to growth, proliferation, and metabolism. Therefore, the exercise-induced increases in c-Myc mRNA observed herein likely indicates that, beyond a potential up-regulation in c-Myc-regulated Pol I and Pol III activity, other c-Myc-regulated genes are likely up-regulated in response to resistance exercise.

Our in vivo data also suggest that, in spite of post-exercise nutrient provision, resistance exercise increased mRNA expression patterns of genes related to rRNA processing (Nop56 and Bop1), ribosome assembly (Ncl and Rpl3) and nuclear ribosome export (Npm1, Fb1, Xpo-5, and Nxt1). While these genes are known to increase 1–7 days in response to synergist ablation-induced skeletal muscle overload (Chaillou et al. 2013), which is an extreme model of muscle hypertrophy, we are the first to report these findings using a rodent resistance exercise model.

In order to glean potential mechanisms whereby resistance exercise affected these ribosome biogenesis markers, we performed a series of in vitro experiments examining if 'exercise-like' signals affect the mRNA expression patterns of these genes. While the 'exercise-like' signals likely do not fully emulate resistance exercise, AMPK activation, intramuscular calcium influx, and ROS generation all increase with resistance exercise (references in "Methods"). Therefore, AICAR, CAFF and H_2O_2 treatments were performed, respectively, to supplement our in vivo exercise model. Interestingly, AICAR treatments increased the mRNA expression patterns of c-Myc as well as genes related to rRNA processing (Nop56 and Bop1), ribosome assembly (Ncl and rpl-3) and nuclear ribosome export (Npm1, Fb1). While acute resistance exercise is known to increase MPS rates up to 48 h following exercise (Phillips et al. 1997), MPS rates are inhibited during exercise due to an activation in AMPK (Dreyer et al. 2006). Thus, given that AICAR increases AMPK activation, we posited that the exercise-induced increase in many of the aforementioned genes related to ribosome biogenesis may have been due to an increase in AMPK activation during resistance exercise. CAFF and H_2O_2 treatments in vitro also affected several of the same genes related to ribosome biogenesis, though CAFF did not affect c-Myc, Ncl or Fb1 mRNA expression and H_2O_2 did not increase Rpl3 mRNA expression. Notwithstanding, our in vitro data may suggest that the generation of reactive oxygen species, increases in intracellular calcium and AMPK activation during resistance exercise may all act in tandem to increase mRNA expression patterns of genes involved with various aspects of ribosome biogenesis.

L-leucine, but not WP, may potentiate rDNA transcription independent of exercise

Interestingly, LEU provision after exercise increased 45S pre-RNA compared to CTL and WP-fed animals. Furthermore, LEU elicited a modest (~60 %) but significant increase in rDNA transcription in vitro compared to the WP and CTL conditions. Given that LEU activates mTOR activity, and mTOR has been posited to enhance rDNA transcription rates, these data continue to suggest that skeletal muscle rDNA transcription is largely regulated by mTOR activation and can be modulated by LEU ingestion.

However, these data are difficult to reconcile given that WP feeding following exercise nor WP treatments in vitro failed to potentiate rDNA transcription rates. Moreover, while LEU sustained myotube RNA compared to the CTL treatment in vitro, WP treatments decreased RNA content. Indeed, WP contains approximately 12–15 % LEU

(Hulmi et al. 2010), and prior data has shown that WP feedings increase the appearance of LEU in circulation within 30 min of ingestion (Tang et al. 2009). However, WP may have other 'bioactives' as suggested above which prevents LEU-mediated increases in rDNA transcription. In this regard, future studies are needed to determine the time course of rDNA transcription rates following resistance exercise with and without nutrient provision to further elucidate how various dietary agents affect ribosome biogenesis processes.

Nuclear RNA was greater in the EX leg of CTL versus LEU and WP-fed rats

A very interesting finding was that nuclear and total RNA content was greater in the exercised leg of CTL versus LEU and WP-fed rats. While past human data demonstrate that total RNA levels do not increase up to 6–24 h following resistance exercise (Roberts et al. 2010; Stec et al. 2015), rodent data suggest that stimulated leg contractions in younger rodents robustly increases gastrocnemius total RNA by ~30 % 24 h following exercise (Haddad and Adams 2006); of note, exercise increased total RNA by 23 % in the CTL group only by 3 h post-exercise. Therefore, species differences may exist whereby exercise-induced increases in rRNA are more rapid in rodents compared to humans.

However, our finding that only the CTL group experienced an increase in nuclear and total RNA following exercise is also difficult to reconcile given that only the LEU EX rats presented a significant increase in 45S pre-rRNA levels compared to the EX legs of the CTL and WP treatments; a finding that would suggest that the LEU EX leg would have presented higher nuclear RNA levels relative to other treatments. In this regard, one of two things could have occurred which include: (1) LEU enhanced the EX-induced increase in 45S pre-rRNA expression in vivo, though this did not lead to an appreciable increase in nuclear RNA by 3 h post-exercise, or (2) LEU and WP increased total RNA turnover, specifically rRNA degradation rates. Both hypotheses are supported by our in vitro data which show that, while LEU increased 47S pre-RNA expression, total RNA did not increase. Moreover, WP did not affect 47S pre-RNA expression in vitro, but total RNA was significantly lower compared to CTL and LEU treatments.

Nutrient and mTOR modulation have been shown to exhibit rapid effects on rRNA turnover in vitro. For instance, mTOR inhibition via rapamycin has been shown to decrease 28S and 18S rRNA in yeast by 50 % within 3 h (Pestov and Shcherbik 2012). Moreover, media nutrient deprivation in protozoan cultures have been shown to rapidly increase rRNA degradation within 6 h and then stabilize synthesis and degradation rates (Eckert and Kaffenberger 1980). Therefore, nutrient provision with exercise may lead to some sort of unidentified cellular stress which, in the case with LEU, increases both rRNA synthesis and degradation rates and, in the case with WP, does not increase synthesis rates but does increase degradation rates. However, this explanation is speculative and more research is needed to determine if rRNA synthesis and degradation rates are increased in response to a combined exercise and nutrient stimulus.

Conclusions

To our knowledge this is the first study to compare the anabolic post-exercise effects of LEU only versus WP. We report that WP elicits a greater post-exercise MPS response compared to LEU, though only LEU increased rDNA transcription rates independent of exercise. Our model of rodent resistance exercise, as well as 'exercise-like' signals in vitro, acutely increase the expression of numerous genes involved with ribosome biogenesis independent of nutrient provision. Due to the acute nature of our data, future longer-term interventions whereby resistance exercise training with and without nutritional provision (i.e., supplementing with intact proteins or amino acid mixtures) is needed to further clarify the role that nutritional provision plays in potentially enhancing ribosome biogenesis.

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Compliance with ethical standards

Conflict of interest Besides M.K. and J.R.M., none of the authors have non-financial and/or financial competing interests. M.K. and J.R.M. are employed by the MusclePharm Research Institute, but both intellectually contributed to study design and data write-up. Therefore, all co-authors agreed that their intellectual input into this project warranted co-authorship.

Statement on the welfare of animals This article does not contain any studies with human participants performed by any of the authors. All of the proposed animal studies were approved by the Institutional Animal Care and Use Committee at Auburn University. When applicable, replacement of rats with cell culture experiments, a reduction in the number of rats used, and refinement of methods to alleviate animal discomfort were used in the animal protocol.

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