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RESEARCH ARTICLE

Presleep dietary protein-derived amino acids are incorporated in myofibrillar protein during postexercise overnight recovery

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[1]*NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands;* [2]*Top Institute Food and Nutrition, Wageningen, The Netherlands;* [3]*Department of Physiology, Australian Institute of Sport, Belconnen, ACT, Australia; and* [4]*Gatorade Sports Science Institute, Leicester, United Kingdom*

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Trommelen J, Kouw IW, Holwerda AM, Snijders T, Halson SL, Rollo I, Verdijk LB, van Loon LJ. Presleep dietary protein-derived amino acids are incorporated in myofibrillar protein during postexercise overnight recovery. *Am J Physiol Endocrinol Metab* 314: E457–E467, 2018. First published May 23, 2017; doi:10.1152/ajpendo.00273.2016.—The purpose of this study was to determine the impact of ingesting 30 g casein protein with and without 2 g free leucine before sleep on myofibrillar protein synthesis rates during postexercise overnight recovery. Thirty-six healthy young men performed a single bout of resistance-type exercise in the evening (1945) after a full day of dietary standardization. Thirty minutes before sleep (2330), subjects ingested 30 g intrinsically L-[1-^{13}C]phenylalanine-labeled protein with (PRO+leu, $n = 12$) or without (PRO, $n = 12$) 2 g free leucine, or a noncaloric placebo (PLA, $n = 12$). 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 net balance, myofibrillar protein synthesis rates, and overnight incorporation of dietary protein-derived amino acids into myofibrillar protein. Protein ingestion before sleep improved overnight whole body protein net balance ($P < 0.001$). Myofibrillar protein synthesis rates did not differ significantly between treatments as assessed by L-[*ring*-$^{2}H_5$]phenylalanine (0.057 ± 0.002, 0.055 ± 0.002, and 0.055 ± 0.004%/h for PLA, PRO, and PRO+leu, respectively; means ± SE; $P = 0.850$) or L-[1-^{13}C]leucine (0.080 ± 0.004, 0.073 ± 0.004, and 0.083 ± 0.006%/h, respectively; $P = 0.328$). Myofibrillar L-[1-^{13}C]phenylalanine enrichments increased following protein ingestion but did not differ between the PRO and PRO+leu treatments. In conclusion, protein ingestion before sleep improves whole body protein net balance and provides amino acids that are incorporated into myofibrillar protein during sleep. However, the ingestion of 30 g casein protein with or without additional free leucine before sleep does not increase muscle protein synthesis rates during postexercise overnight recovery.

sleep; recovery; exercise; stable isotopes; casein

A SINGLE SESSION of resistance-type exercise stimulates both muscle protein synthesis and breakdown rates (5, 34, 40). Protein ingestion after exercise stimulates muscle protein synthesis and inhibits muscle protein breakdown, resulting in net muscle protein accretion during the acute stages of postexercise recovery (6, 9, 25). Therefore, postexercise protein ingestion is widely applied as a strategy to increase postexercise muscle protein synthesis rates and, as such, to stimulate postexercise recovery and facilitate skeletal muscle reconditioning. Various factors have been identified that can modulate the postexercise muscle protein synthetic response including the amount (29, 46), type (39, 45), leucine content (13, 43), and timing (1, 26) of protein ingestion.

In general, most studies assess the effect of food intake on the muscle protein synthetic response to exercise performed in an overnight-fasted state. Such postabsorptive conditions differ from normal everyday practice, in which recreational sports activities are performed in the late afternoon or evening after a full day of habitual physical activity and food intake. We have previously shown that protein ingestion after a bout of resistance-type exercise performed in the evening increases whole body protein synthesis rates during the first few hours of recovery but does not increase mixed muscle protein synthesis rates throughout overnight sleep (3). In a follow-up, proof-of-principle study, we demonstrated that casein protein ingested immediately before sleep allows protein-derived amino acids to be released in the circulation, thereby stimulating mixed muscle protein synthesis during overnight sleep (36).

It has been well established that the ingestion of 20 g protein is sufficient to maximize muscle protein synthesis rates during the acute stage of postexercise recovery in young adults (29, 46). This is considerably less than the 40 g casein protein we provided before sleep to increase muscle protein synthesis rates throughout overnight sleep (36). Furthermore, we have recently reported that well-trained athletes consume only ~5–10 g of protein [7 ± 8 (SE) g protein] before sleep (19). We questioned whether a more moderate amount (<40 g) of protein can significantly increase muscle protein synthesis rates during overnight recovery. We hypothesized that presleep ingestion of 30 g dietary protein increases postexercise overnight muscle protein synthesis rates and provides amino acids that are incorporated into myofibrillar protein during overnight sleep. In addition, we hypothesized that leucine coingestion would further enhance the muscle protein synthetic response to the ingestion of 30 g protein before sleep.

In the present study, we tested our hypotheses by selecting 36 healthy young subjects who ingested 30 g casein protein with (PRO+leu) or without (PRO) an additional 2 g of crystalline leucine, or a placebo (PLA), before going to sleep. By combining contemporary stable isotope methodology with the ingestion of intrinsically L-[1-^{13}C]phenylalanine- and L-[1-

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Table 1. *Subjects' characteristics*

	PLA	PRO	PRO+leu
n	12	12	12
Age, yr	24 ± 1	23 ± 1	23 ± 1
Weight, kg	75.4 ± 2.3	73.0 ± 1.7	72.7 ± 2.2
BMI, kg/m^2	22.7 ± 0.6	22.1 ± 0.5	22.9 ± 0.4
Fat, %	15 ± 1	14 ± 1	14 ± 1
Lean body mass, kg	61.2 ± 1.8	59.5 ± 1.6	63.7 ± 1.4
Leg volume, l	9.1 ± 0.3	9.0 ± 0.3	9.3 ± 0.4

Values are means ± SE. PLA, placebo; PRO, 30 g dietary protein; PRO+leu, 30 g dietary protein plus 2 g crystalline leucine. BMI, body mass index. Data were analyzed with a one-way ANOVA. There were no differences between treatments.

^{13}C]leucine-labeled protein we were able to assess *1*) the postprandial release of dietary protein-derived amino acids into the circulation, *2*) their impact on overnight whole body protein kinetics, *3*) myofibrillar muscle protein synthesis rates, and *4*) the incorporation of dietary protein-derived amino acids into muscle protein during overnight sleep (10, 33, 42).

METHODS

Subjects. A total of 36 healthy, recreationally active young men [age, 23 ± 1 yr; body mass index (BMI), 22.6 ± 0.3 kg/m^2; participating in exercise other than structured resistance-type exercise training, for 1–3 days/wk for ≥12 mo] were selected to participate in this study. Subjects' characteristics are presented in Table 1. Subjects were randomly assigned to ingest a placebo (PLA), 30 g casein protein (PRO), or 30 g casein protein plus 2 g of crystalline leucine (PRO+leu) before going to sleep. The experiments were performed in a randomized, double-blind manner. 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 presleep protein feeding on overnight muscle protein synthesis. The project was registered at Netherlands Trial Register as NTR3885, was approved by the Medical Ethical Committee of Maastricht University Medical Centre, Maastricht, 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. During screening, body weight and body composition (fat and fat-free mass) were determined by dual-energy X-ray absorptiometry (DEXA; Discovery A; Hologic, Bedford, MA). Leg volume was determined by anthropometry measurements as described by Jones and Pearson (23). 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 one-repetition maximum (1 RM) on the leg press and leg extension exercises using the multiple-repetitions testing procedure (27). 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 days.

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 days before the experimental day. Food intake and physical activity questionnaires were collected for 2 days before the experiment. All subjects received a standardized diet throughout the experimental day [0.16 MJ/kg, providing 62 energy percentage (En%) carbohydrate, 13 En% protein, and 22 En% fat]. The energy content of the standardized diet was based on individual energy requirements based on the Harris-Benedict equation and adjusted using a physical activity factor of 1.6 to ensure ample energy intake. During the experimental day, all participants ingested 1.2 ± 0.01 g protein/kg body wt via the standardized diet with an additional 20 g (0.28 ± 0.01 g/kg) of protein provided immediately after cessation of exercise (2045). Participants receiving the PRO and PRO+leu treatments ingested an additional 30 g (0.42 ± 0.01 g/kg) of protein immediately before sleep (2330). Prior to intake of the test drink and in the morning after the intervention, hunger ratings were assessed using the visual analog scale (VAS; 35). In the morning, subjects were invited to an ad libitum breakfast, and food intake at breakfast was recorded for all subjects.

Experimental protocol. An outline of the study protocol is provided in Fig. 1. At 1730, participants reported to the laboratory and had Teflon catheters inserted into the antecubital veins of each arm. At

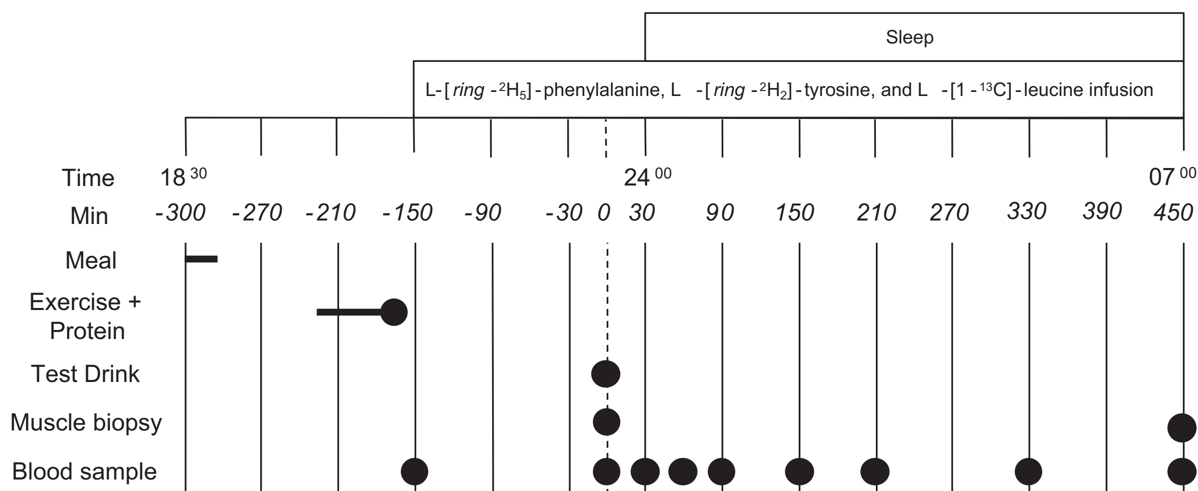

Fig. 1. Experimental protocol. A drink with 20 g milk protein isolate and 45 g carbohydrate was ingested at 2045 immediately after cessation of exercise. Subjects were randomly assigned to ingest either 30 g intrinsically labeled casein protein, 30 g intrinsically labeled casein protein with an additional 2 g of crystalline leucine, or placebo (water) at 2330.

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1830 ($t = -300$ min), all the subjects consumed a standardized dinner (Sligro, Maastricht, The Netherlands) under supervision (0.04 MJ/kg, providing 55 En% carbohydrate, 21 En% protein, and 20 En% fat), after which no more solid food was consumed. A single bout of resistance-type exercise was performed between 1945 and 2045. Drinks providing 20 g milk protein and 45 g carbohydrates were ingested immediately after exercise and were ingested within 2 min. The purpose of this recovery drink was to optimize muscle protein synthesis rates in the early stages of recovery (3, 29, 46). Following protein ingestion, a background blood sample was taken before the initiation of the tracer infusion protocol, which was started at 2100 ($t = -150$ min). The plasma and intracellular phenylalanine and leucine pools were primed with a single intravenous dose (priming dose) of L-[*ring*-$^2H_5$]phenylalanine (2.0 μmol/kg), L-[*ring*-$^2H_2$]tyrosine (0.615 μmol/kg), and L-[1-^{13}C]leucine (4.0 μmol/kg). Once primed, the continuous stable isotope infusion was initiated (infusion rate: 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, L-[*ring*-$^2H_5$] phenylalanine; 0.015 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, L-[*ring*-$^2H_2$]tyrosine; and 0.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, L-[1-$^{13}C$]leucine; Cambridge Isotope Laboratories, Andover, MA). Participants rested in a supine position for 2.5 h until 2330 ($t = 0$ min), after which the first muscle biopsy was taken. Subsequently, subjects ingested the beverage PLA, PRO, or PRO+leu within 5 min and went to sleep afterward at ~0000. 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 0700 ($t = 450$ min).

Blood samples were collected in tubes containing EDTA and centrifuged at 1,000 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 vastus lateralis muscle, 15 cm above the patella and ~4 cm below entry through the fascia, using the percutaneous needle biopsy technique (4). Muscle samples were dissected carefully and freed from any visible nonmuscle 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, Rotterdam, The Netherlands) and 6 sets of 10 repetitions on the leg extension machine (Technogym). The first two sets of both exercises were performed at 55 and 65% of the subjects' 1 RM, respectively. *Sets 3–6* were performed at 75% of 1 RM, and there were 2-min rest intervals between all sets. Immediately after the exercise session, all subjects received drinks providing 20 g protein and 45 g carbohydrate (Gatorade G-series 03 Recover protein recovery shake; Gatorade, Chicago, IL).

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) and L-[1-^{13}C]leucine (200 μmol/min) maintained for 96 h in a lactating dairy cow. The milk was collected, processed, and fractionated into the casein protein concentrate as previously described (11, 33, 42). 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-C-IRMS; MAT 252; Finnigan, Bremen, Germany) and averaged 38.7 and 9.3 molar percent excess (MPE), respectively. The proteins met all chemical and bacteriological specifications for human consumption. Each subject was randomized to participate in one experiment, in which water (PLA) with 30 g (PRO and PRO+leu) of intrinsically L-[1-^{13}C]phenylalanine- and L-[1-^{13}C]leucine-labeled casein protein, which added up to an equal volume of 450 ml, was ingested before sleep. In the PRO+leu treatments, an additional 2 g crystalline leucine was added. All test beverages were flavored by adding 1.5-ml vanilla flavor (Dr. Oetker, Amersfoort, The Netherlands).

Tracer preparation. The stable isotope tracers L-[*ring*-$^2H_5$]phenylalanine, L-[1-^{13}C]leucine, and L-[*ring*-$^2H_2$]tyrosine were purchased from Cambridge Isotope Laboratories 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).

Plasma and muscle analysis. Plasma glucose and insulin concentrations were analyzed using commercially available kits (GLUC3, cat. no. 05168791190, and Immunologic, cat. no. 12017547122; Roche). Plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry analysis (Agilent 7890A GC/5975C MSD; Wilmington, DE). Myofibrillar protein-bound L-[*ring*-$^2H_5$]phenylalanine enrichments were determined by GC-C-IRMS analysis (Trace GC Ultra, IRMS model MAT 253; Thermo Fisher Scientific). Complete details have been described previously (41).

Western blot analyses were performed as described previously (17). In short, ~30 mg of muscle tissue were homogenized, and protein quantification was performed. After protein quantification, the gels were transferred onto a nitrocellulose membrane. Total protein staining of the membrane (via Ponceau S staining) was used as the normalization control (whole lane) for all blots. Specific proteins were detected by overnight incubation with the following antibodies: anti-mammalian target of rapamycin (anti-mTOR; 289 kDa, dilution 1:1,000, no. 2972; Cell Signaling Technology, Danvers, MA), anti-phospho-mTOR (Ser^{2248}, 289 kDa, dilution 1:1,000, no. 2971; Cell Signaling Technology), anti-P70S6 kinase (anti-P70S6K; 70 kDa, dilution 1:1,000, no. 9202; Cell Signaling Technology), anti-phospho-P70S6K (Thr^{389}, 70 kDa, dilution 1:1,000, no. 9206; Cell Signaling Technology), anti-ribosomal protein S6 (anti-rS6; 32 kDa, dilution 1:1,000, no. 2217; Cell Signaling Technology), and anti-phospho-rS6 (Ser^{235}/Ser^{236}, 32 kDa, dilution 1:1,000, no. 4856; Cell Signaling Technology). The complimentary secondary antibodies applied were IRDye 680 donkey anti-rabbit (cat. no. 926-32223, dilution 1:10,000; Li-Cor Biotechnology, Lincoln, NE) and IRDye 800CW donkey anti-mouse (cat. no. 926-32212, dilution 1:10,000; Li-Cor Biotechnology). Protein quantification was performed by scanning on an Odyssey Infrared Imaging System (Li-Cor Biotechnology).

Calculations. Ingestion of L-[1-^{13}C]phenylalanine-labeled protein, intravenous infusion of L-[*ring*-$^2H_5$]phenylalanine and L-[*ring*-$^2H_2$] 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 (8, 15). Total rate of disappearance of phenylalanine equals the rate of phenylalanine hydroxylation (first step in phenylalanine oxidation) and utilization for protein synthesis. Myofibrillar protein fractional synthetic rate (FSR) was calculated with the standard precursor-product method. The complete details have been described previously (41).

Sleep quality assessment. The Pittsburg Sleep Quality Index (PSQI; Sleep Medicine Institute, University of Pittsburgh) was used to assess habitual sleep quality during pretesting (12). PSQI scoring (global scores 0–21 points; higher scores indicate worse sleep quality) was used to classify all subjects as very good, good, poor, or very poor sleepers. Subjects that scored >5 (poor sleepers) were not included in the trial. Prior to sleep, and in the morning after the intervention, tiredness ratings were assessed using a VAS. Sleep behavior during the test night was monitored using wrist activity monitors (Philips Respironics, Murrysville, PA). Additionally, the start and end times 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

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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 ± SE. 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 0.05 to detect a 20% difference in FSR between groups. Baseline characteristics between groups were compared using one-way ANOVA. Time-dependent variables (i.e., plasma metabolite concentrations, plasma enrichments, whole body protein kinetics, and signaling proteins) were analyzed by two-factor repeated-measures ANOVA with time as within-subjects factor and treatment group as between-subjects factor. The analysis was performed for the period starting at the time of protein administration, i.e., between $t = 0$ and 450 min. Non-time-dependent variables (i.e., FSR values) were compared between treatment groups using one-way ANOVA. In case of significant interaction between time and treatment group, one-way ANOVA was performed to compare between treatments at different time points. In case of significant main effects of treatment, a Bonferroni post hoc test was applied to locate group differences. Statistical significance was set at $P < 0.05$. All calculations were performed using SPSS (version 21.0; IBM, Armonk, NY).

RESULTS

Plasma analysis. Plasma glucose and insulin concentrations are shown in Fig. 2. Both plasma glucose and plasma insulin concentrations were slightly higher in the PRO+leu group compared with the PLA and PRO groups throughout the overnight period (main effect treatment, $P < 0.05$). Plasma glucose concentrations at $t = 0$ min averaged 5.5 ± 0.1, 5.2 ± 0.1, and 5.9 ± 0.1 mmol/l in PLA, PRO, and PRO+leu, respectively, and showed an overall decline throughout the overnight period. Plasma insulin concentration at $t = 0$ min averaged 7.8 ± 1.8, 5.4 ± 0.7, 9.5 ± 2.7 mU/l in PLA, PRO, and PRO+leu, respectively, and showed a transient increase at $t = 30$ min when casein protein was provided before sleep.

Plasma phenylalanine, tyrosine, and leucine concentrations are shown in Fig. 3. Plasma amino acid concentrations (μmol/l) were not different between treatments at baseline but increased following casein protein ingestion before sleep (PRO and PRO+leu treatments, time × treatment interaction, $P < 0.001$) and remained elevated throughout the overnight period compared with PLA. In addition, plasma leucine concentrations were significantly higher from $t = 30$ until $t = 210$ min after ingestion in PRO+leu compared with PRO (Fig. 3*B*).

Figure 4 shows the plasma L-[*ring*-$^2H_5$]phenylalanine, L-[1-^{13}C]phenylalanine, and L-[1-^{13}C]leucine enrichments (MPE). Plasma tracer enrichments did not differ between treatments before ingesting the casein protein ($t = 0$ min). Plasma L-[*ring*-$^2H_5$]phenylalanine increased slightly over time, and this increase was somewhat lower following casein protein ingestion (time × treatment interaction, $P < 0.001$). L-[1-^{13}C]leucine increased slightly over time, and this increase was somewhat higher following casein protein ingestion (time × treatment interaction, $P < 0.001$). Plasma L-[1-^{13}C]phenylalanine concentrations, originating from the ingested casein protein, increased in the PRO and PRO+leu treatments, but not in the PLA treatment (Fig. 4*B*; time × treatment interaction, $P < 0.001$).

Whole body phenylalanine kinetics are presented in Fig. 5. Exogenous phenylalanine rates of appearance increased following casein protein ingestion in PRO and PRO+leu but did not change in PLA (Fig. 5*A*). Endogenous phenylalanine rates

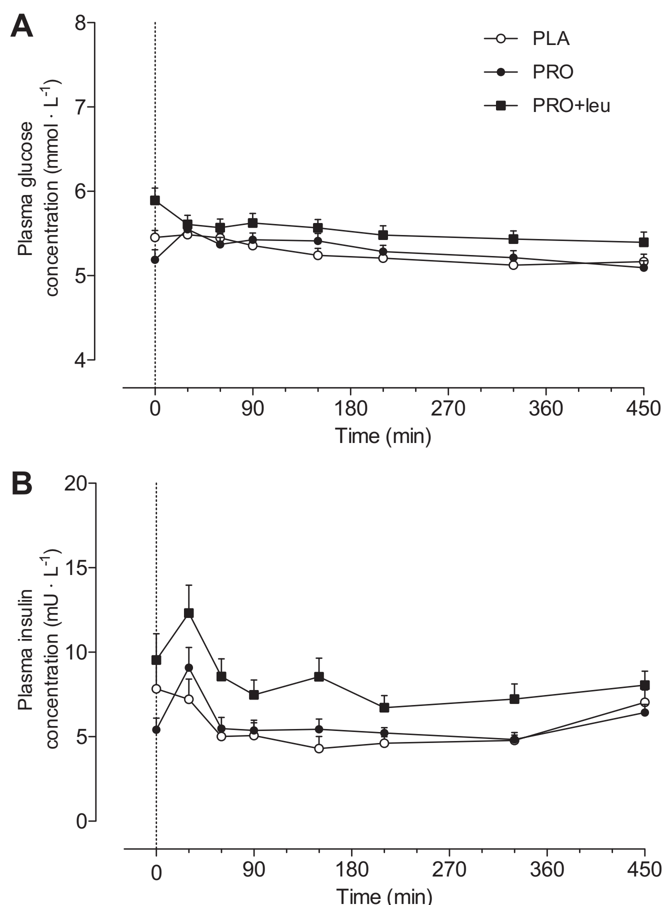

Fig. 2. Overnight plasma glucose (mmol/l; *A*) and insulin concentration (mU/l; *B*). The dotted lines represent the ingestion of the casein protein or placebo drinks. Values represent means ± SE. Data were analyzed with a two-way repeated-measures (time × treatment) ANOVA. Glucose: time effect, $P < 0.001$; treatment effect, $P = 0.037$; time × treatment interaction, $P = 0.004$. Insulin: time effect, $P < 0.001$; treatment effect, $P = 0.008$; time × treatment interaction, $P = 0.148$. PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

of appearance decreased following the ingestion of the experimental test drinks but did not differ between treatments (Fig. 5*B*). As a result, total phenylalanine rates of appearance were higher in PRO and PRO+leu compared with PLA (Fig. 5*C*). Likewise, total phenylalanine rates of disappearance were increased following casein protein ingestion (Fig. 5*D*). The amount of dietary protein-derived phenylalanine that appeared in the circulation over the 7.5-h overnight period was 56 ± 2 and 60 ± 2% in PRO and PRO+leu, respectively, with no differences between treatments ($P = 0.099$).

Data on whole body protein metabolism are expressed in Fig. 6. Whole body protein synthesis and oxidation rates were higher in PRO and PRO+leu compared with PLA ($P < 0.05$), whereas breakdown rates did not differ between treatments. As a result, whole body protein net balance was higher following casein protein ingestion (PRO, 53 ± 2; PRO+leu, 59 ± 3; and PLA, −6 ± 1 μmol phenylalanine·kg^{-1}·7.5 h^{-1}; $P < 0.001$).

Myofibrillar fractional synthesis rates and protein-bound enrichments. Myofibrillar protein FSR based on L-[*ring*-$^2H_5$]phenylalanine infusion with plasma L-[*ring*-$^2H_5$] enrichments as precursor (Fig. 7*A*) or using L-[1-^{13}C]leucine inges-

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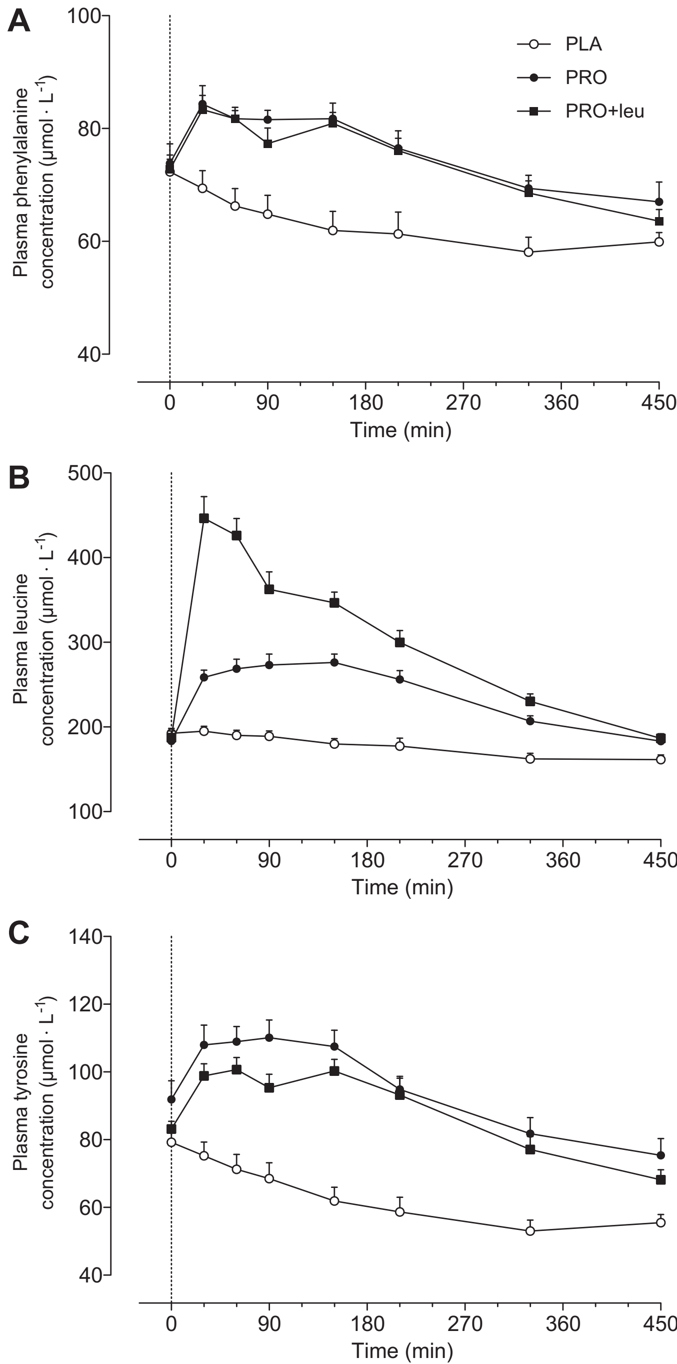

Fig. 3. Overnight plasma phenylalanine (*A*), leucine (*B*), and tyrosine (*C*) concentrations (μmol/l). The dotted lines represent the ingestion of the casein protein or placebo drink. Values represent means ± SE. Data were analyzed with a two-way repeated-measures (time × treatment) ANOVA. Phenylalanine: time effect, $P < 0.001$; treatment effect, $P = 0.001$; time × treatment interaction, $P < 0.001$. Leucine: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P < 0.001$. Tyrosine: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P < 0.001$. PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

tion and infusion with plasma L-[1-^{13}C]leucine enrichments as precursor (Fig. 7*B*) are displayed in Fig. 7. Myofibrillar protein FSR as calculated over the 7.5-h overnight period did not differ between treatments based on the L-[*ring*-$^{2}H_5$]phenylalanine tracer (0.057 ± 0.002, 0.055 ± 0.002, and 0.055 ± 0.004%/h for PLA, PRO, and PRO+leu, respectively; $P = 0.850$). In agreement, myofibrillar muscle protein FSR did not differ between treatments based on the L-[1-^{13}C]leucine tracer (0.080 ± 0.004, 0.073 ± 0.004, and 0.083 ± 0.006%/h for PLA, PRO, and PRO+leu, respectively; $P = 0.328$). Myofibrillar L-[1-^{13}C]phenylalanine enrichments were higher following protein ingestion compared with PLA ($P < 0.001$), reach-

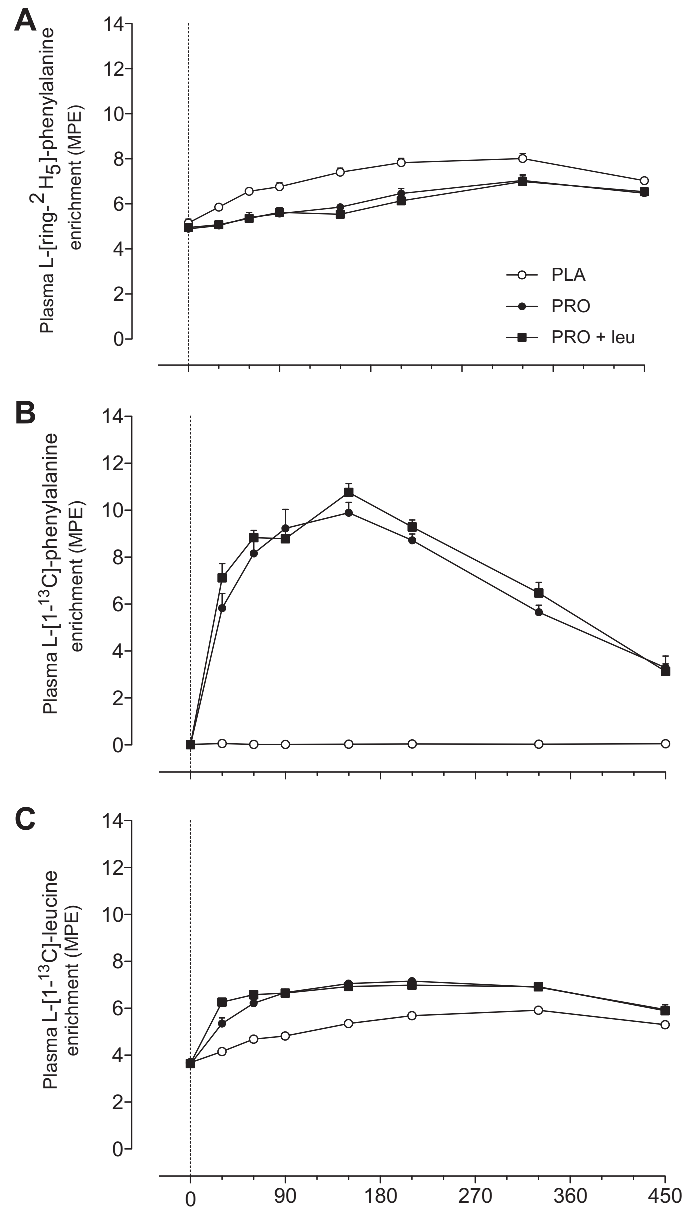

Fig. 4. Overnight plasma L-[*ring*-$^{2}H_5$]phenylalanine (*A*), L-[1-^{13}C]phenylalanine (*B*), and L-[1-^{13}C]leucine (*C*) enrichments in mole percent excess (MPE). The dotted lines represent the ingestion of the casein protein or placebo drinks. Values represent means ± SE. Data were analyzed with a two-way repeated-measures (time × treatment) ANOVA. L-[*ring*-$^{2}H_5$]phenylalanine enrichment: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P < 0.001$. L-[1-^{13}C]phenylalanine enrichment: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P < 0.001$. L-[1-^{13}C]leucine enrichment: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P < 0.001$. PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

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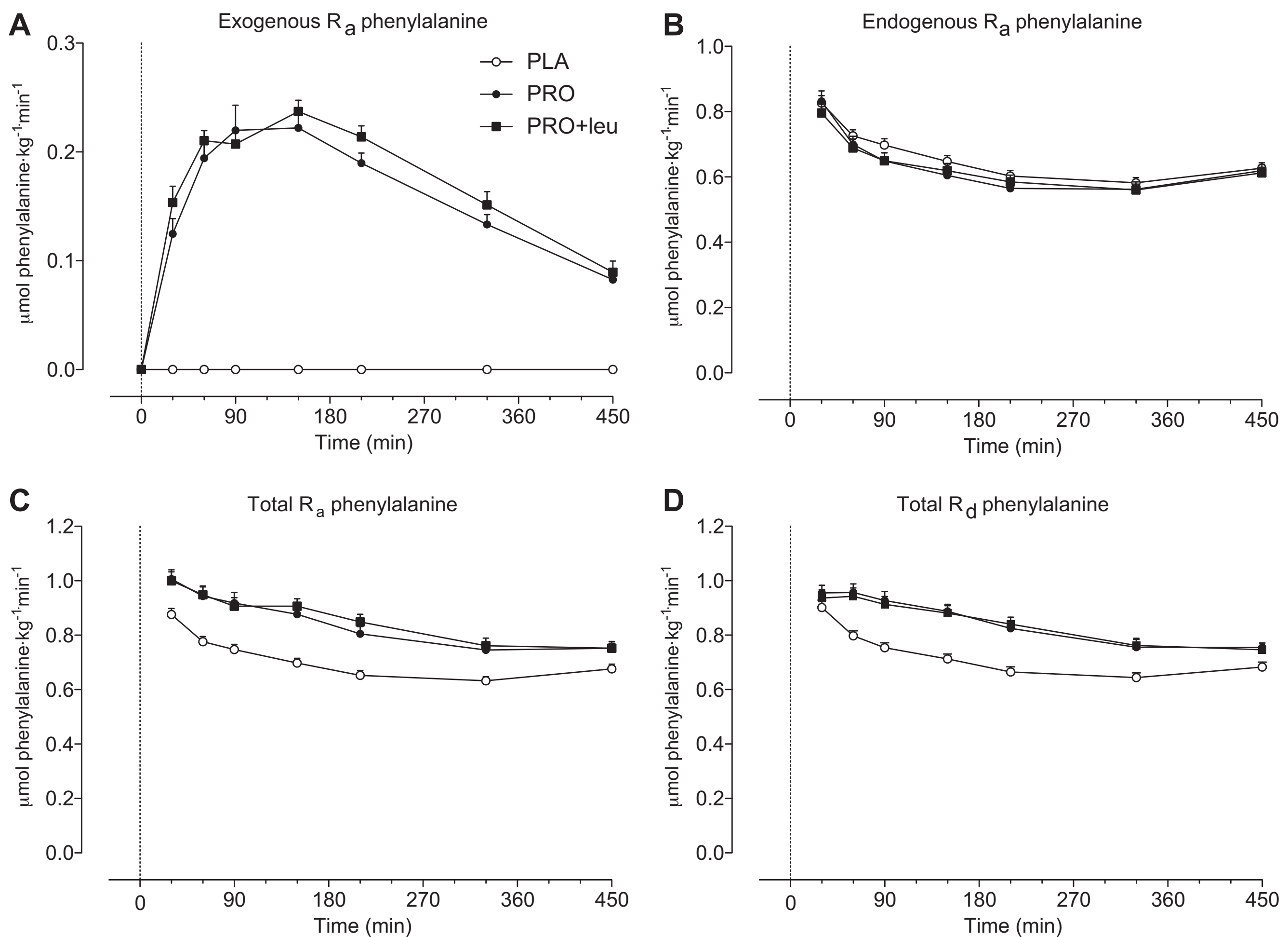

Fig. 5. Overnight exogenous phenylalanine rate of appearance (R_a; *A*), endogenous phenylalanine R_a (*B*), total phenylalanine R_a (*C*), and total phenylalanine rate of disappearance (R_d; *D*). The dotted lines represent the ingestion of the casein protein or placebo drinks. Values represent means ± SE. Data were analyzed with a two-way repeated-measures (time × treatment) ANOVA. Exogenous phenylalanine R_a: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P < 0.001$. Endogenous phenylalanine R_a: time effect, $P < 0.001$; treatment effect, $P = 0.479$; time × treatment interaction, $P = 0.512$. Total phenylalanine R_a: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P = 0.008$. Total phenylalanine R_d: time effect, $P < 0.001$; treatment effect, $P < 0.001$; time × treatment interaction, $P = 0.001$. PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

ing values of 0.026 ± 0.003 and 0.031 ± 003 MPE in the PRO and PRO+leu treatments, respectively (Fig. 8).

Signaling proteins. The muscle phosphorylation status of selected proteins involved in the regulation of muscle protein synthesis is displayed in Fig. 9. Data are expressed as the ratios between the phosphorylated proteins and the total protein content. Directly before presleep drink ingestion ($t = 0$ min), a higher phosphorylation status was observed for P70S6K and rS6 compared with the next morning after 7.5 h of overnight sleep ($P < 0.01$ and $P < 0.001$, respectively). Changes over time did not differ between treatments (time × treatment interaction, $P > 0.05$).

Sleep data. There were no differences in VAS tiredness scores between treatments before sleep or in the morning. Sleep analysis data are presented in Table 2. Total sleep duration did not differ between the treatments [PLA, 5:56 ± 0: 08; PRO, 6:00 ± 0:11; PRO+leu, 6:06 ± 0:06 (h:min); $P = 0.770$]. In addition, there were no significant differences 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) between the treatments.

Hunger and satiety in the morning. The VAS hunger and satiety scores before casein protein ingestion or following the overnight period before (ad libitum) breakfast did not differ between treatments. Energy intake at breakfast averaged 3.1 ± 0.1 MJ (14 ± 1 En% protein, 53 ± 2 En% carbohydrate, and 30 ± 2 En% fat) during the ad libitum breakfast with no differences between treatments ($P = 0.986$).

DISCUSSION

The present study demonstrates that protein ingested before sleep is rapidly digested with ~55% of the ingested protein-derived amino acids appearing in the systemic circulation throughout overnight sleep. These protein-derived amino acids improve whole body protein balance and are taken up and incorporated into myofibrillar protein. However, the ingestion of 30 g casein protein with or without an additional 2 g leucine did not increase muscle protein synthesis rates during a 7.5-h postexercise overnight period.

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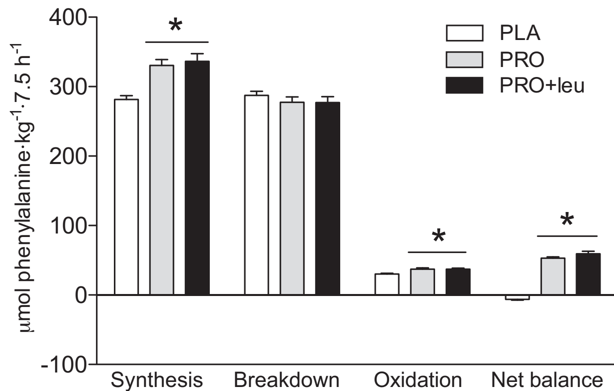

Fig. 6. Calculated rates of overnight whole body protein synthesis, breakdown, oxidation (using phenylalanine hydroxylation to tyrosine as a proxy for phenylalanine oxidation), and net balance (μmol phenylalanine·kg^{-1}·7.5 h^{-1}). Values represent means ± SE. Data were analyzed with one-way ANOVA, and Bonferroni post hoc test was used to locate differences between treatments. Synthesis: main treatment effect, $P < 0.001$; post hoc, *PRO and PRO+leu different compared with PLA, $P < 0.01$. Breakdown: main treatment effect, $P = 0.553$. Oxidation: main treatment effect, $P = 0.005$; post hoc, *PRO and PRO+leu different compared with PLA, $P < 0.05$. Net balance: main treatment effect, $P < 0.001$; post hoc, *PRO and PRO+leu different compared with PLA, $P < 0.001$. PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

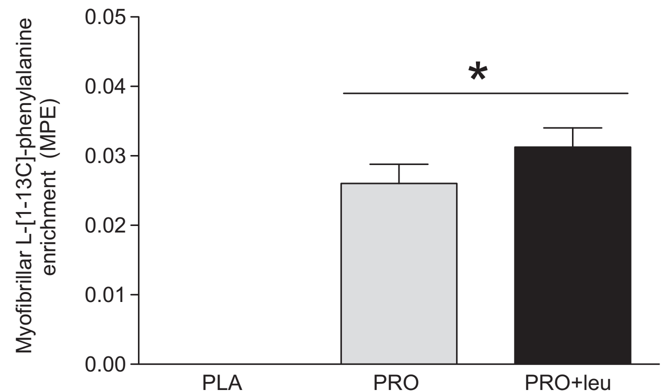

Fig. 8. Overnight L-[1-^{13}C]phenylalanine incorporation in myofibrillar protein [mole percent excess (MPE)]. Values represent means ± SE. Data were analyzed with one-way ANOVA, and Bonferroni post hoc test was used to locate differences between treatments. Main treatment effect, $P < 0.001$; post hoc, *PRO and PRO+leu different compared with PLA, $P < 0.001$. PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

In the present study we combined the ingestion of specifically produced intrinsically L-[1-^{13}C]phenylalanine-labeled protein with continuous infusion of L-[*ring*-$^{2}H_5$]phenylalanine to assess the rate of dietary protein-derived amino acids being released in the circulation and their impact on whole body protein kinetics during overnight sleep. Exogenous dietary protein-derived phenylalanine appearance rates remained elevated throughout overnight sleep (Fig. 5*A*), with ~55% 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 casein protein becoming available in the systemic circulation during a 5–7-h postprandial period (21, 22, 36).

We have previously shown that ingestion of 40 g casein protein before sleep increases whole body protein synthesis rates, resulting in a positive protein net balance during overnight sleep (36). In the present study, we observed that ingestion of a more moderate dose of 30 g casein protein also improves whole body protein synthesis rates and allows for a more positive overnight whole-body protein net balance (Fig. 6). As whole body protein kinetics do not necessarily reflect skeletal muscle metabolism, we also collected skeletal muscle biopsies before and after overnight sleep to assess the overnight muscle protein synthetic response to presleep casein protein feeding.

In contrast to our hypothesis, the ingestion of 30 g casein protein did not significantly increase myofibrillar protein synthesis rates when assessed over a 7.5-h overnight period (Fig. 7). It could be speculated that the absence of increased myofibrillar protein synthesis rates following presleep protein is due to the prior protein feedings. The muscle-full effect proposes that protein ingestion stimulates muscle protein synthesis for 1–3 h, after which muscle protein synthesis rates rapidly return to baseline levels even when plasma amino acid concentrations remain elevated (2, 7). However, we have previously shown that the ingestion of 40-g casein protein before sleep increases overnight mixed muscle protein synthesis rates under near-identical conditions (36). It is possible that such a proposed refractory period is less evident in a setting of postexercise recovery (14, 30). An alternative explanation

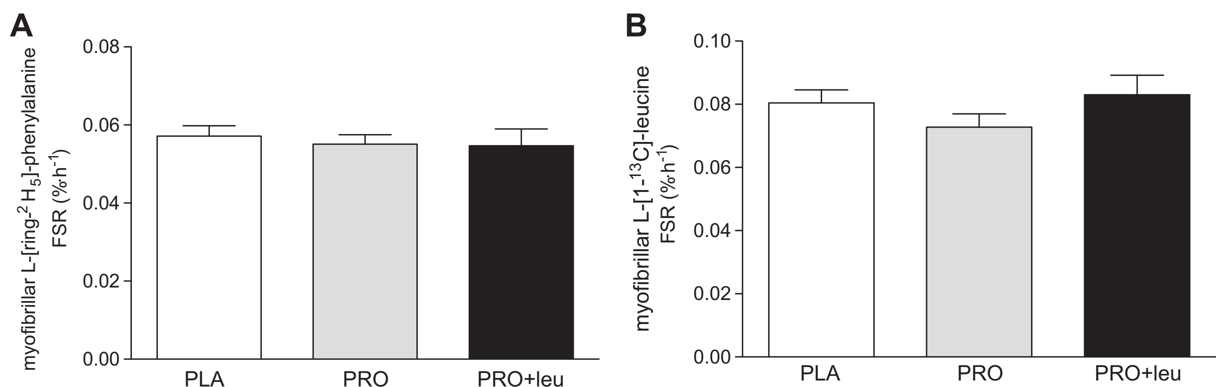

Fig. 7. Overnight myofibrillar protein fractional synthetic rates (FSR in %/h) as calculated using L-[*ring*-$^{2}H_5$]phenylalanine (*A*) or L-[1-^{13}C]leucine (*B*) as tracer. Values represent means ± SE. Data were analyzed with one-way ANOVA. No significant differences between treatments ($P > 0.05$). PLA, placebo; PRO, 30 g casein protein; PRO+leu, 30 g casein protein plus 2 g crystalline leucine.

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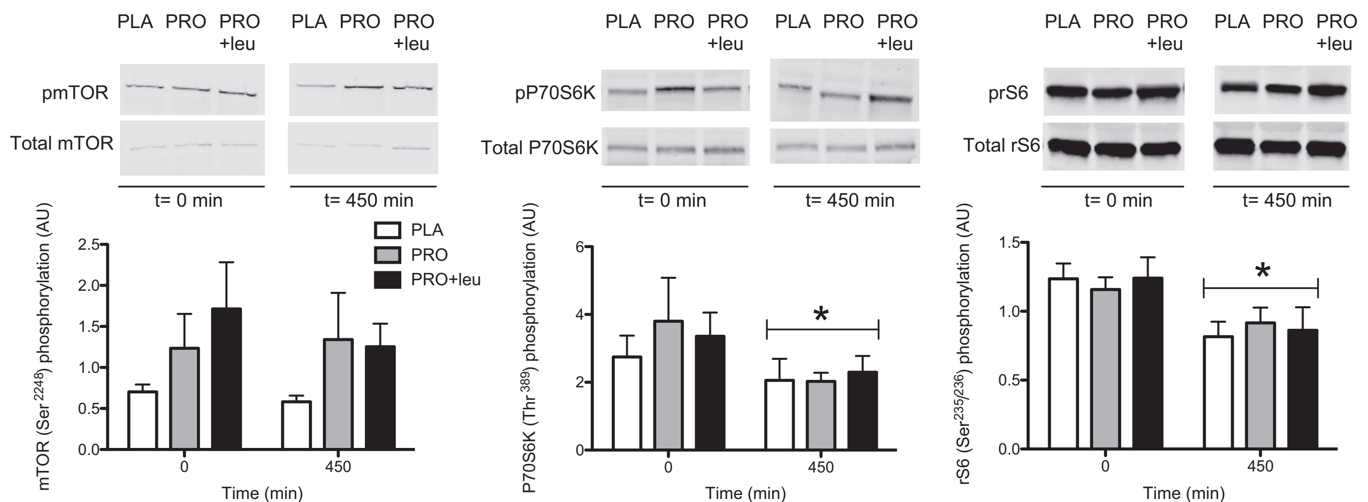

Fig. 9. Skeletal muscle phosphorylation status of selected proteins. Muscle samples were taken directly before presleep drink ingestion ($t = 0$ min) and after 7.5 h of overnight sleep ($t = 450$ min). mTOR: time effect, $P = 0.403$; treatment effect, $P = 0.467$; time × treatment interaction, $P = 0.467$. P70S6K: time effect, $P < 0.01$; treatment effect, $P = 0.839$; time × treatment interaction, $P = 0.516$. rS6: time effect, $P < 0.001$; treatment effect, $P = 0.989$; time × treatment interaction, $P = 0.250$. *Significant time effect ($P < 0.05$). AU, arbitrary units.

could be the measurement of myofibrillar as opposed to mixed muscle (36) protein synthesis rates. However, the protein dose-response relationship appears to be similar for mixed muscle and myofibrillar protein synthesis rates (29, 46), and 30 g of casein protein should be more than sufficient to stimulate myofibrillar protein synthesis rates in younger men (46). Therefore the most likely explanation for the absence of a significant increase in overnight myofibrillar protein synthesis rate following the ingestion of 30 g protein before sleep is the more moderate amount of protein ingested. This implies that >30 g casein protein may be required to significantly increase myofibrillar protein synthesis rates during a 7.5-h postexercise overnight period.

In addition to the applied continuous intravenous infusions of L-[*ring*-$^2H_5$]phenylalanine and L-[1-^{13}C]leucine to measure myofibrillar protein synthesis rates, the ingestion of highly enriched intrinsically L-[1-^{13}C]phenylalanine-labeled protein allowed us to also assess the metabolic fate of the presleep dietary protein-derived amino acids. Muscle protein-bound L-[1-^{13}C]phenylalanine enrichments increased following protein ingestion (Fig. 8), demonstrating that the casein protein ingested before sleep provided amino acids that are incorporated into myofibrillar protein during overnight sleep. As we did not observe a concomitant increase in myofibrillar protein synthesis rates assessed over the entire 7.5-h overnight period, we can only speculate that an early postprandial rise in presleep protein-derived amino acid incorporation into muscle protein does not suffice to elevate overnight muscle protein synthesis rates when expressed over the entire 7.5-h overnight recovery period. In support, we have previously shown that daily ingestion of ~30 g protein before sleep augments the gains in muscle mass and strength during 3 mo of prolonged resistance-type exercise training in young men (38).

Table 2. *Sleep analysis data*

	PLA	PRO	PRO+leu	P
n	11	12	11	
Sleep time, h:min	0:02 ± 0:05	23:57 ± 0:06	23:59 ± 0:03	0.849
Wake time, h:min	6:52 ± 0.08	6:53 ± 0:07	6:51 ± 0:04	0.951
Sleep onset latency, h:min	0:09 ± 0:02	0:11 ± 0.02	0:12 ± 0:04	0.830
Sleep duration, h:min	5:56 ± 0:08	6:00 ± 0:11	6:06 ± 0:06	0.770
Time awake/light sleep, h:min	0:26 ± 0:05	0:30 ± 0:08	0:24 ± 0:03	0.789
Sleep efficiency, %	87 ± 3	87 ± 3	89 ± 2	0.772
Wake bouts	12 ± 2	11 ± 2	11 ± 2	0.911

Values are means ± SE. Data were analyzed with one-way ANOVA.

Previous work from our group (43) as well as others (13, 32) has shown that increasing the leucine content in a dose of protein/amino acids can further augment the postprandial muscle protein synthetic response to protein feeding and increase the efficacy by which dietary protein-derived amino acids are used for incorporation into muscle protein. Despite the greater plasma leucine availability following leucine coingestion, we observed no significant increase in overnight muscle protein synthesis rates (Fig. 7). Likewise, we failed to observe greater protein-derived phenylalanine incorporation rates into skeletal myofibrillar protein following leucine coingestion (Fig. 8). The absence of such a stimulating effect following leucine coingestion could be attributed to the relatively long period over which the muscle protein synthetic response was assessed. Leucine coingestion produces a rapid, but transient, increase in muscle protein synthesis (13), which may not have been detectable when assessed over an extended 7.5-h period. Although the leucine content of a meal is an important determinant of postprandial muscle protein synthesis rates (31), other essential amino acids are also capable of stimulating muscle protein synthesis (14, 37). Our previous and present work combined suggest that the total presleep protein/essential amino acid content is a more important determinant of overnight muscle protein synthesis than merely the leucine content.

The postprandial stimulation of muscle protein synthesis is initiated by a phosphorylation cascade in which mammalian target of rapamycin (mTOR) and its downstream effectors P70S6 kinase (P70S6K) and ribosomal protein S6 (rS6) are key players (44). Here we show that following a day of standardized nutrition and a single bout of resistance-type

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exercise performed in the evening, the phosphorylation status of P70S6K and rS6 is higher immediately before sleep (t = 0 min) compared with the overnight-fasted state the following morning (t = 450 min, Fig. 9). The ingestion of 30 g casein protein with or without additional free leucine before sleep did not lead to additional changes in the activation of mTOR, P70S6K, or rS6 the following morning (t = 450 min) compared with the placebo treatment. This is not surprising considering previous work showing the peak of this translation initiation process to occur 1–2 h following protein ingestion, after which it subsides (14, 18, 20). Of course, the timing of our muscle biopsy collection was chosen to allow 7.5 h of uninterrupted sleep and was therefore not designed to detect temporary changes in either anabolic signaling or muscle protein synthesis during the early as opposed to the late postprandial phase. Leucine plays an important role in the activation of the mTOR pathway and muscle protein synthesis (24, 28). It could be speculated that the absence of increased myofibrillar protein synthesis rates following leucine coingestion is due to the anabolic signaling already being fully activated during the early stages of the overnight period.

The present study shows that ingestion of 30 g casein protein with or without additional free leucine before sleep is not sufficient to stimulate overnight muscle protein synthesis during postexercise overnight recovery. However, we do show that casein protein ingested before sleep provides amino acids that are incorporated into myofibrillar protein and improves overnight whole body protein net balance during sleep. The increase in whole body protein synthesis following presleep protein ingestion may reflect an increase in protein synthesis in tissues other than muscle, i.e., splanchnic tissues. In support, feeding has been shown to improve gut tissue protein net balance (16). However, it is not clear whether a more positive protein net balance in splanchnic tissues results in organ growth and whether this has any functional relevance. Furthermore, it should be noted that on a whole body level the rate of amino acids disappearing from the circulation (i.e., tissue uptake) minus amino acid oxidation rates is assumed to reflect whole body protein synthesis rates. This is not necessarily true, as amino acids may be temporarily stored in tissue free amino acid pools without actual incorporation into tissue protein (i.e., true protein synthesis) and be released back into the circulation at a later stage. In contrast, the use of skeletal muscle biopsies allowed us to assess the actual incorporation of dietary protein-derived amino acids into myofibrillar protein and determine myofibrillar protein synthesis rates. The observed presleep protein-derived amino acid incorporation appears to translate into a measurable benefit when applied during a more prolonged period of resistance-type exercise training. Specifically, we have previously demonstrated that the ingestion of ~30 g casein protein before sleep augments muscle mass and strength gains during 3 mo of resistance-type exercise training in healthy young men (38).

In contrast to the present study, we have previously shown that the ingestion of a larger amount of casein protein before sleep (i.e., 40 g) increases overnight muscle protein synthesis rates (36). These data suggest that a presleep protein dose-response relationship exists, which differs from the immediate postexercise recovery period, during which 20 g seem sufficient to maximize muscle protein synthesis rates (29, 46). Future work should identify a maximal stimulatory protein dose for overnight muscle protein synthesis and aim to find strategies that allow smaller amounts of protein to be more effective. Such work may provide interesting opportunities for the design of feeding strategies that optimize overnight muscle reconditioning in healthy or more clinically compromised populations.

In conclusion, casein protein ingestion before sleep provides amino acids that are incorporated in myofibrillar protein during sleep. However, the ingestion of 30 g casein protein with or without additional free leucine before sleep does not increase myofibrillar protein synthesis rates during overnight postexercise sleep.

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DISCLAIMERS

The views expressed in this manuscript are those of the authors and do not necessarily reflect the position or policy of PepsiCo, Incorporated.

DISCLOSURES

I. Rollo is an employee of the Gatorade Sports Science Institute, a division of PepsiCo, Incorporated. The other authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

J.T., I.R., and L.J.C.v.L. conceived and designed research; J.T., I.W.K.K., A.M.H., and T.S. performed experiments; J.T. and S.L.H. analyzed data; J.T. and L.J.C.v.L. interpreted results of experiments; J.T. prepared figures; J.T. drafted manuscript; J.T., I.W.K.K., A.M.H., T.S., S.L.H., I.R., L.B.V., and L.J.C.v.L. edited and revised manuscript; J.T., I.W.K.K., A.M.H., T.S., S.L.H., I.R., L.B.V., and L.J.C.v.L. approved final version of manuscript.

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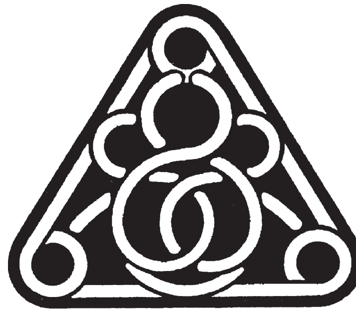

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