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Pre-sleep protein ingestion does not compromise the muscle protein synthetic response to protein ingested the following morning

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Running title: Protein intake and post-prandial protein handling

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Abstract

Background: Protein ingestion before sleep augments post-exercise muscle protein synthesis during overnight recovery. **Purpose:** It is unknown whether post-exercise and pre-sleep protein consumption modulates post-prandial protein handling and myofibrillar protein synthetic responses the following morning. **Methods:** Sixteen healthy young (24±1 y) men performed unilateral resistance-type exercise (contralateral leg acting as a resting control) at 20:00 h. Participants ingested 20 g protein immediately after exercise plus 60 g protein pre-sleep (PRO group; *n*=8) or equivalent boluses of carbohydrate (CON; *n*=8). The subsequent morning participants received primed-continuous infusions of L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[1-^{13}C]leucine combined with ingestion of 20 g intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine labelled protein to assess postprandial protein handling and myofibrillar protein synthesis in the rested and exercised leg in CON and PRO. **Results:** Exercise increased post-absorptive myofibrillar protein synthesis rates the subsequent day (P<0.001), with no differences between CON and PRO. Protein ingested in the morning increased myofibrillar protein synthesis in both the exercised- and rested-leg (P<0.01), with no differences between treatments. Myofibrillar protein bound L-[1-^{13}C]phenylalanine enrichments were greater in the exercised (0.016±0.002 and 0.015±0.002 MPE in CON and PRO, respectively) versus rested (0.010±0.002 and 0.009±0.002 MPE in CON and PRO, respectively) leg (P<0.05), with no differences between treatments (P>0.05). **Conclusions:** The additive effects of resistance-type exercise and protein ingestion on myofibrillar protein synthesis persist for more than 12 h after exercise and are not modulated by protein consumption during acute post-exercise recovery. This work provides evidence of an extended window of opportunity where pre-sleep protein supplementation can be an effective nutrient timing strategy to optimize skeletal muscle reconditioning.

Introduction

Resistance-type exercise training forms an effective interventional strategy to increase skeletal muscle mass and strength (e.g. 37). A single bout of resistance-type exercise increases both muscle protein synthesis and breakdown rates, albeit the latter to a lesser extent (2, 31). Though exercise improves net muscle protein balance, the balance remains negative in the absence of protein ingestion (2, 31). Dietary protein ingestion in close proximity to exercise further augments the exercise induced increase in muscle protein synthesis rate and inhibits exercise induced proteolysis, resulting in a positive post-exercise protein balance (3, 5). This interaction between exercise and nutrition on the postprandial muscle protein synthetic response during recovery from exercise has been well-established, and forms a fundamental principle by which gains in muscle mass and strength are achieved in both an athletic and rehabilitative setting(e.g. 8, 41).

Studies examining the synergy between exercise and nutrition generally administer protein immediately before (38, 39), during (1, 21) or immediately after (3, 23, 29, 32) exercise. Recently, we showed that protein administration prior to (33) or during (19) sleep can also augment overnight muscle protein synthesis rates. However, the influence of protein ingestion after exercise and/or before sleep on the myofibrillar protein synthetic response to subsequent meals has not yet been investigated. We reasoned that protein ingested immediately after exercise and/or prior to subsequent sleep would reduce the muscle protein synthetic response to the consumption of a meal-like amount of protein the following morning. Discovery of the existence (or absence) of such a negative feedback loop would be of key importance to our understanding of post-prandial protein handling and could have great relevance for nutritional intervention strategies in both a sports and rehabilitative setting.

In the present study, we determined if protein ingestion immediately after a single bout of resistance-type exercise and prior to subsequent sleep modulates the postprandial myofibrillar

protein synthetic response to protein consumed the subsequent morning in both resting and exercised skeletal muscle tissue of healthy, young men. We hypothesized that ingesting large amounts of protein during acute and overnight recovery from resistance type exercise would modulate post-prandial protein handling and lower the post-prandial muscle protein synthetic response to protein feeding the following morning. We applied a unilateral one-legged exercise protocol (9) and combined the ingestion of specifically produced intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine labelled dietary protein with continuous intravenous L-[*ring*-$^{2}H_5$] phenylalanine and L-[1-^{13}C]leucine infusions, using a recently validated triple tracer approach (6). This allowed us to simultaneously assess post-absorptive and post-prandial muscle protein synthesis rates as well as directly assess the accretion of the dietary protein derived amino acids into *de novo* myofibrillar protein in both resting and exercised skeletal muscle tissue. These data are the first to show that there is a window of opportunity during which protein feeding will augment post-exercise muscle protein synthesis rates without negative feedback inhibition of the post-prandial muscle protein synthetic response to protein consumed the following day.

Methods

Participants and ethical approval

Sixteen healthy, young men (age: 24±1 y; body mass: 74.7±2.6 kg; BMI: 22.7±0.7 kg/m^2) volunteered to participate in this study. Characteristics of the participants are presented in **Table 1**. Participants were recreationally active and engaged in exercise at least 2 times per week for ≥1 y. All participants were deemed healthy based on their response to a routine medical screening questionnaire. Participants were informed of the purpose of the study, experimental procedures, and all its potential risks prior to providing written consent to participate. Participants had no prior history of participating in stable isotope labelled amino acid tracer experiments. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre+, Maastricht, the Netherlands and conformed to standards for the use of human participants in research as outlined in the sixth Declaration of Helsinki.

Pretesting

All participants underwent two pretesting sessions. Participants reported to the laboratory for familiarization with the exercise equipment and for determination of unilateral maximum strength as determined by their one repetition maximum (1RM) on leg extension and leg press machines for the right and left legs. In addition, body mass, height and body composition by dual-energy X-ray absorptiometry (Discovery A; Hologic Corp, Bedford, MA) were measured. In a subsequent session, 10RM was confirmed by using 70% of the previously established 1RM and this was the exercise load that was used in the experimental trial. Subsequently, participants were randomly assigned and counterbalanced for leg strength to

either the protein (PRO; *n*=8) or carbohydrate (CON; *n*=8) treatments. All beverages used in the study were prepared in coded containers by an independent research assistant.

Diet and physical activity control

Participants were instructed to refrain from vigorous physical activity and to report their dietary intake in a food diary for two days prior to and on the first day of the experimental protocol. All participants consumed a standardized meal of the same composition (32±1 $kJ \cdot kg^{-1}$ body weight: providing 51 energy% (En%) carbohydrate, 33 En% fat, and 16 En% of protein) the evening prior to the experimental protocol.

Experimental protocol

An overview of the experimental protocol is shown in **Figure 1**. On day 1, participants were provided with standardized meals of identical composition (consisting of 57 Energy percentage (En%) carbohydrate, 13 En% protein, and 30 En% fat) to be consumed for breakfast, lunch, and dinner. Dinner was provided after the participants arrived at the laboratory at 17:00 h and was consumed under supervision. Subsequently, participants rested until 20:00 h when the exercise protocol was started. The exercise protocol consisted of unilateral resistance-type exercise performed for 4 sets × 10-12 repetitions to volitional fatigue with a load that corresponded to their previously established 10RM (~70% 1RM) on the horizontal leg press and leg extension machines (Technogym BV, Rotterdam, the Netherlands). There was a resting period of 2 min between each set and a 5 min rest between exercises. The contralateral leg did not perform resistance-type exercise and, as such, served as a rested control. To optimize muscle protein synthesis during acute recovery from exercise we provided subjects with 20 g whey protein immediately after cessation of exercise, which is currently advised in guidelines for optimal post-exercise recovery (23, 44) (PRO; Bulk

153 powders Pure Whey Isolate 97, Sports Supplements Ltd, Colchester, Essex, UK) or 20 g
154 carbohydrate (CON; 50% dextrose monohydrate, Avebe Food, Veendam, the Netherlands,
155 50% maltodextrin, AppliChem GmbH, Darmstand, Germany) dissolved in 400 mL of water.
156 At 23:00 h, the participants in the PRO group were provided with an additional 60 g of whey
157 protein dissolved in 400 mL water to stimulate overnight muscle protein synthesis rates (33).
158 The CON group received a 400 mL beverage containing 60 g carbohydrate instead.
159 Afterwards, participants slept for 7.5 h within the laboratory. The next morning, participants
160 were woken up at 07:00 h and a Teflon catheter was inserted into an antecubital vein for
161 stable isotope infusion (Day 2; **Figure 1**). A second Teflon catheter was inserted into a dorsal
162 hand vein of the contralateral arm and placed in a hot-box (60°C) for arterialized blood
163 sampling. After baseline blood collection (t=-180 min), the phenylalanine, tyrosine, and
164 leucine pools were primed with a single intravenous dose of L-[ring-$^{2}H_5$]phenylalanine (2
165 μmol·kg^{-1}), L-[3,5-$^{2}H_2$]tyrosine (0.615 μmol·kg^{-1}), and L-[1-^{13}C]leucine (4 μmol·kg^{-1}).
166 Subsequently, continuous L-[ring-$^{2}H_5$]phenylalanine (infusion rate: 0.05 μmol·kg^{-1}·min^{-1}), L-
167 [*ring*-$^{2}H_2$] tyrosine (0.15 μmol·kg^{-1}·min^{-1}), and L-[1-^{13}C]leucine (0.10 μmol·kg^{-1}·min^{-1})
168 infusions were initiated and maintained throughout the protocol. To provide a reference value
169 for postabsorptive myofibrillar protein synthesis rates (7, 10) a single muscle biopsy was
170 collected from the exercised (EX-FAST) and rested (REST-FAST) legs after 180 min of
171 infusion. Immediately after the muscle biopsies, participants ingested a single bolus of 20 g
172 intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine-labelled whey protein dissolved in
173 350 mL vanilla flavored water. Additional biopsies were collected at *t*=180 min for the
174 measurement of postprandial muscle protein synthesis rates in the exercised (EX-FED) and
175 non-exercised (REST-FED) legs. The biopsies were collected from the middle region of the
176 *vastus lateralis* (15 cm above the patella) with a Bergström needle under local anesthesia. All
177 biopsy samples were freed from any visible adipose tissue and blood, immediately frozen in

liquid nitrogen, and stored at –80˚C until subsequent analysis. Arterialized venous blood samples were drawn every 30 or 60 min during the post-absorptive and postprandial states and were processed as previously described (**Figure 1**) (7, 10).

Intrinsically labelled whey protein

Intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine labelled milk protein was obtained by a constant infusion of L-[1-^{13}C]phenylalanine (455 μmol/min) and L-[1-^{13}C]leucine (200 μmol/min) maintained for 96 h in a lactating dairy cow (6, 30, 40). The milk was heated to 50˚C and converted to skim milk before being microfiltrated using a membrane with a pore size of 1.4 μM at 50˚C to remove microbes. Subsequently, the skim milk was microfiltrated on a 0.2 μM pore size diameter membrane to separate the casein micelles from the soluble whey proteins at 55˚C. The whey proteins were collected and cooled. The soluble whey protein fraction was concentrated (~96% protein), sterile filtrated, and stored at room temperature prior to use. The L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine enrichments in the whey protein were measured by gas chromatography mass spectrometry (Agilent 6890N GC coupled with a 5973 inert MDS; Little Falls, DE) after hydrolysis and averaged 36.1 MPE and 8.9 MPE, respectively. The proteins met all chemical and bacteriologic specifications for human consumption.

Plasma analyses

Plasma glucose and insulin concentrations were analysed using commercially available kits (Glucose HK Gen.3, Roche, Ref: 05168791190, and Elecsys Insulin assay, Roche, Ref: 12017547122, respectively). Mixed plasma proteins, plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry analysis (Agilent 6890N GC coupled with a 5973 inert MDS; Little Falls, DE) as previously described (7, 10)

Muscle analyses

Myofibrillar protein enriched fractions were isolated as described in our previous work (6). Myofibrillar protein bound enrichments were determined by GC-MS analysis. To reduce the signal-to-noise ratio during GC-MS analysis at low tracer enrichments, the phenylalanine from the myofibrillar protein hydrolysates were enzymatically decarboxylated to β-phenylethylamine (12) prior to tBDMS derivatization (35, 36). Enrichments of the protein bound samples were determined by selected ion monitoring for β-phenylethylamine-tBDMS mass to charge ratio at 183 (*m*+5) to 180 (*m*+2) and a single linear standard curve (to avoid slope influences on the measured TTR) from mixtures of known *m*+5 to *m*+2 ratios. To avoid saturation of the MS and eliminate bias due to any potential concentration dependencies (27), the split ratio was adjusted prior to the injection of each sample so that nearly equal amounts of phenylalanine were injected for all samples and standards. The remaining aliquot of purified amino acids were converted to their N(O,S)-ethoxycarbonyl ethyl esters derivatives to determine the L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine labelling of the myofibrillar proteins by gas chromatography combustion-isotope ratio mass spectrometry analysis (GC-C-IRMS; Trace GC Ultra, IRMS model MAT 253, Thermo Scientific, Bremen, Germany). The derivatized amino acids were separated on a 30m × 0.25mm × 0.25µm DB-5MS column (temperature program: 120˚C for 10 min; 3˚C min^{-1} ramp to 150˚C; 30˚C min^{-1} ramp to 300˚C; hold for 5 min) prior to combustion. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Total RNA was isolated from 10-20 mg of frozen muscle tissue using Trizol Reagent (Life Technologies, Invitrogen) and quantitative RT-PCR was performed to determine skeletal

muscle mRNA expression of LAT1, CD98, SNAT2, PAT1, FOXO1, myostatin, MuRF1, and MAFBx as described in our previous work (42, 43). All genes of interest were labelled with the fluorescent reporter FAM (6-carboxyfluorescein). The thermal cycling conditions used were: 2 min at 50˚C, 10 min at 95˚C, followed by 50 cycles at 95˚C for 15 s and 60˚C for 1 min. The housekeeping gene 18S was used as an internal control as we and others have used this as a reliable housekeeping gene in previous studies similar studies (13, 20, 42). Values of the target gene were normalized to C_t values of the internal controls and final results were calculated as relative expression against a standard curve.

Calculations

Whole-body amino acid kinetics were assessed in non-steady conditions by the ingestion of intrinsically L-[1-^{13}C]phenylalanine labelled whey protein combined with the intravenous infusion of L-[ring-$^{2}H_5$]phenylalanine and L-[ring-3,5-$^{2}H_2$]tyrosine. Exogenous and endogenous phenylalanine rate of appearance (R_a), total rate of disappearance (R_d), and plasma availability of dietary protein-derived phenylalanine (the fraction of the dietary phenylalanine that appeared in systemic circulation, Phe_{plasma}) were calculated using modified Steele equations (15, 28).

The fractional synthetic rates (FSR) of myofibrillar protein were calculated using standard precursor-product methods by dividing the increment in L-[ring-$^{2}H_5$]phenylalanine, L-[1-^{13}C]leucine, or L-[1-^{13}C]phenylalanine enrichment in the myofibrillar protein by the tracer enrichments of the plasma free precursor pool over time (7, 10). The single biopsy approach for the determination of the postabsorptive myofibrillar protein synthetic rates in the exercised and non-exercised legs was only used for the L-[ring-$^{2}H_5$]phenylalanine tracer as the modified prime with the L-[1-^{13}C]leucine tracer (4 μmol/kg versus the more commonly used 7.6 μmol/kg priming dose) did not allow for muscle protein labeling that was immediately linear

after initiating the infusion and invalidated its use for the determination of postabsorptive muscle protein synthesis rates with the L-[1-^{13}C]leucine tracer (7, 10).

Statistics

Differences in plasma amino acid, insulin and glucose concentrations, and tracer enrichments, and myofibrillar L-[1-^{13}C]phenylalanine enrichments were tested by two-factor (treatment × time) repeated measures analysis of variance (ANOVA). Myofibrillar FSRs and muscle gene expression were analysed using a three-factor (treatment, protein ingestion and exercise conditions) ANOVA. When significant interaction effects were observed in the ANOVA, Bonferroni post-hoc tests were performed to locate these differences. Statistical significance was set at $P<0.05$. All calculations were performed using IBM SPSS Statistics Version 20. All data are expressed as means±SEM.

Results

Participants' characteristics

Participants' characteristics within the two experimental groups and their habitual dietary intakes are presented in **Table 1**. No differences in age, weight, height, BMI, body composition, strength or habitual diet were detected between groups. All subjects completed the required protocol for the single bout of one-legged resistance type exercise training, and consumed both the post-exercise and pre-sleep protein beverages without problem. In addition, all subjects reported being able to sleep well during the overnight stay in the laboratory.

Plasma analyses

Plasma glucose concentrations declined over time ($P<0.001$) during the experimental visit from ~4.9 to ~4.5 $mmol \cdot L^{-1}$ without any group differences (*not shown*). Plasma insulin concentrations (**Figure 2A**) showed a rapid and brief increase following protein ingestion (at $t=0$ min) in both groups up to 20-30 $mU \cdot L^{-1}$ after 30 min, before returning to baseline levels after 90 min (time effect; $P<0.001$). However, there was no effect of treatment ($P=0.27$) or any interaction ($P=0.59$) detected. Both plasma phenylalanine (**Figure 2B**) and leucine (**Figure 2C**) concentrations increased following protein ingestion in both groups (time effect; $P<0.001$) and remained above basal levels for 60 and 120 min, respectively. The time course of plasma L-[*ring*-$^{2}H_5$]phenylalanine (**A**), L-[1-^{13}C]leucine (**B**) and L-[1-^{13}C]phenylalanine (**C**) enrichments are illustrated in **Figure 3**. During the post-absorptive period, plasma L-[*ring*-$^{2}H_5$]phenylalanine and L-[1-^{13}C]leucine remained in steady-state at ~7-8 and ~5-6 MPE, respectively. Following protein ingestion (at $t=0$ min), plasma L-[*ring*-$^{2}H_5$]phenylalanine enrichments decreased for 60 min before returning to fasting, steady-state levels (time effect; $P<0.001$), while plasma L-[1-^{13}C]leucine enrichments increased in response to protein

ingestion (time effect; $P<0.001$) and remained at an elevated steady state of ~8 MPE for the duration of the post-prandial period. No treatment effects were observed for plasma L-[*ring*-$^{2}H_5$]phenylalanine and L-[1-^{13}C]leucine enrichments, although an interaction effect was detected for L-[*ring*-$^{2}H_5$]phenylalanine ($P<0.05$) without any individual differences evident following post-hoc analysis. Following protein ingestion, plasma L-[1-^{13}C]phenylalanine enrichments increased rapidly in both groups (time effect; $P<0.001$) from ~0 MPE to ~13-14 MPE after 30 min in both groups and began declining thereafter, though remaining elevated above fasting levels for the entirety of the post-prandial period. However, no treatment or interaction effects were observed for plasma L-[1-^{13}C]phenylalanine enrichments.

Whole body phenylalanine kinetics

Whole body phenylalanine kinetics are presented in **Figure 4 (A-D)**. Exogenous phenylalanine rates of appearance (R_a) (i.e. the rate at which dietary protein-derived phenylalanine enters the circulation) (**A**) increased after protein ingestion (time effect; $P<0.001$) and to a similar extent in both groups. The amount of dietary protein-derived phenylalanine that appeared in the circulation over the 3-hour postprandial period was equivalent in both groups (56±1% vs 60±5% in CON and PRO, respectively; $P=0.39$). Endogenous phenylalanine R_a (i.e. the rate at which phenylalanine derived from whole body protein breakdown enters the circulation) (**B**) decreased after protein ingestion (time effect: $P<0.001$) with no differences observed between groups. Total phenylalanine R_a (C) and rates of disappearance (R_d) (**D**) increased after protein ingestion (time effect: both $P<0.001$). While total phenylalanine R_a was unaffected by nutritional intervention, a significant time × nutritional intervention effect was detected for R_d ($P<0.05$), with no individual effects located following post-hoc tests.

Skeletal muscle tracer analyses

Mean postabsorptive and postprandial myofibrillar protein fractional synthetic rates (FSR) based on L-[*ring*-$^{2}H_5$]phenylalanine are presented in **Figure 5**. Based on the L-[*ring*-$^{2}H_5$]phenylalanine tracer, post-absorptive myofibrillar FSRs in resting muscle did not differ between groups (0.021±0.001 and 0.026±0.004 %·h^{-1} in the CON and PRO groups, respectively; P>0.05) but were higher in exercised muscle (0.044±0.003 and 0.043±0.005 %·h^{-1} in the CON and PRO, respectively) in both groups (main effect of exercise; P<0.001) without any interaction (P>0.05) or impact of the nutritional intervention (P>0.05). Based on the L-[*ring*-$^{2}H_5$]phenylalanine tracer, the ingestion of 20 g dietary protein stimulated myofibrillar FSR in resting (increased to 0.043±0.004 and 0.041±0.005 %·h^{-1} in CON and PRO, respectively) and exercised (increased to 0.060±0.006 and 0.058 %·h^{-1} in CON and PRO, respectively) muscle to a similar degree (main effect of protein ingestion; P<0.01) without any interaction or effect of the nutritional treatment evident (P>0.05). Due to higher post-absorptive FSR, exercised muscle retained higher myofibrillar FSR in the post-prandial state compared with resting muscle (main effect of exercise: P<0.001). Based on L-[1-^{13}C]leucine, post-prandial myofibrillar FSR was lower in resting (0.043±0.004 and 0.041±0.005 %·h^{-1} for CON and PRO, respectively) compared with exercised (0.060±0.006 and 0.058±0.005 %·h^{-1} for CON and PRO, respectively) muscle (main effect of exercise; P<0.001) without any differences detected between groups.

Myofibrillar L-[1-^{13}C]phenylalanine enrichments 3 h following the ingestion of 20 g intrinsically L-[1-^{13}C]phenylalanine labelled whey protein are presented in **Figure 6.** Following protein ingestion, myofibrillar L-[1-^{13}C]phenylalanine enrichments increased from background in resting (to 0.010±0.002 and 0.009±0.002 MPE in CON and PRO, respectively) and exercised (to 0.016±0.002 and 0.015±0.002 MPE in CON and PRO, respectively) muscle (main effect of protein ingestion; P<0.001) and by a greater degree in exercised compared

with resting muscle (P<0.001). However no effect of nutritional intervention or any interaction effects were detected.

Gene expression

The skeletal muscle mRNA expression of genes implicated in the regulation of intracellular amino acid transport and muscle protein breakdown are presented in **Figure 7 (A-H)**. Muscle LAT1 mRNA expression (**A**) was increased with protein ingestion (P<0.001) exercise (P<0.05) and in the CON group compared with PRO (P<0.05). Muscle PAT 1 mRNA expression (**B**) was higher in exercised compared with resting muscle (P<0.001) and decreased in response to protein ingestion in exercised muscle only (exercise × protein ingestion interaction; P<0.01). However, PAT1 expression did not differ between CON and PRO groups (P>0.05). Muscle SNAT2 mRNA expression (**C**) was higher in exercised muscle in the CON group only (exercise × nutritional intervention interaction; P<0.05), and decreased in response to protein ingestion in exercised muscle only (exercise × protein ingestion interaction; P<0.05). Muscle CD98 mRNA expression (**D**) was greater in exercised compared with resting muscle (P<0.01) and increased in response to protein ingestion (P<0.05) without any interaction or effect of nutritional intervention. Muscle myostatin mRNA expression (**E**) was lower in exercised compared with rested muscle (P<0.05) with the effect more profound in the PRO group (exercise × nutritional intervention interaction; P<0.05). Muscle MAFBx mRNA expression (**F**) was lower in exercised compared with resting muscle (P<0.001) and a three way interaction was observed (exercise × protein ingestion × nutritional intervention; P<0.05) such that expression increased in response to protein ingestion in the PRO group in exercised muscle, whereas expression decreased in response to protein ingestion in the CON group in resting muscle. Muscle MuRF1 mRNA expression (**G**) was lower in exercised compared with resting muscle in the fasting state only

(exercise × protein ingestion interaction; $P<0.05$). Muscle FOXO1 mRNA expression (**H**) increased in response to protein ingestion in resting muscle only (exercise × protein ingestion interaction; $P<0.05$).

Discussion

The present work is the first to show that feeding large amounts of protein after a single bout of resistance-type exercise performed in the evening did not attenuate the post-prandial muscle protein synthetic response to protein consumed the following morning in either exercised or rested muscle tissue. Regardless of the ingestion of large amounts of protein immediately after exercise and prior to sleep the day before, the protein ingested the following morning was effectively digested and absorbed, stimulating post-postprandial muscle protein accretion, with the protein derived amino acids being used as precursors for *de novo* muscle protein synthesis. In addition, the stimulating effect of prior exercise on the myofibrillar protein synthetic response to protein ingestion persists the day after exercise was performed, regardless of whether large amounts of protein were consumed during acute and overnight recovery.

Our previous work has established that 40 g protein ingestion before sleep represents an effective nutritional strategy to augment overnight muscle protein synthesis rates (33) and, consequently, the skeletal muscle adaptive response to prolonged resistance-type exercise training (37). Our current work offers the mechanistic underpinning of how pre-sleep protein supplementation acts as a nutrient timing strategy to facilitate skeletal muscle reconditioning (repair, remodelling, and/or muscle protein accretion). Specifically, the consumption of ample amounts of protein immediately after cessation of exercise (20 g) and prior to sleep (60 g) did not modulate digestion and absorption kinetics (**Figure 4**) or 'desensitize' the muscle protein synthetic response to protein ingested the following morning in either the exercised or non-exercised leg (**Figure 5**). In support, we also demonstrated that the use of dietary protein derived amino acids for *de novo* postprandial muscle protein accretion did not differ between the PRO and CON groups in the previously exercised or non-exercised leg (**Figure 6**). As such, these data infer that exercise augments the muscle protein synthetic response to each

and every meal consumed within a given post-exercise time period, which would explain why pre-sleep protein feeding further augments muscle mass (and strength) gains during more prolonged resistance type exercise training [39].

Contrary to exercise, pre-sleep protein feeding (i.e. when examining the non-exercised leg) did not modulate basal muscle protein synthesis rates determined the following morning. This is not surprising as the stimulatory effect of protein ingestion is temporary, lasting for approx. 2-5 h (24). However, our work provides insight into the interactive effects of nutrition and exercise during late recovery, which is an area that, so far, has received little attention (11). Previous work has shown that the synergistic effects of exercise and protein ingestion on muscle protein synthesis rates occur immediately after exercise (4) and may persist for at least 24 h during recovery from resistance-type exercise (11). Here, we show that protein ingested in the morning further increases muscle protein synthesis rates beyond the already elevated (post-absorptive) myofibrillar protein synthesis rates in the previously exercised leg, without any interference from prior ingestion of large amounts of protein during acute and overnight recovery. Moreover, we extend the time course of our previous work (29) by showing that exercise prior to protein ingestion allows for greater use of dietary protein derived amino acids for *de novo* muscle protein accretion for up to 17 h of post-exercise recovery (**Figure 6**). Collectively, these data provide evidence supporting the existence of a 'window of anabolic opportunity' for protein ingestion to further increase muscle protein synthesis rates during post-exercise recovery. This window of opportunity extends for at least 17 h of post-exercise recovery, where the ingestion of protein results in greater net muscle protein accretion. Feeding protein within this window likely supports the skeletal muscle adaptive response to training in a variety of populations or environments, resulting in greater net gains in muscle mass and/or strength.

In an effort to understand how protein before sleep may modulate the skeletal muscle adaptive response, we measured the mRNA abundance of amino acid transporters (LAT1, PAT1, SNAT2, CD98), markers of muscle proteolysis (FOXO1, MAFBx, MuRF1) (26) and a known key regulator of skeletal muscle mass (myostatin) (**Figure 7**) (22, 25). We extend on previous findings (14, 16-18) by demonstrating that the co-ordinated increase in gene expression of the amino acid transporters induced by resistance-type exercise persists the day after exercise. This was most evident in the contraction sensitive transporter PAT1 (16) which, in contrast, seemed remarkably resistant to any synergistic effects of nutritional stimuli. It is therefore likely that the prolonged elevations in muscle protein synthesis induced by resistance-type exercise (**Figure 5**) are supported by an increased intracellular availability of amino acids due to increased muscle amino acid uptake. Acute protein ingestion also led to a general increase in amino acid transporter expression, most notably that of LAT1. This is in line with previous findings (14, 16-18) and consistent with the role of LAT1 as a leucine specific amino acid transporter. However, LAT1 expression was greater in fasting muscle in response to protein ingestion in the CON group. It may be speculated that this result is serving as a compensatory mechanism to scavenge limited amino acid supply for transport into skeletal muscle tissue in the CON condition. Worthy of comment, it has been shown that the muscle protein synthetic response to protein ingestion generally subsides after 2-4 h (24) which would argue against the present observation of a far more sustained rise in amino acid transporter expression playing a strong modulatory role in the prolonged regulation of post-prandial muscle protein metabolism. Irrespective, it should be acknowledged that we did not measure protein levels or subcellular location of the amino acid transporters, and therefore their exact role(s) towards modulating the postprandial muscle protein synthetic response remains to be established, but will require carefully designed and specific future experiments.

We revealed an additional novel finding concerning the regulation of muscle myostatin gene expression. Myostatin gene expression was reduced in exercised muscle when protein was ingested during acute and overnight post-exercise recovery. This finding seems to be in line with the concept that low myostatin expression facilitates an anabolic environment (34), which our data suggest persists the day after exercise and is augmented by increased protein intake in the recovery period. Although exercise led to a general decrease in the expression of the atrogenes the subsequent day, presumably supporting muscle anabolism, this was not modulated by post-exercise nutrition.

In conclusion, the consumption of large amounts of protein immediately after exercise and before sleep does not modulate dietary protein digestion and absorption kinetics or postprandial myofibrillar protein synthesis rates to the subsequent morning protein meal with or without prior exercise in healthy, young males. Our work provides insight into the effectiveness of night-time protein supplementation as an effective nutrient timing strategy to augment skeletal muscle reconditioning during prolonged resistance-type exercise training.

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FIGURE LEGENDS

Figure 1. Schematic of the experimental protocol.

On Day 1, participants ingested a standardized meal and performed unilateral resistance exercise. Participants ingested either whey (PRO group; *n*=8) or carbohydrate (CON group; *n*=8) immediately after exercise and prior to sleep. On Day 2, all participants were roused from sleep, received primed continuous IV infusions, and ingested 20 g of intrinsically labeled whey protein. The drink can represents labeled protein ingestion. Asterisks indicate blood samples, and double upward arrows indicate bilateral biopsies were collected at corresponding time points representing the exercise (EX) and non-exercise (REST) legs.

Figure 2 Mean (±SEM) plasma insulin (**A**), phenylalanine (**B**) and leucine (**C**) concentrations during a stable isotope experimental test day in the morning after overnight recovery from a unilateral bout of resistance-type exercise that was performed the evening before with (PRO; *n*=8) or without (CON; *n*=8) dietary protein consumed immediately after exercise (20 g) and prior to subsequent sleep (60 g). The vertical line on each graph indicates the transition from fasting to fed conditions via the consumption of 20 g dietary protein in all subjects. Data were analysed with a two-way repeated measures ANOVA with Bonferonni post hoc tests applied to locate individual differences: A, B and C all showed significant time effects ($P<0.001$) and * indicates values significantly different compared to '0'. No treatment or interaction effects were detected.

Figure 3 Mean (±SEM) plasma L-[*ring*-$^{2}H_5$]phenylalanine (**A;** *intravenously infused tracer only*), L-[1-^{13}C]leucine (**B;** *intravenously infused and ingested within the intrinsically labelled dietary protein*) and L-[1-^{13}C]phenylalanine (**C;** *ingested within the intrinsically labelled*

673 *dietary protein only*) enrichments during a stable isotope experimental test day in the morning
674 after overnight recovery from a unilateral bout of resistance-type exercise that was performed
675 the evening before with (PRO; *n*=8) or without (CON; *n*=8) dietary protein consumed
676 immediately after exercise (20 g) and prior to subsequent sleep (60 g). The vertical line on
677 each graph indicates the transition from fasting to fed conditions via the consumption of 20 g
678 intrinsically labelled dietary protein in all subjects. Data were analysed with a two-way
679 repeated measures ANOVA with Bonferonni post hoc tests applied to locate individual
680 differences: A, B and C all showed significant time effects (P<0.001) and * indicates values
681 significantly different compared to '0'. A significant interaction was detected for A (P<0.05),
682 but no individual differences were observed. No treatment or interaction effects were detected
683 for B or C.
684
685 **Figure 4** Mean (±SEM) whole body phenylalanine kinetics (**A**: exogenous rate of appearance
686 [R_a] phenylalanine. **B**: Endogenous R_a phenylalanine. **C**: Total R_a phenylalanine. **D**: Total rate
687 of disappearance [R_d] phenylalanine) during a stable isotope experimental test day in the
688 morning after overnight recovery from a unilateral bout of resistance-type exercise that was
689 performed the evening before with (PRO; *n*=8) or without (CON; *n*=8) dietary protein
690 consumed immediately after exercise (20 g) and prior to subsequent sleep (60 g). The vertical
691 line on each graph indicates the transition from fasting to fed conditions via the consumption
692 of 20 g dietary protein in all subjects. Data were analysed with a two-way repeated measures
693 ANOVA with Bonferonni post hoc tests applied to locate individual differences: A, B, C and
694 D all showed significant time effects (P<0.001). D also showed a significant time × treatment
695 interaction (P<0.05) though no individual differences were detected.
696

Figure 5 Mean (±SEM) post-absorptive (fast) and post-prandial (fed; ingestion of 20 g dietary protein) fractional myofibrillar protein synthesis rates (FSR) calculated from L-[*ring*-$^{2}H_5$]phenylalanine during a stable isotope experimental test day in the morning after overnight recovery from a unilateral bout of resistance-type exercise that was performed the evening before with (PRO; *n*=8) or without (CON; *n*=8) dietary protein consumed immediately after exercise (20 g) and prior to subsequent sleep (60 g). Data were analysed with a three-way (treatment, protein ingestion and exercise conditions) repeated measures ANOVA with Bonferonni post hoc tests applied to locate individual differences: significant main effects of protein ingestion ($P<0.01$) and exercise ($P<0.001$) were detected with no effect of nutritional intervention or any interactions. † indicates value significantly different compared with corresponding fasting value. # indicates value significantly different compared with corresponding resting value.

Figure 6 Mean (±SEM) delta myofibrillar protein enrichment (MPE) of L-[1-^{13}C]phenylalanine 3 h after the ingestion of 20 g intrinsically L-[1-^{13}C]phenylalanine labelled protein the morning after overnight recovery from a unilateral bout of resistance-type exercise that was performed the evening before with (PRO; *n*=8) or without (CON; *n*=8) dietary protein consumed immediately after exercise (20 g) and prior to subsequent sleep (60 g). Data were analysed with a two-way repeated measures ANOVA with Bonferonni post hoc tests applied to locate individual differences: significant main effect of exercise ($P<0.001$) with no effect of nutritional intervention or any interaction. # indicates value significantly different compared with corresponding resting value.

Figure 7 Mean (±SEM) skeletal muscle mRNA expression of LAT1 (**A**), PAT1 (**B**), SNAT2 (**C**), CD98 (**D**), myostatin (**E**), MAFBx (**F**), MuRF1 (**G**) and FOXO1 (**H**) in the post-

absorptive (fast) and post-prandial (fed; 3 h following ingestion of 20 g dietary protein) state the morning after an evening bout of one-legged resistance-type exercise in the previously exercised (EX) and rested (REST) muscle of two groups of healthy young men who previously had (PRO; n=8) or had not (CON; n=8) consumed post exercise (20 g) and pre-sleep (60 g) dietary protein. Data were analysed with a three-way (treatment, protein ingestion and exercise conditions) repeated measures ANOVA with Bonferonni post hoc tests applied to locate individual differences: A: Significant effect of protein ingestion (P<0.001), exercise (P<0.05) and nutritional intervention (P<0.05). B: Significant effect of exercise (P<0.001) and exercise × protein ingestion interaction (P<0.01). C: Significant exercise × nutritional intervention interaction (P<0.01) and exercise × protein ingestion interaction (P<0.05). D: Significant effect of protein ingestion (P<0.05) and exercise (P<0.01). E: Significant effect of exercise (P<0.05) and exercise × nutritional intervention interaction (P<0.05). F: Significant effect of exercise (P<0.001) and exercise × protein ingestion × nutritional intervention interaction (P<0.05). G: Significant exercise × protein ingestion interaction (P<0.05). * denotes significantly different from corresponding CON value; † denotes significantly different compared with corresponding fasting value; # denotes significantly different compared with corresponding resting value.

747 **Table 1. Participants' characteristics**

748

Variable	CON group (*n*=8)	PRO group (*n*=8)	Significance
Age (y)	25±2	23±1	NS
Height (m)	1.81±0.02	1.82±0.02	NS
Body mass (kg)	76.8±3.9	72.6±3.5	NS
BMI (kg/m^2)	23.6±1.2	21.9±0.7	NS
Whole body lean mass (kg)	63.1±3.1	59.9±2.8	NS
Body fat (%)	14.0±1.6	13.4±0.8	NS
Single leg 1-RM leg extension [left] (kg)	66±6	63±6	NS
Single leg 1-RM leg extension [right] (kg)	68±5	62±4	NS
Single leg 1-RM leg press [left] (kg)	109±8	103±8	NS
Single leg 1-RM leg press [right] (kg)	113±7	104±8	NS
Habitual energy intake ($MJ \cdot d^{-1}$)	12.8±1.8	11.5±1.1	NS
Habitual protein intake ($g \cdot d^{-1}$)	135±24	115±13	NS
Habitual protein intake ($g \cdot kg^{-1} \cdot d^{-1}$)	1.73±0.26	1.52±0.15	NS

749 Values represent means±SEM. NS: non-significant. $g \cdot kg^{-1} \cdot d^{-1}$: grams per kilogram body mass

750 per day.

Figure 1

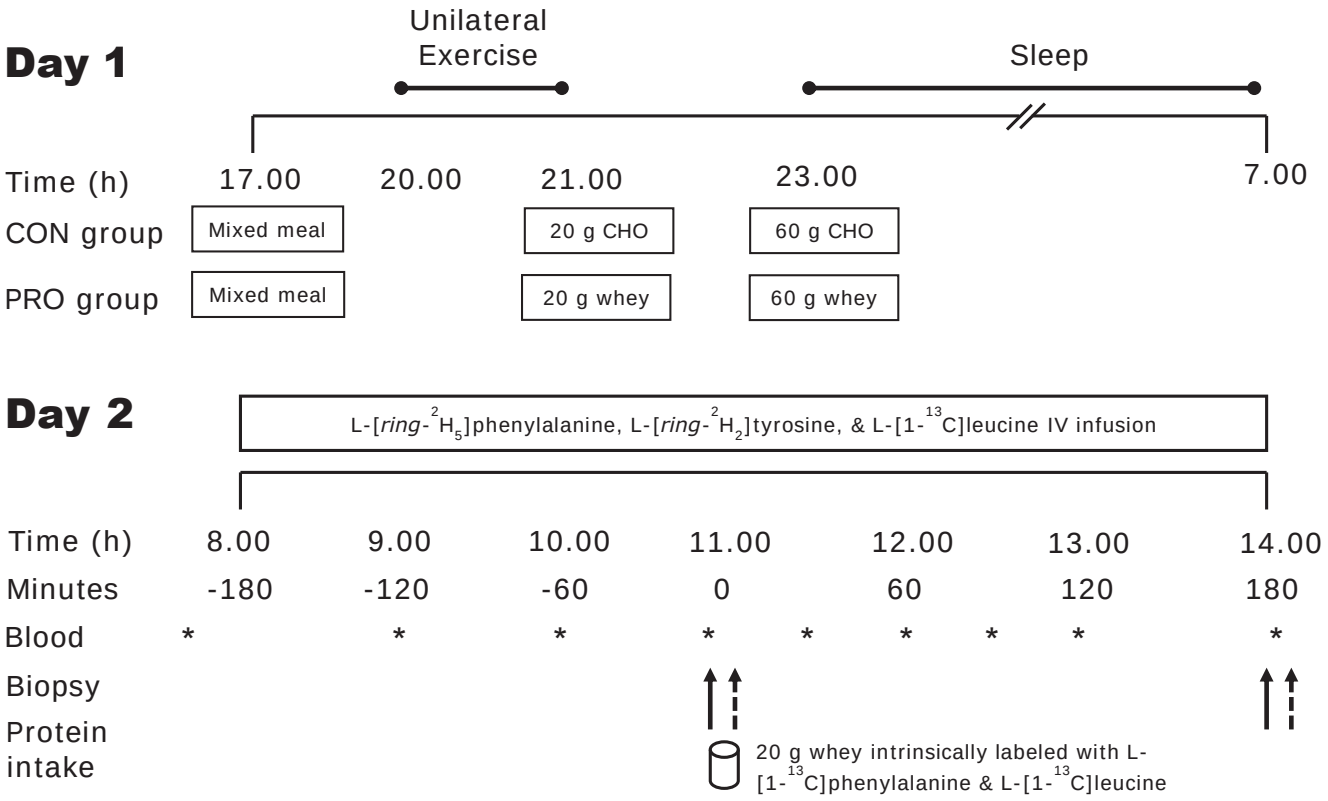

Day 1
Unilateral Exercise
Sleep
Time (h)
17.00
20.00
21.00
23.00
7.00
CON group
Mixed meal
20 g CHO
60 g CHO
PRO group
Mixed meal
20 g whey
60 g whey
Day 2
L-[ring-2H5]phenylalanine, L-[ring-2H2]tyrosine, & L-[1-13C]leucine IV infusion
Time (h)
8.00
9.00
10.00
11.00
12.00
13.00
14.00
Minutes
-180
-120
-60
0
60
120
180
Blood
Biopsy
Protein intake
20 g whey intrinsically labeled with L-[1-13C]phenylalanine & L-[1-13C]leucine

Figure 2

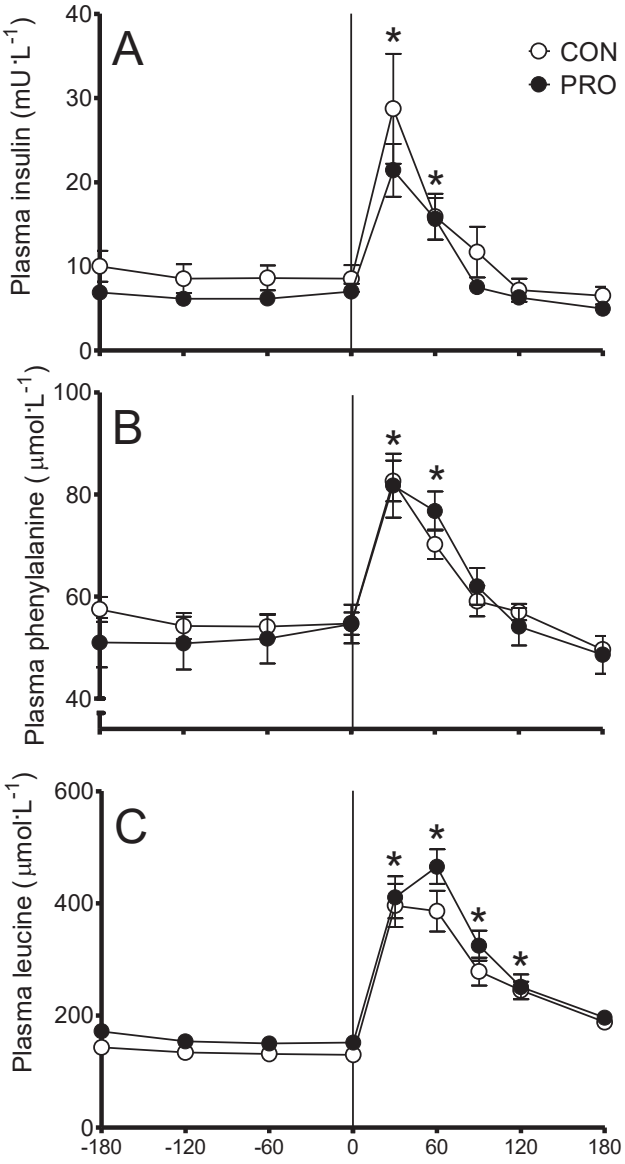

A
B
C
CON
PRO
Plasma insulin (mU·L-1)
Plasma phenylalanine (µmol·L-1)
Plasma leucine (µmol·L-1)
40
30
20
10
0
100
80
60
40
600
400
200
0
-180
-120
-60
0
60
120
180
*

Figure 3

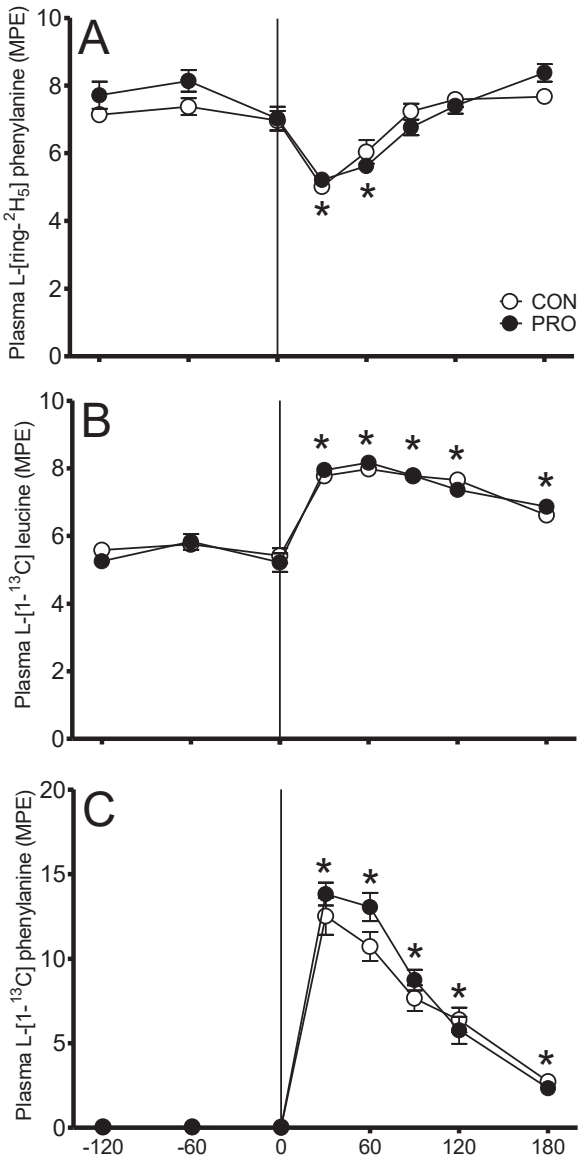

A
Plasma L-[ring-$^{2}H_5$] phenylanine (MPE)
10
8
6
4
2
0
CON
PRO
B
Plasma L-[1-^{13}C] leucine (MPE)
C
Plasma L-[1-^{13}C] phenylanine (MPE)
20
15
10
5
0
-120
-60
0
60
120
180

Figure 4

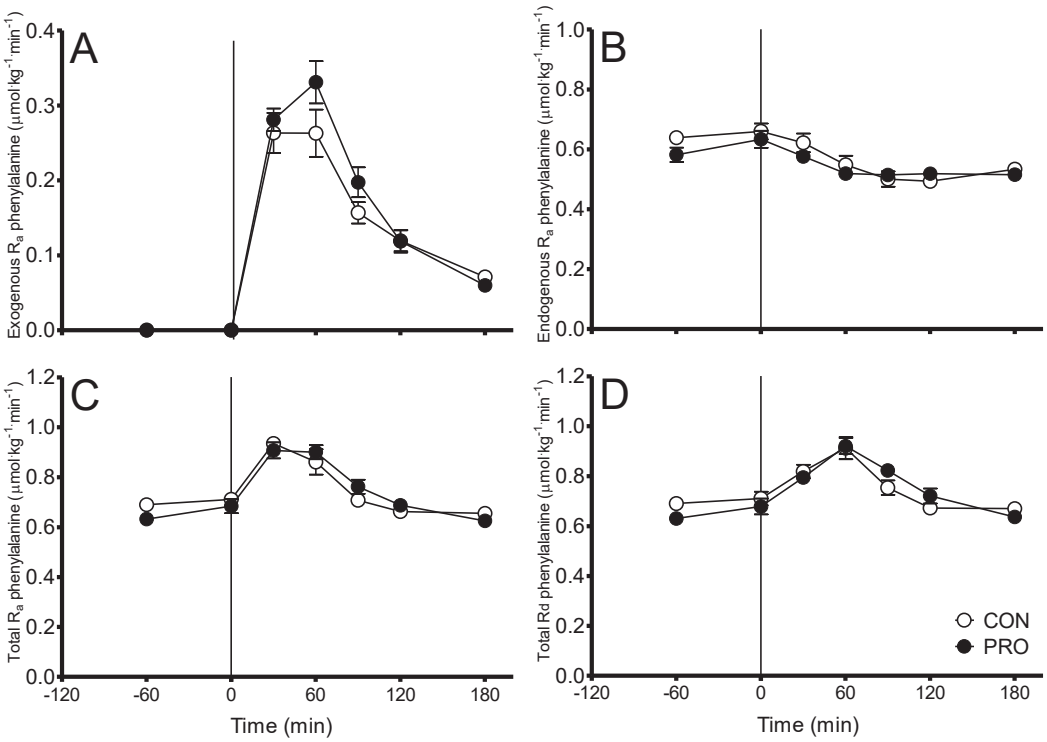

A
B
C
D
Exogenous R_a phenylalanine (μmol·kg^{-1}·min^{-1})
Endogenous R_a phenylalanine (μmol·kg^{-1}·min^{-1})
Total R_a phenylalanine (μmol·kg^{-1}·min^{-1})
Total Rd phenylalanine (μmol·kg^{-1}·min^{-1})
0.0
0.1
0.2
0.3
0.4
0.6
0.8
1.0
1.2
-120
-60
0
60
120
180
Time (min)
CON
PRO

Figure 5

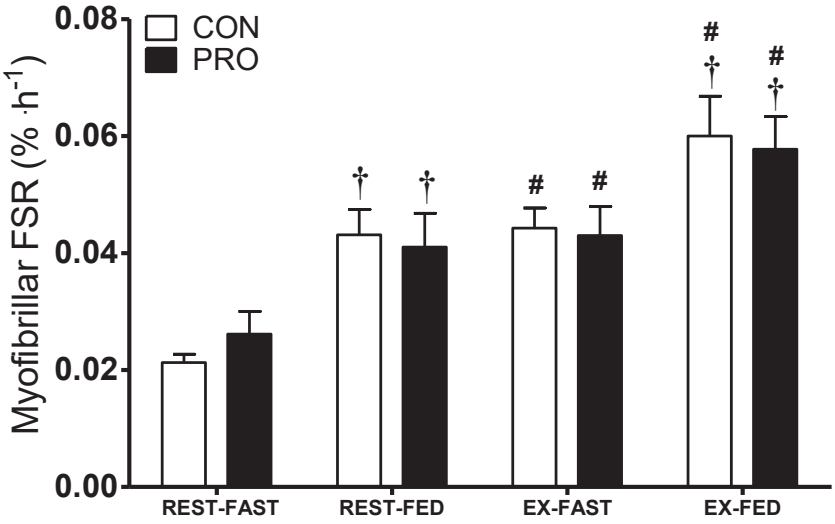

0.08
0.06
0.04
0.02
0.00
Myofibrillar FSR (% · h-1)
CON
PRO
†
†
#
#
†
†
#
#
REST-FAST
REST-FED
EX-FAST
EX-FED

Figure 6

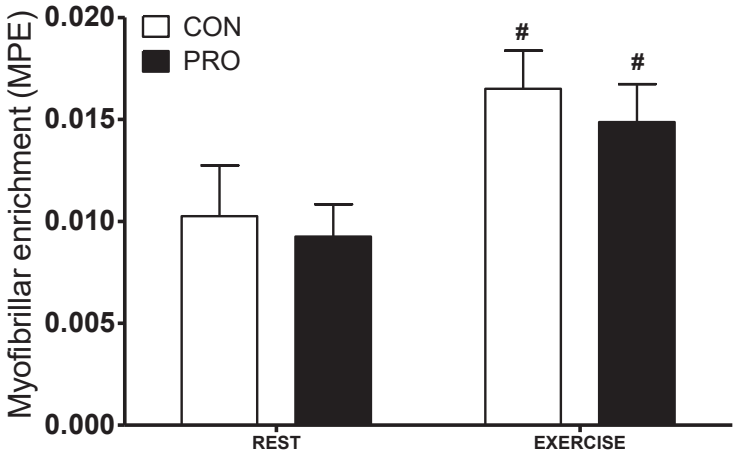
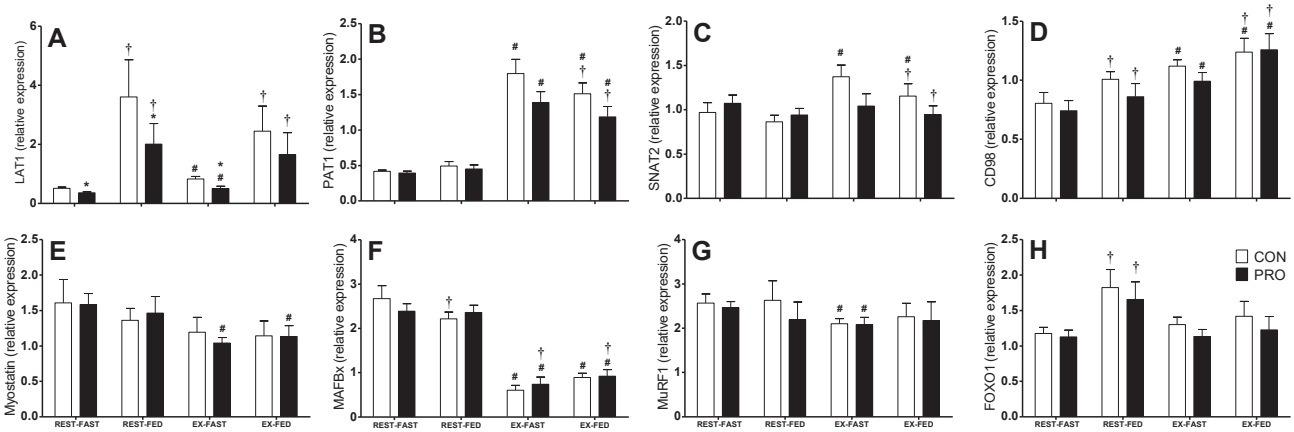

Myofibrillar enrichment (MPE)
0.020
0.015
0.010
0.005
0.000
CON
PRO
#
#
REST
EXERCISE

Figure 7


A
LAT1 (relative expression)
B
PAT1 (relative expression)
C
SNAT2 (relative expression)
D
CD98 (relative expression)
E
Myostatin (relative expression)
F
MAFBx (relative expression)
G
MuRF1 (relative expression)
H
FOXO1 (relative expression)
CON
PRO
REST-FAST
REST-FED
EX-FAST
EX-FED