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Cumulative responses of muscle protein synthesis are augmented with chronic resistance exercise training

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Abstract

Aim: The purpose of this study was to determine the anabolic response of a single bout of high intensity resistance exercise (RE) following 5 weeks of RE training.

Methods: To complete these studies, Sprague–Dawley rats were assigned by body mass to RE, exercise control (EC), or sedentary cage control (CC) groups and studied over 36 h after 5 weeks of RE (squat-like) training. Cumulative (final 36 h) fractional rates of muscle protein synthesis (FSR) were determined by ^2H_2O, and acute (16 h post-RE) rates of muscle protein synthesis (RPS) were determined by flooding with L-[2,3,4,5,6-^{3}H]phenylalanine. Regulators of peptide-chain initiation, 4E-BP1, eIF4E and the association of the two were determined by Western blotting and immunoprecipitation respectively.

Results: No differences were observed with acute measures of RPS obtained 16 h following the final exercise bout in the plantaris or soleus muscles ($P > 0.05$). Consistent with this observation, 4E-BP1 was similarly phosphorylated and bound to eIF4E among all groups. However, upon determination of the cumulative response, FSR was significantly increased in the plantaris of RE vs. EC and CC (0.929 ± 0.094, 0.384 ± 0.039, $0.300 \pm 0.022\%$ h^{-1} respectively; $P < 0.001$), but not the soleus.

Conclusion: With the advantage of determining cumulative FSR, the present study demonstrates that anabolic responses to RE are still evident after chronic RE training, primarily in muscle composed of fast-twitch fibres.

Keywords anabolism, cumulative muscle protein synthesis, exercise training.

Resistance exercise (RE) training improves skeletal muscle function and in most, muscle mass. The observed muscle hypertrophy occurs as a result of an increase in muscle protein synthesis to a greater extent than muscle protein breakdown. This process has been well documented following acute RE in animals (Fluckey *et al.* 1996, 2006, Farrell *et al.* 1999a,b, Kubica *et al.* 2005) and humans (Chesley *et al.* 1992, Biolo *et al.* 1995, Phillips *et al.* 1997, 1999), and it is believed that these acute anabolic effects are adequate to affect the diurnal patterns of protein synthesis and responsible for growth over time. However, when compared with acute RE, chronic RE training appears to result in an attenuated muscle protein synthesis response (Farrell *et al.* 1999a, Phillips *et al.* 1999, 2002, Kim *et al.* 2005). The reasoning for these observations could be due to an adaptation occurring within muscle serving to blunt growth beyond a mammalian organism's potential, which can only be surpassed with altered stimuli. Alternatively, chronic exercise training may result in a shift in the diurnal pattern of the anabolic response.

As indicated above, studies examining muscle protein synthesis have used acute alterations in anabolic

responses to predict the total effect of the intervention, such as RE or amino acid dosing, on the total cumulative (or diurnal) response. At the onset of exercise, typical responses include an elevation in muscle protein synthesis, which, we rightly conclude should lead to an increase in muscle mass over time. On the other hand, the diminished acute responses observed with exercise training lead to the speculation that the muscle is no longer sensitive to the anabolic stimuli, or the stimulant is no longer adequate to facilitate anabolic responses. To better interpret alterations in muscle physiology with prolonged exercise training, we have adapted methodologies that allow us to systematically assess diurnal anabolic responses to RE. The purpose of this study was to determine if rates of protein synthesis (RPS) were augmented in response to an acute bout of exercise after 5 weeks of RE training. Our hypothesis was that while the short-term assessment of rates of synthesis would appear to be 'normalized' following chronic RE training, the anabolic effect of RE would still be evident when measured cumulatively. Results from this study will have important implications towards our understanding of muscle anabolic responses to RE training.

Materials and methods

Chemicals and supplies

Unless otherwise specified, all chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA). $^{2}H_2O$ (99.9 atom percent excess) was purchased from Cambridge Isotopes (Andover, MA, USA). L-[2,3,4,5,6-^{3}H]phenylalanine (Phe) was purchased from GE Healthcare Life Science-Amersham (Buckinghamshire, UK). Gas chromatography–mass spectrometry (GC–MS) supplies were purchased from Agilent Technologies (Wilmington, DE, USA) and Alltech (Deerfield, IL, USA). Antibodies, rabbit (polyclonal) anti-eIF4E (9742), anti-phospho-4E-BP1-Thr^{70} (9455), anti-4E-BP1 (9452), anti-rabbit IgG, HRP (7074) and kinase buffer (10X; 9802) were purchased from Cell Signaling Technologies (Danvers, MA, USA).

Animals and operant conditioning

Forty-two male Sprague–Dawley rats (5–6 months old) were individually housed in a climate-controlled small animal facility with a 12 h light (06:00–18:00 hours) and dark cycle (18:00–06:00 hours). Rats were fed *ad libitum* an experimental diet (Purina Test Diet, 5001) that was composed of 24% protein, 12% fat, 54% carbohydrate, 7% ash, 5% fibre and vitamins. Cholesterol content was manipulated so that half of the animals received 180 ppm and the other half received 1800 ppm to determine if cholesterol influenced protein synthesis and growth. At this time there are inadequate data to support an interactive effect of cholesterol and exercise on anabolism; therefore, out of 42 animals 30 were used for this analysis and randomized by body mass to sedentary cage control (CC, $n = 10$), exercise control (EC, $n = 10$), or RE ($n = 10$). Additional work (studies) is required to accurately assess if cholesterol is a potent mediator of skeletal muscle hypertrophy. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

The RE and EC animals were operantly conditioned to depress an illuminated bar located high on a Plexiglas exercise cage (Fluckey *et al.* 1995). Negative reinforcement via a brief electrical foot shock (1 mA, 60 Hz) was used to train the rats to perform the desired movements. After the animals were conditioned (four sessions), a non-weighted Velcro vest was strapped over the scapulae and the rats were required to touch the illuminating bar 50 times for two additional sessions. The CC animals did not engage in controlled physical activity and remained single-housed over the course of the study.

Experimental protocol

The RE and EC rats performed 15 training sessions (three sessions per week interspersed by at least 48 h of rest) over a 5-week period, modified from Westerlind *et al.* (1998) and Farrell *et al.* (1999a). The RE group completed a progressive resistance training programme with a starting weight of 80 g for 50 repetitions on session 1 and increasing to 410 g for 16 repetitions on session 15. The total volume was decreased (increased resistance and decreased repetitions) by ~5% per week for a total of 25% over the 5 week training period. The EC rats completed the same protocol following the resistance training sessions, performing the same number of repetitions and receiving the same number of electrical foot shocks, without resistance, to elicit a similar stress response experienced by the RE group (adrenal mass did not differ among groups ($P > 0.05$); data not shown).

Twenty hours (Fig. 1) prior to the final exercise bout (36 h total), all animals received an intraperitoneal injection (IP) of 1.5 mL, 99.9 atom per cent excess, of $^{2}H_2O$ and allowed free access to drinking water enriched to 4.0% $^{2}H_2O$ (modified from Gasier *et al.* 2009). Food was withdrawn 4 h prior to the animals being anaesthetized to reduce the effect of prior feeding on muscle protein synthesis, and the left carotid artery and right jugular vein were cannulated with sterile, heparinized polythene tubing (PE-50). Rats remained sedated after the insertion of catheters and a flooding dose of L-[2,3,4,5,6-^{3}H]Phe (~1 mCi/rat) in unlabelled

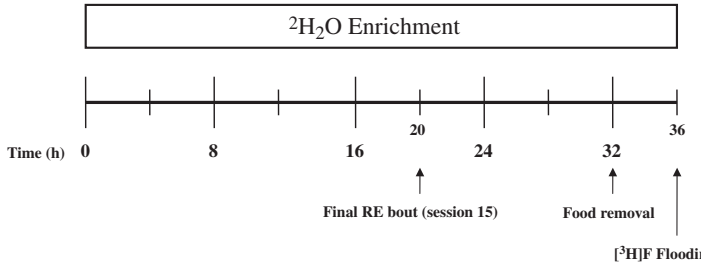

Figure 1 Schematic display of study protocol. Following 5 weeks of resistance exercise (RE), all animals were studied over 36 h. Rats received an intraperitoneal injection of 1.5 mL, 99.9 atom percent excess, of ^2H_2O with drinking water enriched to 4.0% at time 0 to determine cumulative fractional synthesis rates of muscle protein. The final RE session was performed 20 h later and food was removed (FR) 32 h later. After 36 h, animals received a flooding dose of L-[2,3,4,5,6-^{3}H]Phe over a 10- to 15-s period to determine rates of muscle protein synthesis.

Phe (150 mM, 1 mL 100 g^{-1} body mass) was injected into the venous catheter over a 10- to 15-s period (Garlick *et al.* 1980). Following injection, arterial blood samples (1 mL) were taken from the carotid cannula during a 12-min period (2, 6 and 12 min) for the determination of Phe-specific radioactivity. After arterial sampling, the fast glycolytic fibre plantaris (Plant) and the slow oxidative fibre soleus (Sol) (Armstrong & Phelps 1984) were rapidly excised and immediately frozen between aluminium blocks and cooled in liquid nitrogen for later analysis. These muscles were selected on the basis of their differing fibre types and knowledge that these muscles are active both concentrically and eccentrically during the voluntary movement (Fluckey *et al.* 1995). It should also be noted that previous studies by us (Fluckey *et al.* 1995, 1996, 2000) and others (Farrell *et al.* 1999a, 1999b, Hernandez *et al.* 2000, Kostyak *et al.* 2001, Bolster *et al.* 2003b, Kubica *et al.* 2005) employing similar flooding dose methodologies and RE protocols have demonstrated elevated RPS in the selected muscles when measured 16 h post-exercise at the onset of training.

Analytical methods

Rates of protein synthesis (RPS). Approximately 0.15 g of muscle tissue was used to estimate the rate of incorporation of radioactive Phe into the Plant and Sol mixed muscle proteins described by Vary *et al.* (1988). Briefly, the samples were pulverized under liquid nitrogen, homogenized in 2.0 mL of 10% trichloroacetic acid (TCA) and centrifuged at 3800 ***g*** for 15 min at 4 °C. The supernatant was discarded and the protein precipitate was washed three additional times in 2.0 mL of 10% TCA, then freed from lipid with 2.0 mL of acetone, washed with 2.0 mL of distilled H_2O and dissolved in 2.0 mL 0.25 N NaOH. A portion of the protein was used to quantify protein content using the bicinchoninic acid (BCA) method (Smith *et al.* 1985). Radioactivity was determined in both tissue and plasma samples (0.5 and 0.05 mL respectively) via liquid-scintillation counting using appropriate correction for quench. The total concentration of plasma Phe was determined using high-performance liquid chromatography (HPLC) with a fluorescence detector after 1.5 mol L^{-1} $HClO_4$ was added to deproteinize the sample according to Buentello & Gatlin (2001). RPS are reported as nmol Phe incorporated per gram wet weight of muscle per hour (Vary *et al.* 1988).

Fractional synthesis rates (FSR). ^{2}H-labelling of body water was assessed by exchange with acetone as described by (Yang *et al.* (1998). We administered (IP) much less ^2H_2O (1.5 mL) than has been described previously (Dufner *et al.* 2005, Gasier *et al.* 2009), but were able to achieve adequate ^{2}H-labelling of body water (≥2.0%) by the addition of 4.0% ^2H_2O to the drinking water over 36 h (Table 1). The reaction occurred with 20 μL of plasma or standard, 2 μL of 10 N NaOH and 4 μL of a 5% (v/v) solution of acetone in acetonitrile for 24 h. Acetone was removed by the addition of 600 μL of chloroform and 0.5 g Na_2SO_4. The samples were vortexed and 100 μL of the chloroform was transferred to a GC–MS vial. The sample was

Table 1 ^{2}H-labelling of body water and skeletal muscle proteins

Group	*n*	Plasma (MPE)	Plantaris (MPE)	Soleus (MPE)
CC	7–8	2.92 ± 0.18	1.16 ± 0.12	1.00 ± 0.12
EC	9–10	2.31 ± 0.11†	1.16 ± 0.10	1.08 ± 0.11
RE	9–10	2.00 ± 0.06*‡	2.53 ± 0.29‡	0.89 ± 0.12

Values are mean ± SEM. MPE, mole percent excess; CC, cage control; EC, exercise control; RE, resistance exercise.
*Significantly different ($P < 0.05$) from EC group.
†Significantly different ($P < 0.01$) from CC group.
‡Significantly different ($P < 0.001$) from CC group in plasma and from CC and EC groups in the plantaris.

analysed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system (Agilent, Santa Clara, CA, USA), and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm). The following temperature programme was used: 60 °C initial, increase by 20 °C min^{-1} to 100 °C, increase by 50 °C min^{-1} to 220 °C, and hold for 1 min. The sample was injected at a spit ratio of 40 : 1 with a helium flow of 1 mL min^{-1}. Acetone eluted at ~1.7 min. The mass spectrometer was operated in electron impact mode (70 eV). Selective ion monitoring of mass-to-charge ratios (*m/z*) 58 (M) and 59 (M + 1) was conducted using a dwell time of 10 ms per ion.

^{2}H-labelling of protein-bound alanine was measured as previously described by Dufner *et al.* (2005). We chose to measure FSR in the plantaris and soleus because of the growth in the plantaris and the difference in fibre types (fast vs. slow). Briefly, ~0.03 g of Plant and Sol was homogenized on ice in 0.3 mL of a 10% (w/v) TCA and centrifuged at 3800 ***g*** at 4 °C for 15 min. The supernatant was discarded and the protein pellet was washed three additional times with 10% TCA prior to dissolving in 6 N HCL (0.1 mL per 0.030 g tissue) and reacting at 100 °C for 18 h. An aliquot (0.1 mL) of the hydrolysate was freeze dried for 24 h. A 3 : 2 : 1 ratio (0.1 mL) of 'Methyl-8' reagent (Pierce, Rockford, IL, USA), methanol and acetonitrile was added to the residue to determine the ^{2}H-labelling of alanine on its methyl-8 derivative. All samples were analysed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all analyses. The following temperature programme was used: 90 °C initial, hold for 5 min, increase by 5 °C min^{-1} to 130 °C, increase by 40 °C min^{-1} to 240 °C, and hold for 5 min. The sample was injected at a spit ratio of 20 : 1 with a helium flow of 1 mL min^{-1}. Alanine eluted at ~12 min. The mass spectrometer was operated in electron impact mode. Selective ion monitoring of mass-to-charge ratios (*m/z*) 99 (M) and 100 (M + 1) was conducted using a dwell time of 10 ms per ion.

We calculated the fractional synthesis rate of mixed muscle proteins (FSR) by measuring the incorporation of ^{2}H alanine into protein (E_A) and using the precursor-product model (Wolfe & Chinkes 2005, Gasier *et al.* 2010):

$$FSR = E_A/(E_{BW} \times 3.7 \times t) \quad (1)$$

where E_A represents amount of protein-bound ^{2}H Ala (%), E_{BW} is the quantity of ^2H_2O in body water (%), 3.7 represents the exchange of ^{2}H between body water and alanine (Previs *et al.* 2004, Dufner *et al.* 2005) and t is time of label exposure (36 h). Additionally, an absolute rate of protein synthesis (APS) was calculated using the product of FSR and protein concentration per g of wet weight (Wolfe & Chinkes 2005, Xiao *et al.* 2008):

$$APS = FSR \times (mg\ protein \times g^{-1}\ wet\ wt) \quad (2)$$

Quantification of 4E-BP1, phospho-4E-BP1 and 4E-BP1/eIF4E association. To complete this aspect of the study, we assessed total expression and phosphorylation (indicative of deactivation) of 4E-BP1 and the binding states of 4E-BP1 and eIF4E. Briefly, ~0.05 g of Plant was homogenized on ice in 400 μL of a 1X kinase buffer (25 mM Tris-HCl, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) with 200 μM ATP, 0.01% Triton and protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) added. Homogenates were centrifuged at 14 000 ***g*** at 4 °C for 30 min. After centrifugation, the supernatant containing the cytosolic and membrane portion was extracted, protein content in the supernatant was assayed using the BCA method (Smith *et al.* 1985), and 20 μg was added to an 10% discontinuous polyacrylamide gel. After electrophoresis, gels were transferred to a PVDF membrane using a semidry method (Immobilon-P; Millipore, Billerica, MA, USA) and immunoblotted with the specific antibodies of interest mentioned above. Samples of mixed cardiac muscle from these animals were prepared identically and an equal quantity of protein was added to each gel to serve as a control. To determine if 4E-BP1 was bound to eIF4E, eIF4E was precipitated out of 50 μg of protein with anit-eIF4E, Super Block (Fisher Scientific) and Protein A (Sigma-Aldrich); modified from Kimball *et al.* (1997). An equal amount (30 μL) was then added to a 10% discontinuous polyacrylamide gel as above, followed by electrophoresis, gel transfer and then immunoblotted with anti-4E-BP1.

Statistical analysis

All data analysis were completed using SigmaStat version 3.5 and values are expressed as mean ± SEM. Comparisons were made using a one-way ANOVA with the effect being physical activity. *Post hoc* testing was performed using the Fisher LSD method when a main effect was observed. If a test of normality or equal variance failed, then ANOVA on ranks followed by a Fisher LSD *post hoc* comparison test was performed. We accepted an analysis as statistically significant if $P < 0.05$.

Results

Acute anabolic response to chronic resistance exercise training

Prior experiments, using similar exercise models, used flooding dose methodologies to assess the efficacy of

prolonged RE training on muscle protein synthesis (Farrell *et al.* 1999a). Consistent with observations from those studies, we found that acute rates of muscle protein synthesis in a subset of animals did not differ ($P > 0.05$) among RE, EC or CC groups in the Plant or Sol muscles after 5 weeks of RE training (Fig. 1).

4E-BP1, phospho-4E-BP1 and 4E-BP1/eIF4E association

To determine if similarities of acutely measured muscle protein synthesis among groups were due to attenuated mammalian target of rapamycin (mTOR) downstream signalling, we determined the phosphorylation status of 4E-BP1 (Thr 70) in the plantaris muscle, which responded favourably to the RE paradigm. Further, we also measured total expression of this protein, as well as whether 4E-BP1 was bound to eIF4E. Consistent with the similar rates of synthesis that were observed among groups (Fig. 1), there were no differences between the RE, EC or CC groups in the expression of 4E-BP1 (Fig. 2a), phosphorylation of 4E-BP1 (Fig. 2b) or the 4E-BP1 and eIF4E complex (Fig. 2c). These results support the notion that acute anabolic effects to RE dissipate with chronic training.

Assessment of protein synthesis with ^2H_2O

The IP injection with 1.5 mL of ^2H_2O and 4.0% ^2H_2O added to the drinking water yielded at least 2.0% plasma ^2H_2O, with the CC and EC animals obtaining higher enrichment (Table 1). The reason for higher body water ^2H_2O in the CC and EC animals could be due to the stressor of the final RE bout in the RE group, preventing them from drinking as much water. The only difference in protein labelling (^{2}H-Ala) was in the Plant; with the RE group possessing much higher labelling (Table 1). FSRs (Fig. 3a) in the RE group were 142 and 210% greater than the EC and CC animals respectively ($P < 0.001$). Because of the time of the labelling with ^2H_2O (36 h) and the hypertrophy of the plantaris, we calculated APS rates (mg of protein synthesized per gram of tissue) to account for any changes that may have occurred in the protein pool (Fig. 4). The trend was similar with RE yielding a 139% greater absolute rate of protein synthesis than EC and 250% greater than CC ($P < 0.001$) (Fig. 3b). No differences were observed for either fractional or absolute synthesis rates in the Sol muscle (Fig. 3a,b respectively).

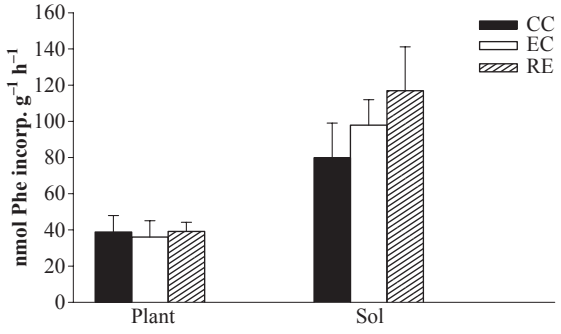

Figure 2 Rates of mixed muscle protein synthesis (RPS) for plantaris (Plant) and soleus (Sol) assessed with L-[2,3,4,5,6-^{3}H] phenylalanine (flooding dose) 16 h following the final bout of RE after 5 weeks of training. Cage control (CC), exercise control (EC) and resistance exercise (RE) rats. Data are presented as mean ± SEM ($n = 5$ for CC and EC, $n = 9$ for RE).

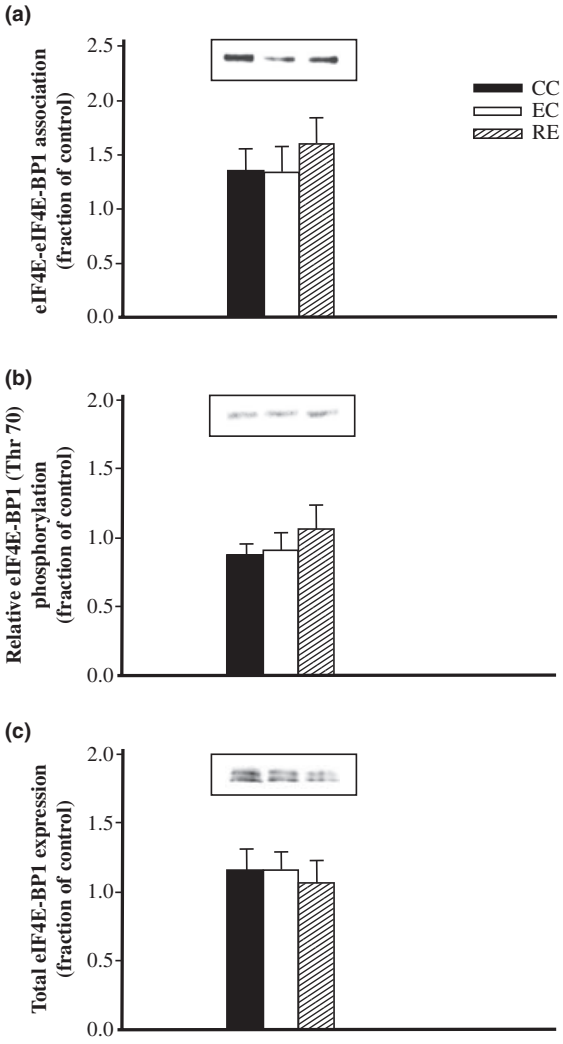

Figure 3 Downstream targets of mammalian target of rapamycin (mTOR) assessed 16 h following the final bout of resistance exercise after 5 weeks of training in the plantaris. Control, heart standards. Western blot analysis and graphical summaries of mean densitometry values for eIF4E-4E-BP1 (panel a), 4E-BP1 phosphorylation (panel b) and total 4E-BP1 (panel c). Lane 1, cage control (CC); lane 2, exercise control (EC); lane 3, resistance exercised (RE). Data are presented as mean ± SEM ($n = 7$–10 per group).

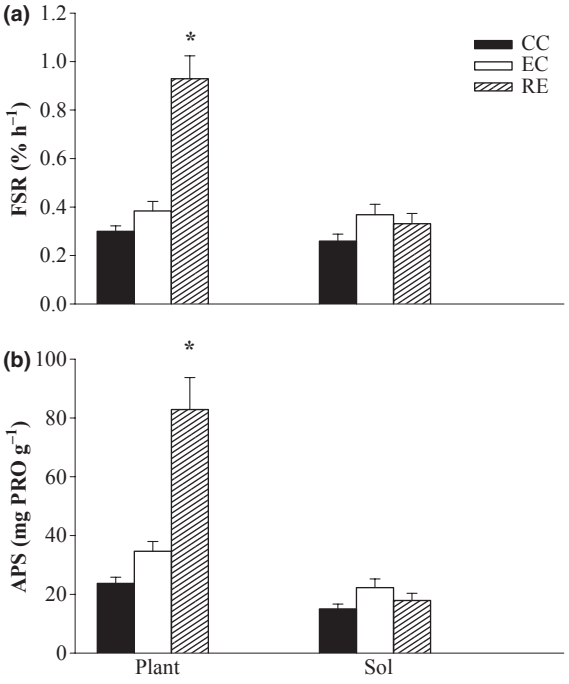

Figure 4 Mixed muscle protein (a) fractional synthetic rate (FSR) and (b) absolute rates of protein synthesis (APS) assessed with ^2H_2O (1.5 mL intraperitoneal injection with 4.0% added to the drinking water) over 36 h following 5 weeks of RE training in the plantaris of cage control (CC), exercise control (EC) and resistance exercised (RE) rats; and soleus of CC, EC and RE muscles. *Significant difference ($P < 0.001$) in RE vs. EC and CC rats. Data are presented as mean ± SEM (n = 7–10 per group).

Discussion

The significance of the present study is that it reports the absence of an acute anabolic response to RE training when rates of protein synthesis are examined in a traditional anabolic window. This is consistent with other studies concluding that acute anabolic responses to RE are diminished with exercise training. On the other hand, the present study demonstrates for the first time, that the acute response to RE is profoundly anabolic when protein synthesis is assessed cumulatively over a 36-h period. These results have important implications towards our understanding of muscle protein metabolism in response to exercise, and may serve to alter our perceptions related to the anabolic potential of RE with chronic exposure.

The lack of an apparent anabolic effect of exercise with RE training is not novel. Farrell *et al.* (1999a) observed the lack of an anabolic response (rates of muscle protein synthesis) in selected hindlimb muscles following 8 weeks of RE using a similar model in rats. In humans, Phillips *et al.* (1999) observed an attenuation in mixed muscle FSR within 4 h following an acute bout of RE in individuals who had been undergoing RE training for more than 5 years (self-reported). Additionally, Kim *et al.* (2005) reported a decrease in mixed muscle FSR and no change in myofibrillar FSR when comparing a trained vs. untrained leg 12 h following an 8 week unilateral RE leg protocol. In the case of the human studies, it should be noted that both studies examined FSR in the fasted state, which may have influenced the lack of an anabolic response with training. However, another study by Phillips *et al.* (2002), which examined the effects of an 8 week RE training programme on mixed muscle FSR in the fed state found that prolonged RE training may reduce the anabolic stimulus of RE. Thus, the use of periodic assessments of muscle protein synthesis typically demonstrates a training-associated loss of the anabolic potential with chronic RE training.

Consistent with our acute measurements of protein synthesis in the traditional anabolic window, both the phosphorylation on 4E-BP1 (Thr 70) and the fraction of 4E-BP1 bound to eIF4E was similar between RE training and sedentary controls. The primary rate-limiting step in mRNA translation is initiation (Roux *et al.* 2007) and a key signalling component for muscle protein synthesis is the binding of eukaryotic initiation factor 4E (eIF4E) to the transcript's 5′ cap structure (Brunn *et al.* 1997, Gingras *et al.* 1999, Bolster *et al.* 2003a, Mochizuki *et al.* 2005, Svitkin *et al.* 2005, Proud 2007). 4E-BP1 (PHAS1) modulates the activity of eIF4E by sharing the same binding site as the scaffolding protein eIF4G. The regulation of 4E-BP1 is via protein kinase B (Akt) mTOR protein kinase, which when activated by external stimuli (mechanical strain, growth factors and nutrients) results in phosphorylation of 4E-BP1 on Thr-37/46 (Gingras *et al.* 1999). This phosphorylation serves as a priming event, which is required for subsequent phosphorylation of carboxy-terminal sites such as Thr-70 resulting in dissociation of eIF4E from 4E-BP1, thus allowing eIF4E to complex with eIF4G. Kubica *et al.* (2005), using an exercise paradigm similar to that of the present study, demonstrated elevated mTOR-dependent (rapamycin-sensitive) 4E-BP1 phosphorylation 16 h after RE without chronic training. Interestingly, with chronic training, the present study did not observe augmented 4E-BP1 activity in response to the last bout of exercise, which is consistent with our inability to assess an anabolic response to exercise during that time period. Together, these results would indicate that the acute anabolic response to RE is dissipated with chronic training and suggest that compensatory hypertrophy with chronic RE must be accomplished via mechanisms that are not dependent on elevations in muscle protein synthesis.

Another possibility for an inability to observe acute anabolic responses to RE is that chronic training may alter the physiological timing of when these assessments should be made. Support for a 'shift in the anabolic window' comes from an investigation by Tang *et al.* (2008), whereby an 8 week unilateral RE leg protocol was followed using an exercise protocol similar to Phillips *et al.* (2002) post-training measurements in FSR at rest, 4 and 28 h following an acute bout of RE. Although we acknowledge that there were methodological differences between those studies, Tang *et al.* (2008) reported significant increases in muscle protein synthesis in the trained leg 4 h post-exercise, but a subsequent attenuation in FSR at 28 h. While these two studies may appear to yield results that are in opposition, comparisons of these two studies may actually represent a shift in the anabolic window with exercise training, and clearly, the identification of this altered window becomes problematic when choosing which time points to examine. More specifically, had Tang *et al.* (2008) not included the 4 h post-exercise measurement, they would have concluded that chronic RE training does in fact diminish the acute anabolic effect. The primary reason for choosing to employ a cumulative vs. an acute assessment was because of our exercise protocol. In human investigations, mixed muscle protein synthesis has been reported to be elevated 1–48 h following acute RE (Phillips *et al.* 1997, Fujita *et al.* 2008), thus a 4 and 28 h measurement was appropriate. However, in our rodent experimental exercise model, muscle protein synthesis does not increase until 12–48 h post-exercise (Hernandez *et al.* 2000), therefore, in our opinion, an earlier acute measurement was not appropriate, whereas a cumulative FSR was.

The assessment of cumulative FSR (see Gasier *et al.* 2010 for methodological description) should greatly enhance our ability to perceive the effect of exercise even when rates of synthesis in the traditional 'anabolic window' may be diminished. This conclusion was based on our ability to assess FSR over the final 36 h period of training, where we observed a significant, cumulative, anabolic response in the plantaris muscle. To our knowledge, this study is the first to utilize this methodological approach in skeletal muscle with chronic RE training, and ultimately, gives us an opportunity to rule out the possibility that we may have missed the 'anabolic window' with the traditional flooding dose methodology. In addition, because the measurement period was over 36 h, we were able to determine the absolute rate of protein synthesis to account for any changes that may occur within the plantaris or soleus protein pool, which yielded a similar pattern as FSR. Our data clearly show that FSR, when assessed for extended periods of time, are greater than twofold in plantaris muscle of RE-trained rats, a finding not observed when we measured over shorter-term periods (i.e. min) at times yielding significant anabolic results without prior training (i.e. 16 h).

We should note that in contrast to the plantaris muscle, the assessment of protein synthesis in soleus muscle demonstrated no differences among groups, regardless of our experimental approach (short-term or cumulative). Unfortunately, at this time we cannot account for why the Soleus was unresponsive to chronic RE training, particularly as this muscle typically responds to this exercise paradigm without prior training (Fluckey *et al.* 1996). However, it is possible that this highly active, postural/locomotor muscle is not responsive to chronic, voluntary training in intact hindlimbs. Alternatively, it is possible that anabolic responses to training may also depend on fibre composition, as plantaris is predominately composed of faster fibres (93%) and Sol is composed of slower fibres (89%) (Armstrong & Phelps 1984). More systematic work will be necessary to determine if muscle composed of slower fibre types do not adequately respond to training or if they become less involved with higher intensity exercises as the organism becomes more extensively trained in the movement.

In summary, this study highlights the importance of assessing total, cumulative anabolic responses to interpret anabolic potential of skeletal muscle with exercise training. When using shorter-term flooding dose procedures, there were no observable differences in mixed muscle RPS of exercised vs. sedentary controls 16 h following the final exercise session. This finding is consistent with the notion that chronic RE training leads to normalized muscle protein synthesis. However, when anabolic responses were assessed cumulatively, the present work demonstrated that exercised muscle fibres display anabolic responses more than twofold higher than those of the control muscles in muscle composed of faster fibres. From the present work, it cannot be inferred if the enhanced cumulative anabolic response in plantaris muscle is due to a temporally distinct anabolic window at another time point(s), or a subtle, but persistent elevation of protein synthesis for greater periods of time. Regardless, our results demonstrate that caution should be taken for the use of acute measures to predict diurnal anabolic responses to exercise, at least under some conditions.

Conflict of interest

The authors declare no conflicts of interest.

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