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Jorn Trommelen[1,2], Andrew M. Holwerda[1,2], Imre W.K. Kouw[1,2], Henning Langer[1], Shona L. Halson[3], Ian Rollo[2,4], Lex B. Verdijk[1,2], and Luc J.C. van Loon[1,2]

[1]NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, the Netherlands; [2]Top Institute Food and Nutrition (TIFN), Wageningen, the Netherlands; [3]AIS Physiology, Australian Institute of Sport, Belconnen, Australia; [4]Gatorade Sports Science Institute, Leicester, United Kingdom

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Correspondence:

Luc J.C. van Loon, Ph.D.

Department of Human Movement Sciences

NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre

P.O. Box 616, 6200 MD Maastricht

The Netherlands

Tel: +31 43 3881397

Fax: +31 43 3670976

E-mail: L.vanLoon@maastrichtuniversity.nl

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ABSTRACT

Introduction: We have previously shown that protein ingestion prior to sleep increases overnight muscle protein synthesis rates. Whether prior exercise further augments the muscle protein synthetic response to pre-sleep protein ingestion remains to be established. **Objective:** To assess whether resistance-type exercise performed in the evening increases the overnight muscle protein synthetic response to pre-sleep protein ingestion. **Methods:** Twenty four healthy young men were randomly assigned to ingest 30 g intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled casein protein before going to sleep with (PRO+EX: *n*=12) or without (PRO: *n*=12) prior resistance-type exercise performed in the evening. Continuous intravenous L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-leucine and L-[*ring*-$^{2}H_2$]-tyrosine infusions were applied. Blood and muscle tissue samples were collected to assess whole-body protein balance, myofibrillar protein synthesis rates and overnight incorporation of dietary protein-derived amino acids into *de novo* myofibrillar protein. **Results:** A total of 57±1% of the ingested protein-derived phenylalanine appeared in the circulation during overnight sleep. Overnight myofibrillar protein synthesis rates were 37% (0.055±0.002 vs 0.040±0.003 %·h^{-1}, *P*<0.001; based upon L-[*ring*-$^{2}H_5$]-phenylalanine) and 31% (0.073±0.004 vs 0.055±0.006 %·h^{-1}, *P*=0.024; based upon L-[1-^{13}C]-leucine) higher in PRO+EX compared to PRO. Substantially more of the dietary protein-derived amino acids were incorporated into *de novo* myofibrillar protein during overnight sleep in PRO+EX compared to PRO (0.026±0.003 vs 0.015±0.003 MPE, *P*=0.012). **Conclusions:** Resistance-type exercise performed in the evening augments the overnight muscle protein synthetic response to pre-sleep protein ingestion and allows more of the ingested protein-derived amino acids to be used for *de novo* myofibrillar protein synthesis during overnight sleep.

Keywords: sleep, recovery, exercise, muscle, stable isotopes, casein

INTRODUCTION

A single session of resistance-type exercise stimulates both muscle protein synthesis and breakdown rates (4, 23, 28). Protein ingestion after exercise stimulates muscle protein synthesis and inhibits muscle protein breakdown, resulting in net muscle protein accretion during the acute stages of post-exercise recovery (5, 7, 15). Therefore, post-exercise protein ingestion is widely applied as a strategy to increase post-exercise muscle protein synthesis rates and, as such, to stimulate post-exercise recovery and facilitate skeletal muscle reconditioning. Various factors have been identified that can modulate the post-exercise muscle protein synthetic response including the amount (20, 31), type (27, 30) and timing (1, 18) of protein ingestion.

Previously, we have demonstrated that protein ingested prior to sleep is properly digested and absorbed, thereby increasing overnight amino acid availability, and stimulating muscle protein synthesis during overnight sleep (13, 24). Based upon these findings we suggested that protein ingestion prior to sleep may represent a practical and effective interventional strategy to support muscle mass maintenance and/or stimulate muscle hypertrophy. In this regard, pre-sleep protein supplementation has recently been applied in a prolonged resistance-type exercise training regimen, allowing greater gains in muscle mass and strength (26). In this study, subjects ingested either ~30 g of additional protein or a noncaloric placebo prior to sleep during a 3 month resistance-type exercise training program, with supplements ingested on both training days (3 times/wk) and non-training days. Though pre-sleep protein supplementation was effective in further increasing muscle mass and strength gains when compared to a placebo, the acute effects of resistance-type exercise on the muscle protein synthetic response to protein ingestion prior to sleep remains to be determined.

The aim of the present study was to assess whether resistance-type exercise performed in the evening increases the overnight muscle protein synthetic response to pre-sleep protein ingestion. We hypothesized that resistance-type exercise performed in the evening augments the muscle protein synthetic response to pre-sleep protein ingestion, allowing more of the ingested protein to be used for *de novo* myofibrillar protein accretion during overnight sleep. Therefore, we studied 24 recreationally active young males who ingested 30 g intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled casein protein before going to sleep with (PRO+EX) or without prior exercise (PRO) being performed in the evening. By combining the use of specifically produced intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled casein with primed continuous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-leucine and L-[*ring*-$^{2}H_2$]-tyrosine, we were able to assess protein digestion and amino acid absorption kinetics, whole-body protein metabolism, muscle protein synthesis rates as well as the metabolic fate of the dietary protein-derived amino acids towards *de novo* myofibrillar protein accretion during overnight sleep.

METHODS

Subjects

A total of 24 healthy, recreationally active (participating in exercise other than structured resistance-type exercise, for 1-3 d·wk^{-}1 for ≥ 12 months), young men were selected to participate in this study. Subjects' characteristics are presented in **Table 1**. Subjects were randomly assigned to ingest 30 g intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled casein protein before going to sleep with (PRO+EX, *n*=12) or without a bout of resistance-type exercise (PRO, *n*=12) being performed in the evening (19:45 – 20:45 PM). All subjects were fully informed of the nature and possible risks of the experimental procedures before their written informed

consent was obtained. This study is part of a greater project investigating the impact of pre-sleep protein feeding on overnight muscle protein synthesis. The project was registered at Netherlands Trial Registry as NTR3885, was approved by the Medical Ethical Committee of the Maastricht University Medical Centre, The Netherlands, and conformed to standards for the use of human subjects in research as outlined in the most recent version of the Helsinki Declaration.

Pretesting

Bodyweight and body composition (fat and fat-free mass) were determined by DEXA (Dual–energy X-ray absorptiometry, DEXA; Discovery A; Hologic, Bedford, USA). Leg volume was determined by anthropometry measurements as described by Jones and Pearson (14). The subjects were then familiarized with the resistance-type exercise protocol and the exercise equipment. All exercises during pretesting and experimental trials were supervised by trained personnel. Subjects started by performing a 10-min cycling warm-up at 150 W before completing an estimation of their 1RM on the leg press and leg extension exercises using the multiple repetitions testing procedure (19). For each exercise, subjects performed 10 submaximal repetitions to become familiarized with the equipment and to have lifting technique critiqued and properly adjusted. Sets were then performed at progressively increasing loads until failure to perform a valid estimation within 3-6 repetitions of the set. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance. A 2 min resting period between subsequent attempts was allowed. The pretesting and experimental trials were separated by at least 7 d.

Diet and physical activity

All subjects were instructed to refrain from exhaustive physical labor and exercise and to keep their diet as constant as possible 2 d before the experimental day. Food intake and physical activity questionnaires were collected for 2 d prior to the experiment. All subjects received a standardized diet throughout the experimental day (0.16 $MJ \cdot kg^{-1}$, providing 62 energy percentage (En%) carbohydrate, 13 En% protein, and 22 En% fat). The energy content of the standardized diet was based upon individual energy requirements based upon the Harris-Benedict equation and adjusted using a physical activity factor of 1.6 to ensure ample energy intake. During the experimental day, participants ingested 1.2±0.01 g protein·kg $bodyweight^{-1}$ via the standardized diet with an additional 20 g (0.27±0.01 $g \cdot kg^{-1}$) of protein provided at 20:45 h and an additional 30 g of protein (0.40±0.01 $g \cdot kg^{-1}$) provided prior to sleep (23:30 h).

Experimental Protocol

An outline of the study protocol is provided in **Figure 1**. At 17:30 h, participants reported to the lab and had Teflon catheters inserted into the antecubital veins of each arm. At 18:30 h (t= -300 min), all the subjects consumed a standardized dinner (Sligro, Maastricht, the Netherlands) under supervision (0.04 $MJ \cdot kg^{-1}$, providing 55 En% carbohydrate, 21 En% protein, and 20 En% fat), after which no more solid food was consumed. Subjects in the PRO+EX group subsequently performed a resistance-type exercise bout between 19:45 and 20:45 h. Subjects in the PRO group rested in a sitting position during this period. Immediately after the exercise or rest session, both the PRO and PRO+EX group received drinks providing 20 g protein and 45 g carbohydrate (Gatorade G-series 03 Recover protein recovery shake, the Gatorade Company, Chicago, USA), which were ingested within 2 min. The purpose of this recovery drink was to optimize muscle

protein synthesis rates in the hours prior to sleep (2). Following protein ingestion, a background blood sample was taken prior to the initiation of the tracer infusion protocol, which was started at 21:00 h (t= -150 min). The plasma and intracellular phenylalanine and leucine pools were primed with a single intravenous dose (priming dose) of L-[*ring*-$^{2}H_5$]-phenylalanine (2.0 μmol·kg^{-1}), L-[*ring*-$^{2}H_2$]-tyrosine (0.615 μmol·kg^{-1}), and L-[1-^{13}C]-leucine (4.0 μmol·kg^{-1}). Once primed, the continuous stable isotope infusion was initiated (infusion rate: 0.05 μmol·kg^{-1}·min^{-1} L-[*ring*-$^{2}H_5$]-phenylalanine, 0.015 μmol·kg^{-1}·min^{-1} L-[*ring*-$^{2}H_2$]-tyrosine, 0.1 μmol·kg^{-1}·min^{-1} L-[1-^{13}C]-leucine): Cambridge Isotopes Laboratories, Andover, MA). Participants rested in a supine position for 2.5 h until 23:30 h (t= 0 min), after which the first muscle biopsy was taken. Subsequently, subjects ingested a 450 mL beverage containing 30 g intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled casein with an added 1.5 mL of vanilla extract (Dr. Oetker, Amersfoort, the Netherlands) within 5 min. Subjects went to sleep at 00:00 h. During the night, blood samples (10 mL) were taken without waking up the subjects at t= 30, 60, 90, 150, 210, 330, and 450 min relative to the intake of the protein drink. A second muscle biopsy was obtained from the contralateral leg 7.5 h later at 07:00 h (t= 450 min).

Blood samples were collected in tubes containing EDTA and centrifuged at 1000 *g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C. Muscle biopsies were obtained from the middle region of the *M. vastus lateralis*, 15 cm above the patella and approximately 4 cm below entry through the fascia, using the percutaneous needle biopsy technique (3). Muscle samples were dissected carefully and freed from any visible non-muscle material. The muscle samples were immediately frozen in liquid nitrogen and stored at –80°C until further analysis.

Exercise protocol

The exercise protocol consisted of 60 min of lower-body resistance-type exercise. After 15 min of self-paced cycling at 150 W with a cadence of 60–80 rpm, subjects performed 6 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, Netherlands) and 6 sets of 10 repetitions on the leg extension machine (Technogym BV). The first two sets of both exercises were performed at 55% and 65% of the subjects' 1RM, respectively. Sets 3–6 were performed at 75% of 1RM and there were 2-min rest intervals between all sets. Immediately after the exercise session, both the PRO and PRO+EX group received drinks providing 20 g protein and 45 g carbohydrate (Gatorade G-series 03 Recover protein recovery shake, the Gatorade Company, Chicago, USA).

Production of intrinsically labeled protein

Intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled casein protein was obtained during the constant infusion of L-[1-^{13}C]-phenylalanine (455 μmol·min^{-1}) and L-[1-^{13}C]-leucine (200 μmol·min^{-1}) maintained for 96 h in a lactating dairy cow. The milk was collected, processed, and fractionated into the casein protein concentrate as previously described (8, 22, 29). The L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments in casein protein were measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; MAT 252, Finnigan, Breman, Germany) and averaged 38.7 molar percent excess (MPE) and 9.3 MPE, respectively. The proteins met all chemical and bacteriological specifications for human consumption.

Tracer preparation

The stable isotope tracers L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-leucine and L-[*ring*-$^{2}H_2$]-tyrosine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9% saline before infusion (Basic Pharma, Geleen, the Netherlands). Continuous intravenous infusions were performed using a calibrated IVAC 598 pump (San Diego, CA, USA).

Plasma analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (GLUC3, Roche, Ref: 05168791 190, and Immunologic, Roche, Ref: 12017547 122, respectively). Plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Wilmington, Delware, USA). Specifically, internal standards of [U-$^{13}C_6$]-leucine, [U-$^{13}C_9{}^{15}N$]-phenylalanine, and [U-$^{13}C_9{}^{15}N$]-tyrosine were added to the samples. The plasma was deproteinized on ice with dry 5-sulfosalicylic acid. Free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin; mesh size: 100-200 µm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, USA). The purified amino acids were converted into *tert*-butyl dimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA before analysis by GC-MS. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass/charge (*m/z*) 302 and 308 for unlabeled and [U-$^{13}C_6$] labeled-leucine, 336 and 346 for unlabeled and [U-$^{13}C_9{}^{15}N$] labeled-phenylalanine respectively, and 466 and 476 for unlabeled and [U-$^{13}C_9{}^{15}N$]-tyrosine, respectively. The plasma leucine, phenylalanine, and tyrosine ^{13}C and ^{2}H enrichments were determined using selective ion monitoring at m/z 302 and 303 for unlabeled and labeled (1-^{13}C) leucine, respectively; m/z 336, 337 and 341 for unlabeled and labeled (1-^{13}C

and *ring*-$^{2}H_5$) phenylalanine, respectively; m/z 466, 467, 468 and 470 for unlabeled and labeled (1-^{13}C, *ring*-3,5-$^{2}H_2$, and *ring*-$^{2}H_4$) tyrosine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometry and to account for any isotopic fractionation.

Muscle analysis

Myofibrillar protein enriched fractions were extracted from ~60 mg of wet muscle tissue by hand-homogenizing on ice using a pestle in a standard extraction buffer (7 μL·mg^{-1}) (16). The samples were spun at 2500 *g* and 4 ºC for 5 min. The pellet was washed with 500 μL ddH2O and centrifuged at 250 *g* and 4 ºC for 10 min. The myofibrillar protein was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50 ºC for 30 min with vortex mixing every 10 min. Samples were centrifuged at 9500 *g* and 4 ºC for 5 min, the supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and spinning at 700 *g* and 4 ºC for 10 min. The myofibrillar protein was washed twice with 70% ethanol and hydrolyzed overnight in 2 mL of 6 M HCL at 110˚C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under nitrogen stream while being heated to 120 ºC. The free amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA), and eluted with 2 M NH_4OH. The purified amino acids were divided into 2 aliquots to determine the L-[*ring*-$^{2}H_5$]-phenylalanine enrichments by GC-MS analysis and the L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments by gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS) analysis. To reduce the signal-to-noise ratio during GC-MS analysis at

low tracer enrichments, the phenylalanine from the myofibrillar protein hydrolysates was enzymatically decarboxylated to β-phenylethylamine prior to derivatization with MTBSTFA. To determine myofibrillar protein L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments by GC-IRMS analysis, the purified amino acids were converted into N-ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate (ECF). The derivatives were then measured by GC-IRMS (Finnigan MAT 253, Bremen, Germany) using a DB5-MS-column (no. 122-5532; Agilent J+W scientific GC Column), GC Isolink, and monitoring of ion masses 44, 45 and 46. Standard regression curves were applied to assess the linearity of the mass spectrometer and to account for isotopic fractionation.

Calculations

Ingestion of L-[1-^{13}C]-phenylalanine labeled protein, intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine, and L-[ring-3,5-$^{2}H_2$]-tyrosine, and blood sample enrichment values were used to assess whole-body amino acid kinetics in non-steady state conditions. Total, exogenous, and endogenous phenylalanine rate of appearance (R_a) and plasma availability of dietary protein-derived phenylalanine that appeared in the systemic circulation as a fraction of total amount of phenylalanine that was ingested (Phe$_{plasma}$) were calculated using modified Steele's equations (6, 10).

These parameters were calculated as follows:

$$\text{Total}Ra = \frac{F - \left[pV \cdot C(t) \cdot \frac{dE_{iv}}{dt}\right]}{E_{iv}(t)} \quad (1)$$

$$\text{Exo}R_a = \frac{\text{Total } R_a \cdot E_{po}(t) + \left[pV \cdot C(t) \cdot \frac{dE_{po}}{dt}\right]}{E_{prot}} \quad (2)$$

$$\text{Endo}R_a = \text{Total}R_a - \text{Exo}R_a - \text{F} \quad (3)$$

$$\text{Phe}_{\text{plasma}} = \left(\frac{\text{AUC}_{\text{ExoRa}}}{\text{Phe}_{\text{prot}}}\right) \cdot 100 \quad (4)$$

where F is the intravenous tracer infusion rate (μmol·kg^{-1}·min^{-1}), and pV (0.125 L·kg^{-1}) is the distribution volume for phenylalanine (6). $C(t)$ is the mean plasma phenylalanine concentration between 2 consecutive time points. dE_{iv}/dt represents the time-dependent variations of plasma phenylalanine enrichment derived from the intravenous tracer and $E_{iv}\ (t)$ is the mean plasma phenylalanine enrichment from the intravenous tracer between 2 consecutive time points. $ExoR_a$ represents the plasma entry rate of dietary phenylalanine, $E_{po}\ (t)$ is the mean plasma phenylalanine enrichment for the ingested tracer, dE_{po}/dt represents the time-dependent variations of plasma phenylalanine enrichment derived from the oral tracer and E_{prot} is the L-[1-^{13}C]-phenylalanine enrichment in the dietary protein. Phe_{plasma} is the percentage of ingested dietary phenylalanine that becomes available in the plasma and is calculated using Phe_{Prot} and AUC_{ExoRa}. Phe_{Prot} is the amount of dietary phenylalanine ingested and AUC_{ExoRa} represents the area under the curve (AUC) of ExoR_a, which corresponds to the amount of dietary phenylalanine that appeared in the blood over a 7.5 h period following ingestion.

Total rate of disappearance of phenylalanine equals the rate of phenylalanine hydroxylation (first step in phenylalanine oxidation) and utilization for protein synthesis. This parameter is calculated as follows:

$$\text{Total}R_d = \text{Total}R_a - pV \cdot \frac{dC}{dt} \quad (5)$$

Because whole-body R_d comprises the rate of phenylalanine disappearance from the free amino acid pool in the blood due to protein synthesis and oxidation, whole-body protein synthesis can be calculated as R_d minus oxidation. Whole-body phenylalanine oxidation can be determined from the conversion (hydroxylation) of L-[*ring*-$^2H_5$]-phenylalanine to L-[*ring*-$^2H_4$]-tyrosine. The rate of hydroxylation was calculated by using the following formula:

$$\mathrm{Phe_{hydroxylation}} = \mathrm{Tyr}R_a \cdot \frac{\mathrm{E_t(t)}}{\mathrm{E_p(t)}} \cdot \frac{\mathrm{PheR_d}}{(\mathrm{F} + \mathrm{PheR_d})} \qquad (6)$$

$TyrR_a$ represents the rate of appearance of L-[*ring*-$^2H_4$]-tyrosine; E_t(t) and E_p(t) are the L-[*ring*-$^2H_4$]-tyrosine and L-[*ring*-$^2H_5$]-phenylalanine enrichments in plasma between 2 consecutive time points, respectively and F is the infusion rate of phenylalanine. Whole-body protein synthesis was calculated using:

$$Protein\ Synthesis = R_d - Phe_{hydroxylation} \qquad (7)$$

Whole-body protein net balance was calculated as AUC over the 7.5 h post-prandial phase using whole-body protein synthesis minus endogenous R_a.

$$Phe_{net\ balance} = Protein\ synthesis - Endo_{Ra} \qquad (8)$$

The fractional synthesis rate (FSR) of myofibrillar protein was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[1-^{13}C]-leucine or L-[*ring*-$^2H_5$]-phenylalanine, by the enrichment of the respective precursor amino acid enrichments (i.e.,

plasma free amino acids). Weighted mean plasma L-[*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine enrichments were used as the preferred precursor pools to estimate myofibrillar protein fractional synthesis rates from the continuously infused L-[*ring*-$^{2}H_5$]-phenylalanine, and L-[1-^{13}C]-leucine tracers. Consequently, myofibrillar *FSR* was calculated as follows (17):

$$FSR = \frac{E_B(t) - E_B(0)}{\int_{t_0}^{t_1} E_F(t) \cdot \Delta t} \cdot 100 \quad (9)$$

where E_B represents muscle protein bound L-[*ring*-$^{2}H_5$]-phenylalanine or L-[1-^{13}C]-leucine. E_F represent the average plasma L-[*ring*-$^{2}H_5$]-phenylalanine or L-[1-^{13}C]-leucine enrichment during the tracer incorporation period. *t* Indicates the time interval (h) between biopsies.

Sleep quality assessment

The Pittsburg Sleep Quality Index (PSQI, Sleep Medicine Institute, University of Pittsburgh) was used to assess habitual sleep quality during pretesting (9). PSQI scoring (global scores 0-21 points; higher scores indicate worse sleep quality) was used to classify all subjects to very good, good, poor or very poor sleepers. Subjects that scored >5 (poor sleepers) were not included in the trial. Sleep behavior during the test night was monitored using wrist activity monitors (Philips Respironics, Bend, USA). Additionally, the start and end time of sleep were recorded throughout the trial. The following parameters were derived from sleep records and activity monitors: sleep time (clock time), wake time (clock time), sleep onset latency (the period of time between bed time and sleep start), sleep duration (h), time awake/light sleep (h), sleep efficiency (%, sleep duration expressed as a percentage of time in bed), and wake bouts.

Statistics

All data are expressed as means±SEM. A sample size of 12 subjects per group including a 10% dropout rate was calculated with a power of 80% and an α-level of .05 to detect a 20% difference in FSR between groups. Baseline characteristics between groups were compared using a Student's *t*-test. Time-dependent variables (i.e. plasma metabolite concentrations, plasma enrichments and whole-body protein kinetics) were analyzed by two-factor repeated measures ANOVA with time as within-subjects factor and treatment group as between-subjects factor. The analysis was carried out for the period starting at the time of protein administration, between t= 0 and 450 min. Non time-dependent variables (i.e. FSR values) were compared between treatment groups using Student's *t*-tests. Statistical significance was set at $P<0.05$. All calculations were performed using SPSS 21.0 (SPSS inc., Chicago, Illinois, USA).

RESULTS

Plasma analysis

Plasma glucose and insulin concentrations are shown in (see Figure, **SDC 1**, plasma glucose and insulin concentrations, http://links.lww.com/MSS/A759). Both plasma glucose and plasma insulin concentrations were slightly lower in the PRO-EX vs PRO group throughout the overnight period (treatment effect: $P<0.05$). However, changes in plasma glucose and plasma insulin over time were not different between groups (time x treatment interaction: ($P=0.476$). Plasma insulin concentrations peaked at 30 min following protein ingestion at 9.1±2.2 and 14.3±1.2 $mU \cdot L^{-1}$ in the PRO and PRO+EX treatment, respectively.

Plasma phenylalanine (**A**), leucine (**B**), and tyrosine (**C**) concentrations are displayed in **Figure 2**. Plasma amino acid concentrations increased over time, with no significant differences in plasma phenylalanine and leucine concentrations between treatments (time x treatment interaction: $P>0.05$). In contrast, a significant time x treatment interaction was found for plasma tyrosine ($P=0.005$).

Figure 3 shows the plasma L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-leucine, and L-[1-^{13}C]-phenylalanine enrichments (MPE). Plasma tracer enrichments did not differ between treatments before ingesting the protein (t= 0 min, $P>0.05$). Following protein ingestion, plasma L-[*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine enrichments increased slightly over time, but this increase over time did not differ between treatments (time x treatment interaction, $P>0.05$). Plasma L-[1-^{13}C]-phenylalanine enrichments, originating from the ingested protein, increased in both treatments, reaching maximal values of 9.39±0.42 MPE at t= 90 min in PRO and 9.89±0.44 MPE at t= 150 min in the PRO+EX treatment, and remained elevated above basal levels for the duration of the night with no differences over time between treatments (time x treatment interaction: $P=0.365$).

Whole-body amino acid kinetics

Plasma amino acid kinetics data are presented in (see Figure, **SDC 2**, plasma amino acid kinetics, http://links.lww.com/MSS/A760). Exogenous phenylalanine appearance rates increased following protein ingestion with no differences between treatments (time x treatment interaction: $P=0.334$). Endogenous phenylalanine appearance rates declined following protein ingestion, with no differences between treatments (time x treatment interaction: $P=0.179$). Over the entire 7.5 h post-prandial period, 59.0±1.8 and 55.8±1.7% (PRO and PRO+EX, respectively) of the ingested

dietary protein-bound phenylalanine appeared in the plasma circulation, with no differences between treatments (*P*=0.205).

Whole-body protein metabolism data are expressed in **Figure 4**. Protein ingestion prior to sleep resulted in positive overnight whole-body protein net balance with no differences observed between treatments (PRO: 51.1±1.5 vs PRO+EX: 53.0±1.9 µmol phenylalanine·kg^{-1}·7.5 h^{-1}, *P*=0.436). Furthermore, resistance-type exercise did not appear to further influence any other parameters of whole-body protein metabolism.

Myofibrillar protein fractional synthesis rates and protein-bound enrichments

Myofibrillar protein fractional synthetic rates (FSR) based upon L-[*ring*-$^{2}H_5$]-phenylalanine infusion with plasma L-[*ring*-$^{2}H_5$] enrichments as precursor (**A**: FSR in %·h^{-1}) or using L-[1-^{13}C]-leucine ingestion and infusion with plasma L-[1-^{13}C]-leucine enrichments as precursor (**B**, FSR in %·h^{-1}) are displayed in **Figure 5**. Exercise prior to protein ingestion before sleep resulted in greater stimulation of overnight myofibrillar FSR calculated based upon L-[*ring*-$^{2}H_5$]-phenylalanine (0.055±0.002 vs 0.040±0.003 %·h^{-1}, *P*<0.001) as well as L-[1-^{13}C]-leucine (0.073±0.004 vs 0.055±0.006 %·h^{-1}, *P*=0.024). Overnight FSR were 37% or 31% higher in the PRO+EX compared with the PRO treatment, based upon L-[*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine infusion, respectively.

Myofibrillar L-[1-^{13}C]-phenylalanine enrichments (MPE) increased following the ingestion of intrinsically L-[1-^{13}C]-phenylalanine labeled protein, reaching values of 0.026±0.003 vs 0.015±0.003 MPE in the PRO+EX and PRO treatment, respectively (*P*=0.012; **Figure 5C**).

Sleep Data

Sleep analysis data are presented in (see Table, **SDC 3**, sleep analysis datahttp://links.lww.com/MSS/A761). Total sleep duration did not differ between the treatments (PRO: 6:05±0:10 vs PRO+EX: 6:00±0:11 (h:mm), P=0.794). In addition, there were no significant differences between the treatments in sleep time, wake time, sleep onset latency (the period of time between bedtime and sleep start), or sleep efficiency (% sleep duration expressed as a percentage of time in bed).

DISCUSSION

The present study demonstrates that protein ingested prior to sleep is properly digested and absorbed, with ~55% of the ingested protein-derived amino acids appearing in the systemic circulation throughout overnight sleep. Resistance-type exercise performed earlier in the evening increases myofibrillar protein synthesis rates during overnight sleep, and improves the efficiency by which the pre-sleep dietary protein-derived amino acids are directed towards overnight *de novo* myofibrillar protein synthesis.

In the present study we combined the ingestion of specifically produced intrinsically L-[1-^{13}C]-phenylalanine labeled protein with continuous intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine to allow us to assess dietary protein digestion and amino acid absorption kinetics during overnight sleep. Ingestion of 30 g casein protein was followed by proper protein digestion and subsequent amino acid absorption, as indicated by the post-prandial increase in plasma phenylalanine concentrations (Figure 2A) and L-[1-^{13}C]-phenylalanine enrichments (Figure 3C). Exogenous dietary protein-derived phenylalanine appearance rates remained elevated throughout overnight sleep (see Figure, **SDC 2**, plasma amino acid kinetics,

http://links.lww.com/MSS/A760), with 57±1% of the ingested protein-derived amino acids being released in the circulation over the entire 7.5 h overnight period. These data are in line with previous observations showing ~50% of ingested protein becoming available in the systemic circulation during a 5-7 h post-prandial period (11, 12, 24). We extend upon our previous work, with the observation that resistance-type exercise performed earlier in the evening does not impair the digestion and absorption of protein ingested prior to sleep.

We have previously shown that ingestion of 40 g protein prior to sleep increases whole-body protein synthesis, resulting in a positive protein net balance during overnight sleep (24). In the current study, we observed that a more moderate 30 g protein dose likewise improves whole-body protein synthesis rates and allows for a positive whole-body protein net balance (Figure 4). Prior resistance-type exercise did not further modulate overnight whole-body protein synthesis, breakdown, amino acid oxidation or net balance following pre-sleep protein ingestion (Figure 4). As whole-body protein kinetics do not necessarily reflect skeletal muscle metabolism, we also collected muscle biopsies before and after overnight sleep to assess the impact of resistance-type exercise on the overnight muscle protein synthetic response to pre-sleep protein feeding.

As hypothesized, resistance-type exercise augmented overnight skeletal muscle reconditioning, with muscle protein synthesis rates being 37% (L-[*ring*-$^{2}H_{5}$]-phenylalanine, Figure 5A) and 31% (L-[1-^{13}C]-leucine, Figure 5B) higher in the exercise compared with the non-exercise control group. These data are in line with the greater anabolic response to protein ingestion following exercise observed in the morning after an overnight fast (21, 25, 31). The present study extends upon these findings with the observation that resistance-type exercise increases the overnight muscle protein synthetic response to pre-sleep protein ingestion. In addition to the applied continuous infusions of L-[*ring*-$^{2}H_{5}$]-phenylalanine and L-[1-^{13}C]-leucine to measure

myofibrillar protein synthesis rates, the ingestion of highly enriched (>35%) intrinsically L-[1-^{13}C]-phenylalanine labeled protein allowed us to also directly assess the metabolic fate of the pre-sleep dietary protein-derived amino acids. We were able to measure L-[1-^{13}C]-phenylalanine enrichments in myofibrillar protein following pre-sleep protein ingestion (Figure 5C), demonstrating that the pre-sleep protein-provided amino acids as precursors for *de novo* myofibrillar protein synthesis during overnight sleep. Substantially more (76%) more of the dietary protein-derived phenylalanine was incorporated in myofibrillar protein when resistance-type exercise was performed earlier in the evening. These data indicate that prior resistance-type exercise increases the efficiency by which pre-sleep protein-derived amino acids are directed towards *de novo* muscle protein synthesis during overnight sleep.

We have previously shown that protein ingestion before sleep increases muscle mass and strength gains during a 12-wk resistance-type exercise training program (26). Our current data show that resistance-type exercise performed earlier in the evening enhances the muscle protein synthetic response to pre-sleep protein ingestion. Therefore, protein ingestion prior to sleep represents an effective strategy to augment overnight skeletal muscle reconditioning and is even more relevant on exercise training days.

In conclusion, resistance-type exercise performed in the evening augments the overnight muscle protein synthetic response to pre-sleep protein ingestion and allows more of the ingested protein-derived amino acids to be used for *de novo* myofibrillar protein synthesis during overnight sleep. Combing pre-sleep protein ingestion with resistance-type exercise represents an effective strategy to maximize overnight skeletal muscle reconditioning.

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FIGURE LEGENDS

Figure 1. Experimental protocol. A drink with 20 g milk protein isolate and 45 g of carbohydrate was ingested at 20:45 h and a drink with 30 g intrinsically labeled casein protein was ingested at 23:30 h. Subjects were randomly assigned to perform a single bout of resistance-type exercise or to remain rested between 19:45 h and 20:45 h.

Figure 2. Overnight plasma phenylalanine (A), leucine (B) and tyrosine (C) concentrations ($\mu mol \cdot L^{-1}$). The dotted line represents the ingestion of the treatment. Values represent means±SEM. Data were analyzed with a two-way repeated-measures (time x treatment) ANOVA. Phenylalanine: time effect: $P<0.001$; treatment effect: $P=0.212$; time x treatment interaction: $P=0.275$. Leucine: time effect: $P<0.001$; treatment effect: $P=0.741$; time x treatment interaction: $P=0.524$. Tyrosine: time effect: $P<0.001$; treatment effect: $P=0.877$; time x treatment interaction: $P=0.005$. PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise.

Figure 3. Overnight plasma L-[*ring*-$^{2}H_5$]-phenylalanine (A), L-[1-^{13}C]-leucine (B), L-[1-^{13}C]-phenylalanine (C) enrichments in mole percent excess (MPE). The dotted line represents the ingestion of the treatment. Values represent means±SEM. Data were analyzed with repeated-measures (time x treatment) ANOVA. L-[*ring*-$^{2}H_5$]-phenylalanine enrichment: time effect: $P<0.001$; treatment effect: $P=0.530$; time x treatment interaction: $P=0.445$. L-[1-^{13}C]-phenylalanine enrichment: time effect: $P<0.001$; treatment effect: $P=0.446$; time x treatment interaction: $P=0.365$. L-[1-^{13}C]-leucine enrichment: time effect: $P<0.001$; treatment effect:

P=0.164; time x treatment interaction: *P*=0.236. PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise.

Figure 4. Calculated rates of overnight whole-body protein synthesis, breakdown, oxidation, and net protein balance (µmol phenylalanine·kg^{-1}·7.5 h^{-1}). Values represent means±SEM. Data were analyzed with a Student's *t*-test. No significant differences were detected (*P*>0.05). PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise.

Figure 5. Overnight myofibrillar protein fractional synthetic rates (FSR) as calculated using L-[*ring*-$^{2}H_5$]-phenylalanine (A), or L-[1-^{13}C]-leucine (B) as tracer, and overnight L-[1-^{13}C]-phenylalanine incorporation in myofibrillar protein (mole percent excess, MPE, C). Values represent means±SEM. Data were analyzed with a Student's *t*-test. *Significantly different from PRO (*P*<0.05). PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise.

Figure 1

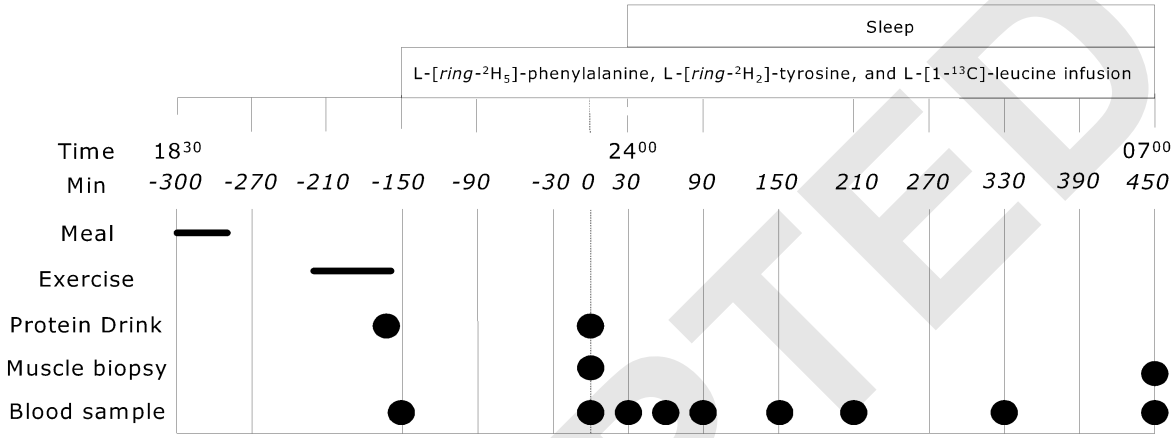


Figure 2

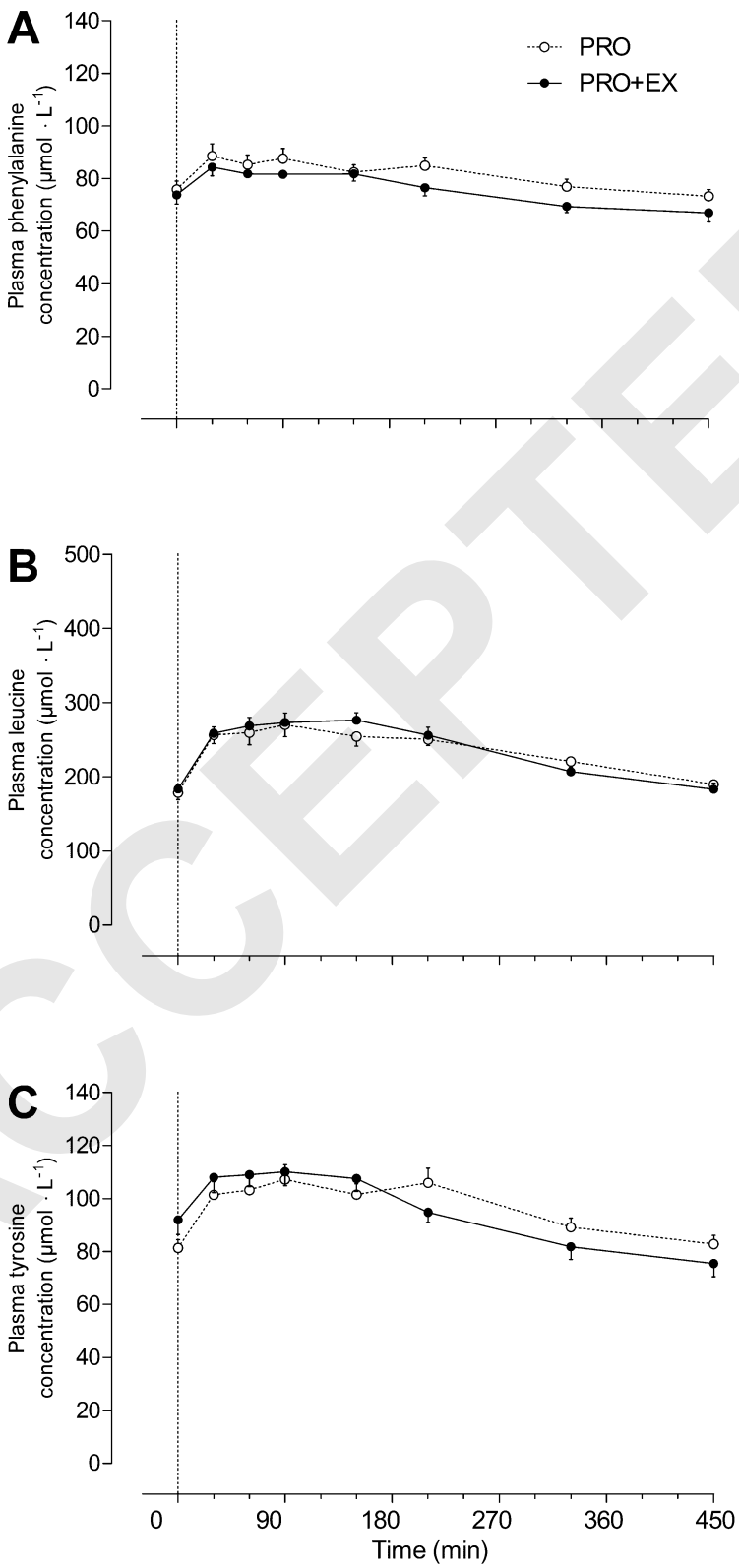


Figure 3

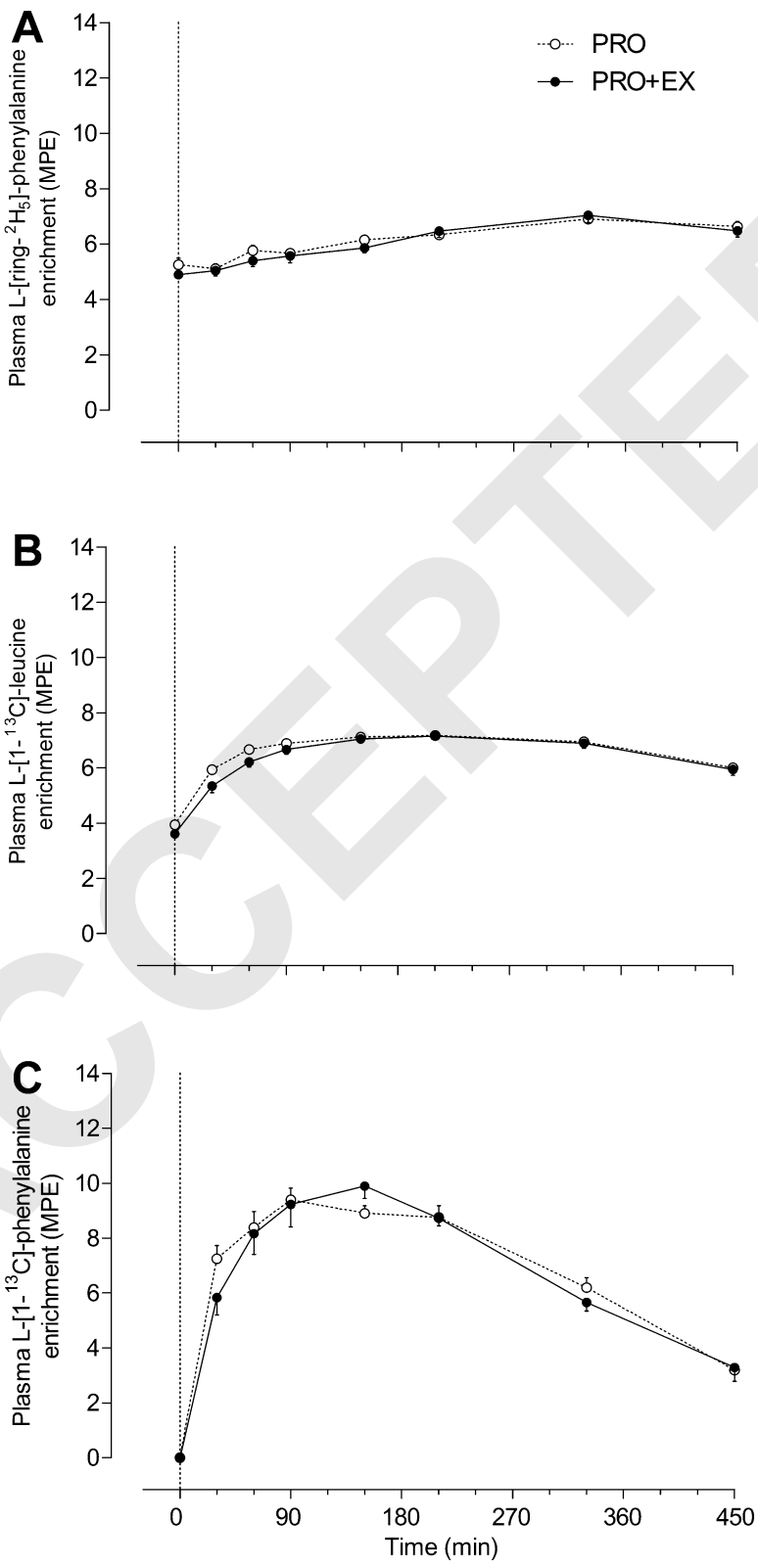


Figure 4

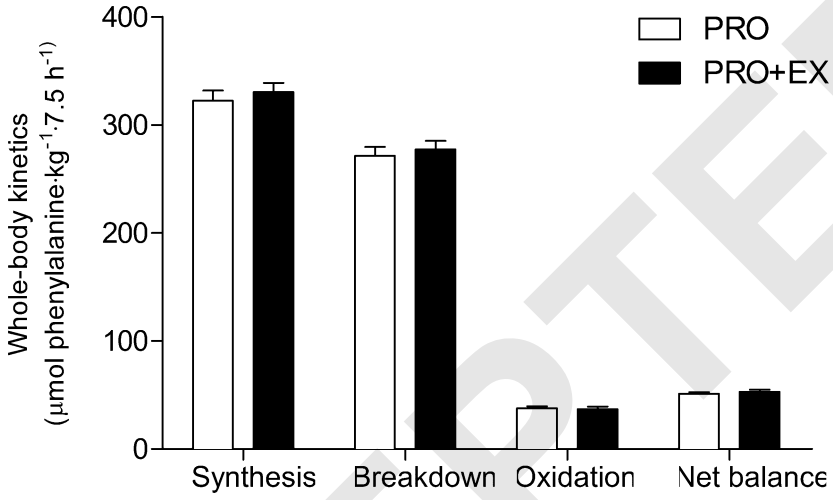
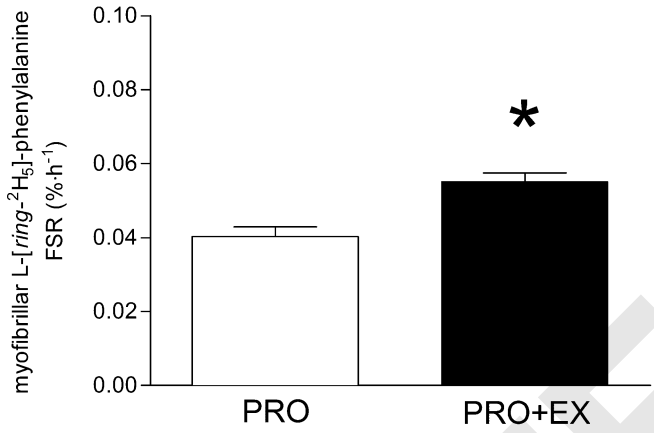
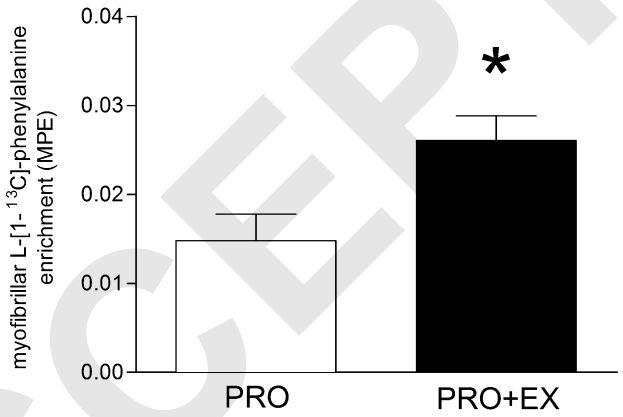


Figure 5





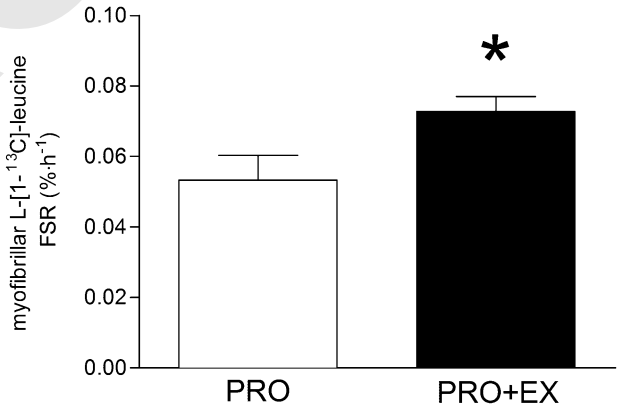


Table 1. Subjects' characteristics

	PRO (*n*=12)	**PRO+EX** (*n*=12)
Age (y)	24±1	23±1
Weight (kg)	77.6±1.2	73.0±1.7*
BMI ($kg \cdot m^{-2}$)	21.9±0.4	22.1±0.5
Fat (%)	13±1	14±1
LBM (kg)	61.9±1.2	59.5±1.6
Leg volume (L)	9.2±0.2	9.0±0.3

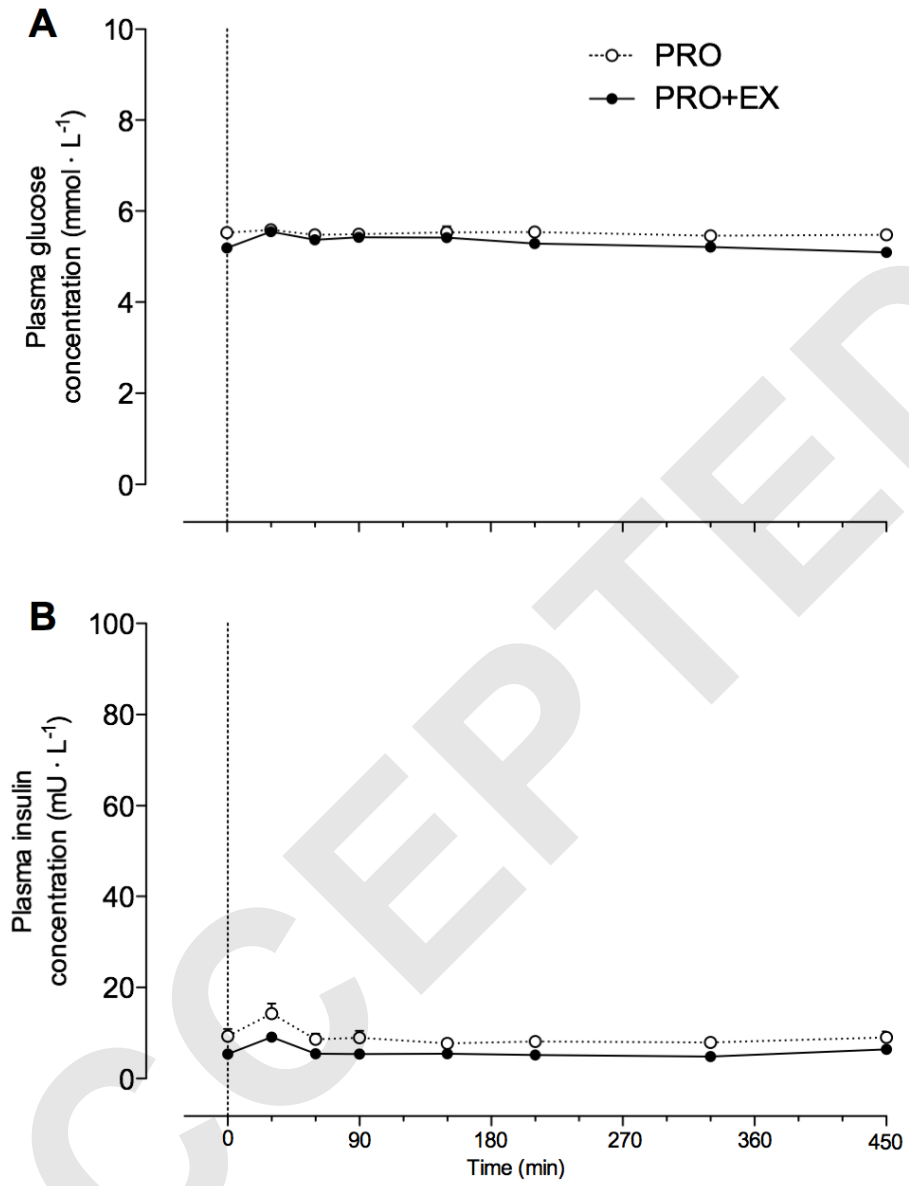
Values represent means±SEM. PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise. BMI: body mass index. * Significantly different from PRO (P=0.036). Data were analyzed with a Students' t-test.

Supplemental material

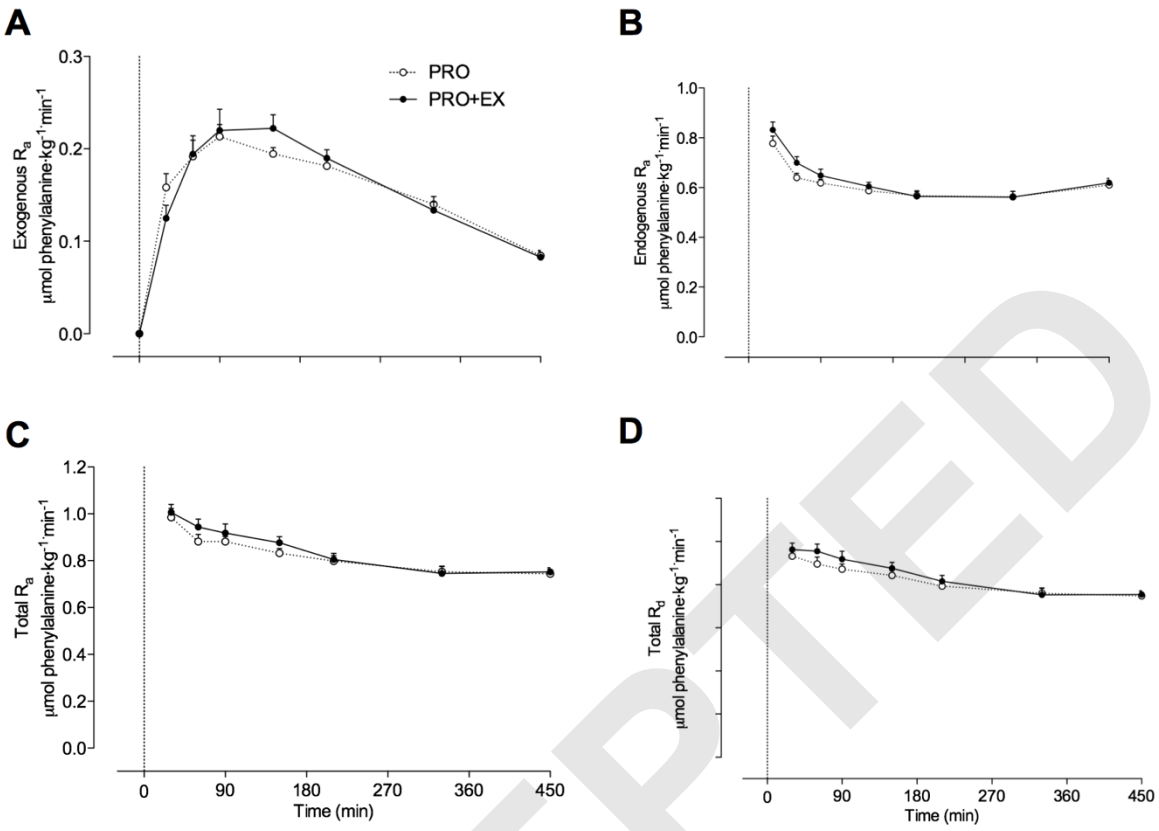
Supplemental Digital Content 1.docx—plasma glucose and insulin concentrations

Supplemental Digital Content 2.docx—plasma amino acid kinetics

Supplemental Digital Content 3.docx—sleep analysis data



Supplemental Figure 1. Overnight plasma glucose (A, mmol·L^{-1}) and insulin concentrations (B, mU·L^{-1}) following protein ingestion with (PRO+EX) and without (PRO) prior exercise. The dotted line represents the ingestion of the treatment. Values represent means±SEM. Data were analyzed with a two-way repeated-measures (time x treatment) ANOVA. Glucose: time effect: P=0.004; treatment effect: P=0.0077; time x treatment interaction: P=0.075. Insulin: time effect: P<0.001; treatment effect: P=0.014; time x treatment interaction: P=0.476.



Supplemental Figure 2. Overnight exogenous phenylalanine rate of appearance (R_a) (A), endogenous phenylalanine R_a (B), total phenylalanine R_a (C), and total phenylalanine rate of disappearance (R_d) (D). The dotted line represents the ingestion of the treatment. Values represent means±SEM. Data were analyzed with repeated-measures (time x treatment) ANOVA. Exogenous phenylalanine R_a: time effect: $P<0.001$; treatment effect: $P=0.957$; time x treatment interaction: $P=0.334$. Endogenous phenylalanine R_a: time effect: $P<0.001$; treatment effect: $P=0.368$; time x treatment interaction: $P=0.179$. Total phenylalanine R_a: time effect: $P<0.001$; treatment effect: $P=0.468$; time x treatment interaction: $P=0.366$. Total phenylalanine R_d: time effect: $P<0.001$; treatment effect: $P=0.421$; time x treatment interaction: $P=0.370$. PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise.

Table 1. Subjects' characteristics

	PRO (*n*=12)	**PRO+EX** (*n*=12)
Age (y)	24±1	23±1
Weight (kg)	77.6±1.2	73.0±1.7*
BMI ($kg \cdot m^{-2}$)	21.9±0.4	22.1±0.5
Fat (%)	13±1	14±1
LBM (kg)	61.9±1.2	59.5±1.6
Leg volume (L)	9.2±0.2	9.0±0.3

Values represent means±SEM. PRO: pre-sleep protein ingestion without prior exercise, PRO+EX: pre-sleep protein ingestion with prior exercise. BMI: body mass index. * Significantly different from PRO (P=0.036). Data were analyzed with a Students' t-test.