Protein co-ingestion stimulates muscle protein synthesis during resistance type exercise

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Running title: muscle protein synthesis during exercise
Abstract

In contrast to the impact of nutritional intervention on post-exercise muscle protein synthesis, little is known about the potential to modulate protein synthesis during exercise. This study investigates the impact of protein co-ingestion with carbohydrate on muscle protein synthesis during resistance type exercise. Ten healthy males were studied in the evening after consuming a standardized diet throughout the day. Subjects participated in 2 experiments, in which they ingested either carbohydrate or carbohydrate with protein during a 2 h resistance exercise session. Subjects received a bolus of test drink prior to and every 15 min during exercise, providing 0.15 g·kg⁻¹·h⁻¹ carbohydrate with (CHO+PRO) or without (CHO) 0.15 g·kg⁻¹·h⁻¹ protein hydrolysate. Continuous intravenous infusions with L-[ring-¹³C₆]phenylalanine and L-[ring-²H₂]tyrosine were applied, and blood and muscle biopsies were collected to assess whole-body and muscle protein synthesis rates during exercise. Protein co-ingestion lowered whole-body protein breakdown rates by 8.4±3.6% (P=0.066), compared to the ingestion of carbohydrate only, and augmented protein oxidation and synthesis rates by 77±17 and 33±3%, respectively (P<0.01). As a consequence, whole-body net protein balance was negative in CHO, whereas a positive net balance was achieved following the CHO+PRO treatment (-4.4±0.3 vs 16.3±0.4 µmol phe·kg⁻¹·h⁻¹, respectively; P<0.01). In accordance, mixed muscle protein fractional synthetic rate (FSR) was 49±22% higher following protein co-ingestion (0.088±0.012 and 0.060±0.004 %·h⁻¹ in CHO+PRO vs CHO treatment, respectively; P<0.05). We conclude that, even in a fed state, protein co-ingestion stimulates whole-body and muscle protein synthesis rates during resistance type exercise.

**Key words:** muscle, protein synthesis, exercise, nutrition.
Introduction

Many studies have assessed the impact of nutritional modulation on muscle protein metabolism during post-exercise recovery. Exercise has been shown to stimulate both muscle protein synthesis (3, 26, 29, 34) and protein breakdown (3, 26, 29), but in the absence of food intake net protein balance remains negative (3, 26). The ingestion of carbohydrate following resistance type exercise attenuates the exercise induced increase in protein breakdown, thereby improving net muscle protein balance (9, 25, 28). However, the ingestion of protein and/or amino acids is essential to stimulate post-exercise muscle protein synthesis and, as such, to achieve a positive net muscle protein balance during recovery from exercise (4, 10, 20, 25, 27, 33).

Previous studies have reported that protein turnover is either decreased or unchanged during resistance (14, 15) and endurance (11, 12, 18, 40, 41) type exercise activities. In contrast to protein metabolism during post-exercise recovery, little is known about the effects of nutritional intervention on muscle protein synthesis during exercise. So far, only 2 studies have examined the role of nutrition on whole-body protein turnover during exercise (18, 35). These studies report an improvement in whole-body protein balance following protein co-ingestion during either prolonged endurance (18) or resistance type exercise activities (35). In the latter study, Tipton et al. (35) suggest that protein ingestion before, as opposed to after, exercise could further augment net muscle protein accretion during recovery. This has been attributed to a more rapid supply of amino acids to the muscle during the acute stages of post-exercise recovery. However, it could also be speculated that protein ingestion prior to and/or during resistance type exercise already stimulates muscle protein synthesis during exercise, thereby creating a larger timeframe for muscle protein synthesis to be elevated.
However, the impact of protein co-ingestion on muscle protein synthesis during exercise has not yet been established.

Studies that have reported on the benefits of protein ingestion on post-exercise recovery generally investigate subjects in the overnight fasted state (4, 10, 20, 25, 27, 33, 35). Under these conditions, it might be speculated that endogenous amino acid availability from the gut (13) and/or the intramuscular free amino acid pool might be limiting. Such post-absorptive conditions differ substantially from normal everyday practice, in which recreational sports activities are generally performed in the evening in a fed state. So far, no data are available on the impact of protein and carbohydrate co-ingestion on muscle protein synthesis during resistance type exercise under normal, fed conditions.

In the present study, we assessed the surplus value of protein co-ingestion with carbohydrate during resistance type exercise on whole-body protein balance and skeletal muscle protein synthesis rate under normal, practical conditions, during which exercise is being performed in the evening after the consumption of a standardized diet throughout the day.
Methods

Subjects
Ten healthy, male volunteers participated in this study (age: 20±1 y, bodyweight: 69.1±2.4 kg, height: 1.79±0.03 m, body mass index: 21.6±0.7 kg/m^2, percentage body fat: 12.0±1.3%, Table 1). All subjects were recreationally active but were not enrolled in any regular exercise program. Subjects were fully informed on the nature and possible risks of the experimental procedures, before their written informed consent was obtained. The study was approved by the Medical Ethical Committee of the Academic Hospital Maastricht, The Netherlands.

Pretesting
All subjects participated in 2 screening sessions, separated by at least 5 days. In the morning following an overnight fast, body composition was determined by the hydrostatic weighing method. Body fat percentage was calculated using Siri's equation (31). Leg volume was measured by anthropometry (16) and averaged 8.2±0.4 L. Subsequently, subjects were familiarized with the exercise equipment and exercise procedure. Proper lifting technique was demonstrated and practiced for each of the upper-body exercises (chest press, shoulder press and lat pulldown) and for the 2 lower-limb exercises (leg press and leg extension). Thereafter, maximum strength for the 2 leg exercises was estimated, using the multiple repetition testing procedure (24).

In the second screening session, subjects’ 1 repetition maximum (1RM) was determined for the 2 leg exercises (22). After warming up, the load was set at 97.5 % of the estimated 1RM, and increased after each successful lift until failure. Between
successive attempts, 5 min rest periods were allowed. A repetition was valid if the subject used proper form and was able to complete the entire lift in a controlled manner without assistance. In addition, subjects performed an incremental exhaustive exercise test on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to measure their maximal oxygen uptake capacity ($\text{VO}_2\text{max}$) and workload capacity ($W_{\text{max}}$) (23).

**Diet and activity prior the experiments**

All subjects received a standardized diet the evening prior to each experimental day (54.3±1.8 kJ·kg bw$^{-1}$, consisting of 62 energy% (En%) carbohydrate, 22 En% fat, and 16 En% protein) and during the entire experimental day (0.16±0.01 MJ·kg bw$^{-1}$·day$^{-1}$, consisting of 62±1 En% carbohydrate, 13±0.4 En% protein and 26±1 En% fat). Subjects were provided with measured amounts of all food products and ingested all meals/snacks at pre-determined time intervals. In both experiments, subjects ingested 78±3 g protein via the standardized diet, with an additional 21±1 g supplemented in the CHO+PRO treatment. All volunteers were instructed to refrain from any sort of heavy physical labour and to keep their diet as constant as possible 2 days before the experimental day. In addition, subjects filled in food intake and physical activity questionnaires for 2 days prior to the start of the first experiment, which were used to standardize food intake and physical activity prior to the second experiment.

**Design**

Each subject participated in 2 treatments separated by at least 2 weeks. During the experimental day, all subjects received a standardized diet (breakfast, lunch, dinner and snacks), participated in their normal daily activities and reported to the lab in the
evening. Subjects performed a 2 h resistance type exercise session, during which either carbohydrate (CHO) or carbohydrate and protein (CHO+PRO) were ingested. Both treatments were performed in a double blind, randomized order. Plasma samples were collected every 15 min and muscle biopsies were taken before and immediately after the cessation of exercise. Tests were designed to simultaneously assess whole-body amino acid kinetics and fractional synthetic rate (FSR) of mixed muscle protein by the incorporation of L-[ring-\textsuperscript{13}C\textsubscript{6}]phenylalanine in the mixed protein pool of muscle tissue samples collected from the \textit{vastus lateralis} muscle.

\textit{Experimental protocol}

At 7.00 p.m., 2 h after ingesting a standard diner, subjects reported to the laboratory, where a Teflon catheter was inserted into an antecubital vein for the primed, continuous infusion of isotopically labelled phenylalanine and tyrosine. A second Teflon catheter was inserted into a contralateral hand vein, which was placed in a hot-box to allow arterialised blood sampling. After a background blood sample was collected (t=-60), tracer infusion was started and subjects rested in a supine position for 1 h. Before engaging in the exercise protocol (t=0 min) the first muscle biopsy was collected, after which the first bolus of test drink was ingested (4.5 ml·kg\textsuperscript{-1}). During exercise, subjects received subsequent boluses (1.5 ml·kg\textsuperscript{-1}) of the test drink every 15 min. The exercise protocol consisted of an interval-cycling program followed by whole-body resistance type exercise training. After a 10 min warm-up on a cycle ergometer (50% \textit{W}\textsubscript{max}), subjects cycled 4 x 5 min at 65% \textit{W}\textsubscript{max}, alternated by 4 x 2.5 min at 45% \textit{W}\textsubscript{max}. No rest periods were allowed between cycling intervals. After a 5 min resting period, subjects started with the resistance type exercise protocol, consisting of an upper- and a lower-body workout. The upper body workout
was performed with a workload set at 40% of the total bodyweight, in which subjects completed 5 sets of 10 repetitions on 3 upper body machines (chest press, shoulder press and lat pulldown). A resting period of 1 min between sets was allowed. This was followed by a lower limb workout, which consisted of 9 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, The Netherlands) and 9 sets of 10 repetitions on the leg extension machine (Technogym). On both machines, 3 sets were completed at 55% of subjects’ 1RM, 3 at 65% 1RM and 3 at 75% 1RM, with 2 min rest between sets. Finally, subjects performed 2 sets of 30 abdominal crunches. During the test, all subjects were verbally encouraged to complete the entire protocol within ~120 min. Immediately after the end of the exercise protocol (t=120 min), an arterialised blood sample from the heated hand vein and a second muscle biopsy from the vastus lateralis muscle were obtained. Arterialised blood samples (8 ml) were taken at t= -60, 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. Muscle biopsies were taken at t= 0 and 120 min.

**Beverages**

Subjects received a beverage volume of 1.5 ml·kg⁻¹ every 15 min during exercise, to ensure a given dose of 0.15 g·kg⁻¹·h⁻¹ carbohydrate (50% glucose and 50% maltodextrin), with or without 0.15 g·kg⁻¹·h⁻¹ protein hydrolysate. The first bolus was provided in a volume of 4.5 ml·kg⁻¹ to stimulate gastric emptying. This supplementation regimen has been shown to allow a continuous supply of glucose and amino acids from the gut and, as such, minimizes perturbations in plasma glucose, amino acid, and circulating insulin concentrations during exercise (18, 37). Glucose and maltodextrin were obtained from AVEBE (Veendam, The Netherlands). The casein protein hydrolysate (PeptoPro®, 85.3% protein) was prepared by DSM Food
Specialties (Delft, The Netherlands) and involved the enzymatic hydrolysis of casein protein by specific endopeptidases, and proline specific endoprotease. To make the taste comparable, all solutions were flavoured by adding 0.05 g·L⁻¹ sodium saccharinate, 0.9 g·L⁻¹ citric acid and 5.0 g·L⁻¹ cream vanilla flavour (Quest International, Naarden, The Netherlands). Treatments were performed in a randomized order, with test-drinks provided in a double-blind fashion.

**Tracer**

The stable isotope tracers, L-[ring-¹³C₆]phenylalanine and L-[ring-²H₂]tyrosine, were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9% saline before infusion. Continuous intravenous infusion (over a period of 3 h, 0.05 µmol·kg⁻¹·min⁻¹ L-[ring-¹³C₆]phenylalanine, 0.02 µmol·kg⁻¹·min⁻¹ L-[ring-²H₂]tyrosine) of the labelled isotopes was performed using a calibrated IVAC 560 pump (San Diego, CA, USA). Both the phenylalanine and tyrosine pool were primed (2 µmol·kg⁻¹ L-[ring-¹³C₆]phenylalanine, 0.775 µmol·kg⁻¹ [²H₂]tyrosine) to enable the calculation of whole body phenylalanine kinetics using established tracer models (30, 32).

**Muscle biopsies**

Muscle biopsies were obtained from the middle region of the vastus lateralis muscle (15 cm above the patella) and approximately 2 cm below the entry through the fascia by means of the percutaneous needle biopsy technique described by Bergström et al (2). The pre- and post-exercise biopsies were taken through the same incision, with the needle pointing in distal and proximal direction, respectively. As such, the biopsies were taken approximately 10 cm apart, to prevent any influence of the pre-exercise biopsy on protein turnover in the post-exercise biopsy (21). All samples were
carefully freed from any visible fat and blood, immediately frozen in liquid nitrogen, and stored at –80 ºC for subsequent analysis.

**Plasma analysis**

Blood samples (8 ml) were collected in EDTA containing tubes and centrifuged at 1000 g and 4°C for 10 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C until analysis. Plasma glucose concentrations were analyzed with the COBAS-FARA semi-automatic analyzer (Uni Kit III, 07367204, La Roche, Basel, Switzerland). Insulin was analyzed by radio immunoassay (Linco, Human Insulin RIA kit, LINCO Research Inc., St. Charles, MO, USA). Plasma (500 µL) for amino acid analyses was deproteinized on ice with 100 µL of 24% (w/v) 5-sulphosalicylic acid, mixed and the clear supernatant was collected after centrifugation. Plasma amino acids concentrations were analyzed on an automated dedicated amino acid analyzer (LC-A10, Shimadzu Benelux, Den Bosch, The Netherlands), using an automated precolumn derivatization procedure and a ternary solvent system. For plasma phenylalanine and tyrosine enrichment measurements, plasma phenylalanine and tyrosine were derivatized to their t-butyldimethylsilyl (TBDMS) derivatives and their ¹³C and/or ²H enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS, Agilent 6890N GC/5973N MSD Little Falls, USA) using selected ion monitoring of masses 336 and 342 for unlabeled and labeled phenylalanine, respectively, and masses 466, 468 and 472 for unlabeled and ²H and ¹³C labeled tyrosine, respectively.
Muscle analyses

For measurement of L-[ring-$^{13}$C$_6$]phenylalanine enrichment in the free amino acid pool and mixed muscle protein, 55 mg of wet muscle was freeze-dried. Collagen, blood and other non-muscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (2-3 mg) was weighed and 8 volumes (8x dry weight of isolated muscle fibers x wet/dry ratio) of ice-cold 2% perchloric acid (PCA) were added. The tissue was then homogenized and centrifuged. The supernatant was collected and processed in the same manner as the plasma samples, such that intracellular free L-[ring-$^{13}$C$_6$]phenylalanine, L-[ring-$^2$H$_2$]tyrosine and L-[ring-$^{13}$C$_6$]tyrosine enrichments could be measured using their TBDMS derivatives on a GC-MS. The free amino acid concentration in the supernatant was measured using an HPLC technique, after precolumn derivatization with o-phthaldialdehyde (36). The protein pellet was washed with 3 additional 1.5 ml washes of 2% PCA, dried and the proteins were hydrolyzed in 6M HCl at 120°C for 15-18 h. The hydrolyzed protein fraction was dried under a nitrogen stream while heated to 120°C, then dissolved in a 50% acetic acid solution, and passed over a Dowex exchange resin (AG 50W-X8, 100-200 mesh hydrogen form, Biorad, Hercules, CA, USA) using 2M NH$_4$OH. Thereafter, the eluate was dried and the purified amino acid fraction was derivatized into the ethoxycarbonyl-ethyl esters to determine the $^{13}$C-enrichment of protein bound phenylalanine using GC-IRMS (Finnigan, MAT 252).

Calculations

Infusion of L-[ring-$^{13}$C$_6$]phenylalanine and L-[ring-$^2$H$_2$]tyrosine with muscle and arterialized blood sampling was used to simultaneously assess whole-body amino acid kinetics and fractional synthetic rate (FSR) of mixed muscle protein. Whole-body
kinetics for phenylalanine and tyrosine were calculated using the equations described by Thompson et al. (32) and Short et al. (30). Briefly, phenylalanine and tyrosine turnover (flux, Q) were measured from the isotope dilution at isotopic steady state:

\[ Q = i \cdot \left( \frac{E_i}{E_p} - 1 \right) \]  

(1)

Where \( i \) is the isotope infusion rate (\( \mu \text{mol.kg body weight}^{-1} \cdot \text{h}^{-1} \)) and \( E_i \) and \( E_p \) correspond to the enrichments of infusate and plasma amino acids, respectively. At isotopic steady state, protein flux (Q) equals the sum of protein synthesis (S) and oxidation (O) as well as the sum of the rate of appearance of meal protein from the gut (I) and protein breakdown (B). The rate of appearance of dietary protein was calculated as total dietary protein intake corrected for a 30% splanchnic extraction rate (38). Whole-body protein synthesis rate was calculated as flux minus oxidation.

\[ Q = S + O = B + I \]  

(2)

\[ S = Q - O \]  

(3)

At isotopic steady state, whole-body phenylalanine oxidation can be determined from the conversion (hydroxylation) of L-[ring-\( ^{13}C_6 \)]phenylalanine to L-[ring-\( ^{13}C_6 \)]tyrosine. The rate of hydroxylation (\( Q_{pt} \)) was calculated (32) using the formula

\[ Q_{pt} = Q_t \cdot \frac{E_i}{E_p} \cdot \frac{Q_p}{(i_p + Q_p)} \]  

(4)

Where \( Q_t \) and \( Q_p \) are the flux rates for L-[ring-\(^2\text{H}_2\)]tyrosine and labeled phenylalanine, respectively, \( E_i \) and \( E_p \) are the L-[ring-\( ^{13}C_6 \)]tyrosine and L-[ring-\( ^{13}C_6 \)]phenylalanine enrichments in plasma, respectively, and \( i_p \) is the infusion rate of the phenylalanine tracer.

Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[ring-
\[ 13\text{C}_6\text{phenylalanine, by the enrichment of the precursor, i.e. plasma } L-[\text{ring-}^{13}\text{C}_6]\text{phenylalanine. Muscle FSR’s were calculated as follows (20):} \]

\[ FSR = \frac{\Delta E_p}{E_{\text{precursor}} \cdot t} \times 100 \quad (6) \]

Where \( \Delta E_p \) is the delta increment of protein bound \( L-[\text{ring-}^{13}\text{C}_6]\text{phenylalanine} \) during incorporation periods, \( E_{\text{precursor}} \) is the average plasma \( L-[\text{ring-}^{13}\text{C}_6]\text{phenylalanine} \) enrichment during the time period for determination of amino acid incorporation, or muscle free \( L-[\text{ring-}^{13}\text{C}_6]\text{phenylalanine} \) enrichment (corrected for contribution of extracellular water (39)) determined in the biopsy taken following exercise, \( t \) indicates the time interval (h) between biopsies and the factor 100 is needed to express the FSR in percent per h (\( \% \cdot \text{h}^{-1} \)).

**Statistics**

All data are expressed as means\( \pm \)SEM. The plasma insulin, glucose and amino acid responses were calculated as area under the curve. A 2-factor repeated measures analysis of variance (ANOVA) with time and treatment as factors was used to compare differences between treatments over time. In case of significant F-ratio’s, Scheffe’s post-hoc tests were applied to locate the differences. For non-time dependent variables, a paired Students’ t-test was used to compare differences in treatment effect. Statistical significance was set at \( P<0.05 \). All calculations were performed using StatView 5.0 (SAS Institute inc., Cary, NC, USA).
Results

Exercise session

The average workload that was applied in the cycling protocol was 152±6 W for the first 10 min and 198±7 and 137±5 W for the interval protocol, respectively. For the upper-body workout, the resistance was set at 40% bodyweight, which averaged 27.5±0.8 kg. For the leg workout, subjects performed 3 sets of 10 repetitions at 55% 1RM, 3 sets at 65% 1RM, and 3 sets at 75% 1RM. This resulted in 119±6, 141±7 and 163±8 kg lifted in the leg press, respectively. On the leg extension 67±2, 79±3, and 90±4 kg were lifted, respectively. Two subjects were unable to complete the last 3 sets of the leg exercises at 75% 1RM, and continued exercise at 65% 1RM. This change in protocol was recorded and repeated during the second experimental day.

Plasma analyses

Plasma glucose and insulin concentrations tended to decrease during the first 30 min of exercise. Thereafter, concentrations increased to 5.9±0.2 and 6.2±0.4 mmol·L⁻¹ (glucose), and 20.9±6.7 mU·L⁻¹ and 30.5±11.7 mU·L⁻¹ (insulin) after 60 min of exercise in the CHO and CHO+PRO treatment, respectively. Total plasma glucose and insulin responses, measured as area under the curve, averaged 10.7±0.2 and 11.0±0.3 mmol·L⁻¹·2h⁻¹ (glucose), and 28.0±8.3 and 37.7±12.7 mU·L⁻¹·2h⁻¹ (insulin), respectively. No significant differences were observed between treatments (P>0.05). Plasma phenylalanine, tyrosine, leucine, valine, and isoleucine concentrations over time are shown in Figure 1. Plasma amino acid concentrations were higher during the entire exercise period in CHO+PRO compared to CHO treatment (P<0.05). Plasma amino acid responses, measured as area under the curve (AUC), for all amino acids
are provided in Table 2. Plasma amino acid responses were higher in the CHO+PRO compared to the CHO experiment for all amino acids (P<0.05), except for glutamic acid, glycine, citrulline and taurine. The time course of the changes in plasma L-[ring-$^{13}$C$_6$]phenylalanine, L-[ring-$^2$H$_2$]tyrosine and L-[ring-$^{13}$C$_6$]tyrosine enrichments are presented in Figure 2. Overall, enrichments were significantly lower during the entire exercise trial in the CHO+PRO compared to the CHO treatment (P<0.01).

**Whole-body protein metabolism**

Phenylalanine flux was higher in the CHO+PRO compared with the CHO experiment and averaged 70±3 and 54±2 µmol·kg$^{-1}$·h$^{-1}$, respectively (P<0.01). Tyrosine flux was also higher in the CHO+PRO experiment, 115±8 vs 58±3 µmol·kg$^{-1}$·h$^{-1}$, respectively (P<0.01). Whole-body protein breakdown was 8.4±3.6% lower (P=0.066), and protein oxidation and synthesis rates were 77±17 and 33±3% higher (P<0.01) in the CHO+PRO compared to the CHO experiment. As a consequence, whole-body net protein balance was negative in CHO, whereas a positive net balance was achieved in the CHO+PRO treatment (-4.4±0.3 vs 16.3±0.4 µmol phe·kg$^{-1}$·h$^{-1}$, respectively; P<0.01, Figure 3A). Without correction for splanchnic extraction of dietary protein (38), whole-body protein breakdown rates would be 26±4% lower in the CHO+PRO compared to CHO experiment. Furthermore, whole-body protein balance would average 25.4±0.4 vs -4.4±0.3 µmol phe·kg$^{-1}$·h$^{-1}$ in the CHO+PRO and CHO experiment, respectively (P<0.01). The latter provides the upper boundary value for net protein balance.
Mixed muscle protein synthesis rates

Mixed muscle protein fractional synthetic rates (FSR), using mean plasma L-[ring-$^{13}$C$_6$]phenylalanine enrichment as the precursor, were 49±22% higher in the CHO+PRO vs CHO experiment, and averaged 0.088±0.012 vs 0.060±0.004 %·h$^{-1}$, respectively (Figure 3B; P<0.05). When applying the intramuscular free intracellular L-[ring-$^{13}$C$_6$]phenylalanine enrichments (corrected for contribution of extracellular water (39)) as the precursor pool, similar results were obtained. FSR values averaged 0.072±0.004 vs 0.104±0.015 %·h$^{-1}$, in the CHO and CHO+PRO experiment, respectively (P=0.088). Plasma and muscle amino acid enrichments are presented in Table 3. In one subject, a post-exercise muscle biopsy could not be obtained. Therefore, FSR and free intracellular tracer enrichments are presented for 9 subjects only.
Discussion

In the present study, we prove that, even in the fed state, protein co-ingestion (0.15 g·kg\(^{-1}\)·h\(^{-1}\)) with carbohydrate (0.15 g·kg\(^{-1}\)·h\(^{-1}\)) improves whole-body protein balance and increases mixed muscle protein synthesis rate during resistance type exercise. Previous studies have reported that an acute bout of resistance type exercise stimulates both muscle protein synthesis and breakdown rates (3, 26, 34). However, in the absence of subsequent food intake net protein balance remains negative (3, 26, 34). Carbohydrate ingestion following exercise has been reported to attenuate the exercise induced increase in protein breakdown rate (9, 25, 28), but protein and/or amino acid administration is prerequisite to stimulate muscle protein synthesis and, as such, to achieve a positive net muscle protein balance following exercise (4, 10, 20, 25, 27, 33).

In contrast to the wealth of data on the impact of exercise on muscle protein metabolism during post-exercise recovery (3-10, 17, 20, 25-29, 33, 34), only little information is available on muscle protein turnover during exercise. Few studies have examined the impact of endurance (12, 18, 40, 41) and resistance (14, 15, 35) type exercise on whole-body and muscle protein synthesis rates during exercise. These studies have reported either a decrease (14, 40, 41) or no effect (12, 15, 41) of exercise on muscle protein synthesis rates. Only 2 studies have addressed the impact of nutrition on muscle protein synthesis during exercise (18, 35). Their results show that the combined ingestion of carbohydrate and protein stimulates whole-body protein synthesis during endurance (18) and resistance (35) type exercise. Koopman et al (18) determined whole-body protein turnover during 5 h of endurance exercise, during which subjects ingested carbohydrate drinks with or without added protein. Protein co-ingestion during exercise was shown to stimulate whole-body protein
synthesis rate and improve net protein balance. Furthermore, Tipton et al (35) reported that protein ingestion prior to resistance type exercise stimulates post-exercise muscle protein synthesis more effectively than protein ingestion after exercise. The latter was attributed to a more rapid provision of amino acids to the muscle during the acute stages of post-exercise recovery (35). However, based on their data, it could also be speculated that protein ingestion prior to and/or during resistance type exercise already stimulates muscle protein synthesis during exercise activities.

In the present study, we observed that protein co-ingestion elevates whole-body protein synthesis rates and, as such, improves whole-body net protein balance during resistance type exercise (Figure 3A). Of course, estimates of whole-body protein synthesis and/or net protein balance do not necessarily reflect skeletal muscle protein synthesis (13). Therefore, we also assessed muscle protein synthetic rates directly by determining the incorporation rate of $^{13}$C-labeled phenylalanine in the mixed muscle protein pool. Our results show that protein co-ingestion substantially increases muscle protein synthesis during exercise, with mixed muscle protein synthesis rates averaging $0.088\pm0.012$ vs $0.060\pm0.004$ %·h$^{-1}$ in the CHO+PRO and CHO experiment, respectively ($P<0.05$; Figure 3B). It should be noted that the choice of the precursor pool, i.e. plasma or muscle free phenylalanine enrichment, can have a substantial impact on the calculated muscle protein synthesis rates. Therefore, we also calculated muscle protein synthesis rates based on the muscle free phenylalanine enrichment (Table 3). The latter resulted in similar findings ($0.104\pm0.015$ vs $0.072\pm0.004$ %·h$^{-1}$, respectively), which did not reach statistical significance ($P=0.088$). We generally prefer the use of plasma enrichment data for precursor pool enrichment because we feel that multiple plasma samples collected throughout the exercise session give a
better estimate of the changes in precursor enrichment over time than the enrichment in the muscle free amino acid pool, which is only measured prior to and immediately after exercise.

In contrast to previous studies investigating subjects in the overnight fasted state, subjects in the present study were investigated in a postprandial state. The latter might explain why the fractional muscle protein synthesis rates during exercise are quite similar to protein synthesis rates observed previously during post-exercise recovery (12, 20, 34). Dreyer et al (14) reported lower muscle protein synthesis rates during resistance type exercise, when compared to rest and post-exercise recovery. The apparent discrepancy might be explained by the fact that subjects in the present study were investigated in the fed state, and ingested carbohydrate with or without additional protein during exercise. In accordance, previous work from our lab has shown that co-ingestion of protein prior to resistance type exercise enables the mTOR signal transduction pathway to be activated during exercise (19). Furthermore, reduced endogenous amino acid availability from the gut and/or intramuscular free amino acid pool in the overnight fasted state could likely attenuate the exercise induced increase in muscle protein synthesis rate.

To increase the practical relevance of the study, we investigated our subjects in a fed state, i.e. during exercise performed in the evening after a full day of standardized dietary practice. To prevent potential flaws in the applied methodology due to large perturbations in circulating plasma amino acid concentrations, we started the exercise session 3 h after ingesting the last meal. Furthermore, during the exercise protocol we administered carbohydrate or carbohydrate-protein beverages every 15 min to allow a more continuous supply of glucose and amino acids from the gut. The latter prevented
perturbations in plasma amino acid concentrations (Figure 1) and enrichments (Figure 2) throughout the latter stages of exercise.

Protein co-ingestion during exercise substantially augmented muscle protein synthesis rates (Figure 3B). Consequently, our findings suggest that protein co-ingestion during exercise could represent an effective dietary strategy to further augment muscle protein accretion by creating a larger timeframe for muscle protein synthesis to be elevated. However, as the progress in metabolomics and proteomics do not yet allow us to assess fractional synthetic rates of individual proteins \textit{in vivo} in human skeletal muscle, we cannot specify which proteins are being synthesized to a greater extent. Furthermore, it remains to be determined whether the observed impact of protein co-ingestion on mixed muscle protein synthesis during exercise is restricted to intermittent, resistance-type exercise activities. It is attractive to assume that AMP kinase is not continually activated throughout intermittent type exercise activities when the exercise is performed in the fed state. The latter could prevent its proposed inhibitory effect on muscle protein synthesis (1, 14, 21) and allow protein synthesis rates to be increased during the resting periods between sets. It would be of interest to address the potential of protein co-ingestion to stimulate muscle protein synthesis during more continuous, endurance-type exercise activities.

In conclusion, even in a fed state, protein co-ingestion prior to and during resistance type exercise improves whole-body protein balance and stimulates muscle protein synthesis during exercise. Protein co-ingestion prior to and/or during resistance type exercise might be advocated to further improve skeletal muscle reconditioning during resistance-type exercise training.
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MB, RK, AKK and LJCvL designed the study. MB organized and carried out the clinical experiments with the assistance of RK. MB performed the statistical analysis and wrote the manuscript together with RK, HK, WHMS and LJCvL. APG and HV performed the stable isotope tracer analyses. WHMS and HK provided medical assistance. AKK is a researcher with DSM Food Specialties, Delft, The Netherlands; no other author had any financial or personal conflicts of interest.
References


Figure legends

Figure 1. Plasma phenylalanine, tyrosine and BCAA (leucine, isoleucine and valine) concentrations (µmol·L⁻¹), in the CHO (n=10) and CHO+PRO (n=10) treatments. Values are means ± SEM. Data were analyzed with ANOVA repeated measures (treatment x time). Plasma phenylalanine: treatment effect, P<0.01; time effect, P<0.01; interaction of treatment and time, P<0.01. Plasma tyrosine: treatment effect, P<0.01; time effect, P<0.01; interaction of treatment and time, P<0.01. Plasma leucine: treatment effect, P<0.01; time effect, P<0.01; interaction of treatment and time, P<0.01. Plasma isoleucine: treatment effect, P<0.01; time effect, P<0.01; interaction of treatment and time, P<0.01. Plasma valine: treatment effect, P<0.01; time effect, P<0.01; interaction of treatment and time, P<0.01. *: significantly different from CHO (Scheffe’s post hoc test, P<0.05).

Figure 2. Plasma L-[ring-¹³C₆]phenylalanine (A), L-[ring-²H₂]tyrosine (B), and L-[ring-¹³C₆]tyrosine (C) enrichment during the CHO (n=10) and CHO+PRO (n=10) experiment. MPE; Mole Percent Excess. Values represent means ± SEM. Data were analyzed with ANOVA repeated measures (treatment x time). Plasma L-[ring-¹³C₆]phenylalanine enrichment: treatment effect, P<0.01; time effect, P<0.05; interaction of treatment and time, P<0.01. Plasma L-[ring-²H₂]tyrosine enrichment: treatment effect, P<0.01; time effect, P>0.05; interaction of treatment and time, P<0.01. Plasma L-[ring-¹³C₆]tyrosine enrichment: treatment effect, P<0.01; time effect, P<0.01; interaction of treatment and time, P<0.01. * significantly different from CHO (Scheffe’s post hoc test, P<0.05).
**Figure 3.** Whole-body protein breakdown, synthesis, oxidation rates and net protein balance (expressed as µmol phenylalanine·kg⁻¹·h⁻¹) (n=10) (A) and fractional synthesis rate (FSR) of mixed muscle protein (n=9) (B) in the CHO and CHO+PRO experiment. Values represent means ± SEM. Data were analyzed with the students’ t-test for paired samples. * significantly different from CHO (Scheffe’s post hoc test, P<0.05).
Table 1  Subjects’ characteristics.

<table>
<thead>
<tr>
<th></th>
<th>(n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179 ± 3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.1 ± 2.4</td>
</tr>
<tr>
<td>BMI (kg·m²)</td>
<td>21.6 ± 0.7</td>
</tr>
<tr>
<td>Body fat % (Siri)</td>
<td>12.0 ± 1.3</td>
</tr>
<tr>
<td>Leg volume (L)</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>1-RM leg press (kg)</td>
<td>217 ± 10</td>
</tr>
<tr>
<td>1-RM leg extension (kg)</td>
<td>122 ± 4</td>
</tr>
<tr>
<td>$W_{\text{max}}$ (watt)</td>
<td>305 ± 11</td>
</tr>
<tr>
<td>$\text{VO}_{2\text{max}}$ (ml·kg⁻¹·min⁻¹)</td>
<td>50.5 ± 2.5</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>CHO (n=10)</th>
<th>CHO+PRO (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>0.130 ± 0.005</td>
<td>0.164 ± 0.007 *</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.130 ± 0.004</td>
<td>0.204 ± 0.011 *</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.246 ± 0.010</td>
<td>0.396 ± 0.013 *</td>
</tr>
<tr>
<td>Valine</td>
<td>0.412 ± 0.020</td>
<td>0.591 ± 0.034 *</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.148 ± 0.010</td>
<td>0.241 ± 0.010 *</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.262 ± 0.021</td>
<td>0.296 ± 0.022</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.124 ± 0.006</td>
<td>0.167 ± 0.009 *</td>
</tr>
<tr>
<td>Serine</td>
<td>0.229 ± 0.012</td>
<td>0.294 ± 0.017 *</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.268 ± 0.028</td>
<td>1.413 ± 0.039 *</td>
</tr>
<tr>
<td>Hystidine</td>
<td>0.175 ± 0.010</td>
<td>0.209 ± 0.008 *</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.463 ± 0.015</td>
<td>0.497 ± 0.023</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.249 ± 0.014</td>
<td>0.344 ± 0.032 *</td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.079 ± 0.010</td>
<td>0.090 ± 0.004</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.186 ± 0.011</td>
<td>0.242 ± 0.009 *</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.984 ± 0.065</td>
<td>1.232 ± 0.055 *</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.276 ± 0.017</td>
<td>0.280 ± 0.025</td>
</tr>
<tr>
<td>α-aminobutyrate</td>
<td>0.025 ± 0.002</td>
<td>0.033 ± 0.004 *</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.046 ± 0.003</td>
<td>0.075 ± 0.003 *</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.104 ± 0.006</td>
<td>0.123 ± 0.004 *</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.112 ± 0.007</td>
<td>0.132 ± 0.007 *</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.298 ± 0.010</td>
<td>0.404 ± 0.020 *</td>
</tr>
</tbody>
</table>

Plasma amino acid responses expressed as area under the curve (in mmol·L⁻¹·2h⁻¹). Values are means ± SEM; * significantly different from CHO (P<0.05).
Table 3  Plasma and muscle AA tracer enrichments.

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th>CHO+PRO</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma AA enrichments (n=10)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-[ring-^{13}C_6]phenylalanine</td>
<td>0.0678±0.0020</td>
<td>0.0565±0.0021</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>L-[ring-^{2}H_2]tyrosine</td>
<td>0.0293±0.0011</td>
<td>0.0219±0.0010</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>L-[ring-^{13}C_6]tyrosine</td>
<td>0.0066±0.0002</td>
<td>0.0050±0.0002</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Muscle AA enrichments (n=9)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-[ring-^{13}C_6]phenylalanine</td>
<td>0.0536±0.0028</td>
<td>0.0467±0.0019</td>
<td>0.056</td>
</tr>
<tr>
<td>L-[ring-^{2}H_2]tyrosine</td>
<td>0.0171±0.0024</td>
<td>0.0144±0.0009</td>
<td>0.337</td>
</tr>
<tr>
<td>L-[ring-^{13}C_6]tyrosine</td>
<td>0.0336±0.0121</td>
<td>0.0138±0.0044</td>
<td>0.102</td>
</tr>
<tr>
<td><strong>δ enrichment muscle protein (n=9)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>L-[ring-^{13}C_6]phenylalanine</td>
<td>0.000092±0.000013</td>
<td>0.000076±0.000006</td>
<td>0.230</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM; CHO, carbohydrate only; CHO+PRO, carbohydrate plus protein; AA, amino acid; Data were analyzed with a paired sample T-test; Plasma AA enrichments represent the average plasma AA enrichment during exercise; Muscle AA enrichments represent the AA enrichment in the muscle biopsy taken after 2 h of exercise; δ enrichment muscle protein represents the increment in muscle protein enrichment from 0 to 2 h of exercise.
Figure 1
159x220mm (150 x 150 DPI)
Figure 3
159x71mm (150 x 150 DPI)