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Running head: Protein ingestion and concurrent training

Protein ingestion increases myofibrillar protein synthesis after concurrent exercise

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## ABSTRACT

**Purpose:** We determined the effect of protein supplementation on anabolic signaling and rates of myofibrillar and mitochondrial protein synthesis after a single bout of concurrent training. **Methods:** Using a randomized cross-over design, 8 healthy males were assigned to experimental trials consisting of resistance exercise (8 × 5 leg extension, 80% 1-RM) followed by cycling (30 min at ~70%  $\dot{V}O_{2peak}$ ) with either post-exercise protein (PRO: 25 g whey protein) or placebo (PLA) ingestion. Muscle biopsies were obtained at rest, 1 and 4 h post-exercise. **Results:** Akt<sup>Ser473</sup> and mTOR<sup>Ser2448</sup> phosphorylation increased 1 h after exercise with PRO (175-400%,  $P < 0.01$ ) and was different from PLA (150-300%,  $P < 0.001$ ). MuRF1 and Atrogin-1 mRNA were elevated post-exercise but were higher with PLA compared to PRO at 1 h (50-315%,  $P < 0.05$ ), while PGC-1 $\alpha$  mRNA increased 4 h post-exercise (620-730%,  $P < 0.001$ ) with no difference between treatments. Post-exercise rates of myofibrillar protein synthesis increased above rest in both trials (75-145%,  $P < 0.05$ ) but were higher with PRO (67%,  $P < 0.05$ ) while mitochondrial protein synthesis did not change from baseline. **Conclusion:** Our results show that a concurrent training session promotes anabolic adaptive responses and increases metabolic/oxidative mRNA expression in skeletal muscle. Protein ingestion after combined resistance and endurance exercise enhances myofibrillar protein synthesis and attenuates markers of muscle catabolism and thus is likely an important nutritional strategy to enhance adaptation responses with concurrent training.

**Keywords:** anabolic signaling; fractional synthetic rate; atrogenes; resistance exercise; endurance exercise

## INTRODUCTION

*Paragraph Number 1* Contraction-induced adaptations in skeletal muscle are largely determined by the mode, volume and intensity of exercise (10). Endurance training leads to multiple adaptations in skeletal muscle including, but not limited to, mitochondrial biogenesis (21) and increases in the maximal activities of oxidative enzymes (21). Resistance training generally promotes a phenotype of myofibrillar protein accretion and increased cross sectional area of type II fibres (13, 33). Exercise-nutrient interactions may also be important in mediating skeletal muscle adaptation and may have the capacity to modulate the specificity of training response (19).

*Paragraph Number 2* The cellular mechanisms regulating the specificity of training adaptations with concurrent resistance and endurance exercise are undoubtedly complex given the capacity for single mode endurance and resistance training to generate divergent phenotypes (13, 39) and the potential confounding factors of exercise order and recovery between bouts. For example, Wilson and colleagues have reported that endurance exercise inhibits hypertrophy/strength with increasing training volume and frequency in a concurrent training paradigm (40). We have previously reported various cell signaling responses related to translation initiation and mRNA expression of mitochondrial/metabolic and myogenic adaptation when endurance or sprint exercise was performed directly before/after resistance exercise in the fasted state (11, 12). While the molecular profile generated by an acute bout of concurrent training has yet to be clearly established, the possibility exists that successive resistance and endurance exercise may have the capacity to promote myofibrillar and mitochondrial protein synthesis.

*Paragraph Number 3* Consumption of high-quality protein in close temporal proximity to resistance exercise enhances translation initiation signaling and maximally stimulates rates of muscle protein synthesis (25) and augments hypertrophy and strength gains (8). Likewise, protein feeding following endurance exercise can increase the transcriptional profile of mitochondrial-related genes

and increase rates of myofibrillar protein synthesis (5, 34). To date no study has determined the effect of protein ingestion following a bout of concurrent training on the acute myofibrillar and mitochondrial protein synthesis rates in skeletal muscle. Accordingly, the primary aim of the present investigation was to examine the acute effects of protein ingestion on rates of myofibrillar and mitochondrial protein synthesis in association with selected cellular/molecular responses following a bout of consecutive resistance exercise and cycling. We hypothesized that, compared to placebo, protein ingestion would enhance anabolic and metabolic signaling and subsequent protein synthesis during the early (1-4 h) recovery period following exercise.

## **METHODS**

### *Subjects*

*Paragraph Number 4* Eight healthy male subjects [age  $19.1 \pm 1.4$  yr, body mass  $78.1 \pm 15.6$  kg, peak oxygen uptake ( $V_{O_{2peak}}$ )  $46.7 \pm 4.4$  ml·kg<sup>-1</sup>·min<sup>-1</sup>, leg extension one repetition maximum (1-RM)  $130 \pm 14$  kg; values are mean  $\pm$  SD] who had been participating in regular concurrent resistance and endurance training ( $\sim 3\times/\text{week}$ ;  $> 1$  year) volunteered to participate in this study. Our subject inclusion criteria were based on subjects with a concurrent training history to quantify the habitual rather than novel adaptation response in skeletal muscle. The experimental procedures and possible risks associated with the study were explained to all subjects, who gave written informed consent before participation. The study was approved by the Human Research Ethics Committee of RMIT University.

### *Study Design*

*Paragraph Number 5* The study employed a randomized counter-balanced, double-blind, cross-over design in which each subject completed two bouts of concurrent resistance exercise and cycling with either post-exercise placebo (PLA) or protein (PRO) ingestion separated by a three week

recovery period, during which time subjects maintained their habitual physical activity pattern. Given the limited data on molecular responses to concurrent training, we chose to undertake resistance exercise prior to endurance exercise based on our previous finding that endurance exercise may attenuate the acute anabolic response if performed before resistance exercise (12). We chose to quantify the fractional synthetic rates in skeletal muscle during the 1-4 h recovery period due to potential complications generated by limb hyperemia with strenuous exercise and that protein digestion and absorption are reduced during the acute post-exercise period (37).

### *Preliminary Testing*

*Paragraph Number 6*  $\text{VO}_{2\text{peak}}$ . Peak oxygen uptake was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Groningen, The Netherlands). The protocol has been described in detail previously (7). In brief, subjects commenced cycling at a workload equivalent to 2 W/kg for 150 s. Thereafter, the workload was increased by 25 W every 150 s until volitional fatigue, defined as the inability to maintain a cadence  $> 70$  revolutions/min. Throughout the test subjects breathed through a mouthpiece attached to a metabolic cart (Parvomedics, USA) to determine oxygen consumption.

*Paragraph Number 7* Maximal Strength. Quadriceps strength was determined during a series of single repetitions on a plate-loaded leg extension machine until the maximum load lifted was established (1 RM). Repetitions were separated by a 3-min recovery and were used to establish the maximum load/weight that could be moved through the full range of motion once, but not a second time. Exercise range of motion was  $85^\circ$  with leg extension endpoint set at  $-5^\circ$  from full extension.

*Paragraph Number 8* Diet/Exercise Control. Before an experimental trial (described subsequently), subjects were instructed to refrain from exercise training and vigorous physical activity, and alcohol and caffeine consumption for a minimum of 48 h. Subjects were provided with standardized prepacked meals that consisted of 3 g carbohydrate/kg body mass, 0.5 g protein/kg body mass, and

0.3 g fat/kg body mass consumed as the final caloric intake the evening before reporting for an experimental trial.

### *Experimental Testing Session*

*Paragraph Number 9* On the morning of an experimental trial, subjects reported to the laboratory after a ~10-h overnight fast. After resting in the supine position for ~15 min, catheters were inserted into the antecubital vein of each arm and a baseline blood sample (~3 mL) was taken (Figure 1). A primed constant intravenous infusion (prime:  $2 \mu\text{mol}\cdot\text{kg}^{-1}$ ; infusion:  $0.05 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) of L-[ring- $^{13}\text{C}_6$ ] phenylalanine (Cambridge Isotopes Laboratories, USA) was then administered. Under local anaesthesia (2–3 mL of 1% Xylocaine) a resting biopsy was obtained 3 h after commencement of the tracer infusion from the vastus lateralis using a 5-mm Bergstrom needle modified with suction. Subjects then completed the exercise intervention (described subsequently). Immediately following the cessation of exercise, subjects ingested 500 mL of either a placebo (PLA: water, artificial sweetener) or protein beverage (PRO: 25 g whey protein). The protein beverage was enriched to 5% L-[ring- $^{13}\text{C}_6$ ] phenylalanine to prevent dilution of the steady-state isotope enrichment implemented by the constant infusion. Subjects rested throughout a 240 min recovery period during which additional muscle biopsies were taken 60 min post-exercise to investigate early cell signaling and mRNA responses and 240 min post-exercise to determine rates of muscle protein synthesis. Each muscle biopsy was taken from a separate site 2-3 cm distal from the right leg for the first trial and left leg for the second trial with all samples stored at  $-80^\circ\text{C}$  until subsequent analysis. Blood samples were collected in EDTA tubes at regular intervals during the post-exercise recovery period.

### *Resistance Exercise*

*Paragraph Number 10* After a standardized warm-up (2 ×5 repetitions at ~50% and ~60% 1 RM, respectively), subjects performed eight sets of five repetitions at ~ 80% 1 RM. Each set was separated by a 3 min recovery period during which time the subject remained seated on the leg extension machine. Contractions were performed at a set metronome cadence approximately equal to 30°/s and strong verbal encouragement was provided during each set. Subjects then rested for 15 min before beginning the cycling protocol.

### *Cycling Exercise*

*Paragraph Number 11* Subjects performed 30 min of continuous cycling at a power output that elicited ~70% of individual  $VO_{2peak}$ . Subjects were fan-cooled and allowed *ad libitum* access to water throughout the ride. Visual feedback for pedal frequency, power output, and elapsed time were provided to subjects.

### *Analytical Procedures*

*Paragraph Number 12* Blood Glucose and Plasma Insulin Concentration. Whole blood samples (5 mL) were immediately analyzed for glucose concentration using an automated glucose analyser (YSI 2300, Yellow Springs, USA). Blood samples were then centrifuged at 1000 g at 4° C for 15 min, with aliquots of plasma frozen in liquid N<sub>2</sub> and stored at -80°C. Plasma insulin concentration was then measured using a radioimmunoassay kit according to the manufacturer's protocol (Linco Research, Inc., St Charles, MO, USA).

*Paragraph Number 13* Plasma Amino Acids and Enrichment. Plasma amino acid concentrations were determined by high performance liquid chromatography (HPLC) from a modified protocol (31). Briefly, 100 µL of plasma was mixed with 500 µL of ice cold 0.6 M PCA and neutralized with 250 µL of 1.25 M potassium bicarbonate (KHCO<sub>3</sub>). Samples were then subsequently derivatized



for HPLC analysis. Plasma [*ring*-<sup>13</sup>C<sub>6</sub>] phenylalanine enrichments were determined as previously described (31).

#### *Mitochondrial and Myofibrillar Protein Synthesis*

*Paragraph Number 14* A piece of frozen wet muscle (~100 mg) was homogenized with a Dounce glass homogenizer on ice in an ice-cold homogenizing buffer (1M Sucrose, 1M Tris/HCl, 1M KCl, 0.5M EDTA) supplemented with a protease inhibitor and phosphatase cocktail tablet (PhosSTOP, Roche Applied Science, Mannheim, Germany) per 10 ml of buffer. The homogenate was transferred to an eppendorf tube and centrifuged (700g, 15 min, 4°C) to pellet a fraction enriched with myofibrillar proteins and collagen that was stored at -80°C for subsequent extraction of the myofibrillar fraction (described below). The supernatant was transferred to another eppendorf tube and centrifuged (12,000g, 20 min, 4°C) to pellet the mitochondrial enriched protein fraction. The supernatant was placed in a separate eppendorf and stored at -80°C for Western Blot analysis (described below). The mitochondrial enriched pellet was then washed, lyophilized and amino acids were liberated by adding 1.5 mL of 6M HCL and heating to 110°C overnight. Rates of mitochondrial protein synthesis were unable to be determined for two subjects due to limited muscle tissue obtained from the biopsy samples.

*Paragraph Number 15* The myofibrillar pellet stored at -80°C was washed twice with the homogenization buffer, centrifuged (700g, 10 min, 4°C) and supernatant was discarded. Myofibrillar proteins were solubilised in 0.3 M sodium hydroxide and precipitated with 1 M perchloric acid. Amino acids were then liberated from the myofibrillar enriched precipitate by adding 2.0 ml of 6 M HCL and heating to 110°C overnight.

*Paragraph Number 16* Free amino acids from myofibrillar and mitochondrial enriched fractions were purified using cation-exchange chromatography (Dowex 50WX8-200 resin; Sigma-Aldrich Ltd) and converted to their N-acetyl-n-propyl ester derivatives for analysis by gas chromatography

combustion-isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnigan, Waltham, MA, USA).

*Paragraph Number 17* Intracellular amino acids (IC) were extracted from a separate piece of wet muscle (~20 mg) with ice-cold 0.6 M PCA for determination of intracellular phenylalanine enrichment. Muscle was homogenized and the free amino acids in the supernatant were purified by cation-exchange chromatography and converted to their heptafluorobutyric (HFB) derivatives before analysis by GC-MS (models 6890 GC and 5973 MS; Hewlett-Packard, Palo Alto, USA) as previously described (31).

#### Calculations

*Paragraph Number 18* The rate of mitochondrial and myofibrillar protein synthesis was calculated using the standard precursor–product method:  $FSR (\% \cdot h^{-1}) = [(E2b - E1b) / (EIC \times t)] \times 100$

Where E2b - E1b represents the change in the bound protein enrichment between two biopsy samples, EIC is the average enrichment of intracellular phenylalanine between the two biopsy samples and t is the time between two sequential biopsies. The inclusion of ‘tracer-naive’ subjects permitted use of the pre-infusion blood sample (i.e. single biopsy method) as the baseline enrichment (E1b) for the calculation of resting MPS. The single biopsy method for quantifying baseline tracer enrichment in tracer-naive subjects has been clearly shown to be a reliable and valid experimental technique in previous studies (15).

*Paragraph Number 19* Western Blots. The supernatant frozen at -80°C from the previous mitochondrial enriched fraction extraction was used for determination of protein concentration using a BCA protein assay (Pierce, Rockford, IL, USA). The supernatant was subsequently resuspended in Laemmli sample buffer and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes and incubated with primary antibody (1:1,000) overnight at 4°C

and secondary antibody (1:2,000), and proteins detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (40 µg) time points for each subject were run on the same gel. Polyclonal anti-phospho-Akt<sup>Ser473</sup> (no.9271), -mTOR<sup>Ser2448</sup> (no. 2971), -eEF2<sup>Thr56</sup> (no. 2331), and monoclonal anti- p70S6K<sup>Thr389</sup> (no.9234) were from Cell Signalling Technology (Danvers, USA). Data are expressed relative to  $\alpha$ -tubulin (no. 3873, Cell Signalling Technology, Danvers, USA) in arbitrary units.

#### *RNA Extraction and Quantification*

*Paragraph Number 20* Briefly, ~20 mg of skeletal muscle was homogenised in TRIzol and chloroform added to form an aqueous RNA phase. This RNA phase was then precipitated by mixing with isopropanol alcohol and the resulting pellet was washed and re-suspended in 50 µl of RNase-free water. Extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia, Cat No Q32852) and on a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) by measuring absorbance at 260 nm and 280 nm with a 260/280 ratio of ~ 1.88 recorded for all samples.

#### *Reverse Transcription and Real-Time PCR*

*Paragraph Number 21* First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia) in a final reaction volume of 20 µL. All RNA and negative control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial dilutions of a template RNA (AMBION; Cat No AM7982) was included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR). Quantification (in duplicate) was performed using a Rotor-Gene 3000

Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes for MuRF-1 (Cat No. Hs00261590), Atrogin (Cat No. Hs01041408), Myostatin (Cat No. Hs00976237), PGC-1 $\alpha$  (Cat No. Hs01016719), Hexokinase (Cat No. Hs00175976) and VEGF (Cat No. Hs00900055) were used in a final reaction volume of 20  $\mu$ L. PCR treatments were 2 min at 50 °C for UNG activation, 10 min at 95 °C then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat No Hs Hs99999905) was used as a housekeeping gene and expression was not different at any time point (data not shown). The relative amounts of mRNAs were calculated using the relative quantification ( $\Delta\Delta$ CT) method (27).

### *Statistical Analysis*

*Paragraph Number 22* Blood, cell signaling and mRNA data were analyzed by two-way ANOVA (two factor: time  $\times$  treatment) with repeated measures and myofibrillar and mitochondrial protein synthesis were analyzed by one-way ANOVA with Student-Newman-Keuls post hoc analysis when  $P < 0.05$  (SigmaStat for windows Version 3.11). All data are expressed as mean  $\pm$  SD. Magnitude-based inferences and Cohen effect sizes (ES) were used to identify physiologically meaningful differences in rates of myofibrillar and mitochondrial protein synthesis (22). The precision of the effect was determined using 90% confidence limits making the same assumptions about sampling distributions that statistical packages use to derive P values. Differences between protein and placebo conditions were interpreted using inferences based on the magnitude of effect as described previously (2). Results are determined using effect sizes similar to Cohen's conventional thresholds values of 0.2 as the smallest effect, 0.5 as a moderate effect, and 0.8 as a large ES (22).

## **RESULTS**

Plasma Insulin, Amino Acids and Blood Glucose

*Paragraph Number 23* There were main effects for plasma insulin and total amino acid concentration with PRO but not PLA ( $P < 0.001$ ; Figure 2A, B). Peak plasma insulin (~535%) and amino acid (~70%) concentrations occurred 40 min post-exercise ( $P < 0.001$ ). The same effect was evident for BCAA concentration (~180%,  $P < 0.001$ ; Figure 2C). Blood glucose was not different at any time in either treatment (data not shown).

#### Intracellular and plasma tracer enrichments

*Paragraph Number 24* Intracellular free phenylalanine enrichments showed a stable precursor pool at rest, and 60 and 240 min post-exercise for PRO (0.0455, 0.0502 and 0.0488 tracer-to-tracee ratio:  $t \cdot T^{-1}$ ) and PLA (0.0468, 0.0468 and 0.0473  $t \cdot T^{-1}$ ). Linear regression analysis indicated that the slopes of the plasma L-[ring  $^{13}C_6$ ] phenylalanine enrichments were not significantly different from zero or between treatments, showing isotopic plateau/steady-state.

#### Cell Signaling

##### Akt-mTOR-p70S6K-eEF2

*Paragraph Number 25* Akt<sup>Ser473</sup> phosphorylation increased above rest with PRO (~175%;  $P < 0.05$ ) but not PLA 1 h after exercise (Figure 3A). This disparity in Akt<sup>Ser473</sup> resulted in a significant difference between treatments at 1 h ( $P < 0.05$ ). Phosphorylation in PRO then returned to resting levels 4 h following recovery from exercise ( $P < 0.05$ ).

*Paragraph Number 26* mTOR phosphorylation increased after PRO (~400%,  $P < 0.001$ ) and PLA (~100%,  $P < 0.05$ ) ingestion at 1 h, and this increase was markedly higher with PRO (~300%,  $P < 0.001$ , Figure 3B). mTOR<sup>Ser2448</sup> phosphorylation remained elevated above rest 4 h post-exercise with PLA only (~130%,  $P < 0.05$ ), resulting in a significant disparity between treatments ( $P < 0.05$ ).

*Paragraph Number 27* p70S6K<sup>Thr389</sup> phosphorylation increased above rest with PRO (~3000%;  $P < 0.001$ ) but not PLA 1 h after exercise (Figure 3C). This disparity in p70S6K<sup>Thr389</sup> resulted in a

significant difference between treatments at 1 h ( $P < 0.05$ ). Phosphorylation of p70S6K after PRO returned to resting levels after 4 h of recovery from exercise ( $P < 0.001$ ).

*Paragraph Number 28* There were main effects for eEF2<sup>Thr56</sup> phosphorylation for time in both treatments ( $P < 0.05$ , Figure 3D). One hour post-exercise, phosphorylation of eEF2 decreased ~60% ( $P < 0.05$ ) with PLA and ~75% ( $P < 0.05$ ) with PRO and remained at this level for the duration of the recovery (4 h).

mRNA expression

MuRF1-Atrogin-1-Myostatin

*Paragraph Number 29* MuRF1 increased significantly above resting levels at 1 h (~315% vs. ~230%,  $P < 0.001$ ) and 4 h (~250% vs. ~140%,  $P < 0.05$ ) post-exercise after both PLA and PRO, respectively (Figure 4A). MuRF1 was higher in PLA compared to PRO at both post-exercise time points (1 h: 78%, 4 h: 105%,  $P < 0.05$ ).

*Paragraph Number 30* Atrogin-1 mRNA expression increased above rest only with PLA 1 h post-exercise (~50%,  $P < 0.05$ ; Figure 4B). The disparity in Atrogin-1 mRNA at 1 h resulted in a significant difference between treatments ( $P < 0.05$ ).

*Paragraph Number 31* There was a main effect of time for myostatin mRNA abundance ( $P < 0.05$ , Figure 4C). Myostatin decreased from rest at 1 h (~40% vs. ~55%,  $P < 0.05$ ) and 4 h (~70% vs. ~80%,  $P < 0.001$ ) after both PLA and PRO, respectively. Myostatin mRNA at 1 h was different from 4 h after PLA (~120%,  $P < 0.05$ ).

PGC-1 $\alpha$ -Hexokinase-VEGF

*Paragraph Number 32* There were main effects for PGC-1 $\alpha$  mRNA abundance for time ( $P < 0.05$ , Figure 5A). PGC-1 $\alpha$  expression increased above resting and 1 h levels following 4 h post-exercise recovery in PLA (~730%,  $P < 0.001$ ) and PRO (~620%,  $P < 0.001$ ).

*Paragraph Number 33* Hexokinase increased above rest at 4 h in PLA only (~120%,  $P<0.05$ ) whereas in PRO there were no changes. This disparity resulted in a significant difference between treatments at 4 h ( $P<0.05$ , Figure 5B).

*Paragraph Number 34* VEGF mRNA expression increased above rest at both 1 h (~200%,  $P<0.001$ ) and 4 h (~210%,  $P<0.001$ ) with PLA (Figure 5C). Likewise, VEGF also increased with PRO at 1 h (~170%,  $p<0.05$ ) and 4 h (~180;  $P<0.05$ ). There were no differences between treatments at any post-exercise time point.

#### Rates of Muscle Protein Synthesis

*Paragraph Number 35* Rates of myofibrillar protein synthesis increased above rest between 1 h and 4 h post-exercise after both PLA (~75%,  $P<0.05$ ) and PRO (~145%,  $P<0.001$ ) (Figure 6A). This post-exercise increase in the rate of myofibrillar synthesis was greater with PRO compared to PLA ( $P<0.05$ ). Magnitude-based inferences revealed the chances that the true value of the statistic is mechanistically or physiologically positive for PRO compared with PLA was 91% and Cohen effect sizes demonstrated a large effect ( $ES >1.0$ ). Rates of mitochondrial protein synthesis ( $n = 6$ ) were unchanged during the acute post-exercise period and there were no differences in post-exercise fractional synthesis rates between treatments (Figure 6B).

## DISCUSSION

*Paragraph Number 36* Athletes from a variety of sports undertake resistance and endurance training concurrently as part of their training. It has been reported that hypertrophy/strength adaptations to concurrent resistance and endurance exercise are ‘compromised’ when compared with training for either exercise mode alone (10, 40). Our results show that in moderately trained individuals the combined effects of resistance and endurance exercise resulted in elevated rates of myofibrillar but not mitochondrial protein synthesis in the early (1-4 h) post-exercise recovery period. In addition,

we provide new information to demonstrate that post-exercise protein ingestion attenuates mRNA expression of markers of muscle catabolism following a concurrent training session.

*Paragraph Number 37* Early studies examining the specificity of adaptation to concurrent training indicate an ‘interference’ in adaptation for hypertrophy and strength relative to resistance training (17, 20). In contrast, there are also reports of little or no decrements in strength gain with combined resistance and endurance training (1, 30). However, the vast majority of studies of concurrent training have concluded that adaptation is compromised compared with each exercise mode undertaken in isolation (26, 40). Our findings indicate resistance exercise appears to generate a sufficient signal to stimulate myofibrillar protein synthesis despite a subsequent bout of endurance exercise (Figure 6A). This increase in myofibrillar protein synthesis is similar to previous maximal levels observed when protein is ingested following resistance exercise (31). What is not known, however, is whether the myofibrillar synthetic response we observed is exclusively the result of the resistance exercise or some interaction with the endurance exercise (24). Regardless, such an acute response may have potential to promote hypertrophy with repeated training bouts in a chronic concurrent training program. Importantly, we show post-exercise protein ingestion was beneficial for promoting myofibrillar protein synthesis with concurrent training and may have the capacity to reduce the potential interference effect of endurance exercise on skeletal muscle hypertrophy.

*Paragraph Number 38* Interestingly, we observed substantial variation in the individual response of myofibrillar protein synthetic rates with placebo and protein ingestion following a bout of concurrent training. The percentage of ‘low’ responders who did not increase rates of myofibrillar protein synthesis with placebo in the present study (25%) is similar to that reported following resistance or endurance training in isolation (32, 36). Following a 21 week training study, Karavirta and colleagues (23) have also shown within a concurrent training paradigm that high ‘responders’



for aerobic adaptation were not also high 'responders' to resistance training (and vice versa) and that simultaneous endurance and resistance training-induced adaptation occurred in only 50% of subjects. Consequently, undertaking concurrent training increases the complexity of the genotype-exercise interaction in promoting skeletal muscle adaptation, with individual nutrient responses further complicating this response. In this regard, a limitation of the present study is the modest subject numbers and understandably further studies are needed to corroborate our findings.

*Paragraph Number 39* We have previously demonstrated a similar selective increase in myofibrillar compared with mitochondrial protein synthesis rates in response to protein-carbohydrate co-ingestion following a high-intensity repeated sprint protocol (9). Given the high load ( $0.75 \text{ Nm}\cdot\text{kg}^{-1}$ ) and subsequent mechanical force required to complete maximal sprint cycling repetitions, the loading stimulus in our previous study was somewhat resistance-like and may have promoted a modest hypertrophy response with protein ingestion (9). However, Breen and co-workers have also recently reported increases in rates of myofibrillar, but not mitochondrial, protein fractional synthetic rates when carbohydrate-protein was co-ingested compared to carbohydrate feeding alone following 90 min of steady state cycling at  $\sim 75\% \text{ VO}_{2\text{max}}$  (5). Our present study shows variable rates of mitochondrial protein synthesis that failed to increase following the concurrent training bout with either treatment (Figure 6B). Few studies have investigated rates of mitochondrial protein synthesis following exercise with, to the best of our knowledge, only one previous study reporting mitochondrial fractional synthetic rates following a bout of concurrent training (15). Specifically, Donges and colleagues (15) showed comparable increases in mitochondrial protein synthesis following concurrent training compared with endurance exercise alone. The exercise intervention was undertaken in an untrained, middle-aged cohort and the results may reflect an adaptive response to unaccustomed contractile stimuli. Consequently, we suggest the training status of subjects in the present study may have required a greater overload stimulus to generate an acute

increase in mitochondrial protein synthesis (14). However, Scalzo and colleagues (35) have recently shown accretion of new mitochondrial protein concomitant with increased PGC-1 $\alpha$  protein and mitochondrial enzyme content after three weeks chronic training. Rowlands and co-workers have also reported an enhanced mitochondrial transcriptome associated with protein ingestion following endurance exercise, an effect that was only evident late (48 h) but not early (3 h) in the post-exercise period (34). Therefore, we cannot rule out that quantification of mitochondrial protein synthesis later in recovery (e.g. 24 h) or following an extended training period may have revealed changes in phenotype that reflect a different adaptation response to exercise and protein ingestion (14).

*Paragraph Number 40* The enhanced myofibrillar protein synthesis was associated with increases in the phosphorylation status of signaling proteins that regulate translation initiation and elongation. We have previously demonstrated a similar time course for Akt-mTOR-S6K phosphorylation during the early recovery period following single bouts of resistance exercise and cycling (6). Others have also previously shown endurance and resistance exercise in isolation activate the insulin/IGF signaling pathway (3, 16). Collectively, these findings indicate specific translational processes in skeletal muscle are not an important factor determining the specificity of training adaptation. More recently, a concurrent training bout has been shown to enhance Akt/mTOR-mediated signaling responses (29, 38). The results of the present study extend these findings by demonstrating that protein ingestion augments Akt-mTOR-S6K phosphorylation following concurrent training (Figure 3). Consequently, we contend Akt-mTOR-S6K signaling may be indicative of nutrient sensitivity and/or muscle overload but fails to discriminate between divergent contraction stimuli. Exercise also generated a decrease in phosphorylation (activation) of the peptide chain elongation factor eEF2 although there were no differences between treatments indicating it may be unresponsive to protein ingestion (Figure 3D). Thus, the nutrient-mediated

increases in muscle protein synthesis following exercise are likely due in part to enhanced translation initiation rather than elongation.

*Paragraph Number 41* Expression of MuRF1 and Atrogin-1 mRNA were elevated above rest following the concurrent training bout, however this increase was attenuated with protein ingestion (Figure 4A and B). Harber and colleagues (18) have previously shown a similar effect on MuRF1 mRNA abundance with ingestion of a protein/carbohydrate supplement following 60 min of cycling and Borgenvik and co-workers (4) demonstrated an amino acid-enriched beverage decreased MuRF1 protein levels at rest and after a resistance exercise bout. Therefore, coordinated attenuation in MuRF1 and Atrogin-1 expression with provision of exogenous amino acids in the present study may have provided substrate for muscle remodelling/hypertrophy that might otherwise be achieved through muscle proteolysis following exercise in the fasted state. However, without direct measures of proteasome activity or muscle protein breakdown the effect of the altered atrogene mRNA expression remains unclear. In contrast, there were no differences between treatments in myostatin mRNA expression during the acute recovery period (Figure 4C). Reduced myostatin expression has been demonstrated following an acute bout of endurance (28) and resistance (7, 28) exercise, and it appears myostatin mRNA expression is responsive to contraction per se rather than a specificity of training response and/or nutrient availability. There were also comparable increases in mRNA abundance of metabolic/mitochondrial proteins following the consecutive resistance and endurance exercise bouts but protein ingestion failed to induce any noteworthy increase in PGC-1 $\alpha$ , hexokinase or VEGF mRNA levels (Figure 5).

*Paragraph Number 42* In conclusion, the results of the present study demonstrate that protein ingestion after consecutive resistance and endurance exercise selectively increased rates of myofibrillar, but not mitochondrial, protein synthesis in the early (1-4 h) recovery period. Protein

ingestion also attenuated post-exercise increases in genetic markers associated with muscle proteolysis. Given endurance exercise undertaken in close temporal proximity to resistance exercise may interfere in hypertrophy/strength adaptation responses with concurrent training, our findings suggest protein intake can be beneficial following successive resistance and endurance exercise by promoting myofibrillar protein synthesis and decreasing ubiquitin ligase expression. Accordingly, we suggest post-exercise protein ingestion may have potential to ameliorate “interference” of endurance exercise on muscle hypertrophy, and represents an important nutritional strategy for concurrent training.

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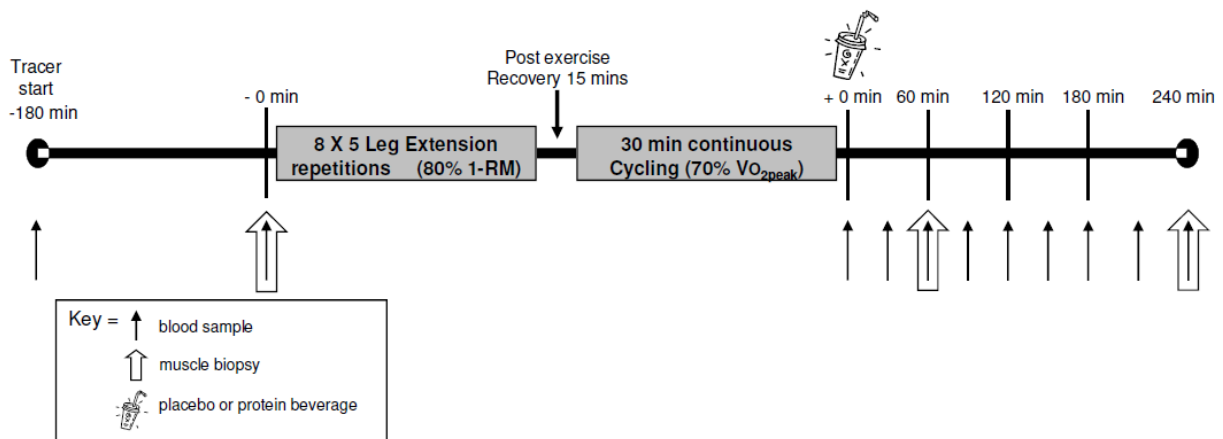
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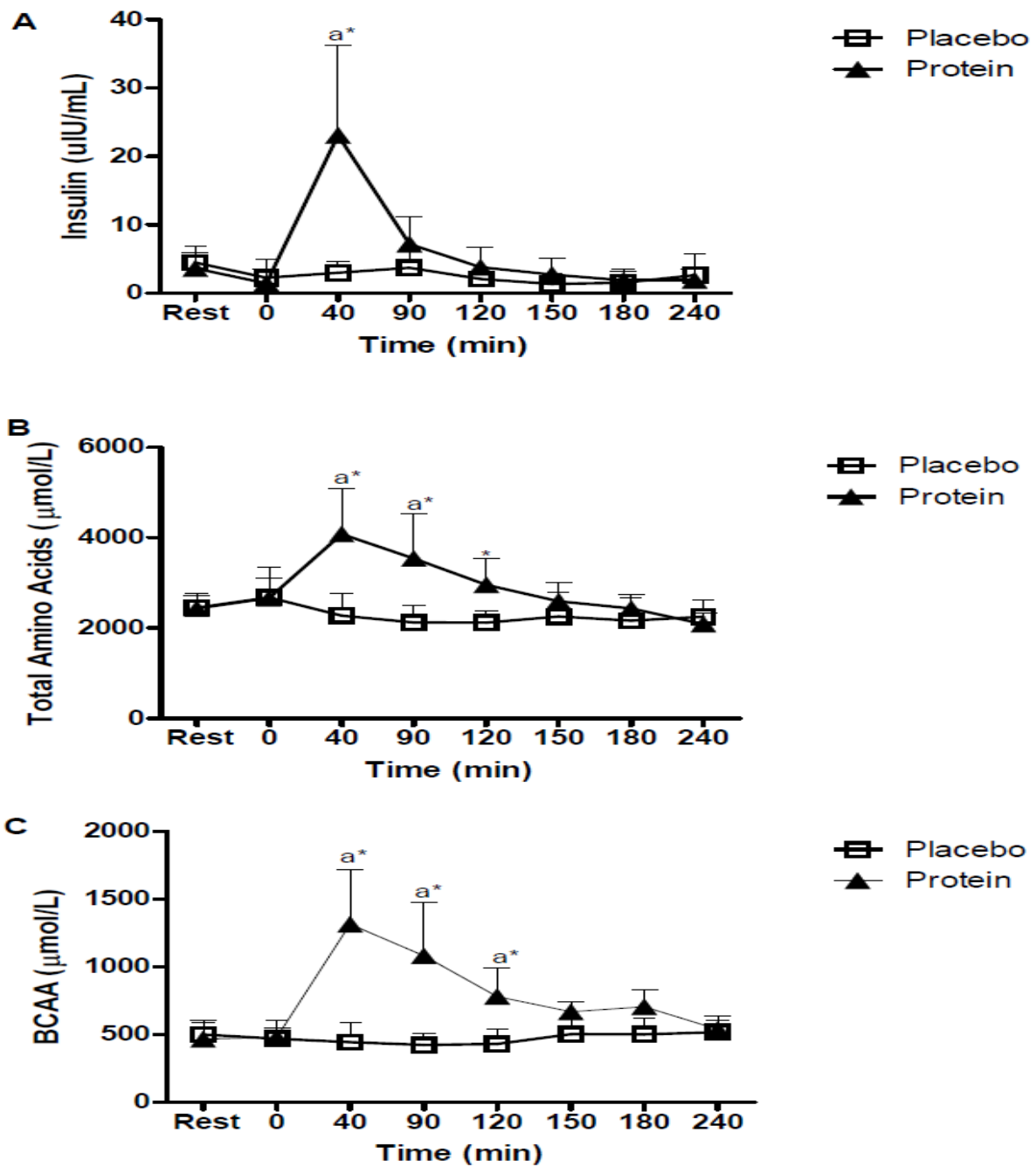
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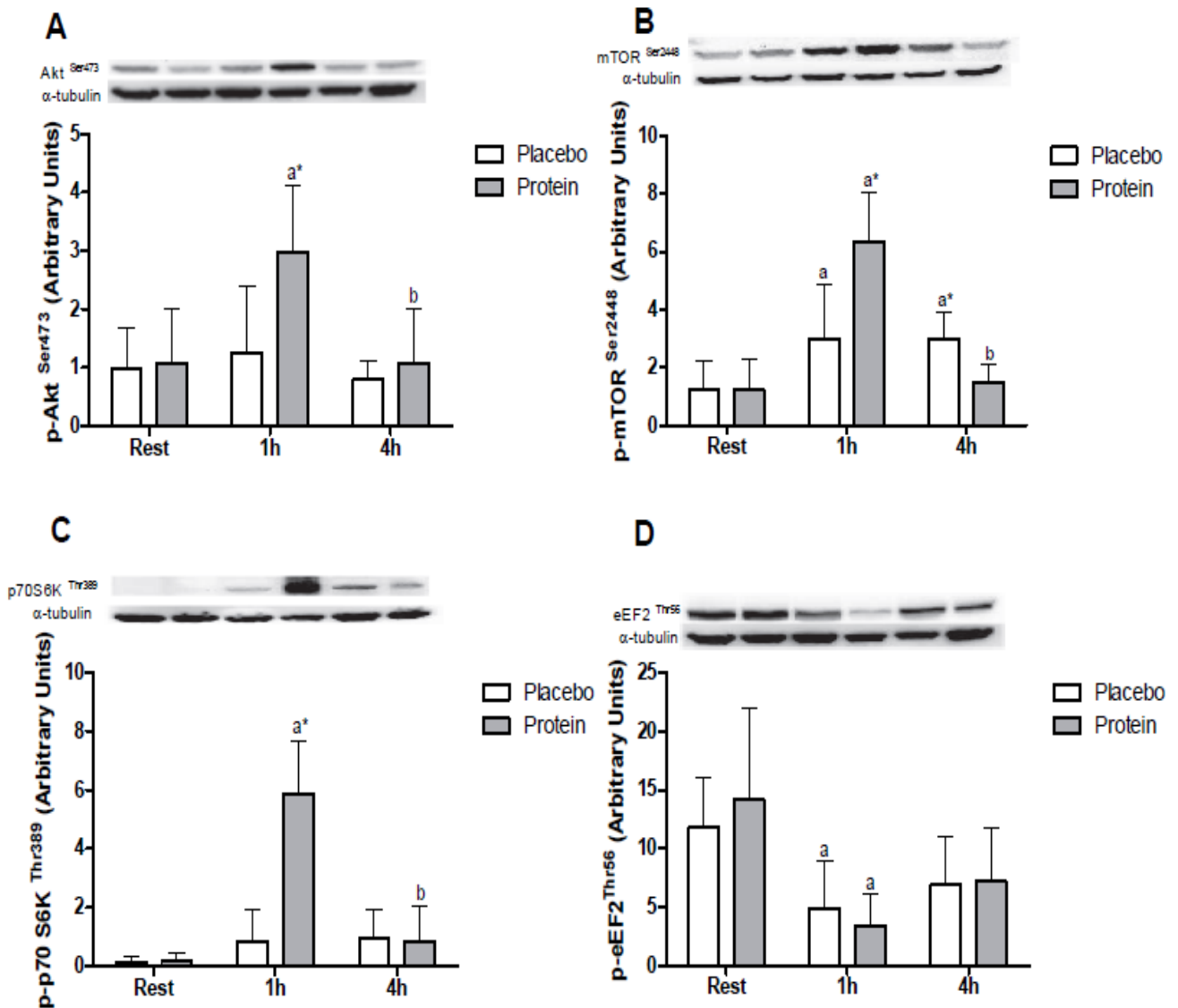
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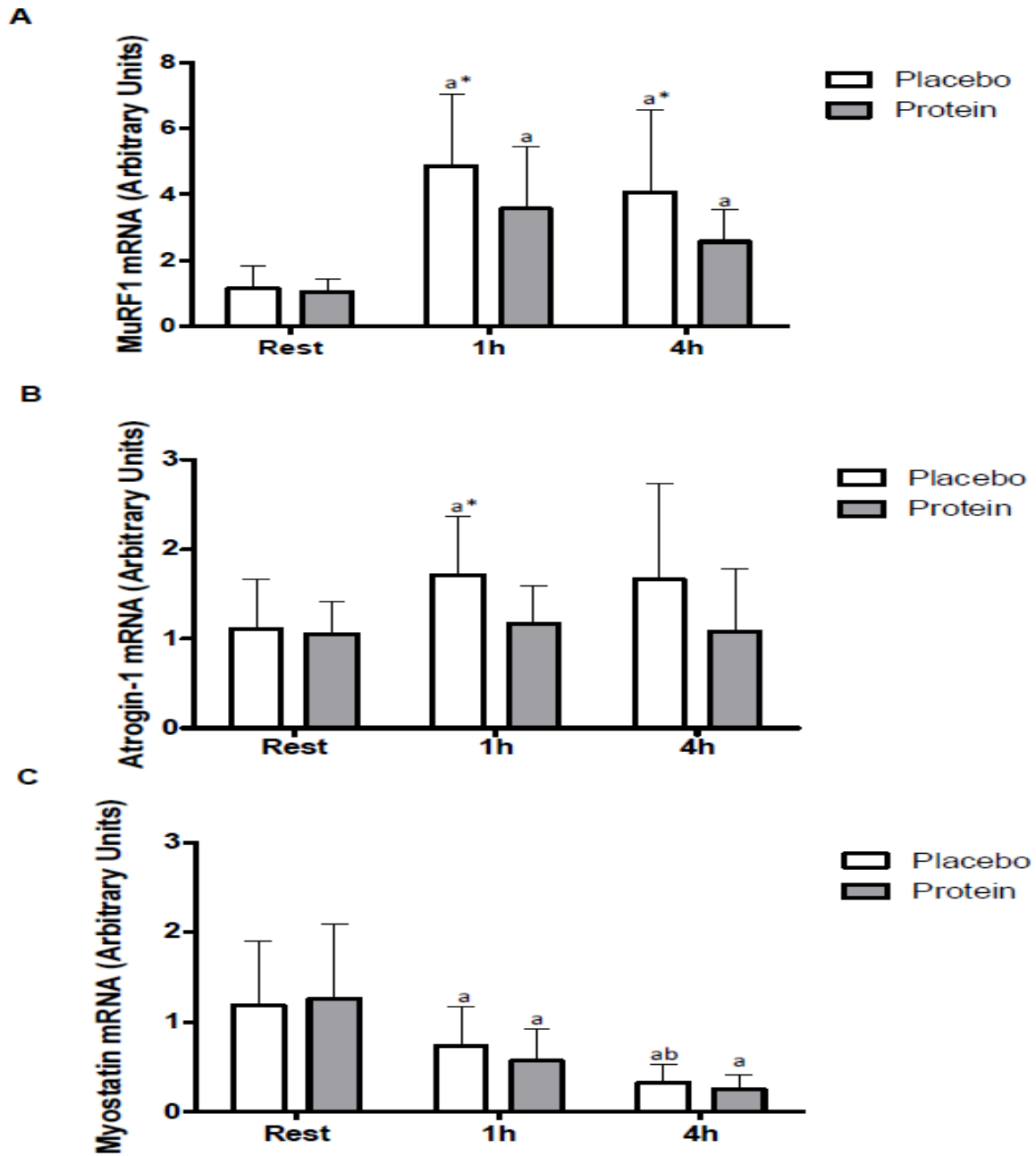
**Figure 1.** Schematic representation of the experimental trial. Subjects reported to the laboratory following an overnight fast and an after initial resting blood sample began a constant infusion of L-*[ring-<sup>13</sup>C<sub>6</sub>]* phenylalanine. 180 min after commencement of tracer infusion, a baseline muscle biopsy (*vastus lateralis*) was obtained, and subjects then completed a concurrent exercise session consisting of resistance (8 sets of 5 leg extension at 80% 1-RM) and endurance (30 min cycling at 70% VO<sub>2peak</sub>) exercise separated by 15 minutes. Immediately after exercise, subjects consumed a 500 mL bolus of protein (25 g whey) or placebo. Additional muscle biopsies were taken at 1 and 4 h post-exercise.



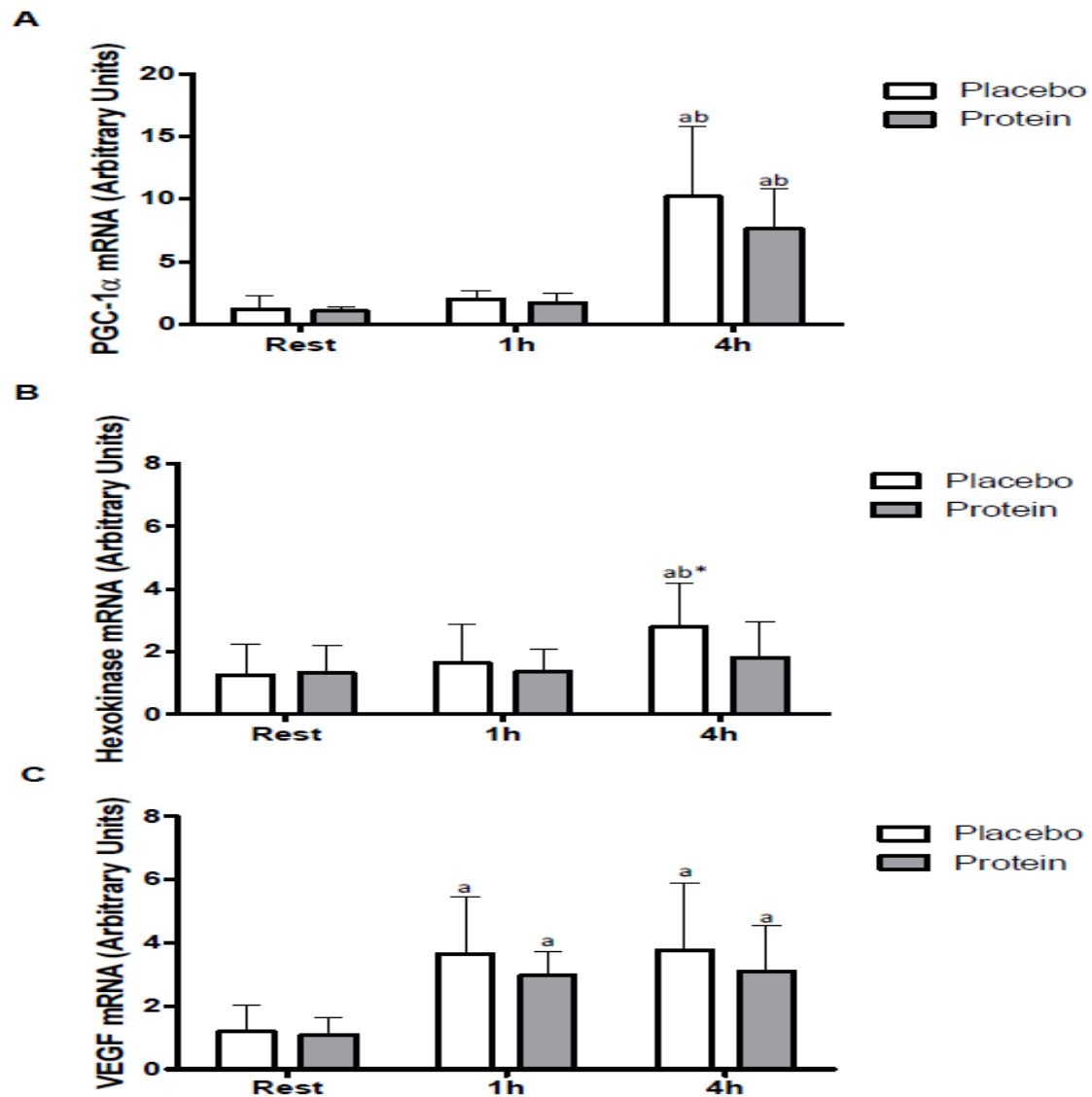
**Figure 2.** Plasma insulin (A), total plasma amino acid (B) and plasma branched chain amino acid concentrations (C) at rest and during 240 min recovery following a concurrent exercise of session resistance (8 sets of 5 leg extension at 80% 1-RM) and endurance (30 min cycling at 70%  $VO_{2peak}$ ) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are mean  $\pm$  SD. Significantly different ( $P < 0.05$ ) versus (a) rest.



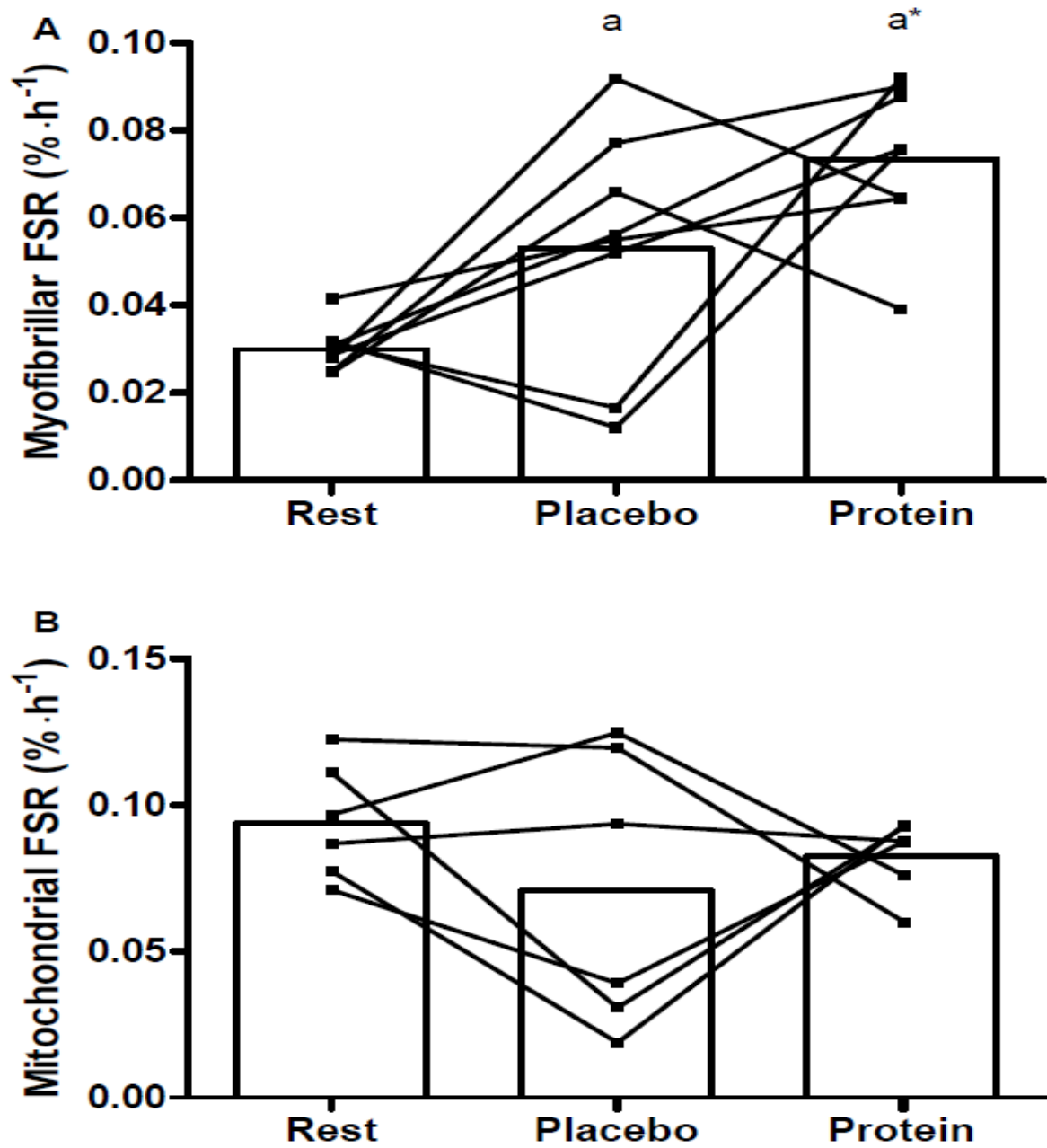
**Figure 3.** (A) Akt<sup>Ser473</sup> (B) mechanistic target of rapamycin (mTOR)<sup>Ser2448</sup> (C) p70S6K<sup>Thr389</sup> and (D) eukaryotic elongation factor 2 (eEF2)<sup>Thr56</sup> phosphorylation in skeletal muscle at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extension at 80% 1-RM) and endurance (30 min cycling at 70%  $VO_{2peak}$ ) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to  $\alpha$ -tubulin and presented in arbitrary units (mean  $\pm$  SD, n=8). Significantly different ( $P < 0.05$ ) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.



**Figure 4.** (A) Muscle RING finger 1 (MuRF1), (B) atrogin and (C) myostatin mRNA abundance at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extension at 80% 1-RM) and endurance (30 min cycling at 70%  $VO_{2peak}$ ) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to GAPDH and presented in arbitrary units (mean  $\pm$  SD, n=8). Significantly different ( $P < 0.05$ ) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.



**Figure 5.** (A) Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), (B) hexokinase and (C) vascular endothelial growth factor mRNA abundance at rest and during 4 h post-exercise recovery following a concurrent exercise session of resistance (8 sets of 5 leg extension at 80% 1-RM) and endurance (30 min cycling at 70%  $VO_{2peak}$ ) exercise and ingestion of either 500 mL placebo or protein beverage immediately post-exercise. Values are expressed relative to GAPDH and presented in arbitrary units (mean  $\pm$  SD, n=8). Significantly different ( $P < 0.05$ ) versus (a) rest, (b) 1 h and (asterisk) between treatments (placebo vs. protein) at equivalent time-point.



**Figure 6.** (A) Myofibrillar (n=8) and (B) Mitochondrial (n=6) protein fractional synthetic rates between 1-4 h recovery following a concurrent exercise session of resistance (8 sets of 5 leg extension at 80% 1-RM) and endurance (30 min cycling at 70%  $VO_{2peak}$ ) exercise and ingestion of either 500 mL placebo or protein (25 g whey protein) beverage immediately post-exercise. Values are expressed as %<sup>·h</sup> and presented as individual data with group mean. Significantly different ( $P < 0.05$ ) versus (a) rest, and (asterisk) placebo vs. protein.