

# Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia

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The present study was designed to assess the effects of dietary leucine supplementation on muscle protein synthesis and whole body protein kinetics in elderly individuals. Twenty healthy male subjects ( $70 \pm 1$  years) were studied before and after continuous ingestion of a complete balanced diet supplemented or not with leucine. A primed ( $3.6 \mu\text{mol kg}^{-1}$ ) constant infusion ( $0.06 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ) of L-[1- $^{13}\text{C}$ ]phenylalanine was used to determine whole body phenylalanine kinetics as well as fractional synthesis rate (FSR) in the myofibrillar fraction of muscle proteins from vastus lateralis biopsies. Whole body protein kinetics were not affected by leucine supplementation. In contrast, muscle FSR, measured over the 5-h period of feeding, was significantly greater in the volunteers given the leucine-supplemented meals compared with the control group ( $0.083 \pm 0.008$  versus  $0.053 \pm 0.009\% \text{h}^{-1}$ , respectively,  $P < 0.05$ ). This effect was due only to increased leucine availability because only plasma free leucine concentration significantly differed between the control and leucine-supplemented groups. We conclude that leucine supplementation during feeding improves muscle protein synthesis in the elderly independently of an overall increase of other amino acids. Whether increasing leucine intake in old people may limit muscle protein loss during ageing remains to be determined.

(Received 2 April 2006; accepted after revision 14 June 2006; first published online 15 June 2006)

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During ageing, a decline in skeletal muscle mass occurs in both humans (Forbes & Halloran, 1976; Dutta & Hadley, 1995) and rodents (Holloszy *et al.* 1991). This atrophy is associated with a loss of muscle strength, which directly affects the mobility and health of elderly people. The mechanisms leading to sarcopenia are still unclear but result from an imbalance between rates of protein synthesis and degradation. This imbalance is not obvious when basal rates of protein turnover are measured (Dardevet *et al.* 1994; Mosoni *et al.* 1995; Volpi *et al.* 2001) but is detected in the postprandial state. An apparent defect in the stimulation of muscle protein synthesis has been shown in old rats (Mosoni *et al.* 1995) and elderly humans (Arnal *et al.* 1999) after the ingestion of a normal protein meal. Moreover, muscle protein breakdown has been shown to be unresponsive to food intake in aged rats (Combaret *et al.* 2005). This defect results in a small daily muscle protein loss, leading in the long term to muscle wasting in the elderly. The origin of this alteration remains obscure because muscle protein

synthesis responded normally if large amounts of amino acids were continuously infused in old rats (Mosoni *et al.* 1993) or given orally in aged volunteers (Volpi *et al.* 1999). Nevertheless, the studies agreed in that aged muscle is less sensitive to the stimulatory effects of amino acids at physiological concentrations but is still able to respond if the increase of aminoacidaemia is large enough (Volpi *et al.* 1999; Arnal *et al.* 1999; Paddon-Jones *et al.* 2004). Recently, the same conclusion was drawn with essential amino acids (EAAs) by Katsanos *et al.* (2005) who showed in the elderly, that a small bolus of EAAs ( $\sim 7$  g) was unable to stimulate muscle protein synthesis whereas an increase in protein synthesis occurred normally when EAA intake was doubled (Paddon-Jones *et al.* 2004).

Among the amino acids, leucine seems to play the major role. Indeed, Anthony *et al.* (2000*a,b*) showed that orally administered leucine stimulated muscle protein synthesis by itself *in vivo* and this was partly independent of insulin. Furthermore, leucine has been shown to act as a true mediator by specifically modulating the

activities of intracellular kinases linked to the translation of proteins such as mammalian target of rapamycin (mTOR)/70 kDa ribosomal protein S6 (p70S6K) kinases (Kimball *et al.* 1999; Anthony *et al.* 2000b; Dardevet *et al.* 2000). We recently demonstrated *in vitro* that protein synthesis in old rat muscles becomes resistant to the stimulatory effect of leucine at its physiological concentration range (Dardevet *et al.* 2000). However, when leucine concentration was increased greatly above its postprandial level, protein synthesis was stimulated normally (Dardevet *et al.* 2002; Rieu *et al.* 2003) and the inhibition of muscle protein breakdown was restored in old rats (Combaret *et al.* 2005). Based on our observations, dietary leucine supplementation may represent a useful nutritional tool for the maintenance of muscle mass and the prevention of sarcopenia in the elderly. To our knowledge, the beneficial effect of a specific leucine supplementation in aged humans has only been shown by Katsanos *et al.* (2006) in combination with a bolus of EAAs. Whether a specific leucine effect on muscle protein synthesis can be obtained with leucine-supplemented meals under normal postprandial conditions (i.e. in the presence of carbohydrates and lipids) remains to be demonstrated. Indeed, Volpi *et al.* (2000) showed that the response of muscle protein anabolism to a large amino acid intake was blunted in combination with other nutrients (especially glucose) in the elderly. The aim of the present study was to evaluate the effect of complete meals (containing protein, carbohydrates and lipids) enriched or not with leucine on whole body protein metabolism and muscle protein synthesis in elderly volunteers.

## Methods

### Subjects

Twenty healthy elderly male subjects ( $69.6 \pm 0.8$  years) participated in the study. The physical characteristics of the subjects are indicated in Table 1. A history of clinical events was recorded for all subjects and a physical examination was performed before recruitment. All subjects recruited had normal blood biochemical profiles and appeared normal in physical examinations, without any chronic diseases. The experimental protocol was approved by the local ethical committee (Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale de Clermont Ferrand) and was conducted according to the Declaration of Helsinki. The nature and potential risks of the study were fully explained to each volunteer and written informed consent was obtained before the study from each participant. To avoid marked differences between protein metabolism between individuals (linked to heterogeneity in their habitual dietary intakes), the volunteers were asked to follow a controlled protein intake adjusted to their body weight providing  $0.8 \text{ g protein kg}^{-1} \text{ day}^{-1}$  during a 4 day period before the study.

**Table 1. Subject characteristics**

	Control	Leucine
Age (years)	$69.7 \pm 1.5$	$69.5 \pm 0.8$
Height (cm)	$172.8 \pm 2.2$	$170.8 \pm 1.4$
Weight (kg)	$75.4 \pm 3.2$	$73.8 \pm 2.4$
BMI ( $\text{kg m}^{-2}$ )	$25.2 \pm 0.7$	$25.3 \pm 0.7$
Fasting glucose ( $\text{mmol l}^{-1}$ )	$4.7 \pm 0.1$	$4.7 \pm 0.2$
Fasting albumin ( $\text{g l}^{-1}$ )	$38.7 \pm 0.8$	$38.2 \pm 0.8$
Fasting creatinine ( $\mu\text{mol l}^{-1}$ )	$87.9 \pm 4.0$	$90.3 \pm 3.8$

Values are expressed as means  $\pm$  S.E.M.

### Materials

L-[1- $^{13}\text{C}$ ]Phenylalanine (99 mole per cent excess, MPE) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). The isotopic and chemical purity was checked by gas chromatography–mass spectrometry. Solutions of the tracer were tested for sterility and pyrogenicity before use and were prepared in sterile non-pyrogenic saline. During each experiment, the tracer was filtered through  $0.22 \mu\text{m}$  filters.

### Experimental design

All subjects were studied in a postabsorptive state after a 12 h overnight fast. A sampling catheter (Venflon 2\*, 20G; Viggo, Helsingborg, Sweden) was inserted retrogradely into a dorsal hand vein. A second catheter was inserted in a contralateral forearm vein for tracer infusion.

Each infusion protocol (Fig. 1) consisted of a 540 min study period throughout which L-[1- $^{13}\text{C}$ ]phenylalanine was infused at a constant rate ( $0.063 \pm 0.001 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ ) after a priming dose of  $3.6 \mu\text{mol kg}^{-1}$ . Each subject was first studied during a 240 min basal period ( $-240$  to  $0$ ) to determine the postabsorptive whole body phenylalanine kinetics. After 240 min, a semiliquid diet was administered for the five remaining hours (from  $0$  to  $300$  min). The composition of the diets is indicated in Table 2. The diet provided 10.2 kcal, 0.4 g protein (in the form of casein), 1.3 g carbohydrate (dextrine maltose) and 0.36 g fat (vegetable oil, Isio 4<sup>TM</sup> Lesieur) per kg body weight, which corresponds to a normal meal at lunch. The leucine diet was supplemented with leucine ( $0.052 \text{ g kg}^{-1}$ ) to increase plasma leucine to twice the normal postprandial plasma leucine concentrations. The leucine diet was also supplemented with isoleucine ( $0.0116 \text{ g kg}^{-1}$ ) and valine ( $0.0068 \text{ g kg}^{-1}$ ) to maintain their plasma levels at normal postprandial values. These amounts were established during pilot experiments. The control diet was supplemented with alanine ( $0.071 \text{ g kg}^{-1}$ ), which did not affect protein metabolism, in order to supply the same amount of nitrogen as the leucine diet. The diets were

prepared on the day of protocol and were ingested as 15 small meals (aliquots of 50 ml) given every 20 min.

Arterialized blood (obtained by placing the forearm and hand in a heating box at 60°C) was taken before starting the infusion of the tracer and every 30 min during the last 1.5 h of the basal period and every 30 min during the entire feeding period. Blood samples were centrifuged at 4°C and the resulting plasma was stored at -20°C for subsequent analyses. Breath samples were collected before infusion and every 30 min during the last 1.5 h of the basal and feeding periods and kept in 10 ml evacuated containers (Vacutainer Becton Dickinson, Grenoble, France). Total CO<sub>2</sub> production rates were measured at isotopic plateau during the last hour of the basal and feeding periods by open-circuit indirect calorimetry (Deltatrac; Datex, Geneva).

Muscle biopsies were taken from the vastus lateralis after local anaesthesia at the end of basal and feeding periods using a percutaneous needle. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

### Analytical procedures

Whole body phenylalanine kinetics were determined from plasma L-[1-<sup>13</sup>C]phenylalanine enrichments and breath <sup>13</sup>CO<sub>2</sub> enrichments. Plasma L-[1-<sup>13</sup>C]phenylalanine enrichments were determined as its *ter*-butyldimethylsilyl derivatives under electron impact ionization by gas chromatography-mass spectrometry (GC-MS) (mass selective detector 5972, coupled with a gas chromatograph 5890 series II; Hewlett Packard, Les Ullis, France) and by monitoring the ions with *m/z* 336 and 337. Breath <sup>13</sup>CO<sub>2</sub> enrichments were measured directly by isotopic ratio mass spectrometry using a VG Isochrom (Micromass HK, Manchester, UK).

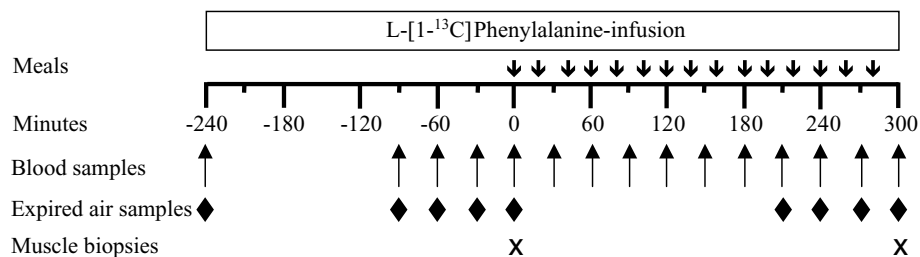
**Table 2. Composition of the experimental meals**

	Control	Leucine supplemented
Casein	0.400	0.400
Leucine	—	0.052
Valine	—	0.0068
Isoleucine	—	0.0116
Alanine	0.071	—
Maltodextrin	1.3	1.3
Oil	0.36	0.36
Glycerol monostearate	0.036	0.036
Raspberry aroma	0.005	0.005

Values are expressed in g (kg body weight)<sup>-1</sup>

For plasma L-[1-<sup>13</sup>C]phenylalanine enrichments, 1 ml of plasma was homogenized in 8 volumes of ice-cold 10% (0.6 M) trichloroacetic acid (TCA) and then centrifuged at 5000 g for 15 min at 4°C. The resultant pellets (TCA-insoluble materials) were washed 2 times in 4 volumes of cold 10% TCA. The combined supernatants, which contained free amino acids, were desalted by cation-exchange chromatography (AG 50 × 8, 100–200 mesh, H<sup>+</sup> form, Bio-Rad, Richmond, CA, USA) in minidisposal columns. Phenylalanine and other amino acids were eluted with 4 M NH<sub>4</sub>OH. After evaporation of NH<sub>4</sub>OH under vacuum, free amino acids were resuspended in 0.01 M HCl for subsequent derivatization and enrichment measurements.

L-[1-<sup>13</sup>C]Phenylalanine enrichment in the free amino acid pool and myofibrillar muscle proteins were determined according to the method previously described (Guillet *et al.* 2004). A 50–100 mg piece of muscle biopsy was homogenized in a 5% ice-cold buffer containing 0.25 M sucrose, 2 mM EDTA and 10 mM Tris-HCl (pH 7.4) using a Potter-Elvehjem homogenizer.



**Figure 1. Experimental design of the study**

Twenty normal elderly men were studied after an overnight fast. After basal samples were taken, a priming dose of L-[1-<sup>13</sup>C]phenylalanine (3.6 μmol kg<sup>-1</sup>) was injected followed by a continuous infusion at a constant rate (0.06 μmol kg<sup>-1</sup> min<sup>-1</sup>) for 540 min. The study was divided into a basal postabsorptive period (-240 to 0 min) and a feeding period (0–300 min). During the feeding period, the subjects ingested a repeated bolus given every 20 min of either a control diet or a leucine supplemented diet (see Table 2 for diet composition). Blood samples were taken every 30 min between -90 and 300 min. Expired air samples were collected every 30 min at the end of the basal and feeding periods (between -90 and 0 min and between 210 and 300 min). Muscle biopsies were taken just before ingestion of the first bolus at time 0 and at the end of the feeding period (time 300 min).

The homogenate was centrifuged at low speed (600 g) and the pellet containing myofibrillar proteins was collected. Myofibrillar muscle proteins were then hydrolysed using 6 M HCl (110°C for 24 h). HCl was removed by evaporation and amino acids purified by cation-exchange chromatography as described above. Amino acids were then derivatized as their *N*-acetyl-propyl residues and [1-<sup>13</sup>C]phenylalanine enrichment was performed using GC-MS. Free muscle amino acids were extracted from muscle tissue by using 10% TCA, purified by cation-exchange chromatography as described above, and derivatized as their *ter*-butyldimethylsilyl residues.

Plasma insulin concentrations were determined using a commercial human RIA kit (Insulin CT Cis Bio International).

The concentrations of free plasma amino acids were measured by ion-exchange chromatography after protein precipitation. Five hundred microlitres of plasma was added to 125 μl of a sulphosalicylic acid solution (1 M dissolved in ethanol with 0.5 M thiodiglycol) previously evaporated to dryness. Samples were incubated on ice for 1 h and centrifuged for 1 h at 3500 g at 4°C. An aliquot (250 μl) of the supernatant was added to 125 μl of 0.1 M lithium acetate buffer, pH 2.2. Amino acid concentrations were determined using an automated amino acid analyser with BTC 2410 resin (Biotronic LC 3000, Roucaire, Velizy, France).

## Calculations

Whole body phenylalanine kinetics were calculated using samples taken during the last 1.5 h of the basal period (at times -90, -60, -30 and 0) and feeding period (at times 210, 240, 270 and 300 min). After checking the isotopic steady state for the last hour of each period, mean plateau enrichment values were used to calculate phenylalanine kinetics.

Total whole body phenylalanine flux ( $Q$ ) ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) was determined using the equation:

$$Q = F \times \text{IE}_{\text{inf}}/\text{IE}_{\text{a}}$$

where  $F$  is the L-[1-<sup>13</sup>C]phenylalanine infusion rate ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ),  $\text{IE}_{\text{inf}}$  is the isotopic enrichment of the infusate (i.e. 99 mol% excess), and  $\text{IE}_{\text{a}}$  (also in mol% excess) is the plasma L-[1-<sup>13</sup>C]leucine enrichment.

Whole body phenylalanine oxidation flux ( $\text{Ox}$ ) ( $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ) was calculated using plasma L-[1-<sup>13</sup>C]phenylalanine enrichment from the following equation:

$$\text{Ox} = (\text{IE}_{\text{CO}_2} \times V_{\text{CO}_2})/\text{IE}_{\text{a}} \times 1/R$$

where  $\text{IE}_{\text{CO}_2}$  (mol per cent excess) is the expired <sup>13</sup>CO<sub>2</sub> enrichment,  $V_{\text{CO}_2}$  ( $\mu\text{mol of CO}_2 \text{min}^{-1}$ ) is the expired CO<sub>2</sub> volume and  $R$  is a factor correcting for incomplete recovery

of labelled bicarbonate ( $R = 0.71$  during basal period and  $0.82$  during feeding period) (Hoerr *et al.* 1989).

According to the model, the following equation applies:

$$Q = I + R_{\text{a}} = \text{Ox} + R_{\text{d}}$$

where  $I$  is the rate of ingested unlabelled phenylalanine ( $I = 0$  in the basal period),  $R_{\text{a}}$  is the endogenous phenylalanine appearance rate (an index of protein breakdown), and  $R_{\text{d}}$  the non-oxidative phenylalanine disposal rate (an index of protein synthesis) from plasma (all in  $\mu\text{mol kg}^{-1} \text{min}^{-1}$ ).

Knowing  $Q$ ,  $I$  and  $\text{Ox}$

$$R_{\text{a}}(\mu\text{mol kg}^{-1} \text{min}^{-1}) = Q - I$$

$$R_{\text{d}}(\mu\text{mol kg}^{-1} \text{min}^{-1}) = Q - \text{Ox}$$

The net phenylalanine balance, an index of protein deposition, is calculated as  $R_{\text{d}} - R_{\text{a}}$ .

Fractional synthesis rate (FSR) of myofibrillar muscle proteins was calculated at the end of feeding period by measuring the incorporation rate of L-[1-<sup>13</sup>C]phenylalanine into proteins according to the equation:

$$\text{FSR} = (\Delta\text{IE}_{\text{pb}}/t)/((\text{IE}_{\text{f1}} + \text{IE}_{\text{f2}})/2) \times 100$$

where  $\Delta\text{IE}_{\text{pb}}$  is the increment of protein bound phenylalanine enrichment between the two biopsies,  $t$  (h) is the time interval between the two biopsies, and  $\text{IE}_{\text{f1}}$  and  $\text{IE}_{\text{f2}}$  are the phenylalanine enrichments in the free muscle pool in the two subsequent biopsies. The results are expressed as per cent per hour.

## Statistical analysis

All data are expressed as means  $\pm$  s.e.m. A two-way ANOVA with repeated measures was used to compare whole body kinetics (Leucine *versus* Control groups and Feeding *versus* Basal periods) and Student's unpaired  $t$  test was used to compare FSR in Leucine and Control groups.  $P < 0.05$  was considered to be significant.

## Results

### Characteristics of the subjects

The two groups of volunteers did not differ with respect to age, height, body weight and body mass index (Table 1). The values of fasting plasma glucose, albumin and creatinine were normal and did not differ between the two groups. The basal insulin : glucose ratio, which is an index of insulin sensitivity, was not different between the control and leucine groups ( $2.82 \pm 0.41$  *versus*  $3.00 \pm 0.28$  mIU l<sup>-1</sup>, respectively,  $P = 0.7$ ).

**Table 3. Mean plasma amino acid concentrations before and after continuous ingestion of control or leucine-supplemented meals in elderly volunteers**

	Control		Leucine	
	Basal	Fed	Basal	Fed
<b>Essential amino acids</b>				
Histidine	68 ± 3	72 ± 3	66 ± 2	67 ± 2
Isoleucine	63 ± 4	82 ± 4†	55 ± 3	93 ± 5†
Lysine	173 ± 10	203 ± 12†	157 ± 6	190 ± 7†
Methionine	7 ± 1	8 ± 1†	7 ± 1	8 ± 1†
Phenylalanine	61 ± 2	71 ± 2†	60 ± 2	66 ± 3†
Threonine	115 ± 7	124 ± 10†	110 ± 7	108 ± 8†
Leucine	130 ± 5	156 ± 6†	111 ± 5	282 ± 14†*
Valine	251 ± 13	280 ± 12†	216 ± 12	270 ± 13†
<b>Non-essential amino acids</b>				
Alanine	232 ± 13	351 ± 23†	288 ± 22	300 ± 20
Arginine	70 ± 4	71 ± 4	67 ± 4	65 ± 5
Asparagine	88 ± 5	104 ± 8†	86 ± 4	92 ± 6†
Aspartate	11 ± 1	12 ± 1	10 ± 1	10 ± 1
Glutamate	212 ± 41	207 ± 37	197 ± 24	198 ± 24
Glutamine	215 ± 30	223 ± 32	223 ± 19	204 ± 20
Glycine	173 ± 9	166 ± 12†	180 ± 15	164 ± 14†
Proline	164 ± 14	244 ± 17†	189 ± 9	248 ± 11†
Serine	107 ± 6	111 ± 7	94 ± 3	91 ± 4*
Tyrosine	52 ± 3	65 ± 3†	52 ± 3	61 ± 3 †

Values are expressed in  $\mu\text{M}$  as means  $\pm$  S.E.M. \*Significantly different from control group at  $P < 0.05$ . †Significantly different from the basal values in the same group at  $P < 0.05$ .

### Plasma insulin concentrations

Plasma insulin levels were similar in the basal period and increased in both groups after the ingestion of the first meal. Insulin concentrations increased during the first 2 h and plateaued for the last 3 h (Fig. 2). The insulin increment after feeding tended to be higher in the group supplemented with leucine but the insulin response, expressed as the area under the curve (above baseline) during the 5 h feeding period was not different between the two groups ( $P = 0.5$ ) (Fig. 2, inset).

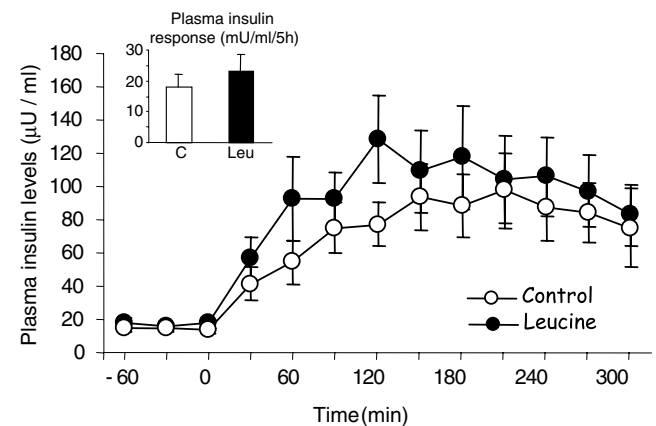
### Plasma amino acid concentrations

As shown in Table 3, plasma concentrations of both essential and non-essential amino acids were similar in both groups at the postabsorptive state.

Plasma leucine levels increased after ingestion of the first meal in both groups ( $P < 0.05$ ). Whereas plasma leucine levels did not further increase in the control group, it strongly increased in the leucine group (Fig. 3A). As shown in Fig. 3B, the plasma leucine response over the entire feeding period was 6-fold higher in the leucine group than in the control group ( $P < 0.0001$ ).

Compared with the postabsorptive state (basal state), control or leucine meal intake slightly increased most

plasma essential amino acid concentrations, except histidine, which did not significantly change, and threonine, which decreased in the leucine group (Table 3). When plasma leucine was omitted, the mean plasma essential amino acid concentrations were not significantly different between control and leucine groups in the fed



**Figure 2. Plasma insulin concentrations (expressed in  $\mu\text{U ml}^{-1}$ ) in elderly volunteers fed a control diet or a leucine-supplemented diet as repeated bolus between 0 and 300 min** The inset represents the plasma insulin response expressed as the area under the curve minus baseline values. Values are means  $\pm$  S.E.M.

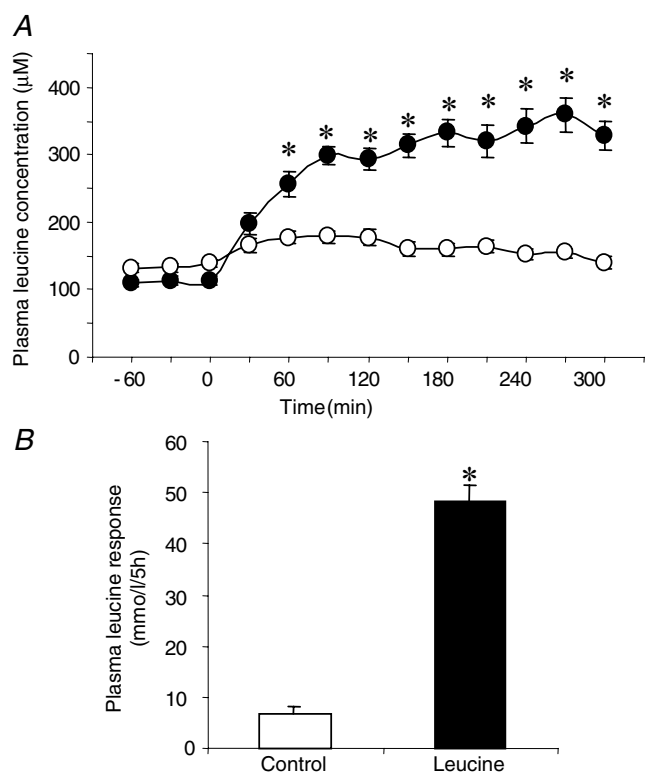
period ( $838 \pm 24$  and  $803 \pm 25 \mu\text{M}$ , respectively). It is important to note that plasma isoleucine and valine concentrations were not decreased in the volunteers fed the leucine-supplemented meals compared with the control group. Moreover, plasma isoleucine and valine concentrations were not significantly different between control and leucine groups (Table 3). Feeding the control meals significantly increased plasma alanine concentration in volunteers whereas the leucine meals did not. Other non-essential plasma amino acid levels were either unchanged (arginine, aspartate, glutamate, glutamine, serine), increased (asparagine, proline, tyrosine) or decreased (glycine) similarly in the control and leucine groups during feeding (Table 3).

### Whole body phenylalanine kinetics

A steady state of  $^{13}\text{CO}_2$  expired and plasma  $^{13}\text{C}$ -phenylalanine enrichments was achieved during the final hours of basal and fed periods in each group of volunteers (Fig. 4). The rate of expired  $\text{CO}_2$  was similar in both groups during the basal period ( $99.7 \pm 2.9$  versus  $100.7 \pm 2.9 \mu\text{mol min}^{-1} \text{kg}^{-1}$  in control and

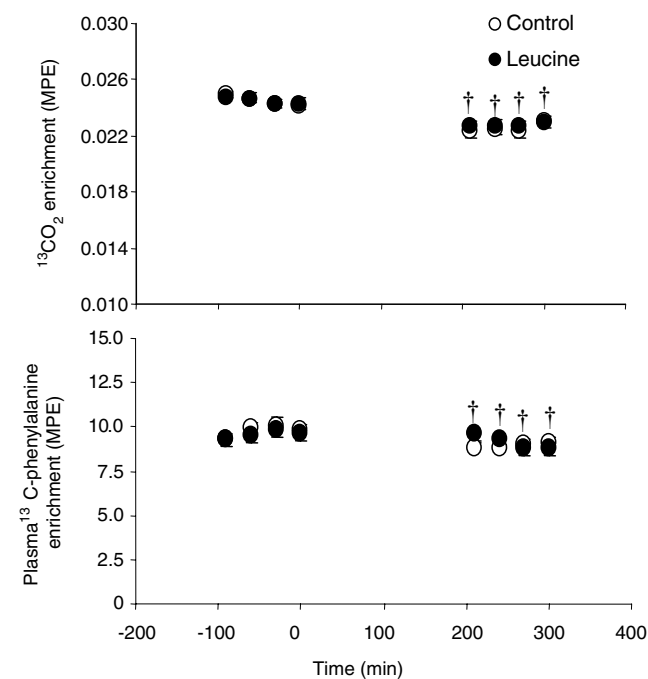
leucine groups, respectively). Feeding increased the rate of expired  $\text{CO}_2$  ( $P < 0.0001$ ) to the same extent in the control and leucine groups ( $117.9 \pm 4.2$  and  $116.5 \pm 2.9 \mu\text{mol min}^{-1} \text{kg}^{-1}$ , respectively,  $P < 0.005$ ). Consequently, expired  $\text{CO}_2$  enrichments decreased in both groups during the feeding period ( $P < 0.0001$ ) (Fig. 4). Similarly, plasma  $^{13}\text{C}$ -phenylalanine enrichments decreased during the feeding period ( $P < 0.005$ ) in both the control and leucine groups.

Whole body phenylalanine kinetics were similar in the control and leucine groups during the basal period (Fig. 5). The effect of feeding on whole body phenylalanine kinetics was similar whatever the composition of the meals given to the volunteers. The rate of whole body phenylalanine oxidation was not changed by feeding whereas whole body protein synthesis was increased ( $P < 0.05$ ) in control and leucine groups. As expected, whole body protein breakdown was decreased during feeding and whole body phenylalanine balance was improved (Fig. 5). The increase in plasma leucine observed with the leucine-supplemented meals had no effect on whole body protein metabolism.



**Figure 3.** *A*, plasma leucine concentrations (expressed in  $\mu\text{mol l}^{-1}$ ). *B*, plasma leucine response (expressed as the area under the curve minus baseline values) in elderly volunteers fed a control diet or a leucine-supplemented diet as a repeated bolus between 0 and 300 min

Values are means  $\pm$  s.e.m. \*Significantly different from control group at  $P < 0.05$ .



**Figure 4.** Expired  $^{13}\text{CO}_2$  enrichments (*A*) and plasma  $^{13}\text{C}$ -phenylalanine enrichments (*B*) in mol per cent excess (MPE) as a function of time in elderly volunteers during the basal postabsorptive period and during ingestion of a control diet or a leucine-supplemented diet given as a repeated bolus between 0 and 300 min

Values are means  $\pm$  s.e.m. †Significantly different from basal period at  $P < 0.05$ .

### Muscle protein synthesis

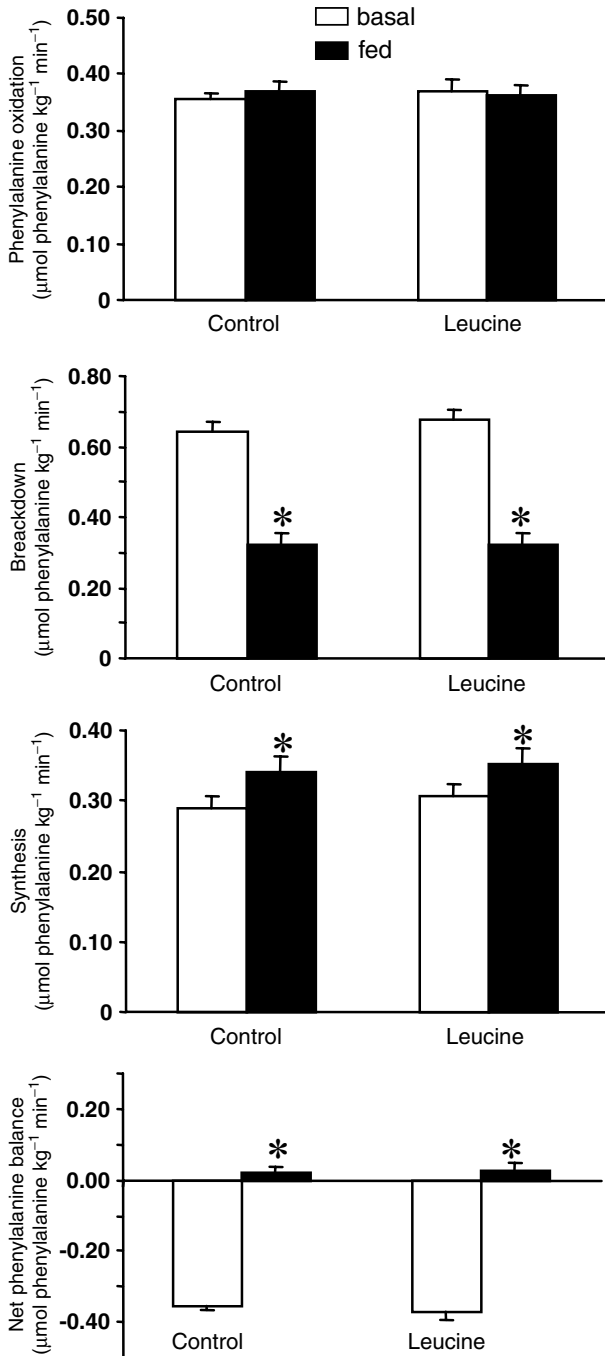
As shown in Fig. 6, leucine supplementation improved myofibrillar muscle protein fractional synthesis rate (FSR) measured at the end of the feeding period ( $0.083 \pm 0.008$

and  $0.053 \pm 0.009\% \text{ h}^{-1}$  in the leucine and control groups, respectively,  $P < 0.05$ ).

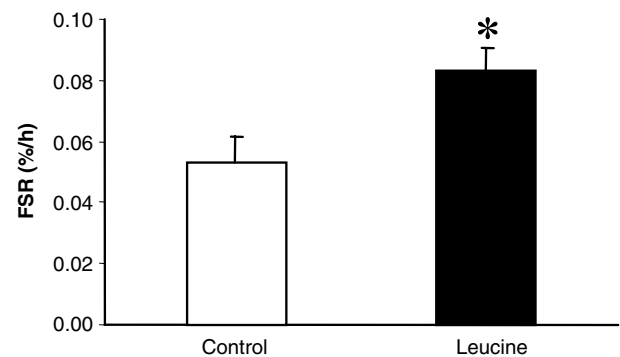
### Discussion

The present study was designed to assess the impact of specific dietary leucine-supplemented meals on protein metabolism in elderly volunteers. For this, we compared the effects induced by mixed semi-liquid meals containing either casein (control group) or casein plus free leucine (leucine group) as the protein source on whole body and muscle protein synthesis in old men. We showed that leucine supplementation during feeding improves muscle protein synthesis in elderly subjects. This beneficial effect was due only to increased plasma leucine concentrations because only plasma free leucine concentration significantly differed between the two groups of volunteers during the feeding period. By contrast, whole body turnover was not affected by leucine supplementation.

Over the past several years, it has become clear that muscle protein loss during ageing may be partly explained by a decreased ability of old muscle to respond appropriately to food intake (Mosoni *et al.* 1995; Arnal *et al.* 1999, 2002; Dardevet *et al.* 2002; Rieu *et al.* 2003). Food intake normally increases muscle protein synthesis and most of this effect results from the stimulatory effect of amino acids (Bennet *et al.* 1989; Fryburg *et al.* 1995; Svanberg *et al.* 1996), whereas insulin has only a permissive effect. We demonstrated in a previous study that the effect of dietary amino acids was blunted in old rats whereas insulin effect was not significantly altered (Prod'homme *et al.* 2005). Wolfe and coworkers have extensively investigated the effect of amino acids on muscle protein synthesis in the elderly. Surprisingly, they showed that amino acids given orally (Volpi *et al.* 1999; Rasmussen *et al.* 2002; Paddon-Jones *et al.* 2004) or intravenously



**Figure 5.** Whole body phenylalanine kinetics (expressed as  $\mu\text{mol phenylalanine kg}^{-1} \text{ min}^{-1}$ ) in elderly volunteers during the basal postabsorptive period and during ingestion of a control diet or a leucine-supplemented diet given as a repeated bolus between 0 and 300 min. Values are means  $\pm$  s.e.m. \*Significantly different from control group at  $P < 0.05$ .



**Figure 6.** Fractional synthesis rates (FSR) of myofibrillar muscle proteins in elderly volunteers fed a control diet or a leucine-supplemented diet as a repeated bolus between 0 and 300 min. Values are means  $\pm$  s.e.m. \*Significantly different from control group at  $P < 0.05$ .

(Volpi *et al.* 1998; Rasmussen *et al.* 2002) were able to stimulate muscle protein synthesis in old as in young adult volunteers. They also demonstrated that essential amino acids are primarily responsible for the amino acid-induced stimulation of muscle protein anabolism (Volpi *et al.* 2003). However it is important to note that the amount of amino acids infused or orally ingested in these experiments led to a sustained large hyperaminoacidaemia. Besides, a more recent study from this group (Katsanos *et al.* 2005) and from Cuthbertson *et al.* (2005) confirmed our observations and showed a decreased sensitivity and responsiveness of muscle protein synthesis to essential amino acids in elderly subjects compared to adults in the case of a milder and more physiological rise of aminoacidaemia.

Among amino acids, branched chain amino acids and especially leucine (Fulks *et al.* 1975; Buse & Reid, 1975; Li & Jefferson, 1978) are the most efficient for protein synthesis stimulation. Indeed, we and others clearly demonstrated that leucine alone is able to stimulate muscle protein synthesis to the same extent as all amino acids (Anthony *et al.* 2000a,b; Dardevet *et al.* 2000; Lynch *et al.* 2002; Crozier *et al.* 2005). In a previous experiment, we clearly showed a decreased sensitivity of protein synthesis to leucine in muscles from old rats compared to adults (Dardevet *et al.* 2000). Indeed, muscle protein synthesis in old rats required greater leucine concentration than young or adult rats to be stimulated. This suggested that at postprandial amino acid levels, muscle protein synthesis was maximally stimulated in adult rats but poorly in old animals. Accordingly, we previously showed that a leucine-supplemented meal corrected the defect of postprandial protein synthesis stimulation in muscle from old rats, suggesting that increased leucine intake in the elderly would be beneficial for maintaining muscle protein mass (Dardevet *et al.* 2002; Dardevet *et al.* 2003; Rieu *et al.* 2003). Recently, Katsanos *et al.* (2006) showed that an enriched leucine bolus of EAAs also stimulated muscle protein accretion in the elderly. However, it has been previously shown that, when given in combination with glucose, which further increased plasma insulin levels, the effect of amino acids on muscle protein accretion was blunted in humans (Volpi *et al.* 2000). This raised the question whether the beneficial effect of leucine supplementation recorded by Katsanos *et al.* (2006) could be maintained if leucine was added in a complex meal associated with proteins, carbohydrates and lipids. Our data clearly demonstrated that the supplementation was still efficient under such conditions despite the fact that plasma insulin was increased to the same extent as in the study of Volpi *et al.* (2000). The reasons for such differences are unclear. It may be related with methodological differences such as ingestion of complete meals *versus* intravenous infusion of an amino acid–glucose mixture. Leucine infusion has been reported to have a transient effect on muscle protein

metabolism if infused alone (Abumrad *et al.* 1982; Escobar *et al.* 2005), probably because of a reduction in other plasma amino acid availability. Indeed, it was suggested that the presence of all amino acids or essential amino acids might be required to sustain protein synthesis stimulated by leucine (Abumrad *et al.* 1982; Frexes-Steed *et al.* 1992; Escobar *et al.* 2005). When leucine was infused alone, the resulting increase in circulating leucine induced a decline of most plasma essential amino acids (Hagenfeldt *et al.* 1980; Nair *et al.* 1992; Tom & Nair, 2006), the effect being more pronounced for isoleucine and valine because of the well-described phenomenon of branched-chain amino acid antagonism (Calvert *et al.* 1982; Harper *et al.* 1984). In the present study, in order to prevent a fall of plasma valine and isoleucine concentrations, which can become rate-limiting for protein synthesis, the leucine-supplemented meals were also supplemented with valine and isoleucine. As in our previous experiment in rats (Dardevet *et al.* 2002; Rieu *et al.* 2003), postprandial plasma valine and isoleucine were maintained at normal postprandial concentrations and were not different in the leucine and control groups. The major relevance of our protocol design was the fact that only plasma leucine concentration was dramatically increased in the leucine group whereas other plasma EAAs were similar between leucine and control volunteers and only slightly increased. Indeed, in our experiment, total plasma EAAs (minus leucine) was only 14–19% increased during feeding whereas it was 2.2-fold increased after a bolus ingestion of EAAs (Volpi *et al.* 1999, 2003; Katsanos *et al.* 2005). Actually, our protocol design reproduced the plasma amino acid pattern occurring after ingestion of a normal single mixed meal in humans (Elia *et al.* 1989). The present experiment demonstrated for the first time that increasing plasma leucine availability alone may favour muscle protein synthesis in old humans and does not require a large increase of other amino acids.

Because leucine has been shown to stimulate insulin secretion, the increase in muscle protein synthesis could result indirectly from an increase in plasma insulin. Despite the fact that plasma insulin concentrations tended to increase more rapidly in the leucine-supplemented group than in the control group, the insulin response during the entire 5 h feeding period was not different between the two groups. Therefore, the increase in muscle protein synthesis that we observed in the leucine-supplemented group was independent of changes in insulin and cannot be attributed to an insulin effect. This was also the case in our previous studies in rats (Dardevet *et al.* 2002; Rieu *et al.* 2003). In agreement with this, Cuthbertson *et al.* (2005) demonstrated that EAAs stimulate muscle protein synthesis independently of increased insulin availability in both young and old men. It has also been shown that leucine supplementation increased protein synthesis in rabbit skin wound and muscle without changes in plasma



insulin (Zhang *et al.* 2004). Whether insulin contributes to the leucine-induced stimulation of muscle protein synthesis remains under debate. Overall, the studies demonstrated that leucine activated protein synthesis through both insulin-independent (Anthony *et al.* 2002*b*) and insulin-dependent mechanisms (Anthony *et al.* 2002*a*). It is likely that insulin is required to cause stimulation of protein synthesis by leucine and that the role of insulin appears to be permissive. This could explain why it has been possible to maintain a stimulation of muscle protein synthesis in the leucine-supplemented group for over 5 h in the present experiment, whereas Bohé *et al.* (2001) could not stimulate muscle protein synthesis for longer than 2 h after the beginning of an amino acid infusion. Indeed, plasma insulin remained elevated during all the feeding period in our study whereas plasma insulin elevation was only transient in the study of Bohé *et al.* (2001). Because muscle protein breakdown was not determined in that experiment, it is unknown whether the improvement of muscle protein synthesis resulted in an anabolic response in volunteers fed the leucine-supplemented meals. We previously demonstrated that the normal fall in muscle protein breakdown induced by feeding was blunted in old rats and was restored by the leucine supplementation as for protein synthesis stimulation (Combaret *et al.* 2005), suggesting an anabolic effect of leucine on muscle proteins in old rats. Moreover, Katsanos *et al.* (2006) demonstrated that increasing the proportion of leucine in a mixture of EAAs (41% versus 25%) improved the muscle balance in old subjects.

Although we found that leucine supplementation improves muscle protein synthesis, we failed to observe an effect on whole body protein turnover in the present experiment. Indeed, both whole body protein synthesis and protein breakdown as well as net balance were similar in both groups whatever the nutritional conditions. This is not surprising because muscle protein synthesis only represents 27% of whole body protein synthesis (Nair *et al.* 1988). Moreover, our data are consistent with those of Koopman *et al.* (2005) who did not show any change in whole body turnover in young male subjects given either carbohydrate and protein or carbohydrate, protein and free leucine despite greatly increased plasma leucine concentrations.

In conclusion, the present experiment demonstrates that dietary leucine supplementation improves postprandial muscle protein synthesis in old humans. Recently, Katsanos *et al.* (2006) also demonstrated such a beneficial effect of leucine in the elderly. However, of most physiological relevance from our work is that the improvement of muscle protein synthesis induced by leucine supplementation (1) occurred after ingestion of a complete meal (i.e. containing proteins, carbohydrates and lipids) instead of a bolus of free amino acids,

(2) was sustained for at least 5 h, and (3) was visible without the large increases of postprandial amino acid levels. Taken together, these data suggest that leucine supplementation may represent an effective nutritional strategy to limit muscle protein losses during ageing. It may be considered as a good alternative to high protein diets, which could have deleterious effects on renal function in the elderly (Fliser *et al.* 1993). However, further experiments will be necessary to determine the best conditions of leucine supplementation in human to obtain protein gain in muscle without negative side effects in old people.

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### Acknowledgements

We thank the volunteers for their participation. The authors thank C. Obled for helpful discussion and advice, F. Laporte and N. Mathieu for their participation in the recruitment of volunteers, M. Brandolini for dietary recall analysis, M. C. Ribeyre for mass spectrometry sample preparations, E. Debras and G. Bayle for amino acid analysis, and H. Lafarge for management of the bibliography. This work was supported by a grant from ESPEN – The European Society for Clinical Nutrition and Metabolism.