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Original article

Differential effects of leucine and leucine-enriched whey protein on skeletal muscle protein synthesis in aged mice

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SUMMARY

Background & aims: It has been suggested that anabolic resistance, or a blunted protein synthetic response to anabolic stimuli, contributes to the failure of muscle mass maintenance in older adults. The amino acid leucine is one of the most prominent food-related anabolic stimuli. However, data on muscle protein synthesis (MPS) after administration of a single bolus of leucine in aged populations is lacking and long-term single leucine supplementation has not been shown to increase muscle mass. This study aimed to determine the MPS response to the administration of a single bolus of leucine or to leucine combined with whey protein, in aged mice.

Methods: Overnight fasted C57/BL6RJ mice at 25-mo of age received an oral gavage with leucine or whey-protein enriched with leucine (0.75 g/kg bodyweight total leucine in both) or 0.5 mL water (fasted control). Subsequently, mice were s.c. injected with puromycin (0.04 μmol/g bw at t = 30, 45 or 60 min) and were sacrificed 30 min thereafter. Amino acid concentrations were determined in plasma and right muscle *tibialis anterior* (TA). Left TA was used to analyse MPS by SUnSET method and phosphorylation rate of Akt, 4E-BP1 and p70S6k by western blot.

Results: In aged mice, leucine administration failed to increase MPS, despite a 6-fold increase in plasma leucine and elevated muscle free leucine levels ($P < 0.05$). In contrast, leucine-enriched whey protein significantly stimulated MPS in aged mice at 60 min after gavage ($P < 0.05$). Muscle free EAA, NEAA and the phosphorylation rate of Akt, 4E-BP1 and p70S6k increased significantly ($P < 0.05$), only after administration of leucine-enriched whey protein.

Conclusions: MPS is stimulated in aged mice by leucine-enriched whey protein but not by leucine administration only. Administration of other amino acids may be required for leucine administration to stimulate muscle protein synthesis in aged mice.

1. Introduction

Skeletal muscle mass is the result of a balance between protein synthesis and protein breakdown of skeletal muscle proteins. With ageing, changes in muscle protein turnover may lead to a decline in skeletal muscle mass and decreased muscle strength and physical endurance [1–3]. This process ultimately leads to a muscle wasting condition known as sarcopenia [4]. The diminished protein

Abbreviations: BCAA, branched chain amino acid; EAA, essential amino acid; MPB, muscle protein breakdown; MPS, muscle protein synthesis; NEAA, non-essential amino acid; TA, *tibialis anterior* muscle; WB, western blot.

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synthetic response to anabolic stimuli (e.g. amino acids and insulin) from a meal [5], known as anabolic resistance, has been suggested to contribute to the inability to maintain muscle mass in older individuals [6]. Therefore, many research groups are now trying to find effective strategies to overcome the higher anabolic threshold associated with aging [7] in order to prevent and treat muscle mass loss in this population.

One of the strategies is related to the branched chain amino acid leucine, which is unique in its ability to activate mTORC1 and its downstream phosphorylation of p70S6k and 4E-BP1. Both factors are involved in mRNA translation initiation and *de novo* muscle protein synthesis (MPS) [8–10]. Moreover, leucine is an insulinotropic amino acid and insulin can affect protein metabolism (reviewed in Ref. [11]). Leucine ingestion has been shown to effectively stimulate MPS in young rat skeletal muscle at single oral doses ranging between 0.135 and 1.35 g kg^{-1} bw [12]. A dose-dependent relationship between leucine and MPS was also observed when isolated muscles from young rats were incubated in an organ bath with various leucine concentrations [13].

Studies in aged rats confirm the presence of anabolic resistance to leucine when administered as a single amino acid in an organ bath [13], but we are not aware of *in vivo* studies with orally administered leucine, not in combination with other nutrients, in aged animals. However, there are reports that leucine-enrichment of a protein meal can effectively stimulate MPS *in vivo* in old rats [13–15]. This observation is similar to what we might find in older adults whose attenuated MPS response can be reversed by increasing the proportion of leucine in an EAA mixture [16] or by adding leucine to a bolus of intact proteins [17–19]. While leucine-enriched amino acid mixtures or proteins can stimulate MPS, the effect of leucine alone on MPS is less well studied in the aged population. This is relevant, since long-term oral supplementation of leucine as standalone intervention, provided with meals, has not been proven effective in preventing muscle wasting or in increasing muscle mass in older adults (reviewed in Refs. [20,21]). Therefore, further understanding of the MPS response to leucine administration in the ageing population is needed to define the most effective strategy.

The objective of our study was to investigate the effect of leucine, either as a single amino acid or in the context of a leucine-enriched whey protein gavage, on MPS and activation of the mTOR signalling pathway in aged mice. MPS was measured at different time points (60, 75 and 90 min postprandial) to explore if aged C57/BL6J mice show a delay in the anabolic response. In a previous study we showed anabolic resistance to a leucine-enriched whey protein gavage in these aged mice (compared with adult mice) that could be overcome by increasing the total amount of protein in the gavage [22]. To further understand the relevance of leucine and availability of other amino acids, we measured plasma and muscle free amino acid concentrations. Additionally, we measured plasma concentrations of insulin and glucose.

2. Methods

2.1. Animals

Aged male C57/BL6J mice of 25 months of age were obtained from Janvier Labs (Saint Berthevin, France). Animals were individually housed in a climate-controlled room (12:12 dark–light cycle (lights on: 7 a.m.) with a constant room temperature of 21 ± 1 °C). Housing consisted of Makrolon Type III cages (Tecniplast, Italy) with bedding and tissues. Mice were fed *ad libitum* with a standard diet (AIN93M) and had free access to tap water. All experimental procedures were approved by an Animal Ethical Committee (DEC consult, Soest, the Netherlands) and complied with the principles of good laboratory animal care following the European Directive for the protection of animal used for scientific purposes. The animals were cared for according to the NIH Guide for the Care and Use of Laboratory Animals. Upon arrival, the mice were allowed to acclimatize for 2 weeks and were fasted overnight before section. At the day of section, mice were randomized to 3 × 3 groups with no greater deviation than 5% from the overall mean bodyweight (n = 8 per group). To investigate a delay in anabolic response, three intervals were studied (60, 75 and 90 min) and per time point 3 groups were analysed (fasted, single leucine or leucine-enriched whey protein).

2.2. In vivo muscle protein synthesis

MPS was measured with the SUnSET method as previously described by Goodman et al. [23] and previously used by us [22,24]. The SUnSET method is an alternative method to determine MPS compared to radioactive isotope or stable isotope tracers. Goodman et al. [23] validated the SUnSET method with a ^{3}H-phenylalanine flooding method in *ex vivo* plantaris muscle from mice that had received synergist ablation (SA). The results showed that the SUnSET technique was indistinguishable from a standard radioactive-based or a standard stable isotope incorporation technique in detecting SA-induced increases in protein synthesis. In addition, in our previous study [24], protein synthesis was measured in myotubes after a 50 min incubation with L-[1-^{13}C]valine by measuring tracer enrichment, or a 30 min incubation with puromycin by quantifying incorporation of puromycin into peptides. Results of both methods were comparable. Furthermore, we used this technique successfully in a previous study were similar dosages of protein showed an anabolic response in adult mice [22]. This proves the reliability of the SUnSET method. This method also made it possible to determine protein synthesis rate and activation of key signalling pathways involved in this process using the same experimental samples.

At section day, mice received an oral gavage (end volume 0.5 mL) containing either 18.8 mg leucine (LEU) (Sigma Aldrich) (0.75 g/kg bodyweight) or 139 mg leucine-enriched whey protein isolate (WHEY + LEU) (Lacprodan 9224, Arla Foods) (5.56 g/kg bodyweight whey protein with total leucine content of 18.8 mg leucine or 0.75 g/kg bodyweight). Concentrations were based on a nutritional supplement (containing 20 g whey protein and 2.8 g total leucine per serving) that was studied in older adults for its effect on MPS [25]. Amounts of whey protein and leucine were subsequently translated to the mouse setting: ratio of daily food consumption of a mouse and a bolus intake. The postprandial condition is compared with the fasting state (0.5 mL tap water) to indicate the levels of postprandial increase. Thirty, 45 or 60 min after oral gavage adult mice received a s.c. injection with 0.04 μmol/g bodyweight puromycin (Calbiochem) [23]. After an additional 30 min, mice were euthanized by cardiac puncture under total isoflurane anaesthesia (isoflurane/N_2O/O_2). As a result, MPS was measured after a 60, 75 and 90 min postprandial period. Plasma and serum samples were prepared by centrifugation and hind limb muscles were excised, weighted, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Western blot analysis

Left *tibialis anterior* muscles were cut into pieces and WB buffer (40 mM Tris pH 7.5; 1 mM EDTA; 5 mM EGTA; 10% glycerol; 1% Triton X-100; PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics) and Protease Inhibitor Ultra Tablets (Roche Diagnostics), 1 tablet per 10 mL buffer) was added in a ratio of 10 μl/mg muscle. Muscles were homogenized using a FastPrep-24 (MP Biomedicals)

with 3 chrome-steel beads per tube and run 3 times for 30 s on 6.5 m/s speed. Between rounds, tubes were placed on ice to prevent heating of the samples. Subsequently, samples were continuously shaken at 4 °C for 1 h. Protein content was determined using the BCA protein assay kit (Pierce). Muscle homogenates were dissolved in Laemmli buffer (BioRad) and 20–35 μg was loaded on a 4–15% gradient gel (Criterion TGX Precast Gels, BioRad). For each experiment, a pool of the homogenates of mice in fasting state was made and loaded in threefold on each gel to be able to compare samples from different gels. Electrophoresis was performed at 100 V. Proteins were transferred to a 0.2 μm PVDF membrane (BioRad). Membranes were blocked with 5% Protifar protein powder (Nutricia) in TBST buffer (25 mM Tris, pH 7.6; 150 mM NaCl; 0.1% Tween-20) for 1 h followed by overnight incubation at 4 °C with anti-puromycin antibody (clone 12D10, 1:5000, Merck Millipore) in 1% BSA in TBST. For mTOR pathway proteins, membranes were incubated with Akt (1:1000); phospho-Akt (Ser473, 1:3000); 4E-BP1 (1:1000); phospho-4E-BP1 (Ser65, 1:1000); p70S6K (1:1000) and phospho-p70S6K (Thr421/Ser424, 1:1000), all purchased from Cell Signalling Technology. Membranes were washed 4 times in TBST and incubated for 1 h at room temperature with HRP-conjugated anti-mouse IgG-Fc2a antibody (1:50.000, Jackson ImmunoResearch) for anti-puromycin antibody or HRP-conjugated anti-rabbit IgG (1:1000, Cell Signaling Technology) for pathway antibodies. After 4 times washing with TBST, membranes were incubated for 5 min with ECL substrate (SuperSignal West FEMTO, Pierce). Protein synthesis was determined by the density of the whole lane using a Chemidoc XRS (BioRad) and calculated as a relative ratio to the fasted control group. mTOR pathway proteins were determined by the density of the specific band representing the phosphorylated or total protein as indicated by the supplier. After analysis, blots were stained with Coomassie Brilliant Blue R-250 (BioRad) for 30 min and destained in destain solution (10% acetic acid, 40% methanol) to correct for protein loading differences between lanes.

2.4. Biochemical measurements

Right *tibialis anterior* muscle was freeze dried, homogenised in 2% perchloric acid, centrifuged (2000 *g* for 20 min at 4 °C) and supernatants were used to determine muscle free amino acid concentrations. Muscle free and plasma amino acid concentrations were measured using ultra-fast liquid chromatography (UFLC) [26]. Serum glucose levels were determined using an enzymatic colourimetric method (GOD-PAP method, Roche Diagnostics) [27]. Serum insulin was analysed by ELISA assay (10-1247-01 Mercodia AB) [26].

2.5. Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were performed using IBM SPSS Statistics (version 19; SPSS Inc, Chicago, IL). Univariate ANOVA followed by LSD *post hoc* analysis was used to compare groups. Data from WB pathway protein 4E-BP1 showed no normal distribution; therefore, all WB data were tested using univariate ANOVA followed by LSD *post hoc* analysis after log-transformation of the data. Statistical significance is defined as $P < 0.05$.

3. Results

3.1. Amino acid concentrations in plasma and TA muscle

Plasma leucine concentrations increased 6-fold at 60 and 75 min after LEU supplementation and 9-fold after 90 min of LEU ($P < 0.05$ for 60, 75 and 90 min vs fasted at same time point; Fig. 1A, Table 1). With WHEY + LEU, plasma leucine concentrations increased 7-fold after 60 min and 4-fold after 75 min ($P < 0.05$ vs fasted at same time point). Plasma concentrations of isoleucine did not change with LEU but increased 7-fold, 4-fold and 3-fold with WHEY + LEU at 60, 75 and 90 min respectively ($P < 0.05$ vs fasted at same time point; Table 1). Plasma valine increased 2-fold with LEU at 90 min vs fasted at same time point, and increased 5-fold, 3-fold and 3-fold with WHEY + LEU at 60, 75 and 90 min respectively ($P < 0.05$ vs fasted at same time point; Table 1). Plasma EAA concentrations (without leucine) increased 2-fold after LEU supplementation compared to the fasted group after 75 and 90 min ($P < 0.05$), but not after 60 min (Table 1). With WHEY + LEU, plasma EAA concentrations increased 5-fold after 60 min and 3-fold after 75 and 90 min ($P < 0.05$ vs fasted at same time point; Table 1). Plasma NEAA increased 2-fold with LEU at 75 and 90 min ($P < 0.05$ vs fasted at same time point) but did not change after 60 min. With WHEY + LEU supplementation, plasma NEAA increased 2.5-fold after 60 min ($P < 0.05$ vs fasted at same time point) but did not change after 75 and 90 min.

Free leucine concentrations in muscle increased in all intervention groups compared to the group that fasted at all postprandial times ($P < 0.05$ vs fasted at same time point, Fig. 1B; Table 1). Muscle free isoleucine concentrations increased 4-fold at 60 min, 2.5-fold at 75 min and 2-fold at 90 min after gavage with WHEY + LEU ($P < 0.05$ vs fasted at same time point, Table 1) but did not change after LEU gavage. Muscle free valine concentrations increased 2.7-fold at 60 and 75 min and 2.4-fold at 90 min after gavage with WHEY + LEU ($P < 0.05$ vs fasted at same time point, Table 1), also no significant changes were observed after LEU

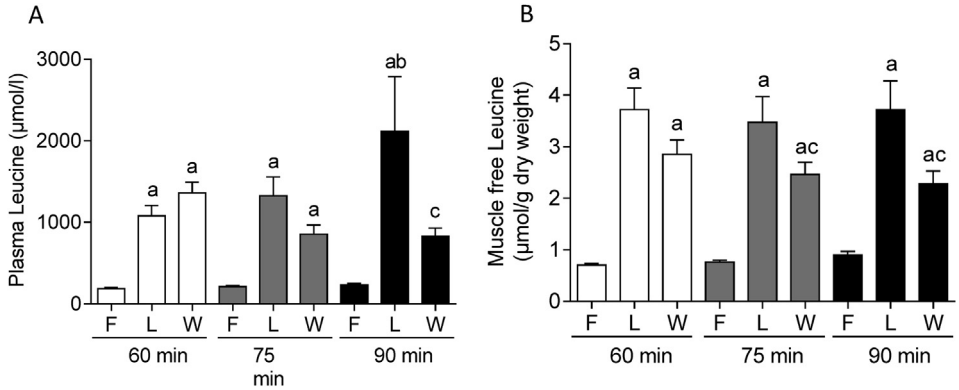

Fig. 1. The effect of leucine and leucine-enriched whey protein on plasma leucine (A) and muscle free leucine concentrations (B) at 60, 75 or 90 min after gavage. F, fasted; L, leucine; W, leucine-enriched whey. Values are means ± SEM. 'a' $P < 0.05$ vs F same time; 'b' $P < 0.05$ vs same supplementation but other time; 'c' $P < 0.05$ vs LEU at same time.

Table 1
Plasma and muscle glucose, insulin and plasma and *m. TA* free amino acid concentrations at 60, 75 and 90 min after oral gavage of leucine or leucine-enriched whey protein in aged mice.

	60 min			75 min			90 min		
	Fasted	Leu	Whey + Leu	Fasted	Leu	Whey + Leu	Fasted	Leu	Whey + Leu
n	8	8	7	8	7	8	8	7	8
Blood concentrations									
Glucose (g/L)	0.9 ± 0.2	0.9 ± 0.2	0.6 ± 0.1	1.0 ± 0.2	1.4 ± 0.3^{b}	0.6 ± 0.2	1.0 ± 0.2	1.3 ± 0.2	0.4 ± 0.1^{a}
Insulin (μg/L)	0.7 ± 0.2	2.2 ± 0.5^{a}	1.1 ± 0.2	0.6 ± 0.1	1.5 ± 0.4^{ab}	0.8 ± 0.1	0.4 ± 0.0	1.4 ± 0.1^{ab}	0.8 ± 0.2
Leucine (μmol/L)	187 ± 13	1081 ± 137^{a}	1362 ± 143^{a}	211 ± 10	1325 ± 251^{a}	854 ± 123^{a}	230 ± 19	2119 ± 719^{abd}	833 ± 103^{d}
ILE (μmol/L)	109 ± 6	125 ± 22	747 ± 73^{ad}	122 ± 6	138 ± 23	473 ± 67^{abd}	129 ± 11	190 ± 49	425 ± 54^{abd}
VAL (μmol/L)	198 ± 10	269 ± 38	1077 ± 77^{ad}	232 ± 18	315 ± 47	794 ± 97^{abd}	233 ± 17	402 ± 90^{a}	765 ± 81^{abd}
EAA (μmol/L)	824 ± 34	1219 ± 140	4004 ± 296^{ad}	948 ± 87	1534 ± 219^{a}	2700 ± 262^{abd}	954 ± 65	2032 ± 442^{ab}	2530 ± 257^{ab}
NEAA (μmol/L)	1017 ± 56	1434 ± 117	2543 ± 253^{ad}	1170 ± 190	2177 ± 370^{ab}	1821 ± 172^{b}	1218 ± 98	2595 ± 593^{ab}	1786 ± 136^{bd}
Muscle free concentrations									
Leucine (μmol/g dw)	0.7 ± 0.0	3.7 ± 0.4^{a}	2.9 ± 0.3^{a}	0.8 ± 0.0	3.5 ± 0.5^{a}	2.5 ± 0.3^{ad}	0.9 ± 0.1	3.7 ± 0.6^{a}	2.3 ± 0.3^{ad}
ILE (μmol/g dw)	0.4 ± 0.0	0.4 ± 0.0	1.5 ± 0.2^{ad}	0.5 ± 0.0	0.3 ± 0.0	1.3 ± 0.2^{ad}	0.5 ± 0.1	0.3 ± 0.1	1.1 ± 0.2^{abd}
VAL (μmol/g dw)	0.9 ± 0.0	0.9 ± 0.1	2.4 ± 0.2^{ad}	0.9 ± 0.1	0.8 ± 0.1	2.3 ± 0.2^{ad}	0.9 ± 0.1	0.8 ± 0.1	2.2 ± 0.2^{ad}
EAA (μmol/g dw)	12.3 ± 0.2	12.3 ± 0.1	16.1 ± 0.5^{ad}	12.5 ± 0.2	11.7 ± 0.2	15.9 ± 0.5^{ad}	12.4 ± 0.3	12.0 ± 0.3	15.3 ± 0.7^{ad}
NEAA (μmol/g dw)	25.3 ± 0.7	27.7 ± 0.7	29.4 ± 0.9^{a}	25.0 ± 1.3	27.5 ± 0.6	31.1 ± 1.0^{ad}	26.0 ± 1.3	28.0 ± 0.9	31.3 ± 1.1^{ad}

Values are means ± SEM. EAA are defined as sum of histidine, isoleucine, methionine, lysine, phenylalanine, threonine, tryptophan and valine; leucine is left out for better comparison. NEAA are defined as sum of alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine and serine. Statistical difference ($P < 0.05$) from fasted control at same time point (^{a}), from 60 min within same supplementation group (^{b}), from 75 min within same supplementation group (^{c}) and from leucine at same time point (^{d}).

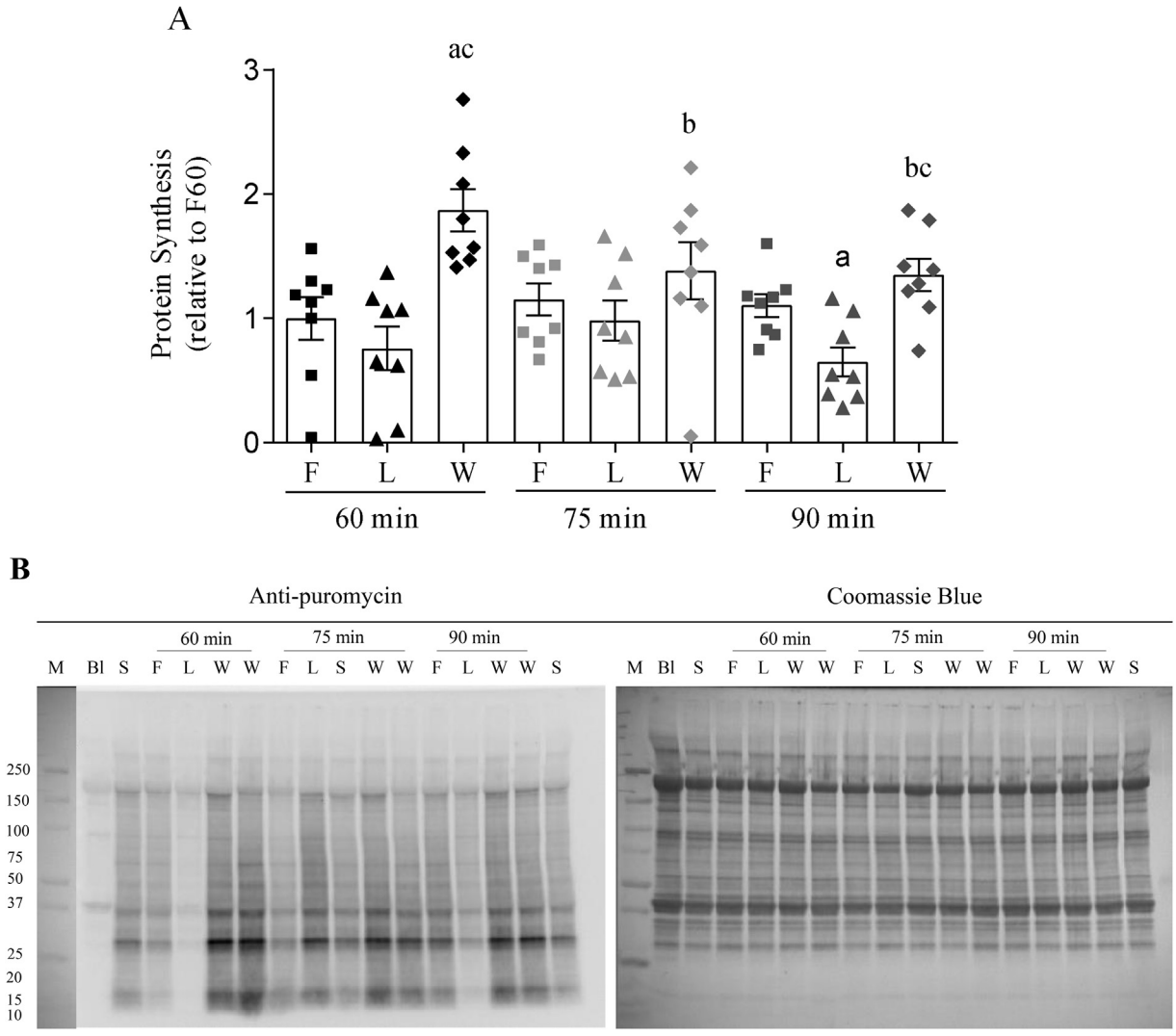

Fig. 2. Muscle protein synthesis assessed by the SUnSET method and measured in the TA after fasting (F) or supplementation with leucine (L) or leucine-enriched whey protein (W) at 60, 75 or 90 min after gavage. A) Scatter blot of puromycin-labelled peptides for each condition. Values (means ± SEM) are expressed as a relative ratio to the fasted control group at 60 min. Statistical difference ($P < 0.05$) to F within same time (^{a}); to same supplementation vs 60 min gavage (^{b}); to LEU same time (^{c}). B) Representative image of blot stained for puromycin and Coomassie Blue to verify equal loading of the samples. 'Bl' indicates control muscle not treated with puromycin to determine non-specific background, 'S' indicates a pooled sample of all 60 min Fasted samples used for relative ratio calculations.

supplementation. Muscle free EAA (without leucine) and NEAA concentrations increased 1.2-fold after supplementation with WHEY + LEU compared to fasted at all postprandial times ($P < 0.05$ vs fasted at same time point) but not after LEU supplementation.

3.2. Serum glucose and insulin concentrations

No changes were observed in glucose concentrations at 60 and 75 min. At 90 min a significant decrease was observed with WHEY + LEU compared to fasted controls. Serum insulin concentrations were significantly increased with LEU compared to fasted mice at all time points ($P < 0.05$ vs fasted at same time point), while no significant changes were observed with WHEY + LEU.

3.3. Muscle protein synthesis

LEU gavage had no significant effect on MPS after 60 and 75 min, but showed a significant decline after 90 min ($P = 0.043$ vs fasted; Fig. 2). MPS after supplementation with WHEY + LEU increased significantly at 60 min compared to fasted control ($P = 0.0001$ at 60 min; Fig. 2), but returned to fasting level after 75 and 90 min ($P = 0.296$ at 75 min and $P = 0.285$ at 90 min; Fig. 2).

3.4. mTOR signalling proteins

Following LEU supplementation, after 60 min no significant changes were observed in the phosphorylated or total 4E-BP1, Akt or p70S6k protein (Fig. 3). A significant increase in phosphorylated 4E-BP1 was observed with WHEY + LEU (2.3-fold, $P = 0.002$ vs fasted and $P = 0.007$ vs LEU) while total 4E-BP1 was significantly lower compared to fasted ($P = 0.039$). This resulted in a significant increase in the phospho-total ratio of 4E-BP1 ($P = 0.001$ vs fasted, $P = 0.004$ vs LEU). Akt phosphorylation was significantly higher in WHEY + LEU compared to LEU and fasted (1.4-fold, $P = 0.037$ vs fasted; $P = 0.033$ vs LEU; Fig. 3) with no changes in total protein contents. The phospho-total ratio of Akt was significant in WHEY + LEU compared to LEU ($P = 0.045$). Phosphorylation of p70S6k was significantly higher in WHEY + LEU ($P = 0.004$ vs fasted) with no changes in total protein or phospho-total ratio.

4. Discussion

This study showed that administration of a single bolus of leucine did not increase MPS in aged mice. However, administration of a bolus of leucine-enriched whey protein with similar leucine content successfully stimulated MPS. The differential effect on MPS stimulation was confirmed by phosphorylation of mTOR signalling pathway proteins. 4E-BP1 and Akt signalling pathway proteins showed significant activation after leucine-enriched whey protein gavage, but not after leucine administration only.

To our knowledge, this non-response of *in vivo* MPS to a single oral bolus of leucine has not been described before in aged animals or humans. This is in contrast to results in young or adult animals that show an anabolic response after single leucine supplementation [8,12,28]. Moreover, in a previous study we also observed that leucine supplementation reaching similar plasma leucine concentrations in adult mice showed a significant increase in MPS (*unpublished data*). Crozier [12] showed that administration of 0.135 g/kg bw until 1.35 g/kg bw of leucine effectively stimulated MPS in food-deprived mature rats. This was accompanied by an increase in 4E-BP1 phosphorylation. When comparing adult rats with aged rats, Dardevet [13] showed that MPS was less responsive to leucine in epitrochlearis muscle of aged rats, measured *in vitro*, as compared to adult rats. This was accompanied by lower p70S6k activation in muscle of aged rats. In our study, the lack of postprandial MPS response in aged mice may be related to anabolic resistance to leucine and insulin [6,13,29,30]. The likelihood of anabolic resistance is supported by the fact that there was no rise in MPS, even though plasma leucine increased ~6-fold and insulin increased ~3-fold. Therefore, it is not expected that a further increase in the leucine dose would have an additional effect on MPS. Since no MPS response was observed at 75 and 90 min either, a delay in MPS response as reported for ageing after anabolic stimuli [31] is also not likely. The results therefore suggest that leucine alone, in the absence of elevated levels of amino acids in the muscle, cannot increase MPS in aged mice [32].

To overcome the lack of available amino acids as precursors to support *de novo* protein synthesis, we combined leucine with whey protein administration, providing a total leucine dose that was like the leucine gavage alone. Indeed, a significant ~1.6-fold MPS increase was observed after leucine-enriched whey protein that peaked at 60 min and gradually diminished to come back to basal level after 90 min. Leucine has also been studied by others in the context of a meal or with other amino acids [14–16,18,33,34]. Rieu [14] showed that a leucine supplemented meal increased postprandial protein synthesis in aged rats after 10 d of supplementation. Dardevet [15] showed that aged rats responded to an acute meal only when it was supplemented with leucine at a dose that doubled the plasma leucine concentrations. Katsanos [16] reported that, compared to younger adults, aged subjects needed additional leucine (in an EAA mixture enriched with 41% leucine) to stimulate protein synthesis. Magne et al. [33] supplemented aged rats for 40 days with a leucine-enriched casein diet (containing 44.5 g/kg leucine) showing no effect on postprandial MPS compared to casein protein. However, whey protein effectively stimulated postprandial MPS. The authors [33] described the lack of MPS response with leucine enriched casein as a desynchronization between the 'leucine signal' and the availability of substrates to induce muscle protein synthesis. With whey protein, this desynchronization was overcome. Such desynchronization may explain the difference between leucine alone and leucine-enriched whey protein in our study. We observed that at 60 min after leucine-enriched whey protein, plasma and muscle free EAA levels were higher. While plasma leucine concentrations were not different, muscle free leucine concentrations were even lower than after single leucine gavage. The transient MPS response after leucine-enriched whey protein coincided with a decrease in plasma EAA levels over time. This argues for the importance of adequate levels of EAA in addition to leucine to produce a significant MPS response. Moreover, while insulin is considered an anabolic stimulus, serum insulin was not increased at 60–90 min after leucine-enriched whey gavage. Unfortunately, we cannot exclude that the insulin peak in the leucine-enriched whey protein group already occurred before the 60 min time point. In humans receiving a leucine-enriched whey protein supplement [25,35], insulin peaks at around 30 min after intake and is back to baseline levels at about 90 min. The lower glucose levels in the leucine-enriched whey protein group (significant at 90 min) may indicate that an insulin-induced glucose lowering indeed has occurred. In contrast, a high insulin response was observed within the 60–90 min timeframe after single leucine gavage. This suggests that leucine-induced insulin is not the exclusive stimulus for MPS but other signalling pathways may be involved, as suggested by others [20,36].

A limitation of our study is that repeated blood sampling is limited in mice and therefore time-dependent changes in plasma concentrations of amino acids and insulin cannot be followed in the same mouse. This could be circumvented in future studies with the use of dry bloodspots to follow concentrations over time in mice. Other limitations are that we did not measure other aspects of protein metabolism, such as protein breakdown, and cannot discriminate between exogenous and endogenous leucine sources

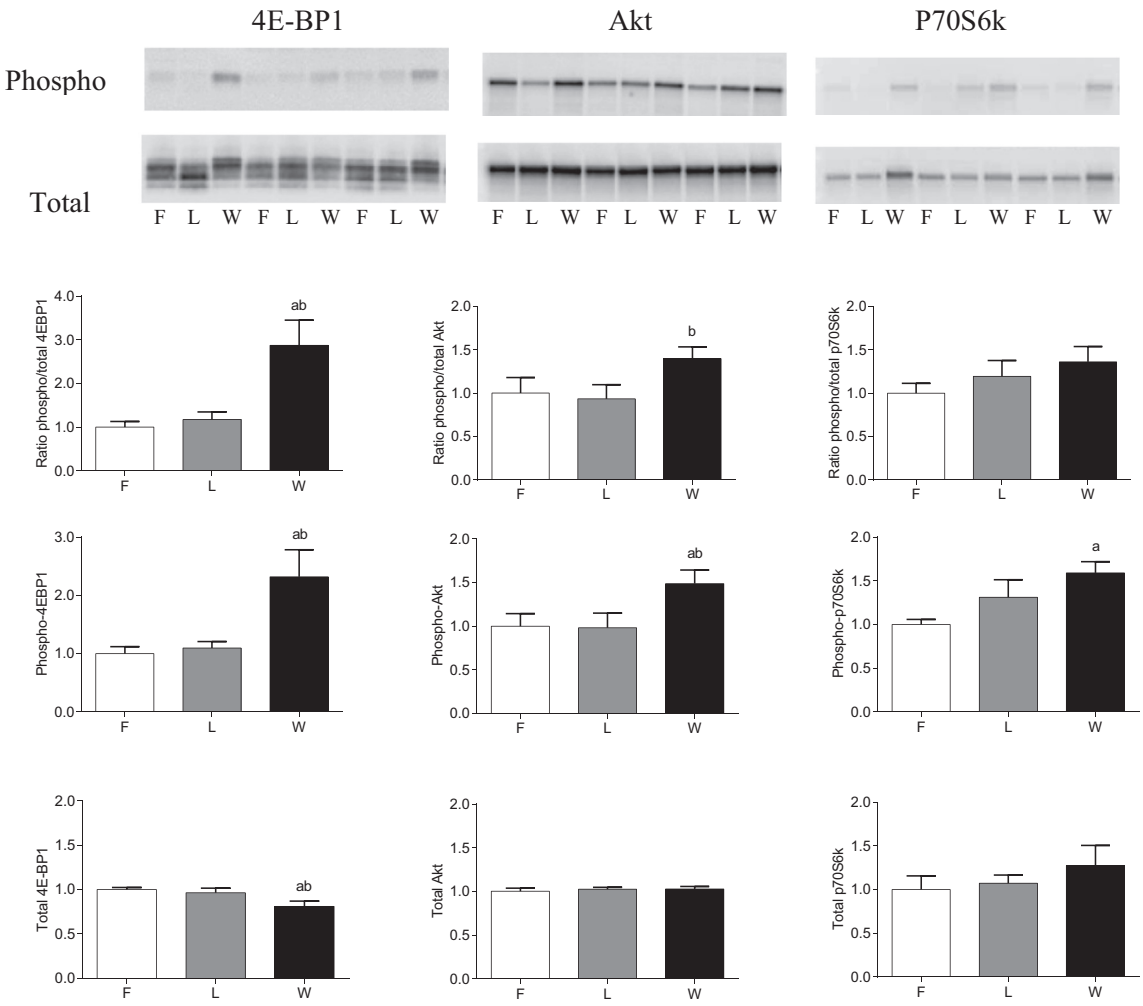

Fig. 3. The effect of leucine and leucine-enriched whey protein on activation of 4E-BP1, Akt and p70S6k at 60 min after gavage. F, fasted L, leucine; W, leucine-enriched whey. Values are means ± SEM. 'a' $P < 0.05$ vs fasted; 'b' $P < 0.05$ vs L.

in our study. The increase in plasma EAA after single leucine gavage at 75 and 90 min suggests increased protein breakdown. Finally, the use of additional control groups, e.g. an isonitrogenous NEAA control providing equal energy or a whey-only group, could further substantiate the importance of other amino acids beyond leucine and the role of leucine for an anabolic response.

A strength of the study is that MPS was measured at three points over time, which is relevant because a time-dependent increase or decrease in MPS and a delayed MPS response with advanced age is known [31,37]. Moreover, we successfully applied the SUnSET method for measuring MPS *in vivo* both in fasted and postprandial conditions. The method is considered a valid and accurate method for measuring *in vivo* protein synthesis [38], but its principle of puromycin-bound peptide formation is different from the flooding dose method [8,12,15,28,39,40].

The clinical relevance of our data lies in the nutritional support for leucine-rich or leucine-enriched amino acid mixtures or proteins as most effective to stimulate MPS in older individuals. Extrapolating to the human situation (e.g. 75 kg male), 5.56 g/kg bodyweight whey protein (~139 mg) in mice equals 24 g protein in humans. For leucine, 0.75 g/kg bodyweight (~18.8 mg) in aged mice represents 3 g leucine [41]. A leucine-enriched whey protein supplement (20 g whey protein, 3 g total leucine) effectively increased MPS in healthy older adults [25] and increased muscle mass after 3 months of intervention [42]. The PRO-TAGE study group also recently published a position paper, recommending the intake of 2.5–2.8 g leucine per meal for older adults [43].

In conclusion, oral administration of leucine-enriched whey protein stimulates MPS, while administration of leucine only does not increase MPS in aged mice. We hypothesize that aged mice require leucine in the presence of other amino acids to induce an anabolic response. These observations may be relevant for developing nutritional strategies to stimulate muscle anabolism and promote muscle mass maintenance with ageing.

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Statement of authorship

F.J. Dijk was involved in designing and executing the experiments, data interpretation, statistical analysis and writing of the manuscript. M. van Dijk was involved in designing and executing the experiments, data interpretation, statistical analysis and writing of the manuscript. Y.C. Luiking was involved in designing the experiments, data interpretation and writing the manuscript. K. van Norren was involved in designing the experiments and reviewed the manuscript. S. Walrand and L.J.C. van Loon were

involved in data interpretation and reviewed the manuscript. All authors critically reviewed the manuscript.

Conflict of interest statement

F.J. Dijk, M. van Dijk and Y.C. Luiking are employees of Nutricia Research. K. van Norren is a former employee of Nutricia Research and an advisor for Nutricia Research. K. van Norren and S. Walrand are advisors for Nutricia Research and L.J.C. van Loon had acted as an advisor for Nutricia Research in the past.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnesp.2017.12.013.

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