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

René Koopman¹*, Lex B. Verdijk¹, Milou Beelen¹, Marchel Gorselink², Arie Nieuwenhuijzen Kruseman³, Anton J. M. Wagenmakers⁴, Harm Kuipers¹ and Luc J.C. van Loon¹.⁵

¹Department of Movement Sciences, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, 6200 MD Maastricht, The Netherlands
²Numico Research B.V., 6700 CA Wageningen, The Netherlands
³Department of Internal Medicine, Academic Hospital Maastricht, 6202 AZ Maastricht, The Netherlands
⁴School of Sport and Exercise Sciences, University of Birmingham, Birmingham B15 2TT, UK
⁵Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, 6200 MD Maastricht, The Netherlands

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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 ± 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-13C6]phenylalanine and L-[ring-2H2]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) µmol/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 sarcopaenia¹. These age-related changes in skeletal muscle mass are attributed to a disruption in the regulation of skeletal muscle protein synthesis and/or degradation². Protein turnover in skeletal muscle tissue is highly responsive to nutrient intake in healthy, young individuals³. In the elderly, the muscle protein synthetic response to food intake seems to be blunted⁴, which is likely due to impaired anabolic signalling in skeletal muscle tissue⁵,⁶. 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 breakdown⁷. However, post-exercise net protein balance will remain negative in the absence of food intake⁸. 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 men⁹. 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 activity¹⁰. A direct stimulating effect of leucine administration on muscle protein synthesis has been reported previously in rodents¹¹–¹⁴. In line with those data, Anthony et al.¹⁵ 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 mTOR¹⁶. 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.

* Corresponding author: Dr René Koopman, Department of Human Biology, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands, fax +31 43 3670976, email R.Koopman@HB.unimaas.nl
affect muscle protein metabolism by decreasing the rate of pro-
tein degradation\textsuperscript{17}, 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 \textit{vivo} in man\textsuperscript{5,10}. 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\textsuperscript{18,19}.
However, the proposed surplus value of leucine co-ingestion under normal living conditions, in which physical activity is fol-
lowed by food intake, has not yet been assessed. We hypothe-
sitized 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-$\text{^{13}}$C\text{$_6$}]phenylalanine and L-[ring-$\text{^{2H}}$\text{$_2$}]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 \textit{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 partici-
pate 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\textsuperscript{20}. Leg volume was determined
as described previously\textsuperscript{21}, 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 practiced 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\textsuperscript{22}.

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 hydroly-
sate (CHO + PRO) or carbohydrate, protein hydrolysate and
additional free leucine (CHO + PRO + leu) were admi-
nistered 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 pro-
tocol 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 incor-
poration of L-[ring-$\text{^{13}}$C\text{$_6$}]phenylalanine in the mixed protein of
muscle biopsies collected from the \textit{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 intra-
venous dose of L-[ring-$\text{^{13}}$C\text{$_6$}]phenylalanine (2 µmol/kg) and
L-[ring-$\text{^{2H}}$\text{$_2$}]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-$\text{^{13}}$C\text{$_6$}]phenylalanine and 0.019 (SEM 0.001)
µmol/kg per min for L-[ring-$\text{^{2H}}$\text{$_2$}]tyrosine) was started and sub-
jects 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\textsuperscript{23,24}.
The energy expenditure during such an activity pattern is esti-
inated to be approximately 650 kJ/30 min\textsuperscript{25}, and was
simulated by combining low-intensity cycling and light resist-

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### Table 1. Characteristics of subjects (eight elderly men)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean</th>
<th>SEM</th>
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<tr>
<td>Age (years)</td>
<td>73</td>
<td>1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.71</td>
<td>0.01</td>
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<td>BMI (kg/m\text{^2})</td>
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<td>Leg volume (litre)</td>
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<td>Basal glucose (mmol/l)</td>
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<td>0.13</td>
</tr>
<tr>
<td>Basal insulin (µU/l)</td>
<td>9.10</td>
<td>1.28</td>
</tr>
<tr>
<td>HOMA-IR</td>
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<td>0.30</td>
</tr>
<tr>
<td>1RM leg press (kg)</td>
<td>154.9</td>
<td>6.6</td>
</tr>
<tr>
<td>1RM leg extension (kg)</td>
<td>77.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

HOMA-IR, homeostasis model assessment of insulin resistance\textsuperscript{29};
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 h26. 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 (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 system27. 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 13C and/or 2H 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-2H2 and ring-13C6) tyrosine, respectively28.

For measurement of L-[ring-13C6]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-13C6]phenylalanine, L-[ring-2H2]tyrosine and L-[ring-13C6]tyrosine enrichments could be measured using their tert-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-phthalaldehyde29. 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-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 using GC–IRMS (MAT 252, Finnigan, Bremen, Germany).

**Calculations**

Infusion of L-[ring-13C6]phenylalanine and L-[ring-2H2]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-[1-13C]leucine tracer to study protein metabolism as it has been shown that amino acid oxidation during and following exercise is overestimated with that particular tracer30. Whole-body kinetics for phenylalanine and tyrosine were calculated using the equations described by Thompson et al.31 and Short et al.32. Briefly, phenylalanine and tyrosine turnover (flux, Q) was measured from the isotope dilution at isotopic steady state:

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

where i is the isotope infusion rate (μmol/kg body weight per h) and $E_i$ and $E_p$ correspond to the enrichments of infused 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.

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

At isotopic steady state, whole-body phenylalanine oxidation can be determined from the conversion (hydroxylaton) of L-[ring-13C6]phenylalanine to L-[ring-13C6]tyrosine. The rate of hydroxylaton (Qp) was calculated (31) using the formula

\[ Q_p = \frac{E_p}{E_p + I_p} \cdot \frac{Q_p}{t_p} \]

where Qp and Ep are the flux rates for tyrosine and phenylalanine, respectively. Ep and Ip are the L-[ring-13C6]tyrosine and L-[ring-13C6]phenylalanine enrichments in plasma, respectively, and ip 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-13C6]-phenylalanine, by the enrichment of the precursor (plasma L-[ring-13C6]-phenylalanine enrichment). Muscle FSR was calculated as follows:

\[ \text{FSR} = \frac{\Delta E_p}{E_{\text{precursor}} \cdot t} \cdot 100 \]

where \( \Delta E_p \) is the delta increment of protein bound L-[ring-13C6]-phenylalanine during incorporation periods; \( E_{\text{precursor}} \) is the average plasma L-[ring-13C6]-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; ti 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% 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)) did not differ between treatments. Plasma leucine, 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)) did not differ between treatments. Plasma leucine, 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, \( P<0.001 \)).
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-13C6]-phenylalanine, L-[ring-2H2]tyrosine and L-[ring-13C6]tyrosine enrichments are shown in Fig. 4. No differences in plasma L-[ring-13C6]phenylalanine, L-[ring-2H2]tyrosine and L-[ring-13C6]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) μmol 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) μmol 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) mmol/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) mmol/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

Mixed muscle protein synthesis rates

Mixed muscle protein FSR, with the mean plasma L-[ring-13C6]-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. Leucine was reported to stimulate protein synthesis effectively in isolated rat diaphragm muscle. The latter has since been confirmed in many follow-up studies in rodents. Moreover, Anthony et al. 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, 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. In addition, more recent studies reported that ingestion of a leucine-enriched amino acid mixture or co-ingestion of leucine with protein 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. 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...
to the greater insulin response that was observed in the CHO + PRO + leu vs. 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.\textsuperscript{17}, showing leucine infusion to reduce...
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). 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-13C6]phenylalanine and measured L-[ring-13C6]phenylalanine and L-[ring-13C6]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). The latter might suggest that phenylalanine is converted to tyrosine in skeletal muscle, as was previously suggested by Van Hall et al. However, these findings are in contrast with previous observations in rodents. 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). 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. 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. 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. 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. 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. Katsanos et al. 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. 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. and Katsanos et al.

Further, 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. (1.7 g over a 3.5 h period) or Rieu et al. (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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma amino acid enrichment*</th>
<th>Muscle amino acid pool enrichment*</th>
<th>Δ Enrichment muscle protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>L-[ring-13C6]phenylalanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO + PRO</td>
<td>0.0743</td>
<td>0.0014</td>
<td>0.0567</td>
</tr>
<tr>
<td>CHO + PRO + leu</td>
<td>0.0752</td>
<td>0.0010</td>
<td>0.0598</td>
</tr>
<tr>
<td>L-[ring-2H2]tyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO + PRO</td>
<td>0.0329</td>
<td>0.0008</td>
<td>0.0210</td>
</tr>
<tr>
<td>CHO + PRO + leu</td>
<td>0.0339</td>
<td>0.0006</td>
<td>0.0217</td>
</tr>
</tbody>
</table>

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

* Enrichment expressed as tracer/tracer ratio.
Anabolic properties of leucine co-ingestion

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 muscle protein synthesis, whole-body protein balance was the same extent in young and elderly lean men. 

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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