

## Co-ingestion of leucine with protein does not further augment post-exercise muscle protein synthesis rates in elderly men

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Leucine has been suggested to have the potential to modulate muscle protein metabolism by increasing muscle protein synthesis. The objective of this study was to investigate the surplus value of the co-ingestion of free leucine with protein hydrolysate and carbohydrate following physical activity in elderly men. Eight elderly men (mean age  $73 \pm 1$  years) were randomly assigned to two cross-over treatments consuming either carbohydrate and protein hydrolysate (CHO + PRO) or carbohydrate, protein hydrolysate with additional leucine (CHO + PRO + leu) after performing 30 min of standardized physical activity. Primed, continuous infusions with L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine and L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine were applied, and blood and muscle samples were collected to assess whole-body protein turnover as well as protein fractional synthetic rate in the *vastus lateralis* muscle over a 6 h period. Whole-body protein breakdown and synthesis rates were not different between treatments. Phenylalanine oxidation rates were significantly lower in the CHO + PRO + leu v. CHO + PRO treatment. As a result, whole-body protein balance was significantly greater in the CHO + PRO + leu compared to the CHO + PRO treatment (23.8 (SEM 0.3) v. 23.2 (SEM 0.3)  $\mu\text{mol/kg}$  per h, respectively;  $P < 0.05$ ). Mixed muscle fractional synthetic rate averaged 0.081 (SEM 0.003) and 0.082 (SEM 0.006) %/h in the CHO + PRO + leu and CHO + PRO treatment, respectively (NS). Co-ingestion of leucine with carbohydrate and protein following physical activity does not further elevate muscle protein fractional synthetic rate in elderly men when ample protein is ingested.

### Protein metabolism: Sarcopaenia: Muscle: Ageing

Ageing is associated with a gradual loss of skeletal muscle mass, often referred to as sarcopaenia<sup>1</sup>. These age-related changes in skeletal muscle mass are attributed to a disruption in the regulation of skeletal muscle protein synthesis and/or degradation<sup>2</sup>. Protein turnover in skeletal muscle tissue is highly responsive to nutrient intake in healthy, young individuals<sup>3</sup>. In the elderly, the muscle protein synthetic response to food intake seems to be blunted<sup>4</sup>, which is likely due to impaired anabolic signalling in skeletal muscle tissue<sup>5,6</sup>. The latter has been proposed to represent a key factor in the aetiology of sarcopaenia.

In addition to food intake, physical activity can effectively modulate muscle protein metabolism, stimulating both muscle protein synthesis and breakdown<sup>7</sup>. However, post-exercise net protein balance will remain negative in the absence of food intake<sup>8</sup>. Recently, we reported that co-ingestion of protein and

leucine with carbohydrate following physical activity can increase muscle protein synthesis to the same extent in young and elderly lean men<sup>9</sup>. The latter indicates that the combined ingestion of carbohydrate and protein with additional free leucine might indeed represent an effective strategy to further increase muscle protein synthesis and/or to inhibit protein degradation following physical activity<sup>10</sup>. A direct stimulating effect of leucine administration on muscle protein synthesis has been reported previously in rodents<sup>11–14</sup>. In line with those data, Anthony *et al.*<sup>15</sup> reported that leucine supplementation enhances muscle protein synthesis in diabetic rats via activation of insulin-independent mechanisms. Follow-up studies have shown that leucine has the ability to function as a nutritional signalling molecule that stimulates muscle protein synthesis at the level of translation initiation through the activation of mTOR<sup>16</sup>. In addition, leucine has also been shown to have the potential to

**Abbreviations:** AUC, area under the curve; EAA, essential amino acid; FSR, fractional synthetic rate; CHO + PRO, carbohydrate and protein hydrolysate; CHO + PRO + leu, carbohydrate, protein hydrolysate and additional free leucine.

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affect muscle protein metabolism by decreasing the rate of protein degradation<sup>17</sup>, most likely by stimulating insulin secretion. In previous studies, we have shown that the combined ingestion of carbohydrate, protein and leucine is more effective than the ingestion of only carbohydrate in stimulating muscle protein synthesis *in vivo* in man<sup>9,10</sup>. More recent data suggest that the intake of amino acid mixtures or proteins with additional leucine can further enhance muscle protein synthesis in the elderly<sup>18,19</sup>. However, the proposed surplus value of leucine co-ingestion under normal living conditions, in which physical activity is followed by food intake, has not yet been assessed. We hypothesized that additional co-ingestion of leucine together with carbohydrate and protein improves whole-body protein balance and further augments muscle protein synthesis rates following physical activity in the elderly.

In the present study, we determined the potential surplus value of free leucine co-ingestion on post-exercise muscle protein synthesis in elderly men (about 75 years old) under conditions where large amounts of whey protein and carbohydrate are being ingested. Continuous intravenous infusions with L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine and L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine were combined with plasma and skeletal muscle tissue sampling to simultaneously measure whole-body protein balance as well as muscle protein fractional synthetic rates *in vivo* in elderly men.

## Methods

### Subjects

Eight healthy, lean elderly men (mean age 73 ± 1 years), with normal glucose tolerance and no history of participating in any regular exercise training programme, were selected to participate in the present study. Subjects' characteristics are shown in Table 1. All subjects were informed about the nature and risks of the experimental procedure before their written informed consent to participate was obtained. The study was approved by the local Medical Ethical Committee.

### Pre-testing

Before selection in the study, all volunteers were subjected to an oral glucose tolerance test<sup>20</sup>. Leg volume was determined as described previously<sup>21</sup>, after which all subjects participated

in an orientation test to become familiarized with the physical activity protocol and the equipment. Proper lifting technique was demonstrated and practised for each of the two lower-limb exercises (leg press and leg extension). Subsequently, maximal strength (one-repetition maximum) was estimated using the multiple repetitions testing procedure<sup>22</sup>.

### Diet and activity prior to testing

All subjects consumed a standardized meal (64.1 (SEM 2.0) kJ/kg body weight, consisting of (energy %): carbohydrate, 65, protein, 15 and fat, 20) the evening before the tests. All volunteers were instructed to refrain from any sort of heavy physical exercise and to keep their diet as constant as possible 3 d before the tests. In addition, subjects were asked to record their food intake for 48 h before the start of the first test and to consume the same diet 48 h before the start of the second test.

### Experimental tests

Each subject participated in two tests, separated by 7 d, in which drinks containing carbohydrate and protein hydrolysate (CHO + PRO) or carbohydrate, protein hydrolysate and additional free leucine (CHO + PRO + leu) were administered in a randomized and double-blind fashion. Each test lasted approximately 8 h. Repeated boluses of a given test-drink were ingested following the physical activity protocol to ensure a continuous supply of glucose and amino acids. Plasma and muscle samples were collected during a 6 h period. These 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-<sup>13</sup>C<sub>6</sub>]phenylalanine in the mixed protein of muscle biopsies collected from the *vastus lateralis* muscle.

### Protocol

At 08.00 hours, after an overnight fast, subjects arrived at the laboratory by car or public transportation to minimize the effect of physical activity before the test on muscle FSR. A Teflon catheter was inserted into an antecubital vein for stable isotope infusion and a second Teflon catheter was inserted in a heated dorsal hand vein of the contra-lateral arm, placed in a hot-box (60°C), for arterialized blood sampling. After basal blood sample collection, a single intravenous dose of L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine (2 µmol/kg) and L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine (0.775 µmol/kg) was administered to prime the phenylalanine and tyrosine pool. Thereafter, tracer infusion (infusion rate of 0.049 (SEM 0.001) µmol/kg per min for L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine and 0.019 (SEM 0.001) mol/kg per min for L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine) was started and subjects rested in a supine position for 1 h, before engaging in the standardized physical activity protocol. The protocol was designed to simulate 30 min of moderate-intensity physical activity (e.g. garden tasks such as lawn mowing) as has been recommended by several public health authorities<sup>23,24</sup>. The energy expenditure during such an activity pattern is estimated to be approximately 650 kJ/30 min<sup>25</sup>, and was simulated by combining low-intensity cycling and light resist-

**Table 1.** Characteristics of subjects (eight elderly men) (Mean values with their standard errors)

	Mean	SEM
<b>Age (years)</b>	73	1
Weight (kg)	71.6	1.4
Height (m)	1.71	0.01
BMI (kg/m <sup>2</sup> )	24.5	0.7
Leg volume (litre)	7.80	0.20
Basal glucose (mmol/l)	5.34	0.13
Basal insulin (mU/l)	9.10	1.28
HOMA-IR	2.16	0.30
1RM leg press (kg)	154.9	6.6
1RM leg extension (kg)	77.5	2.5

HOMA-IR, homeostasis model assessment of insulin resistance<sup>34</sup>;  
1RM, one-repetition maximum.

ance-type exercise. After 5 min of self-paced cycling, subjects performed six sets of ten repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, The Netherlands) and six sets of ten repetitions on the leg extension machine (Technogym BV). The first two sets of both resistance exercises were performed at 40% of the subjects' one-repetition maximum. Sets 3–4 and 5–6 were performed at 55 and 75% of one-repetition maximum, respectively, with 2 min rest intervals between sets. At the end of the exercise protocol ( $t$  0 min), subjects rested supine and an arterialized blood sample and a muscle biopsy from the *vastus lateralis* muscle were collected. Subjects then received an initial bolus (1.33 ml/kg) of a given test-drink. Repeated boluses (1.33 ml/kg) were ingested every 30 min until  $t$  330 min. Arterialized blood samples were collected at  $t$  15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min with a second muscle biopsy taken at  $t$  360 min from the contralateral limb.

#### Beverages

Subjects received a beverage volume of 1.33 ml/kg every 30 min to ensure a given dose of 0.49 g carbohydrate/kg (50% as glucose and 50% as maltodextrin) and 0.16 g/kg of a whey protein hydrolysate with or without the addition of 0.03 g/kg leucine every h. The total amount of protein (0.16 g/kg per h or in total 0.96 g/kg) provided in the CHO + PRO and CHO + PRO + leu treatments exceeds the calculated amount of protein needed to provide sufficient precursor substrate to sustain maximal protein synthesis rates for at least 6 h<sup>26</sup>. Repeated boluses were administered to reach/maintain steady-state conditions. The whey protein hydrolysate (68.8% protein) contained 10.3% leucine and consequently the total amount of leucine administered in the CHO + PRO and CHO + PRO + leu treatments averaged 0.011 v. 0.041 g/kg per h, respectively.

Glucose and maltodextrin were obtained from AVEBE (Veendam, The Netherlands). Whey protein hydrolysate was prepared by DSM Food Specialties (Delft, The Netherlands). Leucine was purchased from BUFA (Uitgeest, The Netherlands). To make the taste comparable in all treatments, beverages were uniformly flavoured by adding 0.2 g sodium-saccharinate solution (25% w/w), 1.8 g citric acid solution (50% w/w) and 5 g of cream vanilla flavour (Numico Research, Wageningen, The Netherlands) for each litre of beverage. Treatments were performed in a randomized order, with test-drinks provided in a double-blind fashion.

#### Analysis

Blood samples were collected in EDTA-containing tubes and centrifuged at 1000 g and 4°C for 5 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C. Plasma glucose (Uni Kit III, 07367204; Roche, Basel, Switzerland) concentrations were analysed with the COBAS-FARA semi-automatic analyser (Roche). Insulin was analysed by RIA (Insulin RIA Kit; LINCO Research Inc., St Charles, MO, USA). Plasma (500 µl) for amino acid analyses was deproteinized on ice with 24% (w/v) 5-sulphosalicylic acid (100 µl), mixed and the clear supernatant was collected after centrifugation. Plasma amino acid concentrations were analysed on an automated dedicated

amino acid analyser (LC-A10; Shimadzu Benelux, Den Bosch, The Netherlands), using an automated precolumn derivatization procedure and a ternary solvent system<sup>27</sup>. The exact phenylalanine and tyrosine concentrations in the infusates were measured using the same method and averaged 4.58 (SEM 0.01) and 1.79 (SEM 0.01) mmol/l, respectively. Plasma phenylalanine and tyrosine were derivatized to their *tert*-butyldimethylsilyl derivatives and their <sup>13</sup>C and/or <sup>2</sup>H enrichments were determined by electron ionization GC–MS (GC, Agilent 6890N; MSD, Agilent 5973N, Little Falls, DE, USA) using selected ion monitoring of masses 336 and 342 for unlabelled and labelled phenylalanine, respectively, and masses 466, 468 and 472 for unlabelled and labelled (ring-<sup>2</sup>H<sub>2</sub> and ring-<sup>13</sup>C<sub>6</sub>) tyrosine, respectively<sup>28</sup>.

For measurement of L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine enrichment in the free amino acid pool and mixed muscle protein, 55 mg wet muscle were freeze-dried. Collagen, blood and other non-muscle fibre material were removed from the muscle fibers under a light microscope. The isolated muscle fibre mass (2–3 mg) was weighed and eight volumes (8 × dry weight of isolated muscle fibres × wet/dry ratio) of ice-cold 2% perchloric acid 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-<sup>13</sup>C<sub>6</sub>]phenylalanine, L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine and L-[ring-<sup>13</sup>C<sub>6</sub>]tyrosine enrichments could be measured using their *t*-butyldimethylsilyl 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<sup>29</sup>. The protein pellet was washed with three additional 1.5 ml washes of 2% perchloric acid, dried and the proteins were hydrolysed in 6 M-HCl at 120°C for 15–18 h. The hydrolysed 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 2 M-NH<sub>4</sub>OH. Thereafter, the eluate was dried and the purified amino acid fraction was derivatized into the ethoxycarbonyl-ethylesters to determine the <sup>13</sup>C-enrichment of protein-bound phenylalanine using GC–IRMS (MAT 252, Finnigan, Bremen, Germany).

#### Calculations

Infusion of L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine and L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine with muscle and arterialized blood sampling was used to simultaneously assess whole-body amino acid kinetics and FSR of mixed muscle protein. We did not use the L-[<sup>13</sup>C]leucine tracer to study protein metabolism as it has been shown that amino acid oxidation during and following exercise is overestimated with that particular tracer<sup>30</sup>. Whole-body kinetics for phenylalanine and tyrosine were calculated using the equations described by Thompson *et al.*<sup>31</sup> and Short *et al.*<sup>32</sup>. Briefly, phenylalanine and tyrosine turnover (flux,  $Q$ ) was 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 (µmol/kg body weight per h) and  $E_i$  and  $E_p$  correspond to the enrichments of infusate and

plasma amino acids (mol % excess), 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 ( $B$ ); 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- $^{13}\text{C}_6$ ]phenylalanine to L-[ring- $^{13}\text{C}_6$ ]tyrosine. The rate of hydroxylation ( $Q_{pt}$ ) was calculated (31) 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 tyrosine and phenylalanine, respectively.  $E_t$  and  $E_p$  are the L-[ring- $^{13}\text{C}_6$ ]tyrosine and L-[ring- $^{13}\text{C}_6$ ]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- $^{13}\text{C}_6$ ]phenylalanine, by the enrichment of the precursor (plasma L-[ring- $^{13}\text{C}_6$ ]phenylalanine enrichment). Muscle FSR was calculated as follows<sup>10</sup>:

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

where  $\Delta E_p$  is the delta increment of protein bound L-[ring- $^{13}\text{C}_6$ ]phenylalanine during incorporation periods;  $E_{\text{precursor}}$  is the average plasma L-[ring- $^{13}\text{C}_6$ ]phenylalanine enrichment to be able to correct for potential changes in precursor enrichment due to protein ingestion during the time period for determination of amino acid incorporation;  $t$  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 and their standard errors. The plasma essential amino acid (EAA), insulin and glucose responses were calculated as area under the curve above baseline values (AUC). A two-factor repeated measures ANOVA with time and treatment as factors was used to compare differences between treatments over time. In case of significant  $F$  ratios, Scheffe *post-hoc* tests were applied to locate the differences. For non-time-dependent variables, paired Student's  $t$  tests were used to compare differences in treatment effect. Statistical significance was set at  $P < 0.05$ . All calculations were performed using StatView version 5.0 (SAS Institute Inc., Cary, NC, USA).

## Results

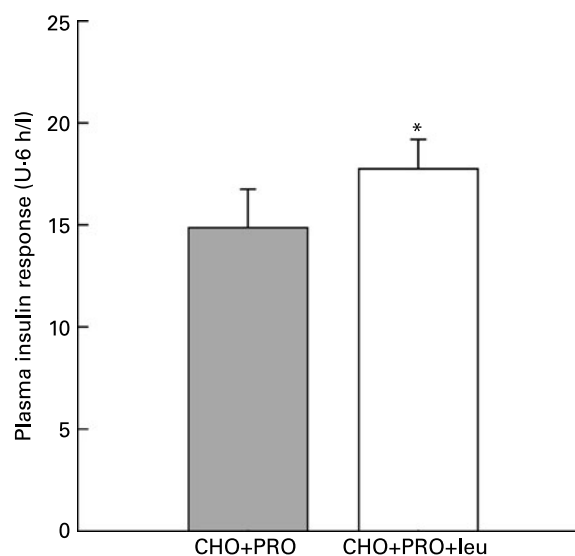
### Plasma insulin and glucose

In both treatments, plasma insulin concentrations increased during the first 45 min following the ingestion of the first beverage, after which insulin concentrations remained elevated

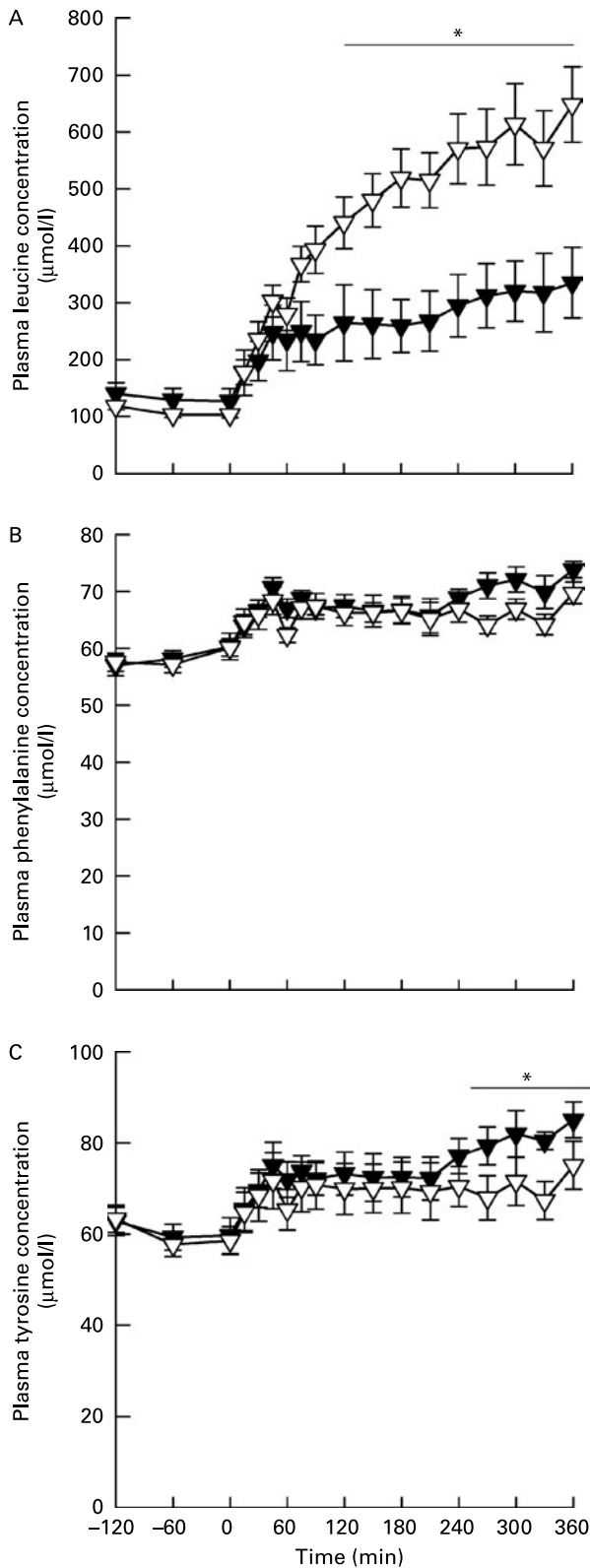
throughout the recovery period. No significant differences in plasma insulin concentrations were observed over time between treatments. The insulin response, expressed as AUC above baseline values during the entire 6 h post-exercise period (Fig. 1), was significantly greater in the CHO + PRO + leu compared with the CHO + PRO treatment (17.7 (SEM 1.5) v. 14.9 (SEM 1.9) U·6 h/l, respectively,  $P < 0.05$ ), and represented a 28 (SEM 14) % difference between treatments. Plasma glucose concentrations increased during the first 30–60 min after ingestion of the first beverage in both treatments, after which concentrations slowly decreased over time. No differences in plasma glucose response were observed between CHO + PRO and CHO + PRO + leu treatment (779 (SEM 48) v. 763 (SEM 63) mmol·6 h/l, respectively, NS).

### Plasma amino acids

Plasma leucine, phenylalanine and tyrosine concentrations over time are reported in Fig. 2. The plasma phenylalanine and tyrosine responses (AUC above baseline values) did not differ between treatments (2.86 (SEM 0.48) v. 2.08 (SEM 0.42) mmol·6 h/l and 5.49 (SEM 1.18) v. 3.89 (SEM 1.18) mmol·6 h/l in the CHO + PRO and CHO + PRO + leu treatment, respectively; NS). The plasma leucine response (AUC) was significantly higher in the CHO + PRO + leu compared to the CHO + PRO treatment (131.04 (SEM 14.16) v. 51.11 (SEM 11.46) mmol·6 h/l, respectively,  $P < 0.001$ ). Plasma histidine, lysine, phenylalanine, threonine and tryptophan responses (Fig. 3(A)) did not differ between treatments. Plasma isoleucine, methionine and valine responses (Fig. 3(A)) were significantly lower in the CHO + PRO + leu compared to the CHO + PRO treatment (10.71 (SEM 1.25) v. 14.40 (SEM 1.29) mmol·6 h/l, 1.02 (SEM 0.56) v. 1.85 (SEM 0.43) mmol·6 h/l and



**Fig. 1.** Plasma insulin responses (expressed as area under the curve minus baseline values) in elderly men ( $n$  8) while ingesting carbohydrate and protein (0.49 and 0.16 g/kg per h, respectively; CHO + PRO) or carbohydrate and protein with additional free leucine (0.49, 0.16 and 0.1 g/kg per h, respectively; CHO + PRO + leu). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the CHO + PRO experiment: \* $P < 0.05$ .



**Fig. 2.** Plasma leucine (A), phenylalanine (B) and tyrosine (C) concentrations ( $\mu\text{mol/l}$ ), in the carbohydrate and protein (CHO + PRO,  $\blacktriangledown$ ) and carbohydrate and protein with additional free leucine (CHO + PRO + leu,  $\nabla$ ) treatments in elderly men ( $n$  8). Values are means with their standard errors depicted by vertical bars. Data were analysed with ANOVA repeated measures (treatment  $\times$  time). Plasma leucine: treatment effect,  $P < 0.05$ ; time effect,  $P < 0.001$ ; interaction of treatment and time,  $P < 0.001$ . Plasma

phenylalanine: treatment effect,  $P = 0.33$ ; time effect,  $P < 0.001$ ; interaction of treatment and time,  $P = 0.291$ . Plasma tyrosine: treatment effect,  $P = 0.451$ ; time effect,  $P < 0.001$ ; interaction of treatment and time,  $P < 0.01$ . Mean values were significantly different from those of the CHO + PRO experiment (Scheffe's test); \* $P < 0.05$ ).

3.83 (SEM 3.23) v. 12.85 (SEM 2.10) mmol·6 h/l, respectively;  $P < 0.05$ ). The plasma EAA response (with the exclusion of leucine) was significantly lower in the CHO + PRO + leu compared to the CHO + PRO treatment (77.12 (SEM 12.37) v. 98.08 (SEM 9.08) mmol·6 h/l, respectively; Fig. 3(B);  $P < 0.05$ ).

The time course of the changes in plasma L-[ring- $^{13}\text{C}_6$ ]-phenylalanine, L-[ring- $^2\text{H}_2$ ]tyrosine and L-[ring- $^{13}\text{C}_6$ ]tyrosine enrichments are shown in Fig. 4. No differences in plasma L-[ring- $^{13}\text{C}_6$ ]phenylalanine, L-[ring- $^2\text{H}_2$ ]tyrosine and L-[ring- $^{13}\text{C}_6$ ]tyrosine enrichments were observed over time between treatments.

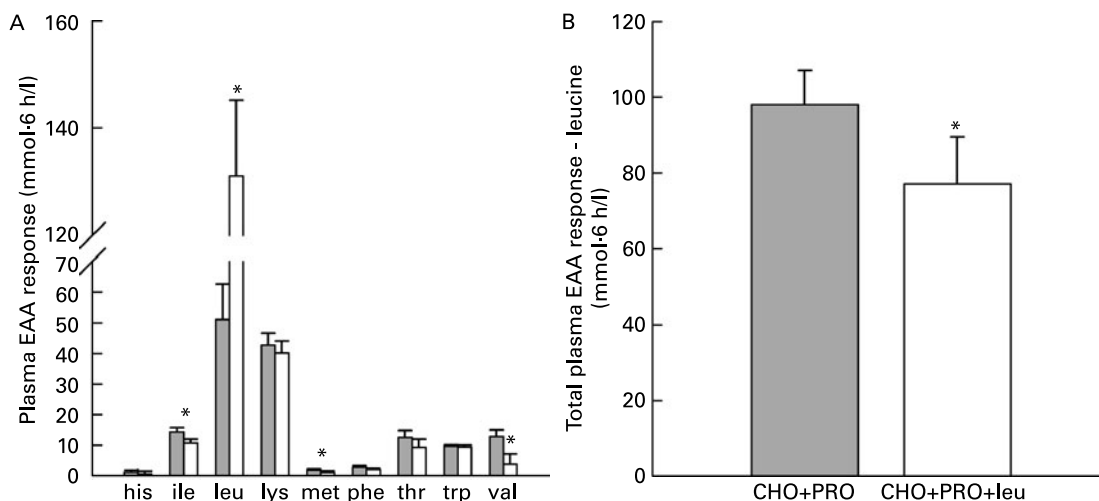
#### Whole-body protein metabolism

Phenylalanine flux was similar in the CHO + PRO and CHO + PRO + leu treatment (49.5 (SEM 1.2) v. 48.8 (SEM 1.1)  $\mu\text{mol}$  phenylalanine/kg per h, respectively; NS). Tyrosine flux was significantly lower in the CHO + PRO + leu compared to the CHO + PRO treatment (49.5 (SEM 1.7) v. 52.3 (SEM 1.6)  $\mu\text{mol}$  tyr/kg per h, respectively;  $P < 0.05$ ). Whole-body protein breakdown and synthesis (Fig. 5), calculated over the 6 h post-exercise recovery, did not differ between treatments. The rate of whole-body phenylalanine oxidation (Fig. 5), calculated from the conversion of phenylalanine to tyrosine, was lower in the CHO + PRO + leu compared with the CHO + PRO treatment (3.63 (SEM 0.31) v. 4.29 (SEM 0.33)  $\mu\text{mol/kg}$  per h, respectively;  $P < 0.05$ ). Whole-body protein balance (Fig. 5) was significantly greater in the CHO + PRO + leu compared with the CHO + PRO treatment (23.8 (SEM 0.3) v. 23.2 (SEM 0.3)  $\mu\text{mol/kg}$  per h, respectively;  $P < 0.05$ ). Protein synthesis efficiency (whole-body protein synthesis as a percentage of the phenylalanine flux) was significantly higher in the CHO + PRO + leu compared with the CHO + PRO treatment (92.6 (SEM 0.6) v. 91.3 (SEM 0.6) %, respectively  $P < 0.05$ ).

#### Muscle analysis

Mean plasma L-[ring- $^{13}\text{C}_6$ ]phenylalanine, L-[ring- $^2\text{H}_2$ ]tyrosine and L-[ring- $^{13}\text{C}_6$ ]tyrosine enrichments during the last 4 h of recovery, muscle free L-[ring- $^{13}\text{C}_6$ ]phenylalanine, L-[ring- $^2\text{H}_2$ ]tyrosine and L-[ring- $^{13}\text{C}_6$ ]tyrosine enrichments in the 6 h post-exercise biopsy and the increments in muscle protein enrichment are presented in Table 2. No differences in free L-[ring- $^{13}\text{C}_6$ ]phenylalanine, L-[ring- $^2\text{H}_2$ ]tyrosine and L-[ring- $^{13}\text{C}_6$ ]tyrosine enrichments in the muscle biopsies collected 6 h after physical activity were observed between treatments. The increase in protein-bound L-[ring- $^{13}\text{C}_6$ ]phenylalanine enrichment during the CHO + PRO and CHO + PRO + leu treatments averaged 0.000341 (SEM 0.000030) and 0.000341 (SEM 0.000045) (tracer/tracee ratio), respectively, with no significant differences between treatments.

phenylalanine: treatment effect,  $P = 0.33$ ; time effect,  $P < 0.001$ ; interaction of treatment and time,  $P = 0.291$ . Plasma tyrosine: treatment effect,  $P = 0.451$ ; time effect,  $P < 0.001$ ; interaction of treatment and time,  $P < 0.01$ . Mean values were significantly different from those of the CHO + PRO experiment (Scheffe's test); \* $P < 0.05$ ).



**Fig. 3.** Plasma response of the individual essential amino acids (EAA; A) and total essential amino acid response with the exclusion of leucine (B) during the carbohydrate and protein (CHO + PRO, □) and carbohydrate and protein with additional free leucine (CHO + PRO + leu, ■) treatments in elderly men ( $n$  8). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the CHO + PRO experiment (paired  $t$  test): \* $P$  < 0.05. his, histidine; ile, isoleucine; leu, leucine; lys, lysine; met, methionine; phe, phenylalanine; thr, threonine; trp, tryptophan; val, valine.

#### Mixed muscle protein synthesis rates

Mixed muscle protein FSR, with the mean plasma L-[ring- $^{13}\text{C}_6$ ]-phenylalanine enrichment as precursor (Fig. 6), averaged 0.081 (SEM 0.003) and 0.082 (SEM 0.006) %/h in the CHO + PRO + leu and CHO + PRO treatment, respectively. No differences were observed in FSR between treatments.

#### Discussion

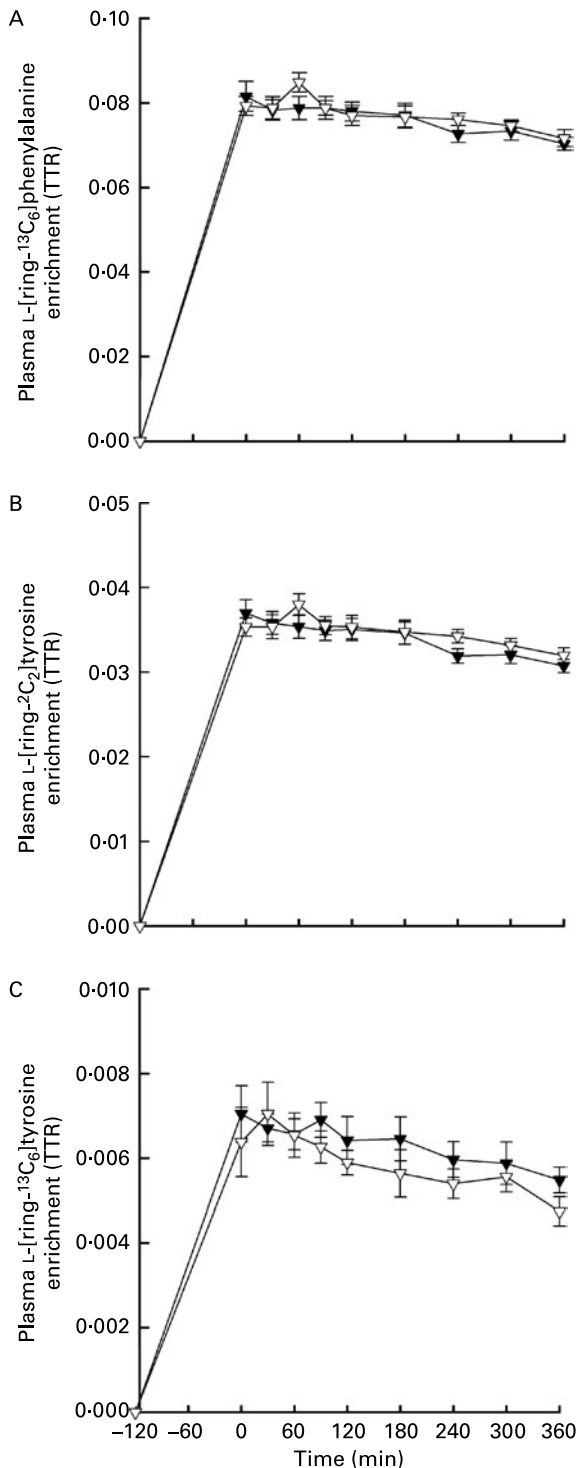
In the present study, we determined the potential surplus value of free leucine co-ingestion on post-exercise muscle protein synthesis in elderly men (approximately 75 years old) under conditions where large amounts of whey protein and carbohydrate are being ingested. Therefore, we assessed whole-body protein turnover and determined mixed muscle protein synthesis rates following the ingestion of carbohydrate and protein with or without additional free leucine in lean, elderly men. Additional intake of free leucine was shown to reduce whole-body protein oxidation, thereby improving whole-body protein balance. Direct measurement of mixed muscle protein synthesis rates revealed that co-ingestion of free leucine does not further enhance muscle protein synthesis rates following physical activity when ample amounts of protein and carbohydrate are ingested.

The potential role of leucine as a regulator of muscle protein metabolism has been studied extensively<sup>33</sup>. Leucine was reported to stimulate protein synthesis effectively in isolated rat diaphragm muscle<sup>33</sup>. The latter has since been confirmed in many follow-up studies in rodents<sup>11–14</sup>. Moreover, Anthony *et al.*<sup>15</sup> provided evidence showing that leucine ingestion can stimulate muscle protein anabolism both by increasing insulin secretion and by stimulating protein synthesis via insulin-independent mechanisms. Such a stimulating effect of leucine on protein synthesis occurs at the level of mRNA translation initiation and involves signalling through mTOR<sup>34</sup>, which is thought to serve as a convergence point for leucine- and insulin-mediated effects on mRNA translation initiation.

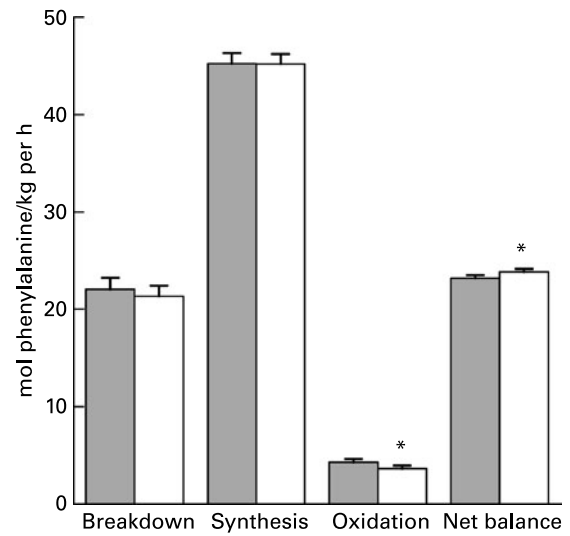
These observations indicate that leucine has the ability to function as a nutritional signalling molecule that can modulate muscle protein synthesis.

As a result, there have been ample recent studies examining the role of leucine in the control of tissue protein metabolism, its mechanism of action, and its proposed applicability in effective nutritional interventions to improve muscle protein balance in the elderly and in cachectic patients. We have recently shown that the combined ingestion of carbohydrate, protein and leucine is more effective in stimulating muscle protein synthesis *in vivo* in man when compared with the ingestion of only carbohydrate<sup>9,10</sup>. In addition, more recent studies reported that ingestion of a leucine-enriched amino acid mixture<sup>18,19</sup> or co-ingestion of leucine with protein<sup>18,19</sup> effectively enhance muscle protein synthesis in the elderly. However, the surplus value of additional leucine ingestion under normal living conditions, in which physical activity is followed by ample food intake, has not been assessed. Therefore, in the present study, we investigated the effect of carbohydrate (0.49 g/kg per h) and protein (0.16 g/kg per h) ingestion with or without additional free leucine (0.03 g/kg per h) on muscle protein synthesis rates following physical activity in lean, elderly men (approximately 75 years old).

Though most *in vitro* and *in vivo* animal studies report that leucine administration stimulates protein synthesis, most *in vivo* human studies report that leucine and/or branched-chain amino acid administration reduces muscle protein breakdown, without stimulating muscle protein synthesis<sup>35</sup>. In line with these findings, we observed lower phenylalanine oxidation rates in the CHO + PRO + leu treatment (expressed both absolute as well as a percentage of total phenylalanine flux) compared with the CHO + PRO treatment (Fig. 5). The latter implies that a greater proportion of the rate of disappearance of phenylalanine is used for protein synthesis (i.e. greater protein synthesis efficiency). As a consequence, net-protein balance was greater in the CHO + PRO + leu *v.* CHO + PRO treatment. The latter could, in part, be attributed

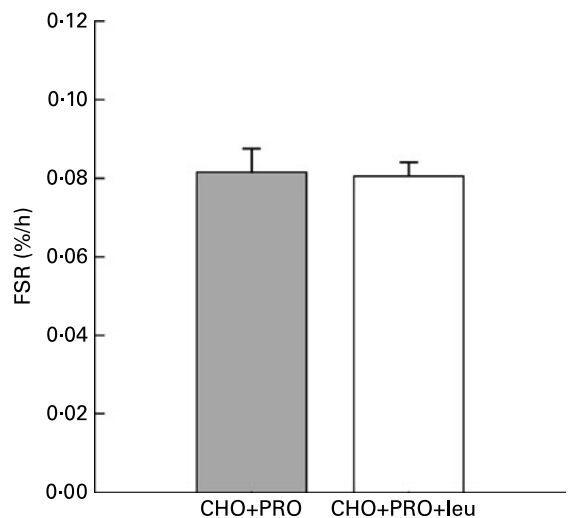


**Fig. 4.** Plasma L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine (A), L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine (B) and L-[ring-<sup>13</sup>C<sub>6</sub>]tyrosine enrichment (C) during the carbohydrate and protein (CHO + PRO, ▼) and carbohydrate and protein with additional free leucine (CHO + PRO + leu, ▽) treatments in elderly men (*n* 8). Values are means with their standard errors depicted by vertical bars. Data were analysed with ANOVA repeated measures (treatment × time). Plasma L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine enrichment: treatment effect, *P*=0.75; time effect, *P*<0.001; interaction of treatment and time, *P*=0.40. Plasma L-[ring-<sup>2</sup>H<sub>2</sub>]tyrosine enrichment: treatment effect, *P*=0.52; time effect, *P*<0.001; interaction of treatment and time, *P*=0.42. Plasma L-[ring-<sup>13</sup>C<sub>6</sub>]tyrosine enrichment: treatment effect, *P*=0.39; time effect, *P*<0.001; interaction of treatment and time, *P*=0.51. TTR, tracer/tracee ratio.



**Fig. 5.** Whole-body protein breakdown, synthesis, and oxidation rates and net protein balance (expressed as μmol phenylalanine/kg per h) during the carbohydrate and protein (CHO + PRO) and carbohydrate and protein with additional free leucine (CHO + PRO + leu) experiments in elderly men (*n* 8). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the CHO + PRO experiment (paired *t* test): \**P*<0.05.

to the greater insulin response that was observed in the CHO + PRO + leu *v.* CHO + PRO treatment (Fig. 1). However, in the present study, we cannot differentiate between the insulin-dependent and/or insulin-independent pathways that might explain the improved protein balance following leucine co-ingestion. Using whole-body tracer methodology, the present study indicates that the co-ingestion of free leucine with protein and carbohydrate further reduces whole-body protein oxidation rates (Fig. 5). The latter is in line with earlier reports by Nair *et al.*<sup>17</sup>, showing leucine infusion to reduce



**Fig. 6.** Fractional synthetic rate (FSR) of mixed muscle protein following the ingestion of carbohydrate and protein (CHO + PRO) or carbohydrate and protein with additional free leucine (CHO + PRO + leu) in elderly men (*n* 8). Values are means with their standard errors depicted by vertical bars. No significant differences were observed between treatments (paired *t* test): *P*>0.05.

**Table 2.** Plasma and muscle tracer kinetics  
(Mean values with their standard errors)

	Plasma amino acid enrichment*		Muscle amino acid pool enrichment*		Δ Enrichment muscle protein*	
	Mean	SEM	Mean	SEM	Mean	SEM
L-[ring- <sup>13</sup> C <sub>6</sub> ]phenylalanine						
CHO + PRO	0.0743	0.0014	0.0567	0.0021	0.000371	0.000029
CHO + PRO + leu	0.0752	0.0010	0.0598	0.0031	0.000371	0.000017
L-[ring- <sup>2</sup> H <sub>2</sub> ]tyrosine						
CHO + PRO	0.0329	0.0008	0.0210	0.0007	NA	NA
CHO + PRO + leu	0.0339	0.0006	0.0217	0.0011	NA	NA
L-[ring- <sup>13</sup> C <sub>6</sub> ]tyrosine						
CHO + PRO	0.0060	0.0002	0.0099	0.0001	NA	NA
CHO + PRO + leu	0.0054	0.0002	0.0111	0.0017	NA	NA

CHO + PRO, carbohydrate and protein hydrolysate; CHO + PRO + leu, carbohydrate, protein hydrolysate and additional free leucine; NA, not applicable.

\* Enrichment expressed as tracer/tracee ratio.

whole-body protein breakdown significantly. Furthermore, data from their study also showed a decline in the plasma concentrations of the other EAA during leucine infusion. In accordance, we observed reduced plasma EAA responses in the CHO + PRO + leu *v.* CHO + PRO treatment, even though the same amount of protein was consumed (Fig. 3). The diminished plasma EAA response following leucine supplementation could be attributed to a reduced release from the muscle, and as such, could be indicative of a reduced protein breakdown and/or oxidation rate (Fig. 5). In addition, it has been suggested that leucine influences the transport of amino acids sharing the same transport system (*i.e.* valine, isoleucine)<sup>36</sup>. Though we did not assess either valine or isoleucine kinetics, it might be speculated that a reduction in their plasma concentration following leucine co-ingestion could prevent a further increase in muscle protein synthesis rate.

In the present study, we infused L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine and measured L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine and L-[ring-<sup>13</sup>C<sub>6</sub>]tyrosine enrichment in plasma and free muscle amino acid pool. Consequently, we observed 50–120% higher tyrosine enrichments in the free muscle amino acid pool compared to values observed in plasma (Table 2)<sup>9,10</sup>. The latter might suggest that phenylalanine is converted to tyrosine in skeletal muscle, as was previously suggested by Van Hall *et al.*<sup>37</sup>. However, these findings are in contrast with previous observations in rodents<sup>38</sup>. The latter should be investigated in more detail in future studies.

The combined ingestion of leucine and protein with carbohydrate has been shown to stimulate endogenous insulin release (Fig. 1)<sup>10,39,40</sup>. In the present study, the greater insulin response following leucine co-ingestion was not accompanied by a reduction in plasma glucose concentration. The latter has been reported before in healthy, normoglycaemic subjects<sup>39</sup>. In contrast, the greater insulin response following protein and/or leucine co-ingestion has been shown to stimulate plasma glucose disposal, and attenuate the postprandial rise in blood glucose under hyperglycaemic conditions in type 2 diabetes patients<sup>39</sup>. Leucine ingestion has been proposed to stimulate muscle protein synthesis, independent of circulating plasma insulin levels, by increasing the phosphorylation (activation) of key proteins involved in the regulation of protein

synthesis<sup>41</sup>. As a consequence, it has been speculated that the administration of an insulinotropic mixture containing carbohydrate, protein and additional free leucine likely represents an effective nutritional strategy to enhance net muscle protein accretion in the elderly<sup>9,18,19</sup>. In addition to whole-body measurements, we measured the incorporation rate of labelled phenylalanine in skeletal muscle tissue to determine the FSR of mixed muscle protein in the *vastus lateralis* muscle. Mixed muscle FSR averaged 0.082 (SEM 0.006%/h) following 6 h after physical activity, during which carbohydrate and protein (0.49 and 0.16 g/kg per h, respectively) were ingested (Fig. 6). Additional intake of free leucine (0.03 g/kg per h) did not further increase muscle protein synthesis rates (Fig. 6), as muscle FSR averaged 0.081 (SEM 0.006) %/h in the CHO + PRO + leu treatment. Therefore, the present data show that co-ingestion of free leucine does not further enhance muscle protein synthesis in lean elderly men.

The present data seem to be in contrast with recently published studies<sup>18,19</sup>. Katsanos *et al.*<sup>19</sup> showed that in elderly men, ingestion of 6.7 g of an EAA mixture containing 41% leucine was more effective in stimulating muscle protein synthesis rates when compared to an EAA mixture containing only 26% leucine. Similarly, Rieu *et al.*<sup>18</sup> showed that co-ingestion of leucine (and small amounts of valine and isoleucine) with protein, carbohydrate and fat further improved muscle protein synthesis in elderly men. The apparent discrepancy could likely be explained by the fact that in the present study, FSR was assessed following physical activity. Furthermore, our subjects were administered a much greater protein/carbohydrate load when compared to these other studies. The total amount of protein ingested in the present study averaged 69 (SEM 1) g *v.* merely 30 and 6.7 g in the studies by Rieu *et al.*<sup>18</sup> and Katsanos *et al.*<sup>19</sup>. Furthermore, the total amount of leucine that was ingested (4.7 and 17.6 g leucine over a 6 h period in the CHO + PRO and CHO + PRO + leu treatment, respectively) was substantially greater than the lowest dose of leucine provided either by Katsanos *et al.*<sup>19</sup> (1.7 g over a 3.5 h period) or Rieu *et al.*<sup>18</sup> (3 g over a 5 h period). Therefore, it could be speculated that the leucine and/or exercise-stimulated protein synthetic response had already reached maximal values in the CHO + PRO treatment.



Though leucine co-ingestion did not further stimulate muscle protein synthesis, whole-body protein balance was shown to be 2.8% greater in the CHO + PRO + leu trial ( $P < 0.05$ ; Fig. 5). The relevance of such small differences in whole-body protein balance and the tissue(s) responsible for these small differences could not be determined in the present study. Long-term intervention studies are warranted to address the efficacy of leucine supplementation as an interventional strategy to attenuate the loss of skeletal muscle mass with ageing.

In conclusion, co-ingestion of leucine with protein and carbohydrate following physical activity does not further augment mixed muscle protein synthesis rates in lean, elderly men under conditions where ample amounts of protein and carbohydrate are being ingested.

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