

Impact of protein coingestion on muscle protein synthesis during continuous endurance type exercise

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Beelen M, Zorenc A, Pennings B, Senden JM, Kuipers H, van Loon LJ. Impact of protein coingestion on muscle protein synthesis during continuous endurance type exercise. *Am J Physiol Endocrinol Metab* 300: E945–E954, 2011. First published March 1, 2011; doi:10.1152/ajpendo.00446.2010.—This study investigates the impact of protein coingestion with carbohydrate on muscle protein synthesis during endurance type exercise. Twelve healthy male cyclists were studied during 2 h of fasted rest followed by 2 h of continuous cycling at 55% W_{max} . During exercise, subjects received either 1.0 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate (CHO) or 0.8 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate with 0.2 $g \cdot kg^{-1} \cdot h^{-1}$ protein hydrolysate (CHO+PRO). Continuous intravenous infusions with L-[ring- $^{13}C_6$]phenylalanine and L-[ring- 2H_2]tyrosine were applied, and blood and muscle biopsies were collected to assess whole body protein turnover and muscle protein synthesis rates at rest and during exercise conditions. Protein coingestion stimulated whole body protein synthesis and oxidation rates during exercise by 22 ± 3 and $70 \pm 17\%$, respectively ($P < 0.01$). Whole body protein breakdown rates did not differ between experiments. As a consequence, whole body net protein balance was slightly negative in CHO and positive in the CHO+PRO treatment (-4.9 ± 0.3 vs. $8.0 \pm 0.3 \mu mol Phe \cdot kg^{-1} \cdot h^{-1}$, respectively, $P < 0.01$). Mixed muscle protein fractional synthetic rates (FSR) were higher during exercise compared with resting conditions (0.058 ± 0.006 vs. $0.035 \pm 0.006\%/h$ in CHO and 0.070 ± 0.011 vs. $0.038 \pm 0.005\%/h$ in the CHO+PRO treatment, respectively, $P < 0.05$). FSR during exercise did not differ between experiments ($P = 0.46$). We conclude that muscle protein synthesis is stimulated during continuous endurance type exercise activities when carbohydrate with or without protein is ingested. Protein coingestion does not further increase muscle protein synthesis rates during continuous endurance type exercise.

carbohydrate; protein metabolism; skeletal muscle; AMP-activated protein kinase; mammalian target of rapamycin

MANY STUDIES HAVE ASSESSED THE IMPACT OF nutrition on muscle protein metabolism during post-exercise recovery (5, 7, 8, 27, 31, 33, 35, 40). Exercise has been shown to stimulate both muscle protein synthesis (4, 32, 36, 41) and breakdown (4, 32, 36), but in the absence of food intake net protein balance will remain negative (4, 32). Carbohydrate ingestion during post-exercise recovery attenuates the exercise-induced increase in protein breakdown, thereby improving net muscle protein balance (7, 31, 35). However, protein and/or amino acid administration is required to stimulate post-exercise muscle protein synthesis, allowing net muscle protein balance to become positive (5, 8, 27, 31, 33, 40).

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In contrast to the wealth of data on the impact of nutrition on post-exercise muscle protein metabolism, little is known about the impact of nutrient intake prior to and/or during exercise. Protein ingestion before as opposed to after exercise has previously been reported to augment net muscle protein accretion during post-exercise recovery (42). The latter has been attributed to a more rapid supply of amino acids to the muscle during the acute stages of post-exercise recovery. However, we recently showed that protein coingestion before and during exercise can stimulate whole body and muscle protein synthesis rates already during resistance type exercise activities (2). Consequently, it was concluded that protein ingestion prior to and during resistance type exercise also stimulates muscle protein synthesis during exercise conditions (2). The latter likely creates a larger time frame for muscle protein synthesis rates to be elevated and might further improve the skeletal muscle adaptive response to exercise training.

It has been speculated that the observed impact of protein coingestion on mixed muscle protein synthesis during exercise conditions is restricted to intermittent, resistance type exercise activities (2, 16). It is attractive to assume that AMPK is not continually activated throughout intermittent, resistance type exercise activities when exercise is performed in the fed state. The latter would allow muscle protein synthesis to become disinhibited during resting periods between sets. As a consequence, it was hypothesized that protein coingestion would not augment muscle protein synthesis rates during more continuous endurance type exercise activities. So far, only one study has examined the impact of protein coingestion on protein metabolism during continuous endurance type exercise (25). In that study, Koopman et al. reported an increase in whole body protein synthesis and a more positive whole body protein balance during ultra-endurance type exercise (25). Unfortunately, in that study, only whole body protein kinetics were assessed, which do not necessarily provide accurate insight into skeletal muscle protein synthesis rates (10).

The present study is the first to assess the impact of protein coingestion with carbohydrate during endurance type exercise on whole body protein balance and skeletal muscle protein synthesis rates. Muscle protein synthesis rates were assessed in 12 healthy young males during 2 h of moderate-intensity cycling exercise while receiving carbohydrate with and without additional protein.

METHODS

Subjects. Twelve healthy male cyclists participated in this study (age 22 ± 1 yr, body weight 70.7 ± 2.1 kg, body mass index 22.2 ± 0.5 kg/m^2 , percent body fat $13.1 \pm 0.8\%$, VO_{2max} 56.5 ± 1.4 $ml \cdot kg^{-1} \cdot min^{-1}$). All subjects were enrolled in regular endurance type exercise training at a recreational competitive level. Subjects

were fully informed of the nature and possible risks of the experimental procedures before their written informed consent was obtained. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre+, Maastricht, the Netherlands.

Pretesting. All subjects participated in a screening session, which was performed at least 5 days prior to the first test. First, subjects' body composition was determined by dual-energy X-ray absorptiometry (DEXA, Discovery A; Hologic, Bedford, MA), and their leg volume (9.0 ± 0.6 liters) was assessed by anthropometry (21). Subsequently, subjects performed an incremental exhaustive exercise test on an electronically braked cycle ergometer (Lode Excalibur; Groningen, The Netherlands) to assess maximal oxygen uptake ($\dot{V}O_{2\max}$) and workload capacity (W_{\max}) (29).

Diet and activity prior the experiments. All subjects received the same standardized dinner [68 ± 2 kJ/kg body wt, consisting of 53 energy percent (En%) carbohydrate, 33 En% fat, and 14 En% protein] the evening prior to each test day. All volunteers refrained from any sort of exhaustive physical labor and/or exercise and kept their diet as constant as possible 2 days prior to 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 then used to standardize food intake and physical activity prior to the second experimental day.

Design. Each subject participated in two treatments separated by at least 1 wk. Each experimental day started with a 4-h resting period, after which subjects performed 2 h of endurance type exercise. In the two experimental tests, subjects ingested either carbohydrate (CHO) or carbohydrate and protein (CHO+PRO) during exercise. Both treatments were performed in a double-blind, randomized order. Plasma samples were collected every 30 min at rest and every 15 min during exercise. Muscle biopsies were taken at rest, prior to the onset of exercise, and immediately after cessation of exercise. Tests were designed to simultaneously assess whole body amino acid kinetics and mixed muscle fractional protein synthetic rate (FSR). The latter was assessed by measuring the incorporation rate of L-[ring- $^{13}\text{C}_6$]phenylalanine in the mixed muscle protein pool of tissue samples collected from the vastus lateralis muscle.

Experimental protocol. At 8:00 AM, following an overnight fast, subjects reported to the laboratory, where a Teflon catheter was inserted into an antecubital vein for a primed, continuous infusion of isotopically labeled phenylalanine and tyrosine. A second Teflon catheter was inserted into a contralateral hand vein, which was placed in a hot box to allow arterialized blood sampling. After a background blood sample was collected ($t = -120$ min), tracer infusion was started, and subjects rested in a supine position for 4 h. After 2 h ($t = 0$ min), the first biopsy was taken from the vastus lateralis muscle, and after 4 h ($t = 120$ min) a second muscle biopsy was taken. Immediately thereafter, subjects ingested the first bolus of test drink (6.0 ml/kg) and started the exercise protocol. The exercise regimen consisted of 2 h of moderate-intensity exercise on a cycle ergometer (Lode Excalibur, Groningen, The Netherlands) at 55% of subjects' individual W_{\max} . During exercise, all subjects ingested boluses (2 ml/kg) of the test drink every 15 min. Immediately after cessation of exercise ($t = 240$ min), a third muscle biopsy was obtained. Blood samples (8 ml) were taken at $t = -120, -60, 0, 30, 60, 90,$ and 120 min (rest) and at $t = 135, 150, 165, 180, 195, 210, 225,$ and 240 min (exercise). Muscle biopsies were taken at $t = 0, 120,$ and 240 min.

Beverages. Subjects received a beverage volume of 2 ml/kg every 15 min during exercise to ensure a given dose of $1.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ carbohydrate only (CHO), or an isocaloric dose of $0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ carbohydrate with $0.2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ protein hydrolysate (CHO+PRO). The first bolus was provided in a volume of 6 ml/kg to stimulate gastric emptying. This supplementation regimen has been proven effective to allow a continuous supply of glucose and amino acids from the gut and, as such, to minimize perturbations in plasma glucose, amino acid, and circulating insulin concentrations during

exercise (25, 44). Glucose and maltodextrin (50% glucose + 50% maltodextrin) were obtained from AVEBE (Veendam, The Netherlands). The casein protein hydrolysate (PeptoPro) 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 tastes comparable, all solutions were flavored by adding 0.05 g/l sodium saccharinate, 0.9 g/l citric acid, and 5.0 g/l cream vanilla flavor (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- $^{13}\text{C}_6$]phenylalanine and L-[ring- $^2\text{H}_2$]tyrosine were purchased from Cambridge Isotopes (Andover, MA) and were dissolved in 0.9% saline before infusion. Continuous intravenous infusion (over a period of 6 h, $0.047 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ L-[ring- $^{13}\text{C}_6$]phenylalanine and $0.019 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ L-[ring- $^2\text{H}_2$]tyrosine) of the isotopes was performed using a calibrated IVAC 598 pump (Cardinal Health, Switzerland). Both the phenylalanine and tyrosine pool were primed ($1.89 \mu\text{mol/kg}$ L-[ring- $^{13}\text{C}_6$]phenylalanine and $0.75 \mu\text{mol/kg}$ L-[ring- $^2\text{H}_2$]tyrosine) to enable the calculation of whole body phenylalanine kinetics using established tracer models (37, 38).

Muscle biopsies. Muscle biopsies were obtained from the middle region of the vastus lateralis muscle (15 cm above the patella) and ~2 cm below the entry through the fascia by means of the percutaneous needle biopsy technique described by Bergström et al. (3). The biopsies at $t = 0$ and 120 min were taken through the same incision, with the needle pointing in distal and proximal directions, respectively. As such, the biopsies were taken ~10 cm apart to prevent any influence of the first biopsy on protein turnover in the second biopsy. The post-exercise muscle biopsy was taken from the contralateral leg. All samples were carefully freed from any visible adipose tissue and blood, immediately frozen in liquid nitrogen, and stored at -80°C 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 (Uni Kit III, 07367204; La Roche, Basel, Switzerland) and lactate (19) concentrations were analyzed with a COBAS-FARA semiautomatic analyzer (Roche). Insulin was analyzed by radio immunoassay (Human Insulin RIA kit, Linco Research, St. Charles, MO). Plasma (500 μl) for amino acid analyses was deproteinized on ice with 100 μl of 24% (wt/vol) 5-sulfosalicylic acid and mixed, and the clear supernatant was collected after centrifugation. Plasma amino acid 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 *tert*-butyldimethylsilyl (TBDMS) derivatives, and their ^{13}C and/or ^2H enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS; Agilent 6890N GC/5973N MSD, Wilmington, DE) 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 ^{13}C -labeled tyrosine, respectively.

Muscle analyses. For measurement of L-[ring- $^{13}\text{C}_6$]phenylalanine enrichment in the free amino acid pool and mixed muscle protein, 55 mg of wet muscle was freeze-dried. Collagen, blood, and other nonmuscle 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 ($8 \times$ dry wt of isolated muscle fibers \times wet/dry ratio) of ice-cold 2% perchloric acid (PCA) was 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- $^{13}\text{C}_6$]phenylalanine, L-[ring- $^2\text{H}_2$]tyrosine, and L-[ring- $^{13}\text{C}_6$]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*-phthalaldehyde (43). The protein pellet was washed with three additional 1.5-ml washes using 2% PCA and dried, and the proteins were hydrolyzed in 6 M HCl at 120°C for 15–18 h. The hydrolyzed protein fraction was dried under a nitrogen stream while being heated to 120°C and then dissolved in a 50% acetic acid solution and passed over a Dowex exchange resin (AG 50W-X8, 100–200 mesh hydrogen form; Bio-Rad, Hercules, CA) using 2 M NH₄OH. Thereafter, the eluate was dried, and the purified amino acid fraction was derivatized into the ethoxycarbonyl ethyl esters to determine the ¹³C enrichment of protein-bound phenylalanine by GC-IRMS (Finnigan, MAT 252).

Another portion of the muscle samples was treated and homogenized using a previously described buffer containing several protease inhibitors (6, 26, 28). Primary phosphospecific antibodies [anti-phospho-ACC (Ser⁷⁹), anti-phospho-AMPK (Thr¹⁷²), anti-phospho-mTOR (Ser²⁴⁴⁸), anti-phospho-S6K1 (Thr³⁸⁹), anti-phospho-S6K1 (Thr⁴²¹/Ser⁴²⁴), anti-phospho-S6 (Ser^{235/236}), anti-phospho-eEF2 (Thr⁵⁶), and anti-phospho-4E-BP1 (Thr³⁷)], and anti-ACC, anti-AMPK, anti-mTOR, anti-S6K1, anti-S6, anti-eEF2, and anti-4E-BP1 were purchased from Cell Signaling Technologies (Beverly, MA). Quantification of phosphorylation status of ACC, AMPK, mTOR, S6K1, S6, eEF2, and 4E-BP1 was performed using Western blotting with phosphospecific and aspecific antibodies as previously described, using α -actin as a loading control (26, 28). Phosphorylation was expressed relative to the total amount of each protein.

Calculations. Infusion of L-[ring-¹³C₆]phenylalanine and L-[ring-²H₂]tyrosine with muscle and arterial blood sampling were used to simultaneously assess whole body amino acid kinetics and FSR of mixed muscle protein. Whole body kinetics for phenylalanine and tyrosine were calculated using the equations described by Thompson et al. (38) and Short et al. (37). Briefly, phenylalanine and tyrosine turnover (flux, *Q*) were measured from the isotope dilution at isotopic steady state:

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

where *i* is the isotope infusion rate ($\mu\text{mol} \cdot \text{kg body wt}^{-1} \cdot \text{h}^{-1}$), 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 66% splanchnic extraction rate (24).

Whole body protein synthesis rate was calculated as flux minus oxidation.

$$Q = S + O = B + I \quad (2)$$

$$S = Q - O \quad (3)$$

At isotopic steady state, whole body phenylalanine oxidation can be determined from the conversion (hydroxylation) of L-[ring-¹³C₆]phenylalanine to L-[ring-¹³C₆]tyrosine. The rate of hydroxylation (*Q_{pt}*) was calculated (38) using the formula

$$Q_{pt} = Q_t \cdot \frac{E_t}{E_p} \cdot \frac{Q_p}{(i_p + Q_p)} \quad (4)$$

where *Q_t* and *Q_p* are the flux rates for L-[ring-²H₂]tyrosine and labeled phenylalanine, respectively, *E_t* and *E_p* are the L-[ring-¹³C₆]tyrosine and L-[ring-¹³C₆]phenylalanine enrichments in plasma, respectively, and *i_p* is the infusion rate of the phenylalanine tracer.

Mixed muscle protein FSR was calculated by dividing the increment in enrichment in the product, i.e., protein-bound L-[ring-¹³C₆]phenylalanine, by the enrichment of the precursor. Plasma L-[ring-¹³C₆]phenylalanine and free muscle L-[ring-¹³C₆]phenylalanine enrich-

ments were used to provide an estimate of the lower boundary (based on plasma precursor enrichments) and higher boundary (based on intracellular muscle precursor enrichments) for the true FSR of mixed muscle proteins. Muscle FSRs were calculated as follows (27):

$$\text{FSR} = \frac{\Delta E_p}{E_{\text{precursor}} \cdot t} \cdot 100 \quad (5)$$

where ΔE_p is the Δ increment of protein-bound L-[ring-¹³C₆]phenylalanine during incorporation periods; *E_{precursor}* is 1) the average plasma L-[ring-¹³C₆]phenylalanine enrichment during the time period for determination of amino acid incorporation, 2) the free muscle L-[ring-¹³C₆]phenylalanine enrichment during the time period for determination of amino acid incorporation, and 3) the free muscle L-[ring-¹³C₆]phenylalanine enrichment during the time period for determination of amino acid incorporation corrected for contribution of extracellular water (45); *t* indicates the time interval (h) between biopsies; and the factor 100 is needed to express the FSR in percent per hour (%/h).

Statistics. All data are expressed as means \pm SE. The plasma insulin, glucose, and amino acid responses were calculated as area under the curve minus baseline values. A two-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* ratios, Bonferroni post hoc tests were applied to locate the differences. For non-time-dependent variables, a paired Student's *t*-test was used to compare differences in treatment effect. Statistical significance was set at *P* < 0.05. All calculations were performed using SPSS Statistics 15.0 (SPSS, Chicago, IL).

RESULTS

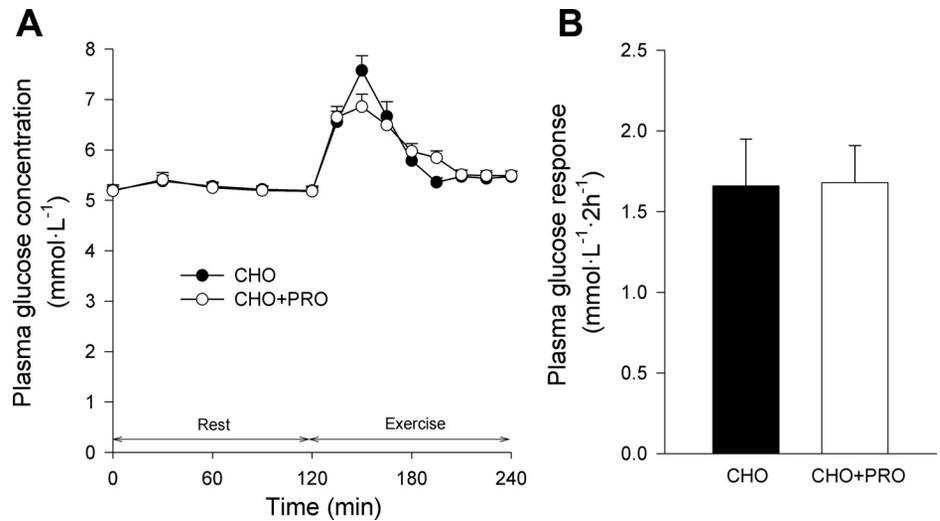
Exercise. The workload for the cycling protocol was set at 185 ± 4 W (55% *W*_{max}). Not all subjects were able to complete the entire 2-h exercise session at 55% *W*_{max}. In 8 of the 12 subjects, workload was reduced to 50% *W*_{max} during the latter stages of the exercise trial. The latter was repeated identically during the second experimental day for each individual subject. The average workload applied in the exercise protocol was 179 ± 4 W.

Plasma analyses. At rest, plasma glucose concentrations averaged 5.3 ± 0.1 and 5.2 ± 0.1 mmol/l in the CHO and CHO+PRO treatments, respectively. Plasma glucose concentrations increased during the first 30 min of exercise to 7.6 ± 0.3 and 6.9 ± 0.2 mmol/l in CHO and CHO+PRO, respectively, after which they returned to baseline values (Fig. 1A). Plasma glucose responses, measured as area under the curve minus baseline values, averaged 1.66 ± 0.29 and 1.68 ± 0.23 mmol \cdot l⁻¹ \cdot 2 h during exercise for the CHO and CHO+PRO treatments, respectively (Fig. 1B). No significant differences were observed between treatments.

Plasma insulin concentrations averaged 4.5 ± 0.5 and 4.5 ± 0.5 mU/l at rest and increased to 17.1 ± 2.7 and 22.3 ± 5.3 mU/l during the first 30 min of exercise in the CHO and CHO+PRO treatments, respectively, returning to baseline values (Fig. 2A). There were significant differences between treatments over time (*P* < 0.05). Total plasma insulin responses, measured as area under the curve minus baseline values, were greater in CHO+PRO compared with CHO (9.1 ± 1.8 and 18.1 ± 4.1 mU \cdot l⁻¹ \cdot 2 h for CHO and CHO+PRO, respectively, *P* < 0.05; Fig. 2B).

Plasma lactate concentrations averaged 0.66 ± 0.03 and 0.68 ± 0.05 mmol/l at rest and increased to 3.0 ± 0.3 and 3.0 ± 0.2 mmol/l after 15 min of exercise in CHO and CHO+PRO,

Fig. 1. Plasma glucose concentrations (mmol/l) over time (A) and plasma glucose responses (mmol·l⁻¹·2 h), measured as area under the curve minus baseline values, during exercise (B) in the carbohydrate (CHO) and carbohydrate + protein (CHO+PRO) treatments. Values are means ± SE. Data were analyzed with repeated-measures ANOVA (treatment × time).



respectively, after which concentrations declined to 1.6 ± 0.2 and 1.5 ± 0.2 mmol/l.

Plasma amino acid concentrations during rest and exercise are listed in Table 1. In general, plasma amino acid responses during exercise were higher in the CHO+PRO compared with CHO treatment. Plasma phenylalanine and tyrosine concentrations increased during exercise in both treatments. Plasma leucine concentrations increased during exercise in the CHO+PRO treatment only. The time course of changes in plasma L-[ring-¹³C₆]phenylalanine, L-[ring-²H₂]tyrosine, and L-[ring-¹³C₆]tyrosine enrichments are presented in Fig. 3. Overall, enrichments were significantly lower during exercise in the CHO+PRO compared with the CHO treatment ($P < 0.01$).

Plasma amino acid concentrations and tracer enrichments were used to calculate whole body protein kinetics. At rest, in the fasting state plasma amino acid kinetics were similar in both trails. Whole body protein breakdown (54.6 ± 2.2 and 56.0 ± 1.8 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), oxidation (6.7 ± 0.4 and 7.6 ± 0.5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), synthesis (49.3 ± 1.9 and 50.2 ± 1.5 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), and net balance (-5.3 ± 0.3 and -5.8 ± 0.3 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) were nearly identical under basal conditions in the CHO and CHO+PRO experiment.

Following CHO or CHO+PRO supplementation during exercise, whole body protein synthesis (46.2 ± 1.3 vs. 56.5 ± 1.8 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) and oxidation rates (6.0 ± 0.4 vs. 10.1 ± 1.2 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) were higher in the CHO+PRO compared with the CHO experiment ($P < 0.01$). Protein breakdown did not differ between treatments (51.1 ± 1.5 vs. 48.4 ± 2.1 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in CHO and CHO+PRO, respectively, $P = 0.06$). As a consequence, whole body protein net balance was slightly negative in CHO and positive in CHO+PRO (-4.9 ± 0.3 vs. 8.0 ± 0.3 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, $P < 0.01$; Fig. 4).

Muscle analyses. Muscle amino acid concentrations and enrichments are presented in Table 2. Muscle free phenylalanine and tyrosine concentrations had decreased following 2 h of fasted rest and subsequently increased during 2 h of cycling with both CHO and CHO+PRO ingestion with no differences between treatments. Similar changes in muscle free leucine concentrations did not reach statistical significance. Muscle free L-[ring-¹³C₆]phenylalanine enrichment was similar over time and between treatments. Muscle protein L-[ring-¹³C₆]phenylalanine enrichment increased during exercise compared with rest, with no differences between the CHO and CHO+PRO treatments. Mixed muscle protein FSR at rest, using mean plasma L-[ring-¹³C₆]phenylalanine enrichment as the precursor

Fig. 2. Plasma insulin concentrations (mU/l) over time (A) and plasma insulin responses (mU·l⁻¹·2 h), measured as area under the curve minus baseline values, during exercise (B) in the CHO and CHO+PRO treatments. Values are means ± SE. Data were analyzed with repeated-measures ANOVA (treatment × time).

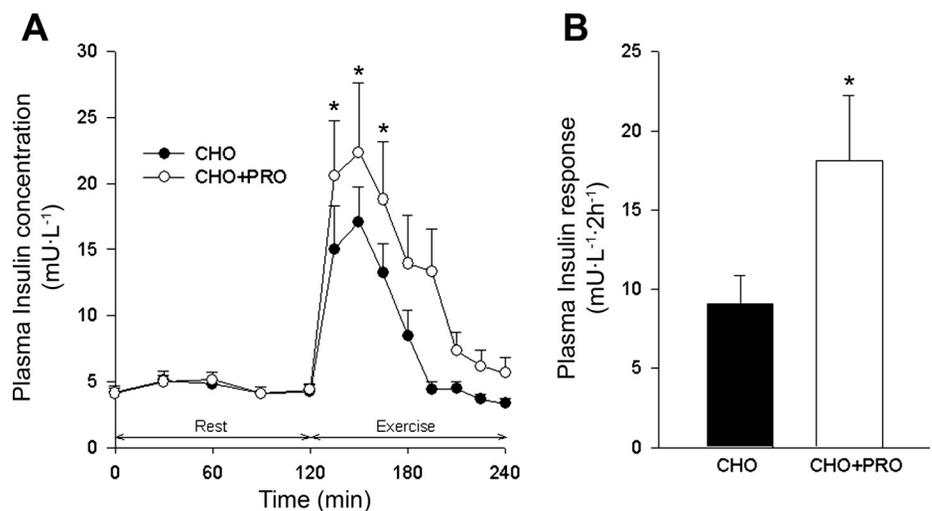


Table 1. Plasma amino acid concentrations

Amino acid	CHO												CHO+PRO											
	Rest				Exercise				AUC	Rest				Exercise				AUC						
	0	60	120	150	180	210	240	AUC	0	60	120	150	180	210	240	AUC								
t, min	40 ± 2	38 ± 2	38 ± 2	44 ± 2	48 ± 2	49 ± 2	49 ± 2	15 ± 2	40 ± 1	39 ± 1	39 ± 1	50 ± 2	56 ± 2	59 ± 2	60 ± 2	29 ± 2*								
Phenylalanine	50 ± 3	48 ± 2	50 ± 2	57 ± 2	60 ± 2	64 ± 3	63 ± 3	19 ± 3	54 ± 2	52 ± 2	52 ± 2	70 ± 3	86 ± 3	102 ± 4	107 ± 6	65 ± 4*								
Tyrosine	112 ± 6	109 ± 6	104 ± 6	115 ± 7	109 ± 7	107 ± 7	102 ± 7	9 ± 5	106 ± 5	103 ± 5	102 ± 5	148 ± 5	166 ± 7	179 ± 6	181 ± 5	113 ± 8*								
Leucine	231 ± 4	222 ± 5	216 ± 5	227 ± 5	223 ± 5	218 ± 6	208 ± 7	8 ± 7	222 ± 4	218 ± 5	213 ± 5	264 ± 5	298 ± 7	322 ± 8	327 ± 6	150 ± 10*								
Valine	52 ± 2	51 ± 3	48 ± 2	54 ± 3	53 ± 3	52 ± 3	49 ± 3	8 ± 3	53 ± 2	52 ± 2	51 ± 2	79 ± 2	91 ± 3	97 ± 3	99 ± 2	69 ± 5*								
Isoleucine	88 ± 4	98 ± 4	92 ± 4	106 ± 5	99 ± 5	108 ± 6	111 ± 7	22 ± 11	87 ± 4	96 ± 3	99 ± 7	117 ± 6	130 ± 7	127 ± 8	139 ± 8	48 ± 12*								
Glutamic acid	58 ± 1	56 ± 2	57 ± 2	58 ± 3	60 ± 2	60 ± 4	56 ± 4	4 ± 2	59 ± 2	57 ± 2	54 ± 2	67 ± 3	74 ± 3	74 ± 3	71 ± 3	30 ± 4*								
Asparagine	120 ± 5	120 ± 5	121 ± 5	115 ± 7	117 ± 5	119 ± 6	114 ± 6	-6 ± 3	121 ± 4	121 ± 5	121 ± 5	128 ± 4	138 ± 5	140 ± 6	141 ± 6	28 ± 5*								
Serine	560 ± 18	546 ± 12	537 ± 15	605 ± 15	609 ± 17	613 ± 26	572 ± 26	117 ± 18	558 ± 22	548 ± 21	540 ± 19	622 ± 22	649 ± 29	670 ± 32	643 ± 27	187 ± 23*								
Glutamine	64 ± 6	63 ± 7	69 ± 5	78 ± 7	80 ± 5	77 ± 8	76 ± 6	15 ± 5	70 ± 4	74 ± 5	73 ± 3	88 ± 6	98 ± 5	84 ± 8	85 ± 7	28 ± 9								
Histidine	225 ± 8	223 ± 9	223 ± 11	239 ± 14	246 ± 11	249 ± 12	243 ± 13	38 ± 10	233 ± 11	229 ± 12	228 ± 10	244 ± 11	254 ± 11	256 ± 11	244 ± 12	40 ± 6								
Glycine	134 ± 4	133 ± 6	134 ± 5	134 ± 7	132 ± 6	132 ± 7	130 ± 6	-2 ± 5	135 ± 5	132 ± 7	129 ± 6	146 ± 7	162 ± 8	166 ± 9	178 ± 12	56 ± 6*								
Threonine	32 ± 3	30 ± 3	29 ± 3	28 ± 2	27 ± 2	27 ± 2	27 ± 2	-3 ± 2	28 ± 1	27 ± 2	30 ± 2	31 ± 3	36 ± 3	39 ± 3	39 ± 3	11 ± 2*								
Citrulline	99 ± 5	94 ± 5	95 ± 5	115 ± 9	121 ± 10	116 ± 10	105 ± 11	37 ± 9	94 ± 4	91 ± 4	90 ± 4	130 ± 7	144 ± 8	145 ± 9	141 ± 7	87 ± 9*								
Arginine	278 ± 10	243 ± 11	243 ± 12	424 ± 21	423 ± 27	411 ± 29	360 ± 32	295 ± 33	274 ± 13	242 ± 16	242 ± 15	453 ± 22	468 ± 23	460 ± 24	410 ± 23	369 ± 22*								
Alanine	116 ± 5	97 ± 9	110 ± 8	142 ± 11	138 ± 13	155 ± 13	151 ± 15	64 ± 17	120 ± 6	101 ± 8	111 ± 7	153 ± 13	167 ± 13	165 ± 16	138 ± 14	82 ± 12								
Taurine	19 ± 1	20 ± 1	20 ± 1	18 ± 1	17 ± 1	16 ± 1	15 ± 1	-6 ± 1	19 ± 1	19 ± 1	20 ± 1	19 ± 1	19 ± 1	19 ± 1	18 ± 1	-2 ± 1*								
α-aminobutyrate	31 ± 1	29 ± 1	31 ± 1	35 ± 1	38 ± 2	38 ± 2	36 ± 2	11 ± 2	32 ± 1	31 ± 1	31 ± 1	42 ± 1	49 ± 2	52 ± 2	52 ± 2	29 ± 2*								
Methionine	29 ± 2	29 ± 2	25 ± 2	28 ± 2	34 ± 2	34 ± 2	35 ± 2	13 ± 2	31 ± 2	31 ± 2	30 ± 2	34 ± 1	38 ± 1	42 ± 1	43 ± 2	16 ± 2								
Tryptophan	51 ± 3	50 ± 3	50 ± 3	46 ± 3	46 ± 3	44 ± 3	43 ± 3	-8 ± 3	50 ± 3	50 ± 3	50 ± 3	54 ± 2	60 ± 3	62 ± 3	62 ± 3	15 ± 4*								
Ornithine	173 ± 6	166 ± 7	160 ± 6	168 ± 6	173 ± 8	178 ± 8	162 ± 8	20 ± 7	176 ± 7	171 ± 9	170 ± 7	224 ± 8	246 ± 14	260 ± 14	261 ± 13	133 ± 14*								
Lysine																								

Plasma amino acid concentrations at rest and during exercise following CHO or CHO+PRO administration. Values are expressed as means ± SE (μmol/l). CHO, carbohydrate treatment; CHO+PRO, carbohydrate and protein treatment; AUC, area under the curve (during exercise) minus baseline values (t = 120 min) (in μmol · l⁻¹ · 2 h); *Significantly different from CHO (P < 0.05).

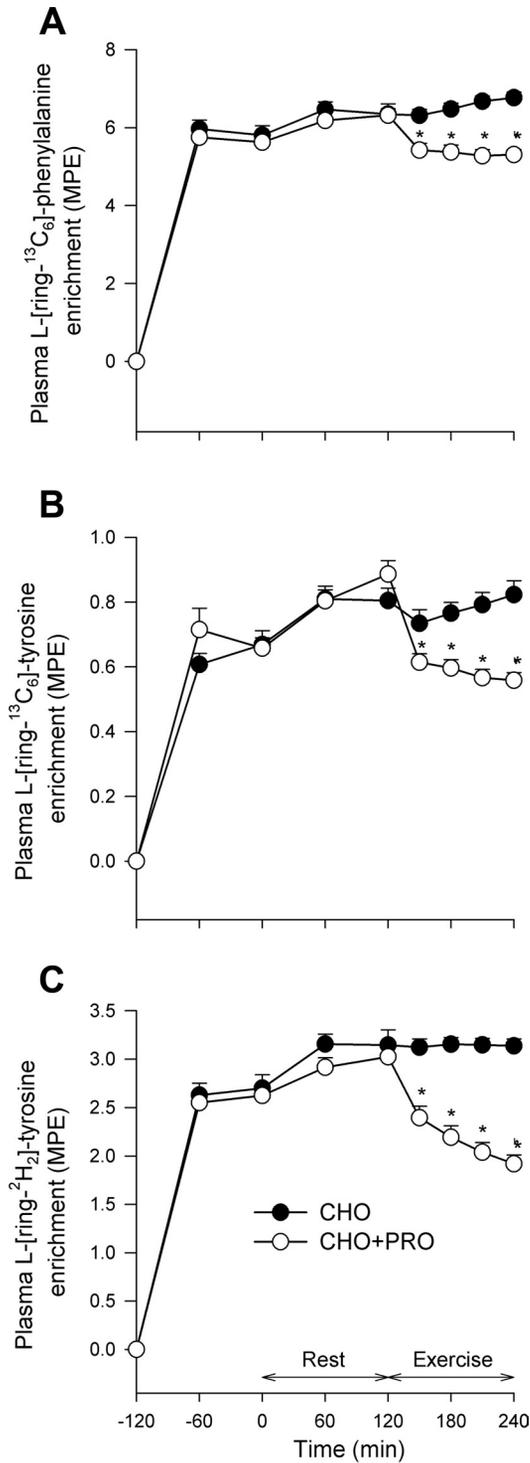


Fig. 3. Plasma L-[ring-¹³C₆]phenylalanine (A), L-[ring-¹³C₆]tyrosine (B), and L-[ring-²H₂]tyrosine (C) enrichment in CHO and CHO+PRO treatments. MPE, mole percent excess. Values represent means ± SE. Data were analyzed with repeated-measures ANOVA (treatment × time). Plasma L-[ring-¹³C₆]phenylalanine enrichment: treatment effect, *P* < 0.01; time effect, *P* < 0.01; 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. Plasma L-[ring-²H₂]tyrosine enrichment: treatment effect, *P* < 0.01; time effect, *P* < 0.01; interaction of treatment and time, *P* < 0.01. *Significantly different from CHO (Bonferroni post hoc test, *P* < 0.05).

sor, was 0.035 ± 0.006 and $0.038 \pm 0.005\%/h$ in the CHO and CHO+PRO experiments, respectively (*P* > 0.05). During exercise, following CHO or CHO+PRO administration, FSR increased to 0.058 ± 0.006 and $0.070 \pm 0.011\%/h$ in the CHO and the CHO+PRO experiments, respectively (Fig. 5). There was a significant increase in FSR during exercise compared with rest (*P* < 0.05), with no differences between treatments. (*P* = 0.46).

When the free intracellular L-[ring-¹³C₆]phenylalanine enrichment was used as precursor (*n* = 10), FSR values were higher but revealed the same intervention effect (exercise). FSR values averaged 0.050 ± 0.013 and $0.062 \pm 0.009\%/h$ at rest and 0.075 ± 0.007 and $0.096 \pm 0.018\%/h$ during exercise in the CHO and CHO+PRO treatments, respectively.

The changes in the phosphorylation status of ACC, AMPK, mTOR, S6K1 (Thr³⁸⁹), S6K1 (Thr⁴²¹/Ser⁴²⁴), S6, eEF2, and 4E-BP1 during endurance type exercise in the CHO and CHO+PRO treatments are presented in Fig. 6. No significant differences in phosphorylation status of mTOR, S6K1 (Thr⁴²¹/Ser⁴²⁴), S6, eEF2, and 4E-BP1 were observed between the CHO and CHO+PRO treatments over time. The phosphorylation status of ACC, AMPK, and S6K1 (Thr³⁸⁹) increased significantly over time, with no differences between treatments.

DISCUSSION

The present study shows that muscle protein synthesis rates are greater during continuous endurance type exercise while one is ingesting carbohydrate or carbohydrate plus protein compared with basal fasting conditions. Protein coingestion during exercise improves whole body protein synthesis and net protein balance but does not further augment mixed muscle protein synthesis rates during exercise.

Few studies have examined the impact of endurance (9, 25, 47, 48) or resistance (2, 13, 14, 16, 42) type exercise on whole

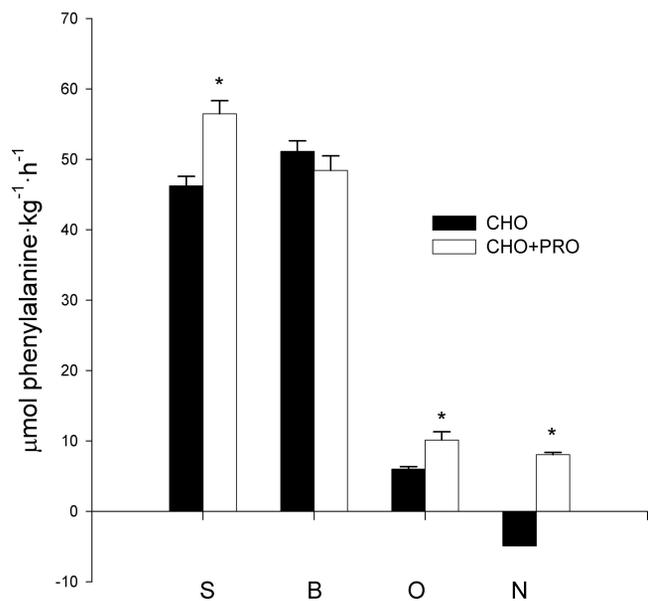


Fig. 4. whole body protein synthesis (S), breakdown (B), oxidation (O) rates, and net protein balance (N) during exercise, expressed as $\mu\text{mol Phe} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. Values represent means ± SE. Data were analyzed with Student's *t*-test for paired samples. *Significantly different from CHO (*P* < 0.05).

Table 2. Muscle amino acid concentrations, tracer enrichments and mixed muscle fractional synthetic rate

	CHO			CHO+PRO			ANOVA Treatment x Time
	0 min	120 min	240 min	0 min	120 min	240 min	
Muscle AA concentrations, μmol/l							
Phenylalanine	164 ± 12	135 ± 12*	168 ± 30	199 ± 30	147 ± 12*	179 ± 15	P = 0.71
Tyrosine	90 ± 8	78 ± 4*	115 ± 11*	103 ± 9	82 ± 5*	154 ± 9*	P = 0.08
Leucine	159 ± 28	139 ± 23	149 ± 28	142 ± 26	123 ± 24	164 ± 33	P = 0.33
Muscle AA enrichments (TTR)							
L-[ring- ¹³ C ₆]phenylalanine	0.0418 ± 0.004	0.0425 ± 0.002	0.0590 ± 0.001	0.0453 ± 0.009	0.0402 ± 0.002	0.0479 ± 0.001	P = 0.19
L-[ring- ² H ₃]tyrosine	0.0164 ± 0.001	0.0200 ± 0.002	0.0240 ± 0.001	0.0240 ± 0.010	0.0169 ± 0.001	0.0154 ± 0.001	P = 0.17
L-[ring- ¹³ C ₆]tyrosine	0.0174 ± 0.005	0.0158 ± 0.003	0.0162 ± 0.002	0.0153 ± 0.003	0.0182 ± 0.004	0.0108 ± 0.002	P = 0.24
Muscle protein enrichment (TTR)							
L-[ring- ¹³ C ₆]phenylalanine	0	0.000046 ± 0.00001*	0.000127 ± 0.00001*	0	0.000049 ± 0.00001*	0.000131 ± 0.00001*	P = 0.97
δ Enrichment muscle protein	0.0000464 ± 0.0000090	0.0000810 ± 0.0000087*	0.0000810 ± 0.0000087*	0.0000487 ± 0.0000069	0.0000826 ± 0.0000132*	0.0000826 ± 0.0000132*	P = 1.00
FSR, %/h							
Plasma precursor		0.035 ± 0.006	0.058 ± 0.006*		0.038 ± 0.005	0.070 ± 0.011*	P = 0.46
Muscle precursor		0.050 ± 0.013	0.075 ± 0.007		0.062 ± 0.009	0.096 ± 0.018	P = 0.68

Values are expressed as means ± SE. TTR, tracer-to-tracee ratio; Muscle AA concentrations represent the average AA concentration in muscle biopsies taken before and after 2-h rest or exercise period. Muscle AA enrichments represent the average AA enrichment in muscle biopsies taken before and after 2-h rest or exercise period; Muscle protein enrichments represent the average tracer enrichment in muscle protein in muscle biopsies taken before and after 2-h rest or exercise period; δ enrichment muscle protein represents the increment in muscle protein enrichment between the 2 biopsies before and after the rest or exercise period. FSR represents the average muscle fractional synthesis rates during 2-h rest or exercise. *Significantly different from rest (P < 0.05).

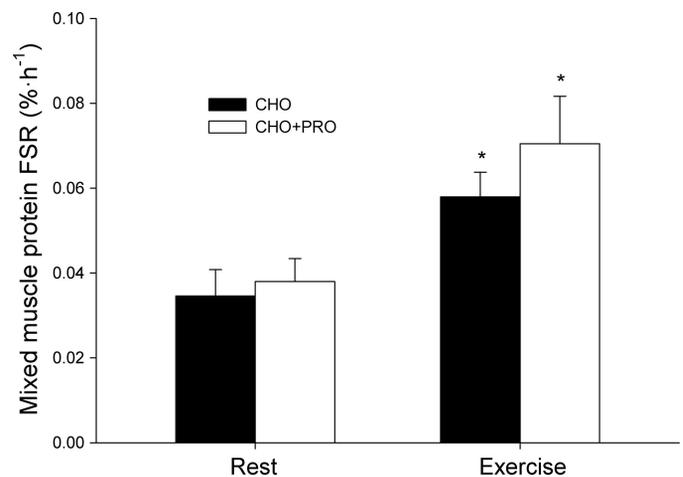


Fig. 5. Fractional synthesis rate (FSR) of mixed muscle protein at rest following an overnight fast and during exercise when CHO or CHO+PRO were ingested. Values represent means ± SE. Data were analyzed with repeated-measures ANOVA. *Significantly different from rest (P < 0.05).

body and/or skeletal muscle protein synthesis rates during exercise conditions. Most of these studies have reported either a decrease (13, 47, 48) or no effect (9, 14, 48) of exercise on muscle protein synthesis rates. In the present study, we clearly show that muscle protein synthesis rates are stimulated during 2 h of continuous endurance type exercise compared with basal fasting muscle protein synthesis rates (0.064 ± 0.006 vs. 0.036 ± 0.004%/h, P < 0.01). Although we may speculate that the greater exercise intensity applied in our study (~60% as opposed to ~30–40% $\dot{V}O_{2max}$ in previous studies) is responsible for the apparent discrepancy in the literature, we believe that this is more likely attributed to the fact that previous studies (9, 13, 14, 47, 48) were all performed in a fasted state. In the present study, we provided the subjects with either carbohydrate (1.0 g·kg⁻¹·h⁻¹) or carbohydrate plus protein (0.8 plus 0.2 g·kg⁻¹·h⁻¹) during 2 h of cycling exercise. Surprisingly, muscle protein synthesis rates were higher during exercise in both the CHO+PRO and CHO trials compared with fasted resting conditions, the latter despite previous findings showing that carbohydrate ingestion does not stimulate muscle protein synthesis (7, 20, 23, 31). We speculate that the greater muscle protein synthesis rates observed during endurance type exercise compared with basal resting values are attributed to the increased provision of plasma-derived amino acids to the working muscle. This hypothesis is supported by the observed increase in muscle free amino acid concentrations during the 2-h cycling exercise bout in both experiments (Table 2). Higher circulating plasma insulin concentrations and increased perfusion have been reported to elevate plasma amino acid delivery to the working muscle (17, 39). The latter suggests that the increase in circulating plasma insulin levels in both the CHO and CHO+PRO experiments, combined with the greater muscle blood flow during exercise, allows for a greater provision of amino acids to the working muscle, thereby stimulating muscle protein synthesis. Furthermore, it could be speculated that the increase in muscle protein synthesis rate during exercise is attributed to the stimulating effect of insulin on muscle protein synthesis itself, rather than a to change in amino acid delivery to the working muscle. Insulin stimulates muscle protein synthesis via the Akt-GSK3-eIF2B pathway (30).

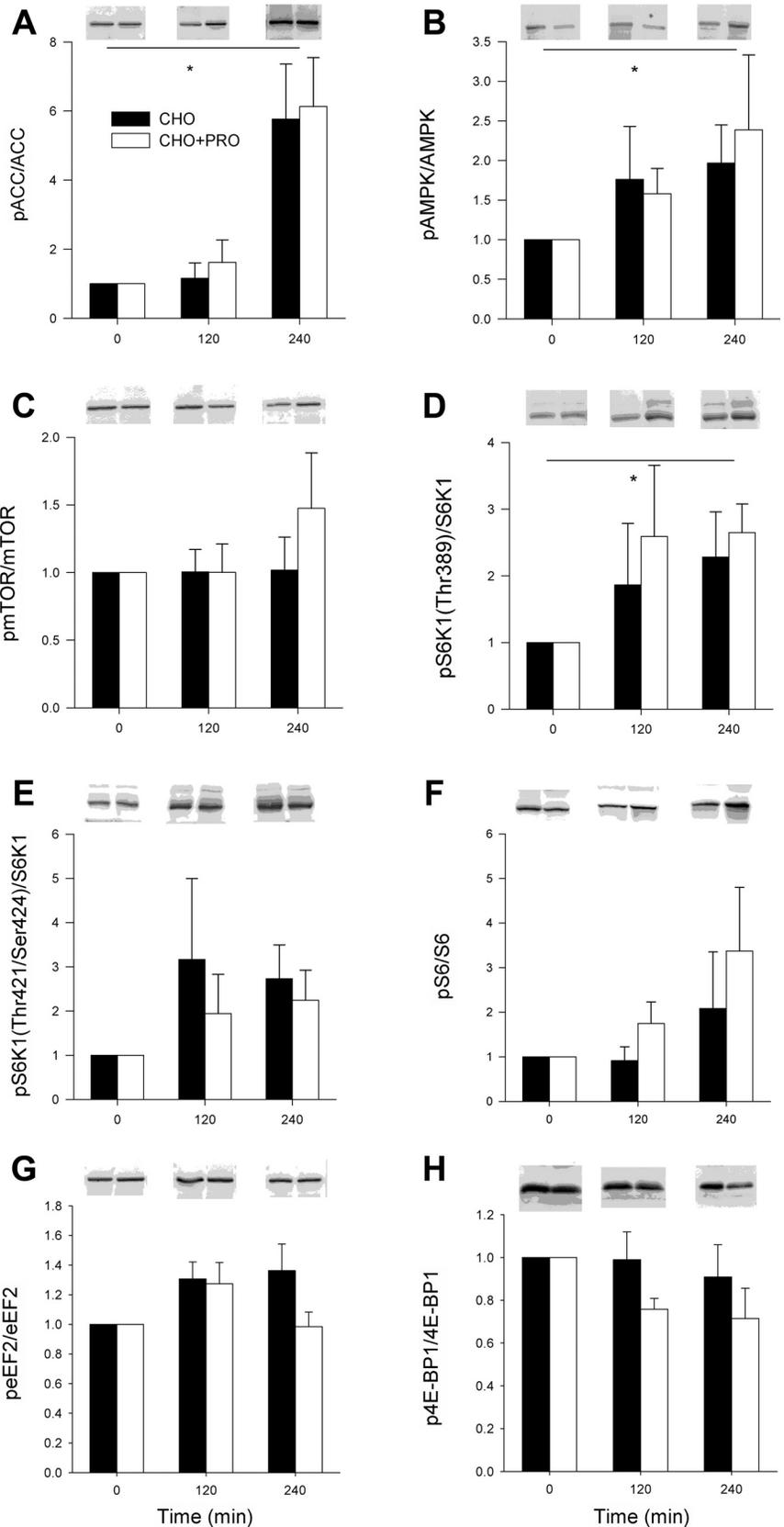


Fig. 6. ACC phosphorylation (A), AMPK phosphorylation (B), mTOR phosphorylation (C), S6K1 (Thr³⁸⁹) phosphorylation (D), S6K1 (Thr⁴²¹/Ser⁴²⁴) phosphorylation (E), S6 phosphorylation (F), eEF2 phosphorylation (G), and 4E-BP1 phosphorylation (H) 2 h before (*t* = 0), prior to (*t* = 120 min), and immediately after (*t* = 240 min) cessation of exercise, when CHO or CHO+PRO were ingested during exercise. Values represent means ± SE and are presented as phosphorylated protein/total protein relative to the amount at *t* = 0. Representative blots of phosphorylated proteins are shown above the bars. Data were analyzed with repeated-measures ANOVA. ACC: treatment effect, *P* = 0.804; time effect, *P* = 0.001; interaction of treatment and time, *P* = 0.973. AMPK: treatment effect, *P* = 0.907; time effect, *P* = 0.044; interaction of treatment and time, *P* = 0.834. mTOR: treatment effect, *P* = 0.524; time effect, *P* = 0.298; interaction of treatment and time, *P* = 0.546. S6K1 (Thr³⁸⁹): treatment effect, *P* = 0.665; time effect, *P* = 0.031; interaction of treatment and time, *P* = 0.845. S6K1 (Thr⁴²¹/Ser⁴²⁴): treatment effect, *P* = 0.639; time effect, *P* = 0.063; interaction of treatment and time, *P* = 0.763. S6: treatment effect, *P* = 0.376; time effect, *P* = 0.090; interaction of treatment and time, *P* = 0.648. eEF2: treatment effect, *P* = 0.304; time effect, *P* = 0.0053; interaction of treatment and time, *P* = 0.190. 4E-BP1: treatment effect, *P* = 0.137; time effect, *P* = 0.308; interaction of treatment and time, *P* = 0.255. *Significant increase over time.

However, while muscle protein synthesis does not occur in the absence of insulin (15), the impact of increasing insulin concentrations on muscle protein turnover is believed to be more related to a decrease in muscle protein breakdown rather than to stimulation of muscle protein synthesis (18, 23).

We (2) recently showed that ingestion of protein with carbohydrate during exercise stimulates muscle protein synthesis during resistance type exercise activities. The latter seems to be in contrast with our current findings in which the same type and amount of protein was coingested during endurance type exercise. It might be speculated that the apparent discrepancy is related to the intermittent character of the resistance type exercise, in which specific muscle groups of the lower limbs could rest during periods of exercise performed by the upper limbs. This is supported by the higher muscle FSR rates that we observed during resistance type exercise in our previous study compared with endurance type exercise in the present study following carbohydrate ingestion (0.060 vs. 0.058%/h), but more particularly when coingesting protein (0.088 vs. 0.070%/h). The latter comparison clearly shows that muscle protein synthesis rates can be further increased during intermittent resistance, as opposed to endurance, type exercise when dietary protein is provided.

It is generally assumed that the energy consuming process of muscle protein synthesis is blunted during exercise activities (1, 6, 11, 12, 28), which is supported by an exercise-induced increase in muscle AMPK and eEF2 and decrease in 4E-BP1 phosphorylation and subsequent inhibition of muscle protein synthesis (6, 12, 28, 34). Therefore, it has been proposed that these signaling proteins play an important role in the regulation of muscle protein synthesis. In the present study, we show a substantial increase in muscle protein FSR during continuous endurance type exercise when one is fed carbohydrate with or without protein. The latter occurred despite a small but significant increase in skeletal muscle AMPK and ACC phosphorylation status. The latter implies that AMPK activation does not (completely) inhibit muscle protein synthesis during exercise performed in the fed state. Furthermore, the elevation in FSR during exercise was accompanied by an increase in S6K1 phosphorylation, which is a downstream target of mTOR and controls the rate of translation initiation by regulating the binding of mRNA to the ribosomal subunits (22). Our observations clearly show that AMPK phosphorylation during exercise does not necessarily block the mTOR pathway, thereby preventing muscle protein synthesis from being stimulated. Furthermore, we do not confirm the increase in eEF2 phosphorylation and decrease in 4E-BP1 phosphorylation, as were previously reported during exercise in the overnight-fasted state (13, 34). As the elongation phase accounts for the majority of energy consumed during protein synthesis, it could be speculated that eEF2 phosphorylation increases during exercise in an energy-depleted (34) but not when exercise is performed in a fed state. The latter might explain the discrepancy between these studies.

The main aim of this study was to examine whether carbohydrate supplementation with or without protein stimulates muscle protein FSR during exercise. However, in practice, exercise is generally performed in a fed, as opposed to an overnight-fasted, state. It could be speculated that consumption of a preexercise meal will already initiate an increase in muscle protein synthesis and, as such, prevent any further increase

during exercise. However, on the basis of our previous work in which a standardized breakfast was provided prior to a single-legged exercise session (46), we speculate that local muscle contraction will further augment FSR during exercise conditions. Future research is warranted to examine the practical relevance of nutritional supplementation during exercise and should also try to quantify the differential impacts of local muscle contraction, insulin release, increased muscle perfusion, and greater amino acid delivery to the working muscle.

In conclusion, this is the first study to show that muscle protein synthesis is stimulated during endurance type exercise activities when carbohydrate or carbohydrate plus protein are ingested compared with basal, fasting muscle protein synthesis rates. Protein coingestion during exercise does not further augment muscle protein synthesis during endurance type exercise activities. The increase in muscle protein synthesis rate during endurance type exercise is accompanied by activation of both the AMPK and mTOR signaling pathways.

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DISCLOSURES

No conflicts of interest are reported by the authors.

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