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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)

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