


# The muscle anabolic effect of protein ingestion during a hyperinsulinaemic euglycaemic clamp in middle-aged women is not caused by leucine alone

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## Key points

- It has been suggested that leucine is primarily responsible for the increase in muscle protein synthesis after protein ingestion because leucine uniquely activates the mTOR-p70S6K signalling cascade.
- We compared the effects of ingesting protein or an amount of leucine equal to that in the protein during a hyperinsulinaemic-euglycaemic clamp (to eliminate potential confounding as a result of differences in the insulinogenic effect of protein and leucine ingestion) on muscle anabolic signalling and protein turnover in 28 women.
- We found that protein, but not leucine, ingestion increased muscle p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup>, although only protein, and not leucine, ingestion decreased muscle p-eIF2 $\alpha$ <sup>Ser51</sup> and increased muscle protein synthesis.

**Abstract** It has been suggested that leucine is primarily responsible for the increase in muscle protein synthesis (MPS) after protein ingestion because leucine uniquely activates the mTOR-p70S6K signalling cascade. We tested this hypothesis by measuring muscle p-mTOR<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup> and p-eIF2 $\alpha$ <sup>Ser51</sup>, as well as protein turnover (by stable isotope labelled amino acid tracer infusion in conjunction with leg arteriovenous blood and muscle tissue sampling), in 28 women who consumed either 0.45 g protein kg<sup>-1</sup> fat-free mass (containing 0.0513 g leucine kg<sup>-1</sup> fat-free mass) or a control drink ( $n = 14$ ) or 0.0513 g leucine kg<sup>-1</sup> fat-free mass or a control drink ( $n = 14$ ) during a hyperinsulinaemic-euglycaemic clamp procedure (HECP). Compared to basal conditions, the HECP alone (without protein or leucine ingestion) suppressed muscle protein breakdown by ~20% and increased p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup> by >50% (all  $P < 0.05$ ) but had no effect on p-eIF2 $\alpha$ <sup>Ser51</sup> and MPS. Both protein and leucine ingestion further

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increased p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup>, although only protein, and not leucine, ingestion decreased (by ~35%) p-eIF2 $\alpha$ <sup>Ser51</sup> and increased (by ~100%) MPS (all  $P < 0.05$ ). Accordingly, leg net protein balance changed from negative (loss) during basal conditions to equilibrium during the HECP alone and the HECP with concomitant leucine ingestion and to positive (gain) during the HECP with concomitant protein ingestion. These results provide new insights into the regulation of MPS by demonstrating that leucine and mTOR signalling alone are not responsible for the muscle anabolic effect of protein ingestion during physiological hyperinsulinaemia, most probably because they fail to signal to eIF2 $\alpha$  to initiate translation and/or additional amino acids are needed to sustain translation.

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## Introduction

Muscle mass is maintained by a tightly controlled balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). During basal, postabsorptive conditions, the rate of MPB exceeds the rate of MPS, causing a net loss of protein (Rennie *et al.* 2004). Meal intake compensates for the postabsorptive loss of muscle protein because dietary protein-derived amino acids stimulate MPS and insulin suppresses MPB (Rennie *et al.* 2004). The postprandial net protein gain is largely determined by the amount of protein ingested because the postprandial increase in plasma amino acids stimulates MPS in a dose-dependent manner, whereas the plasma insulin concentration necessary to achieve maximal suppression of MPB (~15–30 mU L<sup>-1</sup>) already occurs after consuming a small amount of protein or carbohydrate (Bohe *et al.* 2003; Greenhaff *et al.* 2008; Moore *et al.* 2009). The exact mechanism(s) responsible for the stimulatory effect of postprandial hyperaminoacidemia on MPS is unclear. Leucine probably plays a key role in stimulating postprandial MPS because it uniquely activates the mTOR-p70S6K signalling cascade (Atherton *et al.* 2010; Iwanaka *et al.* 2010; Moberg *et al.* 2014), which is essential for myocellular hypertrophy (Goodman *et al.* 2011; Walker *et al.* 2011; Moro *et al.* 2016). Furthermore, increasing the leucine content of mixed meals or protein beverages augments the postprandial increase in MPS (Rieu *et al.* 2006; Wall *et al.* 2013; Churchward-Venne *et al.* 2014; Murphy *et al.* 2016; Devries *et al.* 2018) and we have found that leucine, but not total protein, ingestion determines the increase in muscle mTOR-p70S6K (Smith *et al.* 2015c). However, it is not known whether leucine alone is responsible for the increase in MPS after protein ingestion.

The present study aimed to compare the effects of ingesting protein or an amount of leucine equal to that in the protein on muscle protein turnover. Participants ingested either 0.45 g whey protein kg<sup>-1</sup> fat-free mass (FFM), containing 0.0513 g leucine kg<sup>-1</sup> FFM, or 0.0513 g leucine kg<sup>-1</sup> FFM alone during

a hyperinsulinaemic-euglycaemic clamp procedure (HECP) to eliminate potential confounding as a result of differences in the insulinogenic effect of protein and leucine ingestion. <sup>2</sup>H-labelled phenylalanine and leucine tracers were infused throughout the study. Leg arteriovenous blood and thigh muscle biopsy sampling was used to determine net protein kinetics.

## Methods

### Human subjects research regulatory compliance

The study was approved and monitored by the Human Research Protection Office at Washington University School of Medicine (St Louis, MO, USA). Written informed consent was obtained from all participants before their participation.

### Study participants

Twenty-eight sedentary (<1.5 h of exercise/week), 50–65-year-old postmenopausal women participated in the present study: 14 in the protein group and 14 in the leucine group. All potential participants completed a comprehensive medical examination, including a history and physical examination, a resting electrocardiogram, standard blood tests, and an oral glucose tolerance test. Exclusion criteria included evidence of chronic illness or significant organ dysfunction (e.g. diabetes, kidney disease), taking medications (including hormone replacement therapy) that could interfere with the action of insulin or protein metabolism, excessive alcohol intake (> 20 g per day), and smoking or chewing tobacco products. Participants' body fat mass and FFM were determined using dual energy X-ray absorptiometry (Lunar iDXA; GE Healthcare Lunar, Madison, WI, USA) and thigh muscle volume was determined using magnetic resonance imaging as described previously (Smith *et al.* 2015a). Details concerning the age, body composition and thigh muscle volume of participants are presented in Table 1.

**Table 1. Age, body composition and thigh muscle volume of participants**

	Protein group	Leucine group	<i>P</i>
Age (years)	57.4 ± 1.2	60.1 ± 0.9	0.09
Body mass index (kg m <sup>-2</sup> )	33.6 ± 0.7	36.2 ± 1.2	0.08
Body mass (kg)	90.7 ± 2.1	98.7 ± 3.6	0.07
Body fat (%)	48.3 ± 0.9	50.4 ± 1.1	0.15
Thigh muscle volume (cm <sup>3</sup> )	3,639 ± 87	3,568 ± 91	0.58

Data are the mean ± SEM; *n* = 14 per group.

### Protein metabolism studies

Each participant completed two HECP in randomized order in conjunction with or without protein ingestion (*n* = 14) or in conjunction with or without leucine ingestion (*n* = 14). Before each HECP, participants were instructed to adhere to their usual diet and to refrain from vigorous physical activity for 3 days. In the late afternoon before the HECP, participants were admitted to the Clinical Translational Research Unit (Washington University School of Medicine, St Louis, MO, USA) where they consumed a standard dinner between 18.00 h and 19.00 h, and then fasted, except for water, until the next morning. At 06.00 h, a catheter was inserted into an arm vein for the infusion of <sup>2</sup>H-labelled phenylalanine and leucine tracers; catheters for blood sampling were inserted into the radial artery of the opposite arm and in retrograde fashion into the femoral vein of one leg. At 06.45 h, primed, constant infusions of [ring-<sup>2</sup>H<sub>5</sub>]phenylalanine (priming dose: 6.0 μmol kg FFM<sup>-1</sup>, infusion rate: 0.10 μmol kg FFM<sup>-1</sup> min<sup>-1</sup>) and [5,5,5-<sup>2</sup>H<sub>3</sub>]leucine (priming dose: 6.3 μmol kg FFM<sup>-1</sup>; 0.14 μmol kg FFM<sup>-1</sup> min<sup>-1</sup>), both purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA), were started and maintained for 7 hours. Four hours after the start of the tracer infusions, a HECP was started and maintained for 3 h. Human insulin (Novolin R; Novo Nordisk, Princeton, NJ, USA) was infused at a rate of 50 mU m<sup>-2</sup> body surface area (BSA) min<sup>-1</sup> (initiated with a 10 min step-down priming infusion of 200 mU m<sup>-2</sup> BSA min<sup>-1</sup> for the initial and 100 mU m<sup>-2</sup> BSA min<sup>-1</sup> for the subsequent 5 min). Euglycaemia (blood glucose ~5.6 mM) was maintained by variable rate infusion of 20% dextrose (Baxter, Deerfield, IL, USA). To adjust for the insulin-mediated suppression of whole body proteolysis, the phenylalanine and leucine tracer infusion rates were reduced to 0.08 μmol kg FFM<sup>-1</sup> min<sup>-1</sup> and 0.10 μmol kg FFM<sup>-1</sup> min<sup>-1</sup>, respectively, during the HECP.

Participants in the protein group consumed either 0.45 g of whey protein (unflavored Unjury; ProSynthesis Laboratories, Inc., Reston, VA, USA) per kg FFM (containing 0.0513 g leucine per kg FFM) dissolved in 270 mL of water or an equivalent volume of water (control

study) in small aliquots every 20 min during the 3 h HECP. The total amount of protein ingested was 20.7 ± 0.5 g (containing 2.4 ± 0.1 g leucine). To minimize changes in arterial plasma leucine and phenylalanine enrichments as a result of an increased amino acid appearance in plasma during protein ingestion, [5,5,5-<sup>2</sup>H<sub>3</sub>]leucine and [ring-<sup>2</sup>H<sub>5</sub>]phenylalanine, equivalent to 5% and 6% of the leucine and phenylalanine content in whey protein, respectively, were added to the protein drinks. Participants in the leucine group consumed either 0.0513 g leucine (Sigma-Aldrich, Inc., St Louis, MO, USA) per kg FFM (total: 2.4 ± 0.1 g) enriched to 5% with [5,5,5-<sup>2</sup>H<sub>3</sub>]leucine or an equivalent volume of the control solution only in small aliquots every 20 min during the 3 h HECP. The protein or leucine ingestion and respective control studies were conducted in randomized order, 1–4 weeks apart.

Arterial and femoral venous blood samples were obtained immediately before starting the tracer infusions, every 6–7 min during the last 20 min of the basal period, 1 h and 2 h after starting the HECP, and every 6–7 min during last 20 min of the HECP. Additional arterial blood samples were obtained every 10 min during the HECP to monitor blood glucose concentration. Leg blood flow in the common femoral artery was measured at regular intervals between 2 h and 3 h after starting the tracer infusions (basal period) and between 1 h and 3 h after starting the HECP using Doppler ultrasound (M-Turbo; Sonosite Inc., Bothell, WA, USA) and a linear array 13 to 6 MHz frequency probe (Sonosite Inc.) (Radegran & Saltin, 1999). In nine of the 14 subjects in each group, muscle tissue samples from the quadriceps femoris were obtained under local anaesthesia (lidocaine HCl, 2%; Hospira Inc., Lake Forest, IL, USA) using a Tilley-Henkel forceps 1 h and 4 h after starting the tracer infusions (basal period) and 3 h after starting the HECP to determine muscle protein fractional turnover and the contents of the key cell growth stimulators p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup>, which sense an increase in amino acids, the eIF2 kinase p-GCN2<sup>Thr899</sup>, which senses uncharged t-RNA, and the inhibitory translation initiation factor p-eIF2α<sup>Ser51</sup>.

### Sample processing and analysis

Blood samples were collected in chilled tubes containing heparin (to determine glucose and insulin concentrations) or EDTA (to determine amino acid concentrations and enrichments). Samples were placed in ice and plasma was separated by centrifugation within 30 min of collection and then stored at -80°C until final analyses. Muscle samples were rinsed in ice-cold saline immediately after collection, cleared of visible fat and connective tissue, frozen in liquid nitrogen and stored at -80°C until final analysis.

Plasma glucose concentration was determined using an automated glucose analyser (Yellow Spring Instruments Co, Yellow Springs, OH, USA). The plasma insulin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (EMD Millipore, St Charles, MO, USA). Plasma phenylalanine and leucine concentrations and enrichments were determined using gas-chromatography/mass-spectrometry (GC-MS; MSD 5973 System, Hewlett-Packard, Palo Alto, CA, USA) after adding known amounts of internal standards to each sample and converting the amino acids to their *t*-butyldimethylsilyl (*t*-BDMS) derivatives (Smith *et al.* 2007). The concentrations of additional amino acids were determined by GC-MS using the EZ:faast Amino Acid Analysis kit (Phenomenex, Torrance, CA, USA).

To determine phenylalanine enrichment in muscle proteins and muscle free phenylalanine and leucine concentrations and enrichments, ~20 mg of each muscle biopsy sample was homogenized in 1 mL of trichloroacetic acid solution (3% w/v) after the addition of known amounts of internal standards. Muscle proteins were then precipitated by centrifugation and the supernatant, containing free amino acids, was collected. The pellet containing muscle proteins was hydrolysed in HCl. The supernatant, containing free amino acids was purified by passing it through a 0.2  $\mu$ m filter. Amino acids in the hydrolysate and eluent were converted to their *t*-BDMS derivative and analysed by GC-MS (Smith *et al.* 2007).

The concentrations of phosphorylated intramuscular amino acid sensing proteins and proteins involved in translation initiation (p-mTOR<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup>, p-GCN2<sup>Thr899</sup> and p-eIF2 $\alpha$ <sup>Ser51</sup>) were quantified by western analysis as described previously (Smith *et al.* 2015c). Frozen muscle tissue was rapidly homogenized in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and proteins were extracted (Yoshino *et al.* 2012). Then, 20  $\mu$ g of protein from each sample was loaded onto gradient (4–20%) polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA), separated by SDS-PAGE and transferred to Immobilo polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The blotted membranes were incubated with the following primary antibodies: rabbit polyclonal anti-phospho-mTOR (Ser2448) (#2971; Cell Signaling Technology), rabbit monoclonal anti-phospho-p70S6K (Thr389) (#9234; Cell Signaling Technology), rabbit monoclonal anti-phospho-GCN2 (Thr899) (#75836; Abcam, Cambridge, UK) and rabbit monoclonal anti-phospho-eIF2 $\alpha$  (Ser51) (#3398 Cell Signaling Technology). All blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies and developed using Amersham ECL Select Western Blotting Detection Reagent (#RPN2235; GE Healthcare

Life Sciences, Piscataway, NJ, USA). The p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup> data have been reported previously in a study evaluating the effects of protein and leucine ingestion on muscle p-mTOR<sup>Ser2448</sup> and the action of insulin (Smith *et al.* 2015c).

### Calculations

Phenylalanine and leucine kinetics across the leg were calculated using a two-pool model and recently described equations (Smith *et al.* 2015b):

$$\text{Leg net balance (NB)} = (\text{CT}_A - \text{CT}_V) \times \text{PF} \quad (1)$$

Leg rate of disappearance (leg Rd)

$$= (\text{CT}_A \times \text{MPE}_A - \text{CT}_V \times \text{MPE}_V) \times (\text{PF}/\text{MPE}_A) \quad (2)$$

$$\text{Leg rate of appearance (leg, Ra)} = \text{Leg Rd} - \text{NB} \quad (3)$$

where  $\text{CT}_A$  and  $\text{CT}_V$  are total (i.e. tracee + tracer) plasma amino acid concentrations in the artery and femoral vein, respectively;  $\text{MPE}_A$  and  $\text{MPE}_V$  represent the mole percent excess of the amino acids in arterial and femoral venous plasma, respectively; and PF is plasma flow ( $\text{mL min}^{-1}$ ) calculated from the measured blood flow adjusted for haematocrit (Hct). Leg Ra represents the rate of amino acid release into the vein from protein breakdown and leg Rd reflects the rate of arterial plasma amino acids taken up by the muscle for protein synthesis (phenylalanine) or protein synthesis and oxidation (leucine).

Phenylalanine intramuscular Ra from proteolysis and intramuscular Rd to protein synthesis were calculated using a three-pool model:

$$\text{Intracellular Ra (F}_{M,O}) = \text{F}_{M,A} \times [(\text{MPE}_A/\text{MPE}_M) - 1] \quad (4)$$

where  $\text{MPE}_M$  is the mole percent excess of phenylalanine in the muscle free pool, and  $\text{F}_{M,A}$  is inward transport, calculated as  $\{[(\text{MPE}_M - \text{MPE}_V)/(\text{MPE}_A - \text{MPE}_M) \times \text{CT}_V] + \text{CT}_A\} \times \text{PF}$

$$\text{Intracellular Rd (F}_{O,M}) = \text{F}_{M,O} + \text{NB} \quad (5)$$

The mixed muscle protein fractional synthesis rate (FSR) was calculated by dividing the increment in [<sup>2</sup>H<sub>5</sub>]phenylalanine enrichment in muscle protein over time by either the [<sup>2</sup>H<sub>5</sub>]phenylalanine enrichment in plasma (weighted average from samples collected during basal conditions and at 1 h and 2 h, as well as during last 20 min of the HECp) or the muscle intracellular free precursor pool. The results were not affected by the choice of precursor.



**Table 2. Arterial plasma glucose and insulin concentrations and leg plasma flow**

	Protein group		Leucine group	
	Control	Protein	Control	Leucine
Glucose (mm)				
Basal	5.05 ± 0.05	5.12 ± 0.06	5.38 ± 0.13	5.27 ± 0.10
HECP	5.62 ± 0.04*	5.59 ± 0.05*	5.58 ± 0.06*	5.57 ± 0.05*
Insulin (mU L <sup>-1</sup> )				
Basal	4.6 (3.5, 6.8)	4.5 (2.7, 6.0)	5.4 (4.8, 7.4)	5.5 (4.0, 6.9)
HECP	58.2 (49.3, 65.7)*	65.4 (57.7, 75.9)*	53.7 (48.8, 65.9)*	52.2 (47.2, 64.3)*
Plasma flow (mL min <sup>-1</sup> )				
Basal	167 ± 18	173 ± 16	204 ± 43	184 ± 23
HECP	206 ± 24*	208 ± 19*	244 ± 46*	230 ± 29*

Data are the mean ± SEM or medians (quartile 1, quartile 4); *n* = 14 per group.

\*Significant main effect of HECP (*P* < 0.05).

### Statistical analysis

Statistical analyses were carried out using SPSS, version 24 (IBM Corp., Armonk, NY, USA). Student's *t* test was used to compare the basic characteristics of participants in the protein and leucine groups. Three-way ANOVA, with group (protein *vs.* leucine) as the between subject factor and study (control *vs.* protein or leucine ingestion) and time (basal *vs.* clamp) as within subject factors, was used to evaluate the effect of the HECP with and without concomitant protein or leucine ingestion on plasma metabolite and hormone concentrations, leg plasma flow, muscle free amino acid concentrations, intramuscular signalling protein contents and muscle protein turnover. When statistically significant interactions were found, Tukey's *post hoc* procedure was used to locate the differences. *P* < 0.05 was considered statistically significant. Data are presented as the mean ± SEM unless otherwise noted.

## Results

### Arterial plasma glucose and insulin concentrations and leg plasma flow (Table 2)

During basal conditions, plasma glucose and insulin concentrations and leg plasma flow were not different among the protein and leucine groups and their respective control studies. During the HECP, the plasma glucose concentration was maintained at the 5.6 mM target in all studies; the insulin concentration increased ~10-fold and leg plasma flow increased by ~20% above basal values in all studies (no difference among groups and studies).

### Arterial and venous plasma and muscle free amino acid concentrations (Fig. 1 and Table 3)

During basal conditions, plasma amino acid concentrations were not different among the protein and leucine groups and their respective control studies.

Arterial plasma total essential and non-essential amino acid concentrations decreased by ~15–30% during the HECP in the control studies and during the HECP with concomitant leucine ingestion but increased by ~65% (essential) and ~10% (non-essential) above basal values during the HECP with concomitant protein ingestion.

Arterial and venous plasma phenylalanine concentrations decreased by ~25% during the HECP in the control studies and during the HECP with concomitant leucine ingestion, although they were not different or slightly greater than basal values during the HECP with concomitant protein ingestion; muscle phenylalanine concentration decreased by ~10–30% in all studies. Arterial and venous plasma leucine concentrations decreased by ~50% during the HECP in the control studies but increased by ~50% (venous) to 100% (arterial) during both protein and leucine ingestion. Muscle leucine concentration decreased by ~40% during the HECP in the control studies, did not change during the HECP with concomitant protein ingestion, and increased by ~50% with concomitant leucine ingestion.

### Amino acid sensing and anabolic signalling in muscle (Fig. 2)

Compared to basal conditions, the HECP alone increased both p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr38</sup> but had no effect on p-GCN2<sup>Thr899</sup> and p-eIF2α<sup>Ser51</sup>. Both protein and leucine ingestion augmented the HECP-induced increase in p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr38</sup>, without a difference between groups. Protein, but not leucine, ingestion decreased p-eIF2α<sup>Ser51</sup>. Neither protein, nor leucine ingestion altered p-GCN2<sup>Thr899</sup>.

### Leg phenylalanine and leucine kinetics (Fig. 3)

During basal conditions, leg phenylalanine and leucine kinetics were not different among the protein and leucine groups and their respective control studies. Leg

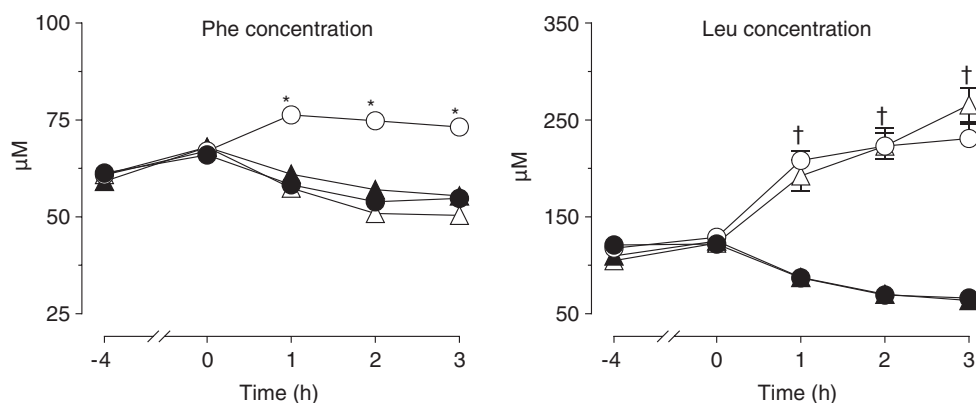
phenylalanine uptake, an index of MPS, increased by >50% during the HECP with concomitant protein ingestion but did not change during the HECP alone or with concomitant leucine ingestion. Intramuscular phenylalanine Rd, a measure of MPS, increased by ~100% during the HECP with concomitant protein ingestion (from  $2119 \pm 339 \text{ nmol min}^{-1}$  during basal conditions to  $4351 \pm 621 \text{ nmol min}^{-1}$ ) but did not change during the HECP alone in the protein and leucine groups or with concomitant leucine ingestion ( $3127 \pm 407 \text{ nmol min}^{-1}$  during basal conditions vs.  $3407 \pm 342 \text{ nmol min}^{-1}$  during the HECP alone or with leucine ingestion; total mean  $\pm$  SEM). Leg phenylalanine release, an index of MPB, decreased by ~20% during the HECP alone and with concomitant protein and leucine ingestion. Intramuscular phenylalanine Ra, a measure of MPB, tended ( $P = 0.09$ ) to decrease during the HECP in all studies (from  $3506 \pm 299 \text{ nmol min}^{-1}$  during basal conditions to  $3235 \pm 255 \text{ nmol min}^{-1}$  during the HECP; total mean  $\pm$  SEM of all studies), although the difference did not reach statistical significance, probably because of a lack of statistical power. Leg net phenylalanine balance changed from negative (net protein loss) during basal conditions to equilibrium during the HECP alone and the HECP with concomitant leucine ingestion, and to positive (net gain) during the HECP with concomitant protein ingestion. Leg leucine uptake was not affected by the HECP alone, whereas both protein and leucine ingestion markedly increased leg leucine uptake. Leg leucine release decreased by ~20–30% during the HECP alone and during the HECP with concomitant protein or leucine ingestion; accordingly, leucine net balance across the leg markedly increased during both protein and leucine ingestion.

### Muscle protein FSR (Fig. 4)

Compared to basal conditions, the muscle protein FSR was not affected by the HECP alone or the HECP with concomitant leucine ingestion but increased by ~100% with protein ingestion.

### Discussion

The muscle anabolic effect of protein ingestion is proposed to be mediated by leucine because of its unique ability to activate mTOR-p70S6K, which is essential for regulating MPS (Atherton *et al.* 2010; Iwanaka *et al.* 2010; Goodman *et al.* 2011; Walker *et al.* 2011; Moberg *et al.* 2014; Moro *et al.* 2016). We compared the effects of ingesting protein or an amount of leucine equal to that in the ingested protein on anabolic signalling and protein turnover in muscle during controlled physiological hyperinsulinaemia, as achieved using the HECP, to eliminate potential confounding as a result of differences in the insulinogenic effect of protein and leucine ingestion. Our data demonstrate that, compared to basal conditions, the HECP alone increased both p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr38</sup> but had no effect on p-eIF2 $\alpha$ <sup>Ser51</sup> or the rate of MPS, and also decreased the rate of MPB. Both protein and leucine ingestion during the HECP caused a similar further increase in p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup>; however, only protein, but not leucine, ingestion decreased p-eIF2 $\alpha$ <sup>Ser51</sup> (which permits translation initiation) and increased the rate of MPS. The protein ingestion-induced decrease in p-eIF2 $\alpha$ <sup>Ser51</sup> occurred in the absence of changes in GCN2 activity. The stimulatory effect of protein, but not leucine, ingestion on



**Figure 1. Arterial plasma phenylalanine and leucine concentration time courses**

Basal conditions: -4 h to 0 h; hyperinsulinaemic euglycaemic clamp procedure: 0 h to 3 h. Data are the mean  $\pm$  SEM. Circles represent the protein ingestion (open circles) and respective control (filled circles) studies; triangles represent the leucine ingestion (open triangles) and respective control (filled triangles) studies. Three-way ANOVA revealed a significant group (whey vs. leucine)  $\times$  study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for plasma phenylalanine and (ii) a significant study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for plasma leucine concentrations. \*Significantly different from the corresponding value in all the other studies ( $P < 0.05$ ). †Significantly different from the corresponding control value ( $P < 0.05$ ).

**Table 3. Arterial and venous plasma and muscle free amino acid concentrations**

	Protein group		Leucine group	
	Control	Protein	Control	Leucine
<b>Total essential (<math>\mu\text{M}</math>)</b>				
Artery				
Basal	807 $\pm$ 29	839 $\pm$ 29	780 $\pm$ 28	780 $\pm$ 31
HECP	526 $\pm$ 30*	1,376 $\pm$ 53*,†,‡	561 $\pm$ 24*	666 $\pm$ 30*,†
<b>Total non-essential (<math>\mu\text{M}</math>)</b>				
Artery				
Basal	934 $\pm$ 45	917 $\pm$ 45	887 $\pm$ 27	935 $\pm$ 26
HECP	692 $\pm$ 42*	1,033 $\pm$ 37*,†,‡	759 $\pm$ 27*	756 $\pm$ 47*
<b>Phenylalanine (<math>\mu\text{M}</math>)</b>				
Artery				
Basal	66 $\pm$ 2	67 $\pm$ 2	68 $\pm$ 2	68 $\pm$ 2
HECP	54 $\pm$ 2*	75 $\pm$ 2*,†,‡	57 $\pm$ 2*	50 $\pm$ 2*
Vein				
Basal	71 $\pm$ 3	71 $\pm$ 2	73 $\pm$ 3	72 $\pm$ 2
HECP	52 $\pm$ 3*	66 $\pm$ 2†,‡	56 $\pm$ 3*	47 $\pm$ 2*,†
Muscle				
Basal	85 $\pm$ 4	91 $\pm$ 3	77 $\pm$ 6	81 $\pm$ 6
HECP	67 $\pm$ 5 <sup>§</sup>	84 $\pm$ 12 <sup>§</sup>	63 $\pm$ 7 <sup>§</sup>	56 $\pm$ 5 <sup>§</sup>
<b>Leucine (<math>\mu\text{M}</math>)</b>				
Artery				
Basal	122 $\pm$ 5	129 $\pm$ 3	125 $\pm$ 5	123 $\pm$ 6
HECP	62 $\pm$ 4*	230 $\pm$ 14*,†	67 $\pm$ 4*	255 $\pm$ 18*,†
Vein				
Basal	127 $\pm$ 5	132 $\pm$ 4	127 $\pm$ 6	127 $\pm$ 6
HECP	58 $\pm$ 4*	176 $\pm$ 10*,†	63 $\pm$ 5*	194 $\pm$ 16*,†
Muscle				
Basal	150 $\pm$ 8	172 $\pm$ 9	151 $\pm$ 7	150 $\pm$ 7
HECP	88 $\pm$ 9*	184 $\pm$ 18†,‡	92 $\pm$ 10*	233 $\pm$ 15*,†

Data are the mean  $\pm$  SEM during the last 20 min of the basal period and the HECP. Three-way ANOVA revealed: (i) a significant group (whey vs. leucine)  $\times$  study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for arterial and venous plasma phenylalanine concentrations, muscle free leucine concentrations, and total essential and non-essential amino acid concentrations; (ii) a significant study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for arterial and venous plasma leucine concentrations; and (iii) a significant main effect of time for muscle free phenylalanine concentrations.

\*Significantly different from corresponding basal value ( $P < 0.05$ ).

†Significantly different from corresponding control value ( $P < 0.05$ ).

‡Significantly different from corresponding value in the leucine group ( $P < 0.05$ ).

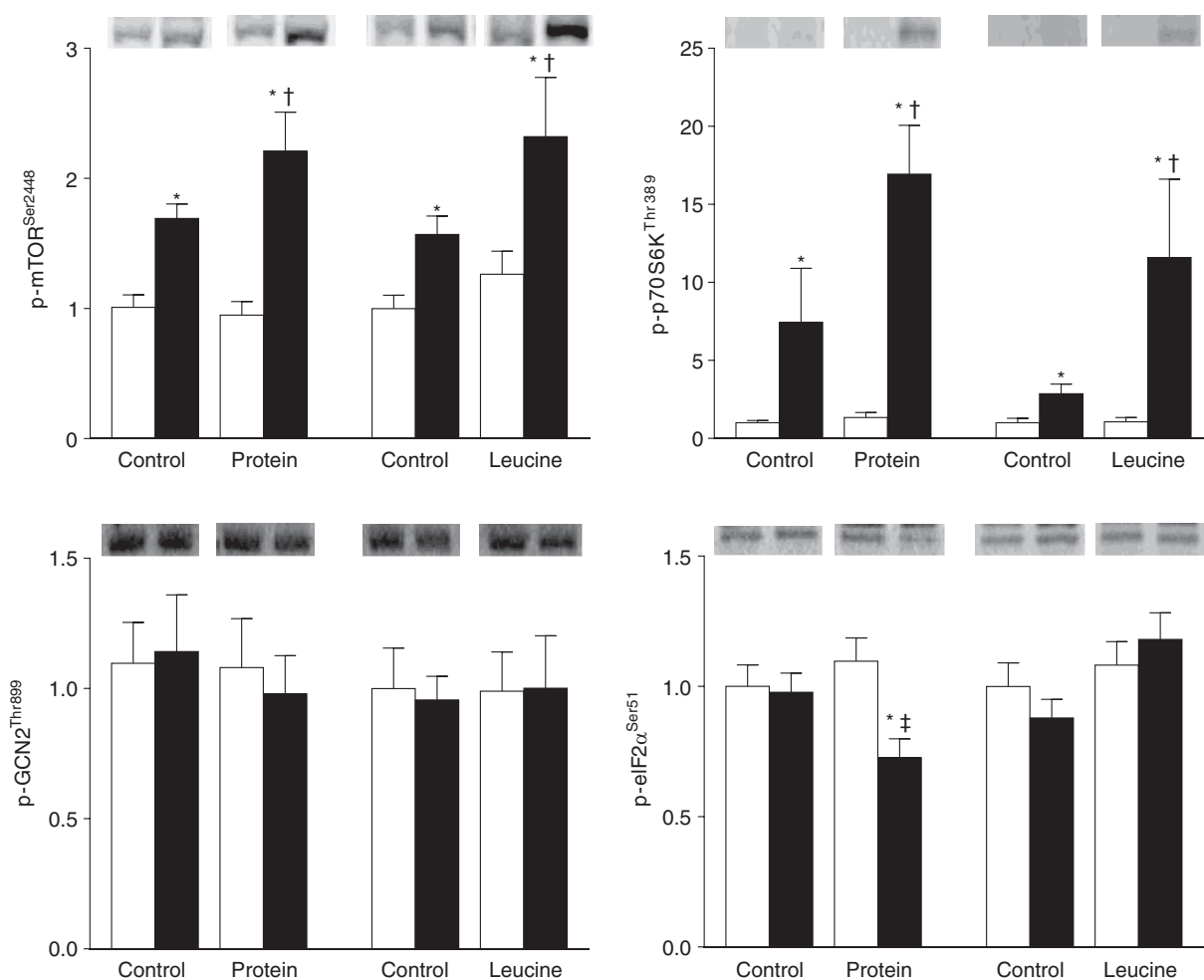
§Significant main effect of the HECP ( $P < 0.001$ ).

MPS was observed independently of the method used to measure muscle protein turnover (i.e. the arteriovenous phenylalanine tracer balance technique in conjunction with either a two- or three-pool modelling approach or by directly measuring the phenylalanine tracer incorporation rate into muscle protein). Neither protein, nor leucine ingestion augmented the insulin-mediated suppression of MPB or leg phenylalanine and leucine rates of release. These results confirm the potent anti-proteolytic effect of insulin and muscle anabolic effect of protein ingestion, as well as the dissociation among key anabolic signalling events in muscle and MPS; they also provide novel insights into the regulation of muscle protein turnover by nutrient ingestion.

The results of the present study are consistent with those obtained in a recent study conducted in very old mice (aged 25 months) reporting that oral gavage with a leucine-enriched protein solution, but not a matched amount of leucine alone, stimulated MPS (Dijk *et al.* 2018). However, our results are inconsistent with the observation that an i.v. or orally administered 'flooding' dose of leucine acutely increased the rate of MPS in healthy young men (Smith *et al.* 1992; Wilkinson *et al.* 2013). The differences in the results among studies could be a result of age-associated anabolic resistance (Cuthbertson *et al.* 2005; Smith *et al.* 2012). However, we consider this doubtful because, unlike leucine, protein ingestion did increase the rate of MPS both in our middle-aged

women and the aged rats studied by Dijk *et al.* (2018). Therefore, the differences in results among studies are probably related to the dose and mode of delivery, comprising a  $\sim 3.5$  g 'flooding' bolus (Smith *et al.* 1992; Wilkinson *et al.* 2013) vs. slow sustained (180 min) delivery of  $\sim 2.4$  g leucine in our protein and leucine groups, and/or the controlled hyperinsulinaemia and ensuing hypoaminoacidemia during the HECP in our study. Hypoaminoacidemia itself inhibits MPS (Kobayashi *et al.* 2003) and the results from our study suggest that leucine alone is insufficient to overcome this blockade because the availability of other (essential) amino acids needed for protein synthesis is rate limiting (Wolfe, 2017). This notion is supported by the results from studies conducted in neonatal pigs and adult rats that found i.v. leucine

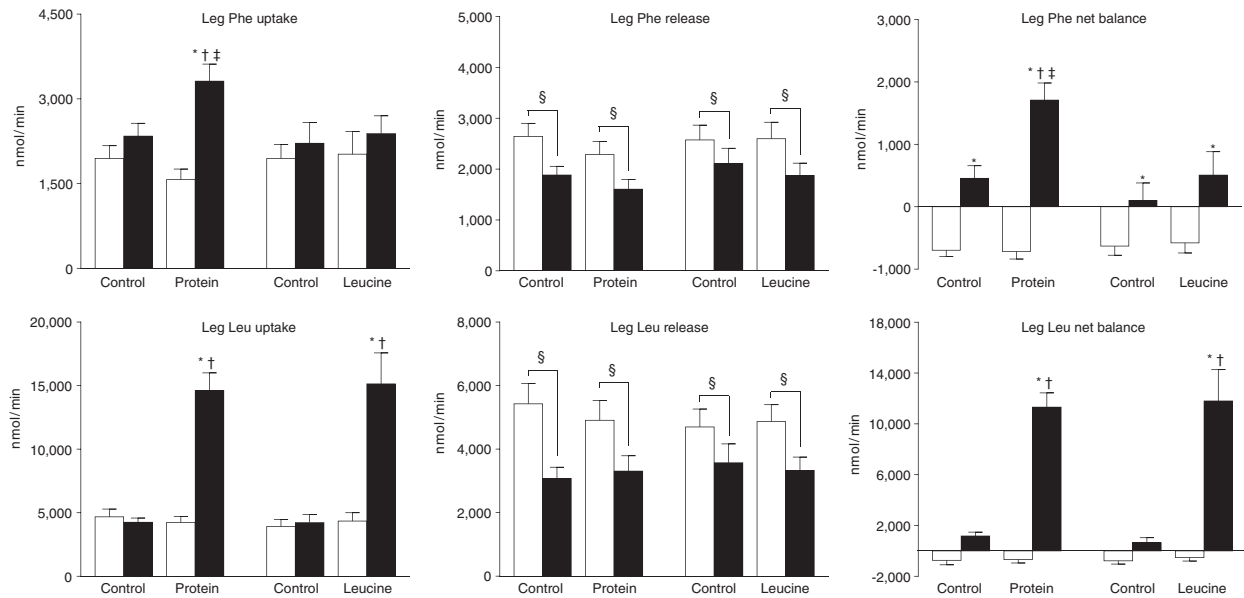
administration transiently increased the rate of MPS and this increase was only sustained when additional amino acids were provided (Anthony *et al.* 2002; Escobar *et al.* 2005; Wilson *et al.* 2010). Indeed, it has been found that leucine infusion causes a decrease in intramyocellular total amino acid concentration (Alvstrand *et al.* 1990), probably because it promotes amino acid incorporation into protein until their availability becomes rate-limiting (Wolfe, 2017). Moreover, there is no dose-response relationship between the amount of leucine given and the initial rate of MPS (Crozier *et al.* 2005). By contrast, protein and essential amino acid ingestion and i.v. mixed amino acid infusion elicit a dose-dependent increase in MPS (Bohe *et al.* 2003; Cuthbertson *et al.* 2005; Moore *et al.* 2009) and increasing the leucine content of mixed meals



**Figure 2. Muscle p-mTOR<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup>, p-GCN2<sup>Thr899</sup> and p-eIF2α<sup>Ser51</sup> contents**

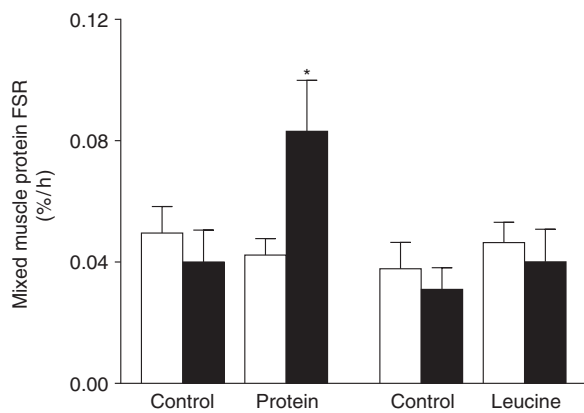
Average contents (arbitrary units) during basal conditions (white bars) and during the hyperinsulinaemic euglycaemic clamp procedure (black bars) expressed as the mean  $\pm$  SEM. Representative blots are shown on top. Three-way ANOVA revealed: (i) a significant group (whey vs. leucine)  $\times$  study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for p-eIF2α<sup>Ser51</sup> and (ii) a significant study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup>. \*Significantly different from corresponding basal value ( $P < 0.05$ ). †Significantly different from the corresponding control value ( $P < 0.05$ ). ‡Significantly different from corresponding value in the leucine group ( $P < 0.05$ ).





**Figure 3. Leg phenylalanine and leucine kinetics**

Leg phenylalanine and leucine kinetics assessed using the two-pool arterio-venous balance model during basal conditions (white bars) and during the hyperinsulinaemic euglycaemic clamp procedure (black bars). Data are the mean  $\pm$  SEM. Three-way ANOVA revealed: (i) a significant group (whey vs. leucine)  $\times$  study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for leg phenylalanine uptake and net balance; (ii) a significant study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction for leg leucine uptake and net balance; and (iii) a significant main effect of time for leg phenylalanine and leucine release. \*Significantly different from corresponding basal value ( $P < 0.05$ ). †Significantly different from corresponding control value ( $P < 0.05$ ). ‡Significantly different from corresponding value in the leucine group ( $P < 0.05$ ). §Significant main effect of the hyperinsulinaemic-euglycaemic clamp ( $P < 0.001$ ).



**Figure 4. Mixed muscle protein FSR**

Mixed muscle protein FSR, calculated using the plasma [ $^2\text{H}_5$ ]phenylalanine enrichment as the precursor pool, during basal conditions (white bars) and during the hyperinsulinaemic euglycaemic clamp procedure (black bars). The results were qualitatively the same when using the muscle free [ $^2\text{H}_5$ ]phenylalanine enrichment as the precursor pool. Data are the mean  $\pm$  SEM. Three-way ANOVA revealed a significant group (whey vs. leucine)  $\times$  study (control vs. protein or leucine ingestion)  $\times$  time (basal vs. clamp) interaction. \*Significantly different from all other values ( $P < 0.05$ ).

or protein beverages dose-dependently augments the post-prandial increase in MPS (Katsanos *et al.* 2006; Rieu *et al.* 2006; Wall *et al.* 2013; Churchward-Venne *et al.* 2014; Murphy *et al.* 2016; Devries *et al.* 2018). Taken together, these data suggest that leucine contributes to but does not cause the muscle anabolic effect of protein ingestion, which requires the presence of additional amino acids.

The canonical mTOR-p70S6K signalling pathway is considered to be the key regulator of MPS (Goodman *et al.* 2011; Walker *et al.* 2011; Moro *et al.* 2016). However, the results from our study suggest that mTOR independent signalling pathways are responsible for translation initiation because the HECP alone and the HECP with both concomitant protein and leucine ingestion increased mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup>, although only protein ingestion decreased p-eIF2 $\alpha$ <sup>Ser51</sup>, which permits translation initiation. The upstream mediators responsible for the differences in eIF2 $\alpha$ <sup>Ser51</sup> phosphorylation during the HECP with and without protein and leucine ingestion are not known. The results from our study suggest that the intracellular amino acid sensor GCN2, which can regulate eIF2 $\alpha$  activity (Kimball, 2002; Gordon *et al.* 2013; Moro *et al.* 2016), is not involved because GCN2 phosphorylation was not different during protein and leucine ingestion. Indeed, GCN2 phosphorylation was not altered by either the HECP or protein and leucine

ingestion, despite marked differences in intramyocellular amino acid concentrations during basal conditions and the HECP with and without protein and leucine ingestion. The most probable explanation for this observation is that intramyocellular amino acid concentrations did not fall below a critical threshold of amino acid 'deprivation' that activates GCN2 (Kimball, 2002; Gordon *et al.* 2013).

The increased leucine uptake by muscle during leucine ingestion was presumably oxidized because leucine ingestion did not increase MPS and the intramyocellular leucine concentration during leucine ingestion was not much greater than that during protein ingestion despite similar leg leucine uptake rates. This finding is consistent with studies that evaluated the fate of leucine during i.v. leucine infusion more directly by measuring the production of  $\alpha$ -ketoisocaproic acid, a leucine oxidation product (Abumrad *et al.* 1982; Alvestrand *et al.* 1990; Nair *et al.* 1992).

Neither protein nor leucine ingestion augmented the insulin-mediated suppression of MPB, assessed as the intracellular appearance of phenylalanine from proteolysis (three-pool model) or leg phenylalanine release (two-pool model). This finding is consistent with the results from previous studies that found the ingestion or infusion of complete or essential amino acid mixtures did not alter MPB (Biolo *et al.* 1997; Glynn *et al.* 2010a; Glynn *et al.* 2013). By contrast, i.v. infusions of a mixture of branched chain amino acids or leucine alone (Louard *et al.* 1990; Nair *et al.* 1992; Louard *et al.* 1995) or ingestion of a leucine-enriched essential amino acid solution (Glynn *et al.* 2010b) inhibited MPB. The reasons for the differences in results among those studies are not clear, although they could be a result of differences in the dose of amino acids/protein administered and/or the prevailing insulin concentration. In the present study, MPB was probably already maximally suppressed by insulin during the HECP alone because plasma insulin increased from  $\sim 5\text{--}6\text{ mU L}^{-1}$  during basal conditions to  $\sim 50\text{--}60\text{ mU L}^{-1}$  during the HECP and MPB is maximally suppressed at plasma insulin concentrations  $> 30\text{ mU L}^{-1}$  (Greenhaff *et al.* 2008).

### Summary and conclusion

The results from the present study confirm the potent anti-proteolytic effect of insulin and the muscle anabolic effect of protein ingestion and provide new insights into the regulation of MPS by demonstrating that leucine and mTOR signalling alone are not responsible for the muscle anabolic effect of protein ingestion during physiological hyperinsulinaemia, probably because they fail to signal to eIF2 $\alpha$  to initiate translation and/or because additional amino acids are needed to sustain translation.

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## Additional information

### Competing interests

The authors declare that they have no competing interests.

### Author contributions

BM designed the experiments. GIS, RR, DNR, and SK conducted the experiments. SvV, GIS, LP, RR, AO, JY, and BM analyzed the data. SvV, GIS, SK, and BM wrote the manuscript. All authors

approved the final version of the manuscript submitted for publication and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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