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Leucine co-ingestion augments the muscle protein synthetic response to the ingestion of 15 g protein following resistance exercise in older men

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Running Title: Post-exercise leucine co-ingestion in older men

Disclosure Summary

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List of abbreviations and their definitions

1RM	1 repetition maximum
AUC	Area under the curve
DEXA	Dual-energy x-ray absorptiometry
ECG	Electrocardiogram
En%	Energy %
EndoRa	Endogenous phenylalanine rate of appearance
ExoRa	Exogenous phenylalanine rate of appearance
FSR	Fractional synthetic rate
MPE	Mole percent excess
OGTT	Oral glucose tolerance test
Phe Plasma	Dietary protein-derived amino acid availability in plasma
15G	15 g protein ingested following resistance exercise
15G+LEU	15 g protein with 1.5 g free leucine ingested following resistance exercise
SEM	Standard error of the mean
W	Watt

ABSTRACT (250 words)

Background: Older adults have shown an attenuated post-exercise increase in muscle protein synthesis rates following ingestion of smaller amounts of protein when compared to younger adults. Consequently, it has been suggested that older adults require the ingestion of more protein to increase post-exercise muscle protein synthesis rates when compared to younger adults.

Objective: We investigated whether co-ingestion of 1.5 g free leucine with a single, 15 g bolus of protein further augments the post-prandial muscle protein synthetic response during recovery from resistance-type exercise in older men.

Design: Twenty-four healthy older men (67±1 y) were randomly assigned to ingest 15 g milk protein concentrate (MPC80) with (15G+LEU; *n*=12) or without (15G; *n*=12) 1.5 g free leucine after performing a single bout of resistance-type exercise. Post-prandial protein digestion and amino acid absorption kinetics, whole-body protein metabolism, and post-prandial myofibrillar protein synthesis rates were assessed using primed, continuous infusions with L-[*ring*-$^{2}H_5$]-phenylalanine, L-[*ring*-$^{2}H_2$]-tyrosine and L-[1-^{13}C]-leucine combined with the ingestion of intrinsically L-[1-^{13}C]-phenylalanine labeled milk protein.

Results: A total of 70±1% (10.5±0.2 g) and 75±2% (11.2±0.3 g) of the protein-derived amino acids were released in the circulation during the 6 h post-exercise recovery phase in 15G+LEU and 15G, respectively (P<0.05). Post-exercise myofibrillar protein synthesis rates were 16% (0.058±0.003 vs 0.049±0.002%·h^{-1}; P<0.05; based upon L-[*ring*-$^{2}H_5$]-phenylalanine) and 19% (0.071±0.003 vs 0.060±0.003%·h^{-1}, P<0.05; based upon L-[1-^{13}C]-leucine) greater in 15G+LEU when compared with 15G.

Conclusion: Leucine co-ingestion further augments the post-exercise muscle protein synthetic response to the ingestion of a single 15 g bolus of protein in older men.

Key words: muscle protein synthesis, sarcopenia, aging, dietary protein, exercise, leucine.

INTRODUCTION

The age-related decline in skeletal muscle mass and strength, termed *sarcopenia*, is accompanied by impairments in functional capacity and an increased risk of developing chronic metabolic diseases (4). Whereas basal muscle protein synthesis and breakdown rates appear to be unaffected by age (33), the muscle protein synthetic response to the main anabolic stimuli, namely food intake and physical activity, seem to be blunted in older individuals (47). This *anabolic resistance* is now considered as a central factor contributing to the progression of sarcopenia.

A single session of resistance-type exercise strongly increases muscle protein synthesis rates (36) and, therefore, represents an effective strategy to compensate for anabolic resistance. For older individuals, ingestion of more than 20 g protein is required to augment post-exercise muscle protein synthesis rates (10, 36, 53). Older individuals possess the capacity to further increase the post-exercise muscle protein synthetic response by ingesting larger protein doses, with the ingestion of 40 g protein further enhancing the muscle protein synthetic response (53, 54). However, older individuals seldom consume 40 g protein in a single meal (40, 41). Therefore, research is warranted to determine nutritional strategies that can augment the muscle protein synthetic response to ingestion of small(er) amounts of protein during recovery from resistance-type exercise in older adults.

Leucine has been established as one of the amino acids with greater anabolic properties due to its ability to stimulate mTORC1 activity to phosphorylate key anabolic signaling proteins (i.e., S6K1) in skeletal muscle tissue (3, 17). Previous work has demonstrated that co-ingestion of free leucine augments the muscle protein synthetic response to protein or amino acid ingestion in older individuals at rest (7, 9, 12, 13, 48) and after a single bout of resistance-type exercise (2, 7, 9, 12, 13). It has also been demonstrated that leucine co-ingested with the main meals augments the integrated muscle protein synthetic response to

resistance-type exercise assessed over multiple days (30). However, as leucine also stimulates splanchnic tissue protein synthesis rates (29, 39), it could be speculated that free leucine co-ingestion stimulates the uptake and incorporation of dietary protein derived amino acids in the splanchnic tissues, thereby attenuating the post-prandial release of dietary protein derived amino acids in the circulation. It remains to be established whether this would preclude the impact of free leucine co-ingestion to further increase post-exercise muscle protein synthesis rates. In short, it remains unclear whether or not free leucine co-ingestion impacts post-prandial protein handling following the ingestion of a small amount of protein during post-exercise recovery in older individuals. Therefore, in the present study we assessed post-prandial protein handling and the muscle protein synthetic response following the ingestion of a single 15 g bolus of protein with or without additional free leucine (1.5 g) during recovery from a single bout of resistance-type exercise in older individuals.

We hypothesized that co-ingestion of 1.5 g free leucine with a single bolus of 15 g protein attenuates the post-prandial release of protein derived amino acids in the circulation when compared to the ingestion of 15 g protein. Furthermore, we hypothesized that despite a potential attenuated rise in post-prandial plasma amino acid availability, free leucine co-ingestion will further increase post-exercise muscle protein synthesis rates and allow for a greater incorporation of the available dietary protein-derived amino acids into myofibrillar protein. To test our hypothesis, we selected 24 healthy older (67±1 y) men who ingested 15 g protein with or without 1.5 g free leucine during recovery from a single bout of resistance-type exercise. By combining the ingestion of specifically produced intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled milk protein concentrate with the administration of primed continuous infusions of L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-leucine and L-[*ring*-$^{2}H_2$]-tyrosine, we were able to assess protein digestion and amino acid absorption kinetics,

75 the increase in muscle protein synthesis rate, and the post-prandial incorporation of dietary
76 protein-derived amino acids during recovery from exercise in older individuals.

MATERIALS AND METHODS

Subjects

A total of 24 healthy, normoglycemic, older men (67±1 y) were selected to participate in the present study. Subjects' characteristics of the study participants are presented in **Table 1**. Subjects were randomly assigned to ingest either 15 g protein (15G: *n*=12) or 15 g protein with 1.5 g crystalline free leucine (15G+LEU: *n*=12) in a double-blind fashion after completing a single bout of whole-body resistance-type exercise. Randomization was performed by an independent researcher who created a table in Excel (Microsoft, USA) using the random number generator function, which was coupled to the different beverages before sorting the number column in order of low to high. The independent researcher also prepared and masked the test beverages on the test day. All subjects were 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 Ethical Committee of the Maastricht University Medical Centre, The Netherlands, and conformed to standards for the use of human subjects in research as outlined in the most recent version of the Helsinki Declaration. This study is part of a greater project, which was registered at the Netherlands Trial Registry as NTR4492. Data from the 15G group have been published previously as part of a protein dose-response study conducted in parallel within the same project (22).

Pretesting

Participants arrived at the laboratory at 0830 h by car or public transport in an overnight fasted state. Upon arrival, body weight, body composition, and bone mineral content were measured with DEXA (Dual-energy X-ray absorptiometry, DEXA; Discovery A; Hologic, Bedford, MA). Thereafter, all participants performed an oral glucose tolerance test (OGTT).

Plasma glucose and insulin concentrations were measured to determine oral glucose intolerance and/or the presence of type 2 diabetes according to 2006 American Diabetes Association guidelines (1). All subjects were screened on medical issues and excluded if any gastrointestinal, neurological or renal diseases were present.

Subjects were cleared to perform resistance-type exercise by a cardiologist who examined electrocardiograms (ECG) measured at rest and during submaximal cycling (performed at 70 % of age-predicted heartrate max). The subjects were then familiarized with the exercise equipment and physical activity protocol. Subjects first performed a 10-min cycling warm-up at 70% of their age-predicted heart rate max before completing an estimation of their 1RM (one repetition maximum) on the leg press and leg extension exercises using the multiple repetitions testing procedure (28). For each exercise, subjects performed 10 submaximal repetitions to warm-up and become familiarized with the equipment and to have lifting technique critiqued and corrected. Subjects then performed sets at progressively increasing loads until failing to complete a valid repetition, judged by their inability to complete the full range of motion for an exercise. Ideally, subjects failed within 3–6 repetitions during the last and heaviest set. A 2-min resting period between subsequent attempts was allowed. The pretesting and experimental trials were separated by a period of at least 7 days.

Diet and physical activity

All volunteers were instructed to refrain from any exhaustive physical activity and to keep their diet as consistent as possible 72 h prior to the trial. Subjects filled in dietary records for 48 h immediately before the experimental trial. Subjects consumed 8.6±0.5 $MJ \cdot day^{-1}$ on average, with 47±1 energy% (En%) as carbohydrate, 33±1 En% as fat, and 18±1 En% as protein. Dietary protein intake averaged 1.1±0.1 $g \cdot kg^{-1}$ bodyweight. On the evening before

126 the experiment, all subjects consumed a standardized meal (22.0±0.6 kJ·kg^{-1} bodyweight,
127 consisting of 55 En% as carbohydrate, 20 En% as protein, and 25 En% as fat).
128
129 *Experimental Protocol*
130 At 0800 h, participants reported to the lab in a fasted and rested state and had Teflon
131 catheters inserted into the antecubital veins of one arm and the top of the opposite hand. At
132 0830 h (t = -150 min), a background blood sample was taken prior to the initiation of the
133 tracer infusion protocol. The plasma and intracellular phenylalanine and leucine pools were
134 primed with a single intravenous dose (priming dose) of L-[*ring*-$^{2}H_5$]-phenylalanine (3.6
135 μmol·kg^{-1}), L-[*ring*-$^{2}H_2$]-tyrosine (1.10 μmol·kg^{-1}), L-[1-^{13}C]-leucine (7.19 μmol·kg^{-1}). Once
136 primed, the continuous stable isotope infusion was initiated (infusion rate: 0.06 μmol·kg^{-}
137 ^{1}·min^{-1} L-[*ring*-$^{2}H_5$]-phenylalanine, 0.018 μmol·kg^{-1}·min^{-1} L-[*ring*-$^{2}H_2$]-tyrosine, 0.12
138 μmol·kg^{-1}·min^{-1} L-[1-^{13}C]-leucine; Cambridge Isotopes Laboratories, Andover, MA).
139 Participants rested for 1.5 h until 1000 h (*t* = -60 min), when the participants completed the
140 resistance-type exercise session. At 1100 h (*t* = 0 min), immediately after the resistance-type
141 exercise session, subjects had a blood sample and muscle biopsy collected from a randomized
142 leg. Subsequently, subjects ingested a 500 mL beverage containing 15 g intrinsically L-[1-
143 ^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled milk protein (MPC80) alone (15G) or with
144 (15G+LEU) an added 1.5 g of crystalline free leucine (**Table 2**). The beverages contained 1.5
145 mL vanilla extract to improve palatability (Dr. Oetker, Amersfoort, the Netherlands). Blood
146 samples (10 mL) were subsequently taken at *t* = 30, 60, 90, 120, 180, 240, 300, 360 min after
147 protein ingestion. A second muscle biopsy was obtained from the contralateral leg at 1700 h
148 (*t* = 360 min), signifying the end of the experimental trial.
149 Blood samples were collected in EDTA containing tubes and centrifuged at 1000*g* for 10 min
150 at 4 °C. Aliquots of plasma were frozen in liquid nitrogen and stored at –80 °C. Muscle

biopsies were obtained from the middle region of the M. *vastus lateralis*, 15 cm above the patella and approximately 4 cm below entry through the fascia, using the percutaneous needle biopsy technique (5). Muscle samples were dissected carefully and freed from any visible non-muscle material. The muscle samples were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis.

Resistance-type exercise protocol

The exercise protocol consisted of 60 min of moderate-to-high intensity whole-body resistance-type exercise. After 10 min of self-paced cycling at 100 W with a cadence of 60–80 RPM, subjects performed 5 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, Netherlands), 2 sets of 10 repetitions on the lat pull down machine (Technogym BV), 2 sets of 10 repetitions on the chest press machine and 5 sets of 10 repetitions on the leg extension machine (Technogym BV). The first set of the lower body exercises were performed at 50 % 1RM and sets 2–5 were performed at 75-80 % 1RM. All sets on the upper body exercises were performed at 75-80 % 1RM. Subjects were allowed to rest for 2 min between all sets.

Preparation of tracer and production of intrinsically labeled protein

The stable isotope tracers L-[*ring*-$^{2}H_5$]-phenylalanine, L-[1-^{13}C]-leucine and L-[*ring*-$^{2}H_2$]-tyrosine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9% saline before infusion (Basic Pharma, Geleen, the Netherlands). Continuous intravenous infusions were performed using a calibrated IVAC 598 pump (San Diego, CA, USA). Intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled milk protein (MPC80) was extracted from whole milk obtained during the constant infusion of L-[1-^{13}C]-phenylalanine (455 μmol·min^{-1}) and L-[1-^{13}C]-leucine (200 μmol·min^{-1}) for 96 h in a

lactating dairy cow (8, 44). The milk was collected, processed, and fractionated into the MPC80 similarly to what has been previously described (19, 37, 44). The L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments in MPC80 were measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; MAT 252, Finnigan, Breman, Germany) and averaged 38.3 mole percent excess (MPE) and 10.8 MPE, respectively. The proteins met all chemical and bacteriological specifications for human consumption.

Plasma and muscle analysis

Plasma glucose and insulin concentrations were analyzed using commercially available kits (GLUC3, Roche, Ref: 05168791 190, and Immunologic, Roche, Ref: 12017547 122, respectively). Plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Wilmington, Delaware, USA). Myofibrillar protein-bound L-[*ring*-$^{2}H_5$]-phenylalanine enrichments were determined by GC-MS analysis, whereas the L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments were determined by GC-C-isotope ratio mass spectrometer analysis (GC-C-IRMS; Trace GC Ultra, IRMS model MAT 253; Thermo Scientific) (20).

Western blotting

Muscle was homogenized as previously described (46), 10 μL of protein was loaded and standard SDS-PAGE procedures were followed. Antibodies included total and phosphorylated mTOR (Cat. no.: Total: #2972, Ser^{2448}: #2971), S6K1 (Cat no.: Total: #9202, Thr^{389}: #9205), RS6 (Cat no.: Total: #2217, Ser$^{235/236}$: #4856), 4E-BP1 (Cat no.: Total: #9452, Thr$^{37/46}$: #9459). α-tubulin (Cat no: #2125) was used as a loading control. All antibodies were purchased from Cell Signaling Technology (Danvers, MA). All samples for a

given protein were detected on the same membrane using chemiluminescence and the FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA, USA).

Calculations

Ingestion of L-[1-^{13}C]-phenylalanine labeled protein, intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine, and blood sample enrichment values were used to assess whole-body amino acid kinetics in non-steady state conditions. Total, exogenous, and endogenous phenylalanine rates of appearance and plasma availability of dietary protein-derived phenylalanine that appeared in the systemic circulation as a fraction of total amount of phenylalanine that was ingested, (Phe_{plasma}) were calculated using modified Steele's equations (6, 11, 52). Myofibrillar protein fractional synthetic rate (FSR) was calculated using the standard precursor-product method (Equation 1).

$$FSR\ \left(\%\cdot h^{-1}\right) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor}\ x\ t}\right) x\ 100 \qquad (1)$$

$E_{m2} - E_{m1}$ represents the change in muscle protein bound L-[1-^{13}C]-leucine or L-[*ring*-$^{2}H_5$]-phenylalanine enrichment. $E_{precursor}$ represents the average plasma L-[1-^{13}C]-leucine or L-[*ring*-$^{2}H_5$]-phenylalanine enrichment during the tracer incorporation period. *t* indicates the time interval (h) between biopsies.

Statistics

Data are expressed as means+SEMs or as box-whisker plots. Baseline characteristics between groups were compared using a *student's* unpaired t-test. A two-factor repeated measures ANOVA (time x treatment) with time as within-subjects factor and treatment group as between-subjects factor was performed for the analysis of plasma amino acid concentrations,

plasma tracer enrichments, whole-body kinetics and glucose and insulin concentrations. The analysis was carried out for the period starting at the time of protein administration, between *t* = 0 and 360 min. Upon identification of a significant time X treatment interaction, Tukey *post hoc* testing was used to identify time points in which the treatments differed. Non time-dependent variables (*i.e.,* Whole-body metabolism, FSR values, L-[1-^{13}C]-phenylalanine myofibrillar enrichments) were compared between treatment groups using *student's* unpaired t-tests. Statistical significance was set at $P<0.05$. All calculations were performed using SPSS 21.0 (IBM, Chicago, Illinois, USA).

RESULTS

Plasma concentrations

Plasma glucose (**Figure 1A**) and insulin (**Figure 1B**) concentrations after protein ingestion did not differ between the 15G and 15G+LEU groups (P>0.05). Plasma insulin concentrations increased after protein ingestion in both treatments, reaching peak levels 30 min after protein ingestion.

Plasma leucine concentrations (**Figure 2A**) increased rapidly following protein ingestion (P<0.01), but were greater in 15G+LEU (peak values: 407±23 μmol·L^{-1}) when compared to 15G (peak values: 234±16 μmol·L^{-1}, P<0.01). Area under the curve (AUC, **Figure 2B**) analysis revealed that plasma leucine availability over the 6 h post-prandial was approximately 1.8-fold greater in the 15G+LEU group when compared to the 15G group (P<0.001). Plasma phenylalanine concentrations (**Figure 2C**) increased rapidly following protein ingestion (Time effect: P<0.01) along with a main effect for treatment (Treatment effect: P<0.01), but no time x treatment interaction (P>0.05). Plasma tyrosine concentrations (**Figure 2D**) increased following protein ingestion (Time effect: P<0.01) along with a main effect for treatment (Treatment effect: P<0.01), but no time x treatment interaction (P>0.05).

Plasma amino acid enrichments

Plasma enrichments from ingested (L-[1-^{13}C]-phenylalanine), infused (L-[*ring*-$^{2}H_5$]-phenylalanine) and ingested and infused (L-[1-^{13}C]-leucine) amino acid tracers did not differ between treatments before protein ingestion (t = 0 min; P>0.05). After protein ingestion, plasma L-[1-^{13}C]-phenylalanine enrichments, originating from the ingested protein, increased in both groups reaching peak values at t = 60 min in 15G (9.6±0.5 MPE) and t = 120 min in 15G+LEU (8.7±0.5 MPE) in 15G+LEU. Plasma L-[*ring*-$^{2}H_5$]-phenylalanine enrichments

decreased after protein ingestion in both groups (P<0.001), but no significant group effect was detected (P>0.05). Plasma L-[1-^{13}C]-leucine enrichments increased after protein ingestion (P<0.001), but no significant group effects were detected (P>0.05).

Whole-body amino acid kinetics

Exogenous phenylalanine appearance rates (**Figure 3A**) increased following protein ingestion with peak levels being reached at t = 60 min in both treatment groups (15G: 0.19±0.01, 15G+LEU: 0.16±0.02 μmol Phe·kg^{-1}·min^{-1}; P>0.05). Dietary protein-derived amino acid availability, calculated as a fraction of the total amount of ingested protein (**Figure 3B**), was higher in 15G (75±2 %) when compared to 15G+LEU (70±1 %; P<0.05).

Whole-body protein synthesis rates did not differ between the treatment groups (15G: 0.60±0.01, 15G+LEU: 0.59±0.01 μmol Phe·kg^{-1}·min^{-1}; P>0.05). Whole-body protein breakdown rates did not differ between the treatment groups (15G: 0.49±0.01, 15G+LEU: 0.49±0.01 μmol Phe·kg^{-1}·min^{-1}; P>0.05). Protein ingestion resulted in a positive whole-body protein net balance, with no differences observed between the treatment groups (15G: 0.108±0.004, 15G+LEU: 0.105±0.003 μmol Phe·kg^{-1}·min^{-1}; P>0.05). Furthermore, leucine co-ingestion did not appear to influence whole-body phenylalanine oxidation rates (15G: 0.049±0.003, 15G+LEU: 0.046±0.002 μmol Phe·kg^{-1}·min^{-1}; P>0.05).

Myofibrillar fractional synthesis rates and protein-bound enrichments

Myofibrillar L-[1-^{13}C]-leucine and L-[*ring*-$^{2}H_5$]-phenylalanine enrichments were measured in muscle samples collected immediately before protein ingestion and after the 6 h post-prandial period. The post-prandial increase in myofibrillar protein bound L-[1-^{13}C]-leucine enrichments tended to be greater in 15G+LEU when compared to 15G (0.0360±0.0016 vs 0.0314±0.0016 MPE, respectively; P=0.055). The post-prandial increase in myofibrillar

protein bound L-[*ring*-$^{2}H_5$]-phenylalanine enrichment was greater in 15G+LEU when compared to 15G (0.0330±0.0015 vs 0.0278±0.0011 MPE, respectively; $P<0.05$).

Myofibrillar protein FSRs (in %·h^{-1}) were calculated using L-[*ring*-$^{2}H_5$]-phenylalanine plasma (**Figure 4A**) and muscle protein-bound enrichments and using L-[1-^{13}C]-leucine (**Figure 4B**) plasma and muscle protein-bound enrichments. When based upon L-[*ring*-$^{2}H_5$]-phenylalanine, myofibrillar protein FSR was approximately 16% greater in 15G+LEU (0.0575±0.0032 %·h^{-1}) when compared with 15G (0.0495±0.0021 %·h^{-1}; $P<0.05$). When based upon L-[1-^{13}C]-leucine, myofibrillar protein FSR was approximately 19% greater in 15G+LEU (0.0710±0.0048 %·h^{-1}) when compared with 15G (0.0598±0.0030 %·h^{-1}; $P<0.05$). L-[1-^{13}C]-phenylalanine myofibrillar protein-bound enrichments (**Figure 5**) were not different in 15G+LEU (0.0205±0.0022 MPE) when compared with 15G (0.0171±0.0017 MPE; $P=0.24$).

Cellular signalling analyses

The phosphorylation status (ratio of phosphorylated to total protein) of key proteins involved in the initiation of muscle protein synthesis are presented in **Figure 6**. Phosphorylation of S6K1 (**Figure 6B**) decreased in both groups over time (time effect, $P<0.01$). Phosphorylation of 4E-BP1 (**Figure 6D**) increased over time and to a greater extent in 15G compared with 15G+LEU ($P<0.01$).

DISCUSSION

In the present study, we examined the impact of free leucine co-ingestion on post-prandial protein handling and the subsequent muscle protein synthetic response following the ingestion of 15 g protein during recovery from resistance-type exercise in older men. We observed that 70-75 % of the dietary-derived amino acids were absorbed into the circulation 6 hours after the ingestion of 15 g protein. Co-ingesting 1.5 g free leucine with 15 g protein further increased post-exercise myofibrillar protein synthesis rates, but did not significantly increase the incorporation of dietary protein-derived amino acids in myofibrillar protein.

We administered a primed, continuous intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine, L-[*ring*-$^{2}H_2$]-tyrosine and L-[1-^{13}C]-leucine throughout a 6 h post-exercise recovery period in older individuals. Following exercise, participants ingested either 15 g of intrinsically L-[1-^{13}C]-phenylalanine labeled milk protein with or without 1.5 g free leucine. With this experimental protocol, we were able to assess *in vivo* protein digestion and amino acid absorption kinetics, whole-body protein metabolism, myofibrillar protein synthesis, and the incorporation of dietary protein-derived amino acids in muscle protein (8). After protein ingestion, we observed a rapid rise in circulating plasma amino acid concentrations (**Figure 2**) and an increase in the rate of exogenous phenylalanine appearance (**Figure 3A**), demonstrating rapid protein digestion and subsequent absorption of dietary protein-derived amino acids during recovery from exercise. As expected, fortification with 1.5 g free leucine resulted in greater peak plasma leucine concentrations (407±23 vs 234±16 μmol·L^{-1}, $P < 0.01$) at $t = 30$ min, and 1.8-fold greater plasma leucine availability over the entire 6 h post-prandial period, when compared to the ingestion of 15 g protein ($P < 0.01$). We observed 70-75 % of dietary protein-derived amino acid absorption into the circulation over the 6 h post-prandial period in both groups. This seems to be much more when compared to recent work from our lab using the same methodology (21, 34, 35). The apparent discrepancy is attributed

to the relatively small amount of dietary protein that was provided in the present study along with the extended 6 h post-prandial assessment period, implying that more protein derived amino acids will be absorbed during such an extended post-prandial period with a relatively smaller bolus of protein being ingested (22). Free leucine fortification seemed to compromise protein digestion and/or amino acid absorption as dietary protein-derived phenylalanine availability was lower following leucine co-ingestion when assessed over the entire 6 h post-prandial period (10.5±0.2 vs 11.2±0.3 g; $P < 0.05$). This was attributed to a mild attenuation of exogenous amino acid appearance rates observed between t = 30-120 min (**Figure 3A**). It could be speculated that the added free leucine may have stimulated splanchnic amino acid retention of dietary-protein derived amino acids during first pass. In agreement, prior work in neonatal pigs has demonstrated that free leucine co-ingested with a low protein dose stimulates an increase in jejunum, but not liver protein synthesis (29, 39). Altogether, our data demonstrate that free leucine co-ingestion further increases the post-prandial rise in leucine concentrations but attenuates the rate of appearance of dietary protein-derived amino acids into the circulation.

By administering a primed, continuous intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine and L-[*ring*-$^{2}H_2$]-tyrosine and providing intrinsically L-[1-^{13}C]-phenylalanine labeled protein, we were able to assess post-prandial whole-body protein synthesis, breakdown, net balance and oxidation. In both groups, protein ingestion resulted in a positive whole-body net protein balance during post-exercise recovery. However, fortification with free leucine did not further impact whole-body post-prandial protein synthesis, breakdown, or net balance. These findings are in agreement with prior work in older men at rest (38) and in younger men during post-exercise recovery (25). Despite previous reports that leucine administration lowers whole-body amino acid oxidation rates (24, 32), we did not observe this effect. These studies achieved far greater plasma leucine availability in comparison to the present study,

which may lead to a reduction in protein breakdown rates (32, 42) and thereby lowering the availability of amino acids for oxidation (25, 42). Our present data align with recent work administering similar, meal-like amounts of leucine (~4.5 g total) (38), and demonstrate that leucine co-ingestion does not impact whole-body phenylalanine oxidation rates.

Changes in whole-body protein metabolism do not necessarily reflect changes on a muscle tissue level. Therefore, we also collected skeletal muscle biopsies to directly assess the impact of leucine fortification of a low protein dose on intramuscular signaling and the muscle protein synthetic response to feeding. Resistance-type exercise and protein ingestion activate intramuscular signaling proteins that regulate protein translation with mTOR and its downstream targets, S6K1, RS6 and 4E-BP1 being of particular relevance. We observed no differences in mTOR or RS6 phosphorylation, but detected a decrease in S6K1 phosphorylation over time. These findings align with previous work showing a rapid increase in S6K1 activity following exercise, which subsides over 3-6 h (26, 51). Considering that biopsy timing was intended to assess the muscle protein synthetic response during the entire post-prandial period, it is most likely that transient increases in signaling activity had subsided by 6 h. However, 4E-BP1 phosphorylation increased over time in both groups, and to a greater extent after the ingestion of 15 g protein when compared with the ingestion of 15 g with leucine. We speculate that the higher leucine availability in 15G+LEU may have transiently activated 4E-BP1 at an earlier time in comparison to 15G (14, 18, 23), which steadily activated 4E-BP1 over the 6 h post-prandial period (26, 49).

Combining stable isotope labeled amino acid infusions with ingestion of intrinsically-labeled protein, we were able to assess muscle protein synthesis rates under both steady-state (L-[1-^{13}C]-leucine) as well as non-steady state (L-[*ring*-$^{2}H_5$]-phenylalanine) precursor conditions (8). Previous work has demonstrated that the ingestion of a low protein dose (< 20 g) following resistance-type exercise does not further stimulate an increase in muscle protein

synthesis rates in older individuals (53, 54). In the present study, free leucine co-ingested with a low protein dose (15 g) increased myofibrillar protein synthesis rates by 16% (L-[*ring*-$^{2}H_5$]-phenylalanine, **Figure 4A**) and 19% (L-[1-^{13}C]-leucine**, Figure 4B**) when compared with the ingestion of 15 g protein. These findings are in line with multiple studies demonstrating that free leucine co-ingestion can further increase the muscle protein synthetic response to protein ingestion in older individuals at rest (2, 9, 12, 13, 30, 48) and during recovery from resistance-type exercise (2, 7, 9, 12, 13, 30). In the present study, participants ingested intrinsically L-[1-^{13}C]-phenylalanine labeled protein, allowing us to directly assess the metabolic fate of the dietary protein-derived amino acids (21, 44, 48). Despite the greater post-prandial muscle protein synthetic response following the co-ingestion of free leucine, we did not observe a significantly greater L-[1-^{13}C]-phenylalanine enrichment in myofibrillar protein in 15G+LEU compared with 15G (**Figure 5**). The absence of a difference in the incorporation of dietary protein-derived amino acids in myofibrillar protein may be related to the mild attenuation in dietary-protein derived phenylalanine availability in the circulation when free leucine was co-ingested (**Figure 3B**). It has been reported that the increase in muscle protein synthesis rates following leucine administration may become limited with inadequate provision of exogenous amino acids as precursors (15, 16). More work will be required to determine the optimal amount of dietary protein that should be co-ingested with free leucine to ensure adequate plasma precursor availability to maximize post-prandial muscle protein synthesis rates.

The muscle protein synthetic response to protein ingestion has been shown to be impaired in older (47) and/or more clinically compromised populations (31, 50). Resistance-type exercise is an effective strategy to improve the sensitivity of skeletal muscle to the anabolic properties of dietary protein. However, recent work from our group has demonstrated that ingestion of less than 30 g protein does not further increase the muscle protein synthetic response during

post-exercise recovery in older men (22). We (34) and others (53, 54) have shown that increasing protein intake can compensate for this anabolic resistance, with as much as 45 g of protein being required to achieve a robust anabolic response during exercise recovery in older individuals. However, ingesting such large protein amounts may not be feasible in older and/or more clinically compromised populations. The current data extend upon previous findings and show that free leucine co-ingestion can further augment the post-exercise muscle protein synthetic response to protein ingestion (2, 7, 9, 12, 13, 30). In particular, the total amount of leucine provided in the 15G+LEU beverage (2.94 g) is equivalent to the amount of leucine contained in 30 g of MPC80. Therefore, increasing the leucine content through leucine co-ingestion may increase the efficiency by which the ingestion of smaller protein doses can augment muscle protein synthesis rates during recovery from exercise. Simply adding leucine to a post-exercise snack, to achieve approximately 3 g total leucine, may represent an effective strategy to support muscle mass maintenance in the older population without the need to ingest large(r) doses of protein. So far only few long-term intervention studies have assessed the anabolic effect of prolonged leucine supplementation. Whereas prolonged leucine supplementation does not seem to increase muscle mass in older individuals (27, 45), it has been suggested that leucine supplementation may augment muscle mass when combined with prolonged resistance-type exercise training (43). Nonetheless, more work is needed to assess the long-term benefits of leucine supplementation in combination with prolonged resistance-type exercise training in the older population.

In conclusion, leucine co-ingestion further augments the post-exercise muscle protein synthetic response to the ingestion of a small amount of protein in older men.

DECLARATIONS

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Ethics approval and consent to participate.

This study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre, The Netherlands (METC 14-3-052). All participants provided written informed consent before participation.

Authors' contributions

A.M.H., L.C.P.G.M.G, and L.J.C.L. designed the research; A.M.H., K.P., M.O. and I.F.K. conducted the research; A.M.H., J.P.B.G., W.K.W.H.W. and L.J.C.L. analyzed the data; A.M.H. and L.B.V. performed the statistical analysis; and A.M.H. and L.J.C.L. wrote the paper and hold primary responsibility for the final content. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Plasma glucose (A, mmol·L^{-1}) and insulin concentrations (B, mU·L^{-1}) following ingestion of 15 g milk protein (15G; *n*=12) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; *n*=12) after resistance-type exercise in older men. The dotted line represents the ingestion of the beverage. Values represent means+SEMs. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P<0.01$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$. B; time effect: $P<0.01$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$.

Figure 2. Plasma leucine (A), phenylalanine (C) and tyrosine (D) concentrations (μmol·L^{-1}) following ingestion of 15 g milk protein (15G; *n*=12) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; *n*=12) during recovery from resistance-type exercise in older men. The dotted line represents the ingestion of the beverage. Values for panels A, C, and D represent means+SEMs. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P<0.01$, treatment effect: $P<0.01$, time x treatment group: $P<0.01$. C; time effect: $P<0.01$, treatment effect: $P<0.01$, time x treatment group: $P>0.05$. D; time effect: $P<0.01$, treatment effect: $P<0.01$, time x treatment group: $P>0.05$. Plasma leucine area under the curves over 360 min (B, μmol·360 min·L^{-1}) are presented as box and whisker plots. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with a student's *unpaired* t-test. *Significant difference ($P<0.05$) from 15G.

Figure 3. Exogenous phenylalanine rate of appearance (A, μmol Phe·kg^{-1}·min^{-1}) following ingestion of 15 g milk protein (15G; *n*=12) or 15 g milk protein co-ingested with 1.5 g free

leucine (15G+LEU; *n*=12) during recovery from resistance-type exercise in older men. The dotted line represents the ingestion of the beverage. Values for panel A represent means+SEMs. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P<0.01$, treatment effect: $P<0.05$, Time x treatment group: $P>0.05$. Dietary protein-derived amino acid plasma availability (panel B), calculated as a fraction of the total amount of ingested protein (% ingested protein), are presented as box and whisker plots. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with a student's *unpaired* t-test. *Significantly different ($P<0.05$) from 15G.

Figure 4. Myofibrillar protein fractional synthetic rates (FSR in $\%\cdot h^{-1}$) assessed using L-[ring-$^{2}H_5$]-phenylalanine (A) and L-[1-^{13}C]-leucine (B) and following ingestion of 15 g milk protein (15G; *n*=12) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; *n*=12) after resistance-type exercise in older men. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with a student's *unpaired* t-test. *Significantly different ($P<0.05$) from 15G.

Figure 5. L-[1-^{13}C]-phenylalanine incorporation into myofibrillar protein following ingestion of 15 g milk protein (15G; *n*=12) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; *n*=12) during recovery from resistance-type exercise in older men. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data

were analyzed with student's *unpaired* t-test. No significant difference between groups (P=0.24).

Figure 6. Muscle phosphorylation status (ratio of phosphorylated to total protein, AU) of mammalian target of rapamycin (mTOR; A) S6 protein kinase 1 (S6K1; B), ribosomal protein S6 (RS6; C), and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1; D) in older men during recovery from resistance-type exercise (0 min) and 360 min after the ingestion of 15 g milk protein (15G; n=12) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; n=12). Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: P>0.05, treatment effect: P>0.05, time x treatment group: P>0.05. B; time effect: P<0.01, treatment effect: P>0.05, time x treatment group: P>0.05. C; time effect: P>0.05, treatment effect: P>0.05, time x treatment group: P>0.05. D; time effect: P<0.01, treatment effect: P>0.01, time x treatment group: P<0.01 *Significantly different (P<0.05) compared to t = 0 min. †Significant difference (P<0.05) from 15G+LEU at the same time point.

TABLES

Table 1. Subjects' characteristics[1]

	15G (*n* = 12)	**15G+LEU (*n* = 12)**	***P***
Age, y	69 ± 2	66 ± 2	0.45
Total body mass, kg	78.8 ± 3.2	79.0 ± 2.4	0.96
Total lean mass, kg	57.6 ± 2.3	58.1 ± 1.5	0.86
Appendicular lean mass, kg	24.9 ± 1.1	25.6 ± 0.7	0.64
Percentage body fat, %	23.9 ± 0.9	23.2 ± 1.2	0.62
Height, m	1.75 ± 0.02	1.78 ± 0.01	0.23
BMI, $kg \cdot m^{-2}$	25.8 ± 0.8	24.9 ± 0.8	0.43
HbA1c, %	5.3 ± 0.1	5.3 ± 0.1	0.80
Resting glucose, $mmol \cdot L^{-1}$	5.8 ± 0.2	6.2 ± 0.2	0.13
Resting insulin, $mU \cdot L^{-1}$	9.3 ± 0.9	8.4 ± 1.2	0.59
HOMA-IR	2.4 ± 0.2	2.4 ± 0.4	1.00
MVPA, min	145 ± 31	160 ± 33	0.95
1RM - Leg press, kg	179 ± 8	166 ± 6	0.23
1RM - Leg extension, kg	86 ± 6	88 ± 2	0.79
1RM - Lat pulldown, kg	60 ± 4	62 ± 4	0.78
1RM - Chest press, kg	60 ± 6	58 ± 5	0.77

[1]Values are mean ± SEM. *n* = 12 per treatment group. 15G: 15 g dietary protein, 15G+LEU: 15 g dietary protein + 1.5 g free crystalline leucine. 1RM: one repetition maximum, HbA1c: glycosylated hemoglobin, MVPA: moderate-to-vigorous physical activity, Resting: resting and fasted values. Data were analyzed with a student's unpaired t-test. No differences were detected between groups.

Table 2. Amino acid composition of the test beverages[1]

	15G	**15G+LEU**
	g	**g**
Alanine	0.45	0.45
Arginine	0.50	0.50
Aspartic acid	0.92	0.92
Glutamic acid	2.51	2.51
Glycine	0.23	0.23
Histidine	0.33	0.33
Isoleucine	0.66	0.66
Leucine	1.44	2.94
Lysine	1.19	1.19
Methionine	0.18	0.18
Phenylalanine	0.63	0.63
Proline	1.38	1.38
Serine	0.70	0.70
Threonine	0.57	0.57
Tyrosine	0.78	0.78
Valine	0.84	0.84

[1]Values are expressed in g. 15G: 15 g dietary protein, 15G+LEU: 15 g dietary protein + 1.5 g free crystalline leucine.