Human skeletal muscle is refractory to the anabolic effects of leucine during the postprandial muscle-full period in older men

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Abstract

Leucine modulates muscle protein synthesis (MPS), with potential to facilitate accrual/maintenance of muscle mass. Animal models suggest leucine boluses shortly after meals may prolong MPS and delay onset of a “muscle-full” state. However, the effects of nutrient “top-ups” in humans, and particularly older adults where deficits exist, have not been explored. We determined the effects of a leucine top-up after essential amino acid (EAA) feeding on anabolic signaling, MPS and muscle energy metabolism in older men. During $^{13}$C$_6$-phenylalanine infusion 16 men (~70y) consumed 15g EAA with (n=8, FED+LEU) or without (n=8, FED) 3g leucine top-up 90min later. Repeated blood and muscle sampling permitted measurement of fasting and postprandial plasma EAA, insulin, anabolic signaling including mTORc1 substrates, cellular ATP and phosphorylcreatine and MPS. Oral EAA achieved rapid insulinaemia (12.5iU.ml$^{-1}$ 25 min post-feed), essential aminoacidemia (3000 µM, 45–65 min post-feed), and activation of mTORC1-signaling. Leucine top-up prolonged plasma EAA (2800 µM, 135 min) and leucine availability (1050µM, 135min post-feed). Fasting FSR’s of 0.046 and 0.056%.h$^{-1}$ (FED and FED+LEU respectively) increased to 0.085 and 0.085%.h$^{-1}$ 90-180min post-feed and returned to basal rates after 180min in both groups. Phosphorylation of mTORc1-substrates returned to fasting levels 240min post-feed in both groups. Feeding had limited effect on muscle high-energy phosphates, but did induce eukaryotic elongation factor 2 (eEF2) phosphorylation. We demonstrate the refractoriness of muscle to nutrient-led anabolic stimulation in the post-prandial period; thus leucine supplements should be taken outside of meals, or with meals containing sub-optimal protein in terms of either amount or EAA composition.

Word count: 250
Perspective

- This study was undertaken to assess the response of human skeletal muscle to leucine top-ups after maximal essential amino acid feeding; rat studies have suggested it may promote muscle protein synthesis.

- Given after adequate doses of essential amino acids, oral leucine did not change skeletal muscle anabolic signalling nor protein synthesis.

- These findings challenge the benefit, in healthy older people, of leucine supplements in close proximity meals of adequate protein/essential amino acids content; leucine supplements should be taken outside of meals, or with meals containing sub-optimal protein. The duration of and mechanisms underlying this refractory period require further investigation.
Introduction

The increase in plasma amino acid (AA) concentration that follows ingestion, digestion and absorption of dietary protein is the major anabolic drive contributing to the reversal of the post-absorptive net efflux of amino acids from human skeletal muscle - shifting the dynamic equilibrium towards net accretion of myofibrillar mass and replacing losses since the last meal [1]. A dose-response relationship exists such that although there is a graded response to small feeds [2], a modest serving of essential amino acids (EAA) or high quality protein (i.e. rich in EAA) achieves the same response as a large serving [3,4]. Recent advances in stable isotope tracer techniques have permitted detailed temporal resolution of muscle metabolic responses to EAA/protein feeding. These techniques have made it apparent that after an initial latent period (c.30 min for intravenous EAA infusion, c.45 min for whey and up to 90 min for free EAAs), muscle protein synthesis (MPS) in healthy young humans approximately doubles for a finite period (~2-3 h) before returning to fasted rates [5–7]. The observation that MPS returns to fasting rates despite ongoing availability of EAA and activity within the mTORC1 signalling pathway [5] suggests a so-called “muscle-full” effect. It is likely that the underlying mechanisms regulating onset of the muscle-full state will depend upon accretion of adequate new muscle protein rather than being simply time-dependent, since the onset of muscle-full appears to be delayed with the provision of a very gradual aminoacidaemia (achieved via comparing pulse to bolus EAA) feeding [8].

Individual amino acids have distinct potencies as muscle anabolic stimulants. Only the EAA stimulate increases in MPS in humans [9–11] and amongst these, branched-chain amino acids (BCAAs) are distinct in being preferentially metabolised in skeletal muscle rather than gut or liver [12–14]. Of the BCAAs, leucine is unique in directly activating the mTOR complex 1 (mTORC1) protein kinase, a master growth controller [15,16], promoting AA uptake by peripheral tissue [17] and maximizing MPS [18,19]; this underpins the academic [20] and commercial [21] interest in its role as a pharmaco-nutrient. Studies in
rats have shown that whilst the leucine content of a mixed meal directs the peak activation (but not
duration) of MPS [22] the provision of leucine in the postprandial period (120 min post feed) can re-
invigorate the maximal stimulation of MPS as it is waning, i.e. delaying the onset of muscle full [23]. As
such, this demands a better understanding of the muscle-full state in humans including assessment of
the refractoriness of skeletal muscle in the postprandial/early postabsorptive period to further anabolic
stimulation, which has not, to our knowledge, been explored. Moreover, given anabolic resistance to
feeding in older age [2] and over which contention remains as to whether this can [24,25] or cannot [2]
be overcome by increasing the dosage, this is a particularly relevant question in older people.

The purpose of this study was to assess the refractoriness of older human skeletal muscle to anabolic
stimulation when in the muscle-full state, by provision of 3 g of oral leucine bolus or “top-up” 90 min
after a 15 g mixed EAA feed, and to contextualise this response in terms of activity within established
anabolic signalling pathways and cellular energy stress markers, within muscle cells. This work provides
new insight into both fundamental nutritional biochemistry and applied nutrition.

Experimental detail

Study Design. With ethical approval from the University of Nottingham Medical School Ethics Committee
(United Kingdom), this work was carried out in accordance with the Declaration of Helsinki, with
prospective registration (clinicaltrials.gov registration no. NCT01890369). Healthy, recreationally active
older males (n=16, 70.3 ± 2.6 y, BMI 25.5 ± 1.8 [Mean ± SD]) were recruited by mail and local advertising.
Recruits were studied after fasting overnight and were asked to avoid heavy exercise for the preceding
48hrs. On the morning of the study (08.00), participants had a 18-g cannula inserted into the dorsum of
the left hand for a primed (0.4mg.kg⁻¹) constant infusion (0.6mg.kg⁻¹.hr⁻¹) of L-[ring-¹³C₆]-phenylalanine
(Cambridge Isotopes Ltd, Cambridge, MA, USA) tracer. Blood samples and muscle biopsies were taken
according to the protocol (Figure 1). Arterialised venous blood was sampled via a retrograde 16-g
intravenous cannula placed in the dorsum of the right hand, with the hand warmed to 55ºC [26]. Muscle biopsies were taken intermittently from m. vastus lateralis using the conchotome technique [27] after infiltration of 5ml 1% lignocaine. Muscle was washed in iced cold saline and visible fat and connective tissue was removed before being frozen in liquid N2 and stored at -80ºC until analysis. Biopsies were taken 1 h and 3 h after commencement of tracer to permit assessment of basal (postabsorptive) MPS. Participants were then provided with 15g mixed essential amino acids (Histidine 1.21g, Isoleucine 1.73g, Leucine 3.59g, Lysine 3.07g, Methionine 0.95g, Phenylalanine 0.91, Threonine 1.13g Tryptophan 0.48g, and Valine 1.86g) in aqueous solution (200 ml), which was consumed in a single draft. Participants were allocated to receive just this EAA bolus feed (FED, N=8) or this feed and an additional leucine “top-up” of 3 g in aqueous solution (200 ml) consumed 90 min after the initial EAA feed (FED+LEU, N=8). Subsequent biopsies, at 90, 180 and 240 min post commencement of feeding allowed assessment of MPS in the intervening periods. After the study, cannulae were removed and the participants fed and monitored for 30 min before departure. Subject demographics are shown (Table 1).

Measurement of plasma AA and insulin concentration. Venous plasma insulin concentrations were measured using undiluted samples on a high-sensitivity human insulin enzyme-linked immunosorbent assay (DRG Instruments GmbH, Marburg, Germany). Area-under-the-curve (AUC) analysis estimated the total insulin response to feeding and was calculated for each individual with a baseline equal to the mean of fasting, +155 min and +195 min insulin concentrations.

For Amino Acid (AA) analyses, equal volumes of arterialised plasma and 10% sulfosalicylic acid were mixed and cooled to 4ºC for 30 mins. Samples were centrifuged at 8000 g to pellet the precipitated protein and the supernatant fluid was passed through a 0.22µm filter before analysis with a dedicated AA analyser, based on ion exchange chromatography, using lithium buffers with post column derivatization with Ninhydrin (Biochrom 30, Biochrom, Cambridge, United Kingdom)) using lithium
buffers. All 20 AA concentrations were measured by comparison to a standard AA mix with norleucine as an internal standard. AUC analysis estimating total EAA exposure was calculated for each individual without baseline correction.

**Measurement of myofibrillar protein fractional synthetic rate.** The fractional synthetic rate (FSR) of myofibrillar protein was derived from the increase in incorporation of L-[ring-\(_{13}\)C\(_{6}\)]-phenylalanine between subsequent muscle biopsies. Muscle intracellular phenylalanine, the average of 2 biopsies, was used as a surrogate of phenylalanyl-tRNA labeling (the immediate precursor for protein synthesis) [28]. The standard precursor-product method was used to calculate FSR (\%·h\(^{-1}\)):

\[
FSR = \left[ \frac{E_{m2} - E_{m1}}{E_p \cdot t} \right] \times 60 \times 100
\]

where \(E_{m1}\) and \(E_{m2}\) are the enrichments of bound L-[ring-\(_{13}\)C\(_{6}\)]-phenylalanine in 2 sequential biopsies, \(t\) is the time interval between 2 biopsies, and \(E_p\) is the mean L-[ring-\(_{13}\)C\(_{6}\)]-free phenylalanine enrichment in the intramuscular pool.

To maintain steady state labeling, the potential dilution of enrichment in plasma (and, in turn, intramuscular) phenylalanine labeling with EAA feeding was offset by provision of 6% of ingested phenylalanine as L-[ring-\(_{13}\)C\(_{6}\)]-phenylalanine.

In relation to quality control (QC) for mass spectrometry (and amino acid analyses); we constructed both concentration and enrichment curves, and ran QC samples alongside each batch of analyses for both plasma and intramuscular Phe by GC-MS - in addition to enrichment curves and QC samples (that bracket the expected enrichment of the myofibrillar protein bound \(^{13}\)C\(_{6}\) Phe) for the GC-C-IRMS analysis. Repeat analyses have coefficients of variation (CV) of <5% and standard curves have R\(^2\) greater than 0.995. All equipment is subject to regular maintenance to maintain specification.
Measurement of intramuscular high-energy phosphates. Approximately 10mg of frozen muscle was cut from each biopsy, freeze-dried and stored at -80°C for subsequent muscle metabolite analysis. Freeze-dried muscle samples were powdered, after removal of macroscopic blood and connective tissue, and metabolites extracted in 0.5 M perchloric acid (containing 1 mM EDTA), followed by neutralisation with 2.2M K$_2$CO$_3$ (Sigma Chemicals). Muscle ATP, phosphorylcreatine (PCr) and free creatine concentrations were determined enzymatically in muscle extracts according to the method of Harris et al, [29] was modified to accommodate use of a 96-well plate spectrophotometer. Muscle total creatine was calculated as the sum of PCr and creatine and subsequently used to correct muscle ATP and PCr values for non-muscle constituents.

Immunoblotting was performed on approximately 30 mg of muscle as described previously [18]. Primary antibodies against phospho-ribosomal protein 70 S6 Kinase 1 (p70 S6K) Thr398, protein kinase B (AKT) Ser473, eukaryotic elongation factor 2 (eEF2) Thr56, eukaryotic initiation factor 2α (eIF2α) Ser51, Acetyl Co-A Carboxylase (ACC) Ser79, AMP-activated protein kinase (AMPK) Thr173, (New England Biolabs), and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) Ser65/Thr70 (Santa Cruz Biotechnology), were used before incubated in HRP-conjugated secondary antibody (New England Biolabs). Membranes were exposed to chemiluminescent HRP substrate (Millipore) and bands were quantified using Chemidoc XRS (BioRad). Protein loading anomalies were normalized to Coomassie-stained membranes and each individual was standardized to fasting. Blot data were analyzed by using peak density and all signals remained within the linear range of detection, avoiding saturation. Immunoblots were run using pre-validated antibodies with specificity verified by positive and negative controls and, where required, peptide competition assays/ in vitro siRNA/ pharmacological ablation of signals for phospho-targets [30]. All samples were run alongside molecular weight standards to verify the expected molecular weight of target proteins.
Statistical analyses. An a priori power calculation suggested we needed 8 subjects per group to detect, with $\alpha$ of 0.05 and $\beta$ of 0.85, a difference between groups of ~20% in the primary end-point of MPS. Data are presented as means ± SE, after D'Agostino & Pearson omnibus normality testing. Demographic and anthropometric comparisons between groups were conducted by unpaired t-tests. Differences for all other analyses were detected by repeated-measures analysis of variance, with Bonferroni correction, using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant, with two-tailed testing to compare between groups and one-tailed tests to detect changes from fasting. Effect size is presented for key comparisons between groups. For a given time point, this is estimated as $\frac{([\text{mean of FED}]-[\text{mean of FED+LEU}]) \times (\text{standard deviation of FED})^1}$.

Results

Plasma concentrations of AAs and insulin. After consumption of 15 g mixed EAA, plasma EAA and leucine concentrations rise rapidly and by a similar amount in both FED and FED+LEU groups, peaking at between 45 and 65 min (EAA +320%, leucine +430%, all $P <0.001$ versus fasting) and falling thereafter. Provision of a 3 g leucine top-up at 90 min after the initial 15 g EAA feed provided a second peak in both plasma EAA (+190% from fasting, effect size 3.8) and leucine (+770% from fasting, effect size 12) concentration at 135 min post initial feeding (45 min post top-up). Thus plasma concentration of EAA and leucine differed between feeding regimens beyond 115 min (Figure 2A and 2B). NEAA concentrations do not differ between groups and modulate little throughout the experimental protocol (Figure 2C). Both groups demonstrated a similar modest plasma insulin response, seen at 25 and 45 min (+160% at 25 min, and +140% at 45 min, all $P <0.001$ versus fasting) and both groups had returned to fasting insulin concentrations at 80 min, with no subsequent divergence detected between groups (Figure 2D).

Myofibrillar protein synthesis. Fasting myofibrillar protein FSR was not different between groups (FED
0.046 ± 0.015%.h⁻¹, FED+LEU 0.056 ± 0.018%.h⁻¹, NS). After a latency of 90 min, FSR increased (FED 0.085 ± 0.003%.h⁻¹ and FED+LEU 0.085 ± 0.009%.h⁻¹, P=0.031 and P=0.004, both versus fasting). Beyond 180 min, FSR returned to basal rate (FED 0.051 ± 0.012%.h⁻¹, NS versus fasting) and the provision of a leucine top-up did not prolong stimulated FSR in the FED+LEU (0.055 ± 0.006%.h⁻¹, NS versus fasting. The effect size of the addition of a leucine top-up, with regard to FSR between 180-240 min, was 0.12. Thus no detectable difference in FSR existed between feeding regimens (Figure 3).

Activity within established signalling pathways. Bolus EAA feeding achieves detectable activity within the mTORC1 complex as evidenced by an increase in phosphorylation of substrates 4EBP1 Ser65/Thr70 (above time 0 min, or trend towards, FED P=0.055, 0.095, FED+LEU P=0.012, 0.031 at +90 and +180 min respectively) and P-p70 S6K Thr389 (above time 0 min, or trend towards, FED P=0.008, 0.079, FED+LEU P=0.007, 0.02 at +90 and +180 min respectively). All returned to fasting levels by 240 min. Leucine top-up failed to achieve detectable prolongation of mTORC1 activity with no difference between treatment arms (Figure 4).

EAA feeding induced phosphorylation of P-eEF2 late in the experimental period (+0.38, +0.36; P=0.007, P=0.09; 0 vs. 240 min; FED, FED+LEU respectively, NS between groups, effect size at 240 min 0.079). Significant changes from fasting were not detected in phosphorylation of P-ACC Ser79, P-Akt Ser473, P-AMPK Thr173, nor P-eIF2α Ser51, with no differences detected between arms.

Intramuscular high-energy phosphates. Muscle PCR content was similar between feeding regimens across the fasting and early to mid-postprandial periods. However, late in the postprandial period they diverged (FED+LEU PCr -40% versus FED, P=0.007, effect size 2.0) Muscle creatine and ATP contents remained unchanged across the study period and were similar between feeding regimens (Figure 5).

Discussion
An accumulating body of evidence demonstrates that a modest amount of dietary protein can achieve a maximal MPS response. The amount of myofibrillar protein accrued after such a meal is in part directed by the onset of a “muscle full” state, of post-absorptive refractoriness to nutritional stimulation [31]. The mechanisms that underlie the onset of the muscle full state have not been elucidated, hampering the development of interventions that could delay the onset of the muscle full state, which may in turn help to build or maintain muscle mass. Research in this area remains current with much interest surrounding the potential of pharmaco-nutrients, e.g. leucine, to boost the anabolic effectiveness of feeding, especially in older populations and in those at risk of sarcopenia [32].

Here, we studied a cohort of older men in the absence and presence of a “late” postprandial period leucine top-up. As expected, provision of 15 g EAA led to sustained hyper-aminoacidaemia and stimulated MPS until the onset of a “muscle full” state 180-240 min after feeding - consistent with a fed-state anabolic window of \( \sim 3 \text{ h} \) [5]. Moreover, as would be expected, 3 g leucine top-up, 90 min after feeding altered plasma aminoacidaemia profiles; increasing the area under the curve for both total EAA and leucine. Nonetheless, despite enhancement of leucinaemia, the temporal profile of MPS was identical between groups. By our demonstrating identical MPS responses to feeding with and without a leucine top-up in the late post-prandial period, this study has an important applied message, which challenges the notion of any benefit of supplementary leucine with, or shortly after, meals of adequate protein content. Thus, supplements of leucine should be guided in between meals or to meals containing sub-optimal protein levels or quality, as we previously demonstrated in a study in older men wherein we were able to stimulate muscle anabolism when participants were given leucine in addition to 10g of protein, following a bout of resistance exercise [33]. Detailed discussion of what should be considered an optimal protein content for a meal is beyond the scope of this paper as this will vary between people and with activity and may demand consideration of (E)AA makeup and digestion/ absorption profile. That said, data from healthy, rested, fasted humans suggests that
a maximal muscle protein synthetic response can be achieved by approximately 10g of an appropriate mix of EAA whether delivered in as dietary protein (eg 113g of beef) [3] or by oral EAA ingestion [2].

We also sought to investigate links between leucine top-ups and the molecular signals controlling MPS. In doing so, we revealed that mTORc1-related signals showed a similar response to that of MPS – albeit consistent with that which we have previously shown, anabolic signaling (i.e. mTORc1 substrates, p70S6K1 and 4EBP1) likely outlasted MPS responses i.e. MPS was at baseline 3-4 h despite signals remaining elevated towards peak at 3 h [5,34]. These data support our MPS data in illustrating that the signals controlling increased MPS in response to nutrition are also refractory to leucine stimulation. We also measured the phosphorylation of AMPK its substrate ACCβ and eEF2, since it had been previously reported that energy stress caused by ATP-consuming processes associated with MPS (i.e. AA transport, tRNA-aminoacylation and translation/elongation stimulated by EAA ingestion) induced a state of ATP depletion leading to a concomitant rise in the AMP/ATP ratio and an ensuing induction of inhibitory AMPK-eEF2 signaling [23,35]. In contrast to this previous study in rodents [23] we find here, and in other similar feeding studies [5] no increases in AMPK-related signaling in either group. Moreover, in measuring ATP:PCr as a sensitive proxy of cellular energy stress [36] we failed to observe a robust induction of stress in mitochondrial energy delivery by feeding – likely ruling this out as a major facet of muscle full in adult humans. That being said, we did observe an induction of eEF2 phosphorylation in both groups which rose to significance by 240 min; since eEF2 phosphorylation puts the brakes on mRNA translation via inhibiting A-P site translocation of ribosomes [37], this could represent an important mediator of the muscle full state (albeit not via AMPK-signaling pathways), since eEF2 is known to be sensitive to nutrient deprivation i.e. eEF2 is upregulated to suppress MPS and switch off energy consuming processes. The phosphorylation of eEF2 is regulated by eEF2 kinase, which is under the regulation of AMPK [38], MAPK [39] mTOR [40] and calcium signaling related pathways [37]. Further
work is needed to define the upstream kinase(s) responsible for the inhibitory induction of eEF2 phosphorylation in response to feeding, and moreover, studies with sufficient temporal definition and/or of a mechanistic nature, to determine a true role for eEF2 in regulating the muscle full state. This is especially important since a statistically significant induction of eEF2 was evident only at 240 min, long after MPS had returned to a postabsorptive values.

Our present findings in relation to energy stress (namely the lack of) are in contrast to previous studies in rats, where the muscle-full state [23,41] appears to represent less profound refractoriness to stimulation and may be overcome by either provision of leucine or carbohydrate top-ups [23]. These differences may reflect differences between basal nutritional patterns (i.e. rats fed 80% of what they would ingest if fed ad libitum may represent a chronically restricted model when compared to healthy community dwelling senior human volunteers); between experimental protocols (i.e. rats fed mixed meals versus humans consuming mixed EAA isolate); between species (rat MPS having been shown to exceed human MPS by a factor of five or more [42]); and, most plausibly, differences between growing animals and weight-stable adult humans. Indeed, the very existence of an analogous muscle full state in neonatal pigs is dubious, with an apparent unlimited stimulation of mTORC1 signalling and MPS provided sustained leucine/ EAA availability [43].

Potential limitations in this study include avoidable variability that has been introduced by our comparison of two age-, weight- and BMI- matched groups of 8 and 8 volunteers rather than undertaking interval studies on each volunteer in a cross-over paradigm. Whilst cross-over studies provide theoretical benefit, the practical challenges of recruitment and completion of two lengthy and invasive acute studies with stable isotope tracers and multiple muscle biopsies in healthy volunteers lead the authors (and their research ethics committee) to choose to employ independent groups as described. Nonetheless, the authors conclude this study questions the theoretical benefit, to healthy
older humans, of leucine supplementation in close proximity to meals containing “adequate” protein i.e. supplements may be most likely to be effective when taken in between meals, perhaps in the form of low dose EAA mixtures, rather than leucine alone; the efficacy of which may be limited in the absence of exogenous EAA to promote whole body and skeletal muscle net balance. Finally, given the absence of overt energy depletion, further work is needed in humans to determine the signals regulating the onset of the muscle full state.

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Author contribution statement

Fig 1. Experimental protocol. During a constant primed infusion of $[^{13}C_6]$phenylalanine, 15 g of essential amino acids were ingested with or without a 3 g leucine top-up 90 minutes later. Blood samples and muscle biopsies allowed assessment of plasma amino acid and insulin concentrations, fractional synthetic rate of myofibrillar proteins, signaling pathways and muscle energy metabolism. EAA, Essential amino acid. Leu, leucine.

Fig 2. Plasma essential amino acids (EAA; A), nonessential amino acids (NEAA; B), leucine (C), and insulin (D) concentrations, in older men after consumption of 15 g of mixed EAA in the absence (FED) and presence (FED+LEU) of a 3g leucine top-up after 90 min. * $P<0.05$, ** $P <0.01$, and *** $P <0.001$, differences between feeding strategies. Values are means +SE; n= 8. Filled arrows represent ingestion of 15 g of EAA; unfilled arrows represent 3 g leucine (EAA+LEU only).

Fig 3. Fractional synthetic rate (FSR) of muscle protein synthesis in older men after consumption of 15 g of mixed EAA in the absence (A) and presence (B) of a 3g leucine top-up after 90 min . Values are means+SE, n=8. * $P <0.05$, ** $P <0.01$, difference from fasting.

Fig 4. Phosphorylation of ribosomal protein 70 S6 Kinase 1 (p70 S6K) Thr398 (A), eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) Ser65/Thr70 (B), Acetyl Co-A Carboxylase (ACCB) Ser79 (C), AMP-activated protein kinase (AMPK) Thr173 (D), protein kinase B (Akt) Ser473 (E), eukaryotic initiation factor 2α (eIF2α) Ser51 (F), and eukaryotic elongation factor 2 (eEF2) Thr56 (G), in older men after
consumption of 15 g of mixed EAA in the absence (EAA) or presence (EAA+LEU) of a 3g leucine top-up. Values are means ± SE; n 8. # trend with P<0.10, *P< 0.05, **P (A) < 0.01, differences from time = 0 min.

Figure 5. Muscle ATP (A), phosphorylcreatine (PCr, B) and creatine (C) in older men after consumption of 15 g of mixed EAA in the absence (EAA) and presence (EAA+LEU) of a 3g leucine top-up after 90 min. All NS versus fasting. Values normalized to muscle total creatine to account for non-muscle constituents.

Table 1.

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Table 1. Volunteer characteristics, values are means ± standard deviations. ASMMI, appendicular skeletal muscle mass index (appendicular muscle mass.height^{-2}); HGS, hand grip strength.


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**Blood sample**

**Muscle biopsies**

**Primed constant infusion of L-[ring-$^{13}$C$_6$]Phenylalanine**

**FED**
- 15g EAA

**FED+LEU**
- 15g EAA
- 3g Leu
Plasma NEAA (μmol.l⁻¹)  

Time post commencement of feeding (min)

Plasma EAA (μmol.l⁻¹)  

Time post commencement of feeding (min)

Plasma leucine (μmol.l⁻¹)  

Time post commencement of feeding (min)

Plasma Insulin (μU.l⁻¹)  

Time post commencement of feeding (min)