Intake of low-dose leucine-rich essential amino acids stimulates muscle anabolism equivalently to bolus whey protein in older women, at rest and after exercise.

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Dysregulated anabolic responses to nutrition/exercise may contribute to sarcopenia; however, these characteristics are poorly defined in female populations. We determined the effects of two-feeding regimes in older women (66±2.5y N=8/group): bolus-whey protein (WP-20 g) or novel low-dose leucine-enriched essential amino acids (EAA) (LEAA-3g [40% leucine]). Using $^{13}$C$_6$-Phenylalanine infusions, we quantified muscle (MPS) and albumin (APS) protein synthesis at baseline and both in response to feeding (FED) and feeding-plus-exercise (FED-EX); resistance exercise (RE): 6×8 knee-extensions at 75%-1RM. We also quantified plasma insulin/AA concentrations, whole-leg (LBF)/muscle microvascular blood-flow (MBF), and muscle anabolic signaling by phospho-immunoblotting. Plasma insulinemia and EAA-aemia were markedly greater after WP than LEAA ($P<0.001$). Neither LEAA nor WP modified LBF in response to FED or FED-EX, while MBF increased to a similar extent in both groups, only after FED-EX ($P<0.05$). In response to FED, both WP and LEAA equally stimulated MPS 0-2h ($P<0.05$), abating thereafter (0-4h; $P>0.05$). In contrast, after FED-EX, MPS increased 0-2h and remained elevated 0-4h ($P<0.05$) with both WP and LEAA. No anabolic signals quantifiably increased after FED, but p70S6K1$^{Thr389}$ increased after FED-EX (2h; $P<0.05$). APS increased similarly after WP and LEAA. Older women remain subtly responsive to nutrition±exercise. Intriguingly though, bolus-WP offers no trophic advantage over LEAA.
INTRODUCTION

Ill-health associated with ageing represents a major socio-economic burden, especially given shifting demographics toward a more aged, populous world. In particular, the loss of skeletal muscle mass associated with ageing, or sarcopenia, is a major clinical issue. For instance, not only are there established links between low muscle mass and all-cause mortality per se (1) but also, lower skeletal muscle mass associated with sarcopenia leads to increased frailty, risk of falls, sedentarism, poor quality-of-life and prevalence of metabolic co-morbidities (17, 53).

The two major extrinsic influences over muscle mass are nutrition and physical activity. For example, oral intake of protein-based foods containing essential amino acids (EAA) leads to a transient (2-3 h; (2)) stimulation of muscle protein synthesis (MPS) in younger men. This brief increase in MPS above post-absorptive rates serves the purpose of replenishing protein stores lost during fasting, ensuring preservation of muscle protein mass. Similarly, physical activity is a prerequisite for maintenance of a healthy muscle mass. For example, inactivity (6, 20) causes muscle atrophy by inducing “anabolic resistance” to nutrition. Conversely physical activity/ exercise potentiates the trophic effects of nutrition i.e. anabolic responses are greater when exercise and nutrient-intake are combined to facilitate adaptation and repair. Thus, investigations into the effects of nutrition and the combination of nutrition and exercise are key to determining the [dys]-regulation of muscle protein metabolism in older age.

The anabolic effects of protein and EAA have been long defined. However, a significant body of recent work has shown that the EAA leucine (LEU) not only serves as substrate for MPS but also serves as an anabolic signal to the MPS machinery (27, 47); this is highlighted by the fact that provision of small quantities of LEU robustly stimulate MPS even in the
absence of other exogenous EAA (61). On this basis the “dose response” of MPS to EAA/protein is not apparently driven by AA quantity per se, but instead, LEU content. This leaves open the possibility that lower doses of EAA enriched with LEU may provide a robust and less satiating (or caloric) anabolic alternative e.g. to large boluses of high-quality protein, in older age.

The rationale for the present study was three-fold. Firstly, older women are grossly underrepresented in terms of studies on the regulation of muscle protein metabolism with ageing. Secondly, since there exists sexual dimorphism in muscle protein turnover in older age (50, 51), with markedly different feeding responses observed between older men and women (50), one cannot assume prior research findings in older men are germane to older women. Therefore, an improved understanding of the regulation of muscle protein metabolism in older women i.e. by nutrition and exercise combinations is needed. Thirdly, in the context of older women, who show an apparent increased level of anabolic resistance (vs. men (51), investigation into novel nutritional interventions, which maximize anabolic response to nutrition are sorely needed. Whilst increasing protein content of meals or providing large boluses of protein are known to maximally stimulate MPS (62) few older individuals may be able or willing to consume such a satiating dose of protein without impacting habitual nutrient intake, hence the need for lower caloric supplements. Finally, albumin is an abundant protein used as a marker of general nutritional status and low levels are associated with low protein intake (36), functional decline (49), reduced muscle mass (4), reduced strength (48) and numerous age-related diseases. Moreover, albumin synthesis is also affected by gender (females have lower synthesis rates than males) (56). Therefore, the albumin synthetic response to anabolic stimuli, such as nutrition, provides another window of insight into gender differences in protein metabolism--- and is linked to skeletal muscle
outcomes in ageing. Therefore, in this study we quantified the effects of two distinct nutritional interventions; i) low-dose leucine enriched (LEAA, 3 g EAA [40% LEU]) nutrition or, ii) a large whey protein (WP) bolus (20g) under rested conditions and in tandem with an acute bout of resistance exercise (which is well known to potentiate the anabolic response (16)) on MPS and plasma albumin synthesis in older women. It was hypothesized that LEAA would provide equivalent stimulation of MPS and albumin protein synthesis (APS), due principally to its leucine content, to WP in older women and the addition of RE would potentiate this response.

MATERIALS AND METHODS

Subject characteristics and ethics

Ethical Approval was obtained from the University of Nottingham Medical School Ethics Committee (United Kingdom), with all studies conducted in accordance with the Declaration of Helsinki and pre-registered at clinicaltrials.gov (registration no. NCT02053441). Sixteen older post-menopausal women (N=8 in each group) matched for age and BMI (66±3y, BMI 29±1 [means±SEM], see Table 1 for subject characteristics) were recruited locally via advertisement through the mail. Exclusion criteria included; impaired mobility, history of diabetes, cardiovascular, pulmonary, liver or kidney disorders, those on contraindicated medications (NSAIDS, acetaminophen, HRT) and those currently undergoing active cancer therapies. All volunteers were then screened by a physician (at least one week prior to the study day) by means of a medical questionnaire, physical examination and resting ECG, with exclusions for metabolic, respiratory, cardiovascular/vascular or claudication related (either symptomatic or on treatment) disorders or other symptoms of ill health. Subjects had normal blood chemistry, were normotensive (BP<140/90) and all subjects performed activities of daily living and recreation but did not routinely participate in any formal strenuous exercise.
regimes and were not on a weight loss diet. During the screening visit knee extension 1-RM was assessed, using the participants’ dominant/preferred leg, on a standard weighted gym knee extension machine (Technogym, Gambettola, Italy). In addition lean body mass was assessed via Dual X-ray Absorptiometry (DXA), and used to measure appendicular muscle mass and calculate skeletal muscle index (SMI) according to the following equation (38):

\[
\text{Skeletal Muscle Index (SMI)} = \left( \frac{\text{Total Body Skeletal Muscle Mass}}{\text{Total Body Mass}} \right) \times 100.
\]

All subjects gave their written, informed consent to participate after all procedures and risks were explained. Following screening subjects were randomly assigned, on the day of the acute study, to one of two groups receiving either; 1) Whey Protein (WP, N=8) or 2) Leucine enriched essential amino acids (LEAA, N=8, Table 2). Subjects were requested not to start any new diet or exercise program between screening and the study day, and to refrain from vigorous exercise for 48 h prior to the acute study visit and to arrive at 0800h on the morning of the study overnight fasted, from 2000h the night before.

Study Procedures

On the morning of the study (0800h), subjects had an 18-g cannula inserted into the antecubital vein of one arm for a primed (0.4 mg·kg\(^{-1}\)) constant infusion (0.6 mg·kg\(^{-1}·h\)^\(^{-1}\)) of L-[ring-\(^{13}\)C\(_6\)]-phenylalanine (Isotec, Sigma Aldrich) tracer, and a retrograde 14-g cannula inserted to sample arterialized blood from the dorsal capillary bed of the hand (using the ‘hot hand’ method). Biopsies were taken 1 h and 3 h after commencement of tracer infusion to permit assessment of basal (post-absorptive) MPS. Subjects then performed a bout of unilateral knee extension RE previously shown (34, 35) to maximally stimulate MPS (6×8 repetitions at 75% of their pre-determined 1-RM using the subjects’ dominant/preferred leg with 2 minute inter-set rest) and also elevate LBF in elderly men (44), before consuming their supplement. Each bout lasted 40-60 seconds. If the subject failed to complete 8 repetitions,
then an inter-set break was allowed before moving on to next set. This happened regularly
with sets 5 & 6. Immediately after the exercise, subjects consumed either 20 g of whey
protein (WP) or 3 g LEAA (“Amino L40”; Ajinomoto Inc.,) prepared in water (250 ml). The
AA composition of each supplement is given in Table 2. This unilateral study design meant
that the non-exercised leg was exposed to the effect of feeding alone (‘FED’), while the
exercised leg was exposed to the combination of feeding and exercise (‘FED-EX’).
Subsequent biopsies were then taken 2 and 4h after feeding to permit assessment of MPS
over and within the intervening periods. Blood samples and blood flow/vascular
measurements were collected as outlined in Figure 1. Muscle biopsies were collected from m.
vastus lateralis using the conchotome technique (14) after induction of local anesthesia via
infiltration of 5ml 1% lignocaine. Muscle was washed in ice-cold phosphate buffered saline
and visible fat and connective tissue removed before being snap frozen in liquid N2 and
stored at -80°C until analysis. After completion of the study, cannulae were removed and
subjects fed and monitored for a further 30 minutes before being provided transportation
home.

**Measurement of plasma insulin and AA concentrations**

Arterialized venous plasma insulin concentration was measured using a high-sensitivity
human insulin enzyme-linked immunosorbent (ELISA) assay (DRG Instruments GmbH,
Marburg, Germany). For AA analyses, equal volumes of arterialized plasma and 10%
sulfosalicyclic acid were mixed and cooled to 4°C for 30 min. Samples were centrifuged at
8000 g to pellet the precipitated protein and the supernatant fluid was passed through a 0.22
µm filter before analysis with a dedicated AA analyzer (Biochrom 30, Biochrom, Cambridge,
United Kingdom) using lithium buffers. All 20 AA concentrations were measured by
comparison to a standard AA mix with nor-leucine as an internal standard.
Measurement of leg blood flow (LBF) and muscle microvascular blood flow (MBF)

Leg blood flow (LBF) was measured using Doppler ultrasound, with a 9-3 mHz probe positioned over the origin of the common femoral artery. Flow was estimated as the product of vessel cross-sectional area and mean velocity over six cardiac cycles. Contrast enhanced ultrasound (CEUS) was used to measure microvascular blood flow (MBF) as previously described (45). Briefly, Sonovue™ microbubbles were infused via an antecubital fossa vein (Bracco, Milan, Italy). An iU22 ultrasound scanner (Phillips Healthcare, Reigate, United Kingdom) with 40 mm linear 9-3 mHz probes (Phillips L9-3) firmly secured on both anterior thighs (and measured individually for the FED and FED-EX legs) was used to register microbubble appearance within the quadriceps muscle at rest and in response to FED and FED-EX. Intermittent high mechanical index (MI) “flashes” disrupted micro-bubbles whilst continuous low MI recording measured the rate of micro-bubble replenishment. Infusion was at 2ml·min⁻¹ for 1 min and 1ml·min⁻¹ for 3 subsequent min. During the last 90 s of this measurement protocol, 3×30 s flash/ replenishment recordings were made. Off line region-of-interest analysis using Q-Lab software (Phillips Healthcare, Surrey UK) was used to measure the plateau (“A” value) linear acoustic intensity and also the rate constant (“β” value); the product of which is proportional to volume blood · volume tissue⁻¹ · second⁻¹ and thus skeletal muscle tissue microvascular blood flow (MBF) (45). In total, 2 measures were performed per participant: first 50-60 min before the exercise and second 50-60 min after the exercise (Figure 1).

Measurement of albumin protein synthesis (APS)

Plasma (200 µl) was precipitated with ice-cold 10% trichloroacetic acid (TCA). Following centrifugation (13,000 rpm, 4°C) to pellet plasma protein, the albumin from the pellet was
then extracted into ethanol to separate it from the non-alcohol soluble proteins. The ethanol extract was dried under nitrogen and solubilized in 0.3 M NaOH at 37°C for 60 min and then precipitated with 1 M Perchloric acid. Isolated albumin bound AA were released by acid hydrolysis with 0.1 M Hydrochloric acid and Dowex H+ ion exchange resin (50W-X8-200, Sigma Aldrich, Poole, UK) heated overnight at 110°C, before purification by ion exchange chromatography; AA were derivatized as their n-acetyl-N-propyl esters and incorporation of L-[ring-$^{13}$C$_6$] phenylalanine into albumin assayed by gas chromatography-combustion-isotope ratio mass spectrometry (Delta Plus XP, ThermoFisher Scientific, Hemel Hempstead, UK). Fractional synthesis rates of albumin were determined using a standard precursor-product model:

$$APS (%/h) = \frac{\Delta E_a}{E_p \cdot t} \times 100$$

Where ΔEa is the change in labelling of albumin phenylalanine between 2 blood samples, $E_p$ is the mean enrichment over time of the precursor (taken as arterialized plasma L-[ring-$^{13}$C$_6$] Phenylalanine labelling) and t is the time in hours between samples.

Measurement of MPS

Myofibrillar proteins were isolated, hydrolyzed and derivatized using our standard techniques (61). Briefly, ~25 mg of muscle biopsy tissue was homogenized in ice-cold homogenization buffer (50 mm Tris-HCl (pH 7.4), 50 mm NaF, 10 mm β-glycerophosphate disodium salt, 1 mm EDTA, 1 mm EGTA, 1 mm activated Na$_3$VO$_4$ (all Sigma-Aldrich, Poole, UK)) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10 μl·μg$^{-1}$ of tissue. Homogenates were rotated for 10 min and the supernatant collected by centrifugation at 13,000 × g for 5 min at 4°C. The resulting pellet was washed three times with homogenization buffer and 0.3 M NaOH was added in order to facilitate the separation of the soluble myofibrillar fraction from the insoluble collagen fraction by subsequent
centrifugation. The myofibrillar fraction was then removed and precipitated using 1 M perchloric acid (PCA) and pelleted by centrifugation. The myofibrillar pellet was then washed twice with 70% ethanol and the protein-bound AA were released by acid hydrolysis using 0.1 M HCl and 1 ml of Dowex ion-exchange resin (50W-X8-200) heated overnight at 110°C. The free AA were purified, derivatized and analyzed as for APS, the fractional synthesis rates (FSR) of the myofibrillar proteins was calculated using the same precursor-product approach:

\[
FSR \text{ (%/h)} = \left[ \frac{\Delta E_m}{E_p \cdot t} \right] \times 100
\]

Where \( \Delta E_m \) is the change in enrichment of bound L-[ring-\text{\textsuperscript{13}C_6}] phenylalanine in two sequential biopsies, \( t \) is the time interval between two biopsies in hours, and \( E_p \) is the mean free L-[ring-\text{\textsuperscript{13}C_6}] Phenylalanine enrichment in the intramuscular pool.

**Immunoblotting for AKT-mTORc1 signalling pathway activity (i.e. phosphorylation)**

Immunoblotting was performed as previously described (3) using the sarcoplasmic fraction collected during MPS preparation described above. Sarcoplasmic protein concentrations were determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE-US) and adjusted to 2 μl·μg⁻¹ in 3× Laemmli buffer. Each sample was loaded onto pre-cast 12% Bis-Tris Criterion XT gels (BioRad, Hemel Hempstead, UK) at 20 μg/ lane and separated electrophoretically at 200 V for 1 h. Proteins were wet transferred at 100 V, 45 min and subsequently blocked in 2.5% non-fat milk in 1× Tris buffered saline/Tween 20 (TBST). Membranes were incubated in primary antibodies (1:2000 dilution in 2.5% BSA in TBS-T) rocking overnight at 4°C, AKT\text{\textsuperscript{Ser473}}, p70S6K1\text{\textsuperscript{Thr389}}, 4EBP1\text{\textsuperscript{Ser65/70}}, eEF2\text{\textsuperscript{Thr56}} (New England Biolabs, Hertfordshire, UK). Membranes were subsequently washed 3×5 min in TBS-T, incubated in HRP-conjugated secondary antibody (New England Biolabs,
Hertfordshire, UK; 1:2000 in 2.5% BSA in TBS-T) at ambient temperature for 1 h, followed by 3×5 min washes in TBS-T. Membranes were exposed to Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA-US) for 5 min and bands quantified by Chemidoc XRS (BioRad, Hertfordshire, UK). All signals were within the linear range of detection; loading anomalies corrected to coomassie (59). Blotting data were captured using peak density analysis.

**Statistical analyses**

Data are presented as means ± SEM, except if indicated. Demographic, anthropometric and CEUS comparisons were analyzed within or between groups by unpaired or paired $t$-tests (all two-tailed), as appropriate. All other data sets were analyzed using two-way repeated measures ANOVA (feed type × time) with differences located via Holm Sidak post-tests using GraphPad Prism Version 5 with $P$ values calculated using GraphPad Quickcalcs (GraphPad Software Inc, San Diego, CA); $P$ values of <0.05 were considered significant.

**RESULTS**

**Plasma AA and insulin concentrations (Figure 2)**

Groups were matched for appendicular lean mass (LEAA: 17±1 vs. WP 17±1 kg) and skeletal muscle index (LEAA: 25±1 vs. WP 26±2) but received markedly different ($P<0.01$) quantities of EAA i.e. LEAA: 0.075±0.003 vs. WP 0.25±0.01 g/kg lean body mass. Plasma AA concentrations increased rapidly in response to LEAA and WP and peaked at ~40-60 min (Figure 2A-E; $P<0.05$). Plasma availability of AA, EAA, BCAA and LEU was significantly greater and more sustained in response to WP than LEAA (i.e. returning to baseline concentrations 60-80 min after LEAA vs. 115-160 min after WP; $P<0.001$). Plasma insulin
concentration increased over postabsorptive values (~5 mU·l\(^{-1}\), both groups) by 20 min both in response to LEAA and WP (to 14±3 vs. 20±3 mU·l\(^{-1}\), respectively; \(P<0.05\)). However, while plasma insulin returned to basal concentration 40 min after LEAA intake, (Figure 2F), WP led to insulin concentrations remaining elevated up to [and beyond] 60 min (18±3 mU·l\(^{-1}\)).

**Leg blood flow (LBF) and microvascular blood flow (MBF) (Figure 3)**

LBF was not different between groups at baseline (LEAA: 0.27±0.03 vs. WP: 0.34±0.04 l·min\(^{-1}\)) and unaltered in response to FED and FED-EX 60-80 min post feed, when AA concentrations peaked (\(P>0.05\) Figure 3 A, C). Similarly MBF, measured 60-80 min post ingestion of supplement, was not increased in the rested leg i.e. FED only, in either group, although MBF was numerically greater in the LEAA group, this was not consistent in all individuals and did not reach significance. However, MBF increased following FED-EX in both LEAA and WP groups (LEAA: +91±22% WP: +229±23%; \(P<0.05\), Figure 3 B, D).

**Effects of WP and LEAA on ASP and MPS (Figures 4, 5)**

Increases in plasma APS were observed in response to WP while only a trend existed for LEAA 0-2h (LEAA Basal to FED 0.19±0.02 to 0.23±0.03%·h\(^{-1}\); WP Basal to FED 0.17±0.01 to 0.26±0.04%·h\(^{-1}\); \(P<0.1\) and \(P<0.05\) respectively, Figure 4). APS remained elevated in both groups 0-4h (LEAA, FED increased to 0.25±0.02%·h\(^{-1}\); WP, FED increased to 0.29±0.02%·h\(^{-1}\); \(P<0.05\)). In response to WP and LEAA, MPS was increased 0-2h (LEAA BASAL to FED 0.071±0.004 to 0.089±0.008%·h\(^{-1}\); WP BASAL to FED 0.066±0.006 to 0.082±0.009%·h\(^{-1}\); both \(P<0.05\)); this anabolic effect abated rapidly, returning to postabsorptive rates (0-4h; \(P>0.05\), Figure 5A). In the exercised leg, MPS also increased 0-2h (LEAA BASAL to FED-EX 0.071±0.004 to 0.085±0.014%·h\(^{-1}\); WP BASAL to FED-EX
0.066±0.006 to 0.095±0.013%·h⁻¹; both \( P<0.05 \), but unlike with FED, MPS remained elevated over the entire 4h study period (LEAA BASAL to FED-EX 0.089±0.005%·h⁻¹; WP BASAL to FED-EX 0.093±0.007%·h⁻¹; both \( P<0.05 \), Figure 5B). Increases in MPS in response to both FED and FED-EX were indistinguishable between WP and LEAA groups (\( P>0.05 \)).

**Effects on muscle anabolic signaling (Figure 6)**

Muscle anabolic signals as determined by measuring the phosphorylation of protein kinase B (AKT), the mechanistic target of rapamycin complex 1 (mTORc1) translational initiation substrates, ribosomal protein S6 kinase (p70S6K1) and 4E-binding protein 1 (4EBP1) and the elongation factor eukaryotic initiation factor 2 (eEF2) (Figure 6 A-F) were not detectably increased in FED. Only in response to FED-EX could we detect significant increases (Figure 6D: +133±22%; \( P<0.01 \)) in phospho-p70S6K1 but only after WP and not LEAA and only 2h post feeding returning to baseline by 4h (Figure 7).

**DISCUSSION**

In the present study we investigated in skeletal muscle of older women, the effects of, and interaction between, the two major extrinsic drivers of muscle maintenance: nutrition and exercise. Furthermore, we have investigated the use of a novel low dose, low calorie, and leucine enriched EAA (LEAA “Amino L-40”) supplement as a nutritional intervention in this group. We found in older women: (i) that both WP and LEAA transiently (\( \leq 2h \)) stimulate MPS, (ii) that RE extends the ‘anabolic window’ \( \geq 4h \), after consumption of WP or LEAA, (iii) that APS is stimulated *similarly* after feeding WP and LEAA, and (iv) that over \( \sim 4h \), WP provides no additional trophic effects to LEAA either in response to FED or FED-EX.
Whether anabolic resistance to nutrition is an *intrinsic* feature of ageing remains contentious (24, 41, 54, 55). In our view, any doubt surrounding the existence of anabolic resistance may be primarily due to inter-study differences e.g. not accounting for differing habitual physical activity, physical status, and pattern of feeding e.g. bolus vs pulse/spread. Many studies measure MPS over periods longer (i.e. 4-6 hours) than the previously demonstrated anabolic window of 90-120 min (2, 5) thereby missing any detectable effect, when the rate is averaged. Nonetheless, whatever the standpoint, few would argue against optimizing muscle anabolic responses to nutrition is paramount in older age. We show here that increasing the quantity of EAA consumed does not necessarily augment MPS over and above ingesting ~1.2 g LEU (*plus* a small 1.8g mixed-EAA) in older women. Therefore, providing greater quantities of AA as meals or supplements to older women may not bolster MPS beyond e.g. LEAA. Nonetheless, since anabolic resistance may be *overcome* by providing larger doses of WP to older men (~40 g; (43)), it does remain entirely plausible that neither 20 g WP nor 3 g LEAA maximally stimulated MPS in skeletal muscles of older women; further studies are needed to define this. Yet despite this, these findings highlight that a small dose of LEAA (of low caloric value) provides adequate EAA substrate to promote robust enhancement of MPS, and thereby, perhaps could promote better maintenance of muscle mass in older women.

While the stimulation of MPS by EAA has been long defined, it is becoming increasingly clear that protein or AA dose-response studies belie the importance of certain *highly* anabolic EAA. For example, LEU is a major driver of MPS (61) to the extent that enrichment of ‘sub-optimal’ doses of protein or EAA with LEU robustly stimulates MPS (10) as does provision of just ~3 g of LEU to ~70 kg younger men, entirely in the absence of other exogenous EAA (61). Herein we extend these findings by demonstrating that LEAA stimulates MPS in older women with equal efficacy to a large (20 g) bolus of WP, despite the latter providing >3
times more EAA per kg of LBM; we anticipate LEU was instrumental in this response given
its unique potent anabolic properties (7, 11, 61). This is somewhat supported by the fact that
despite WP having greater EAA and total AA content, both WP and LEAA showed the same
peak in leucine concentration (figure 2E), and despite the WP maintaining significantly raised
leucine concentrations for longer than the LEAA, there was no additional stimulation of MPS
with WP. On this basis, research is needed to define the effects of LEU in the context of AA
requirements for muscle health in old age (7, 8, 15, 19, 46, 55, 58).

Physical activity, in the form of RE (9, 26, 29, 35, 42) or even maintaining habitual
movement (57) remains the best-known countermeasure against sarcopenia. Nonetheless,
ageing is associated with impaired anabolic responses to exercise meaning it is essential to
study how exercise and nutrition can be best combined to yield maximal MPS. For example,
increases in MPS in response to exercise (18, 34, 35) and the combination of exercise and
nutrition (18) are blunted in older men, as is muscle hypertrophy in both men (32) and
women (23). In the present study, we report that older women exhibit prolonged MPS
responses to FED-EX (vs. FED) and that responses are equal between LEAA and WP.
Therefore, intake of low doses of EAA enriched with LEU robustly stimulates MPS
following exercise in older women, similar to what has been shown in younger men at rest
(10) and after exercise (11). It is noteworthy in the latter study that WP bolus, but not LEAA
led to sustenance of the anabolic effects of the nutrient/ exercise combination. In contrast,
we found similarly prolonged anabolic responses to FED-EX after both LEAA and WP; this
is in agreement with what has been observed in older men with analogous leucine-enriched
AA supplementations post RE (13). We speculate the lack of difference in MPS between WP
and LEAA might reflect blunted hypertrophy in older vs. young individuals (23, 37, 60) i.e.
manifesting as diminished uptake of excess AA substrate into muscle proteins. This is also
substantiated by the lack of robust increases in anabolic signaling (as reported in older men (34) and women (50)) and leg/muscle blood flow, facets which may underlie the moderate anabolic response to raised AA availability after FED and FED-EX in older women.

When studying leg and muscle blood flow, we hypothesized that WP may elicit greater blood flow responses compared to LEAA i.e. due to the vasodilatory properties of arginine, and greater insulin responses associated with higher AA concentrations i.e. through promoting secretory effects of AA upon pancreatic β-cells. Nonetheless, while plasma insulin [and arginine] concentrations were expectedly higher in the WP group (figure 2F) there were no differences in LBF in line with work we recently completed in older men (39, 44); that is older people exhibit “vascular resistance” to nutritional intake. In contrast, there were significant increases to both WP and LEAA in MBF, but only in the FED-EX leg (Figure 3), suggesting this may have some role in the sustainment of MPS beyond 2 h in the FED-EX leg. Indeed despite the fact we have previously shown that pharmacological enhancement of leg and muscle blood flow above response to FED does not enhance anabolism (45), when superimposed onto a metabolic background of a prior bout of RE, our observed sustainment in MBF with FED-EX may facilitate sustaining anabolic responses beyond >2 h.

An important discussion point is to what extent, and under what conditions, such WP or LEAA supplements yield efficacy e.g. on muscle mass/function. Since protein supplements have significant effects to positively influence muscle hypertrophy in response to RET (28, 63), we speculate based on the information provided by the present study that low dose LEAA would be a similarly effective supplement (at least for older women) without the need for more satiating higher protein doses that may act as meal replacements, perhaps negatively impacting overall energy/protein intake. Moreover, most studies focus on the effects of
supplements on muscle gains; however given the incipient nature of sarcopenia, longer-term studies are needed to determine the efficacy of supplements in offsetting losses in muscle mass/ function. Nonetheless, those studies, which have been conducted, have indeed shown promise (30). Additionally, while caution should be applied in terms of the extrapolation of one-off metabolic studies to the chronic scenario, we contend these acute metabolic readouts of protein anabolism form a solid platform for identifying candidate interventions for testing in longer-term supplement studies.

Further to the muscle specific effects observed in the present study, the influence of nutrition on APS is important particularly with age due to the known associations between low serum albumin, health, morbidity and mortality (12). Indeed, albumin concentrations decrease with age (31), and females have slower rates of APS (56). In this context, we have demonstrated that LEAA led to significant stimulation of APS equivalent to a large dose of WP, which is sustained for up to 4 h post feed. This suggests that the LEAA supplement (and also the larger WP bolus) may also have implications for sustaining APS in older women. Given the known links to muscle and general health this could transpire to be an important observation.

Potential study limitations warrant comment. Firstly, we are unable to define the specific importance of LEU in mediating the efficacy of LEAA. Previous work by one of the current authors using I.V flooding dose techniques revealed anabolic effects of other EAA in our LEAA-feed (i.e. valine, phenylalanine) in man (52). Nonetheless, given potent mTORc1 signaling actions attributed to LEU (3, 21, 25) it is speculated LEU was central. Secondly, we neither measured muscle protein breakdown nor whole-body protein turnover i.e. age-related differences in splanchnic metabolism (40) and insulin handling may also impact muscle and whole-body responses to feeding/exercise, particularly due to the anti-proteolytic effect of
insulin on MPB (33). Also, although 20 g of WP led to a significant stimulation of MPS,
larger amounts of protein (>40 g) have been shown to assist in potentiating MPS responses in
older men (63), therefore the MPS response to WP in the current group of older women may
be suboptimal; that said: our aim was to assess the efficacy of a low dose, lower satiating
EAA nutritional supplement rather than testing the absolute dose of WP for maximal MPS
stimulation. Finally, with regards to anabolic signaling (responses in which were negligible),
we may have missed the “peak” best reflecting MPS responses; nonetheless, we designed the
studies to acquire biopsies at times most appropriate to our primary outcome measure of
MPS. Moreover given “peak signaling” event(s) may differ between distinct feeding
regimens such as those applied herein (20g WP or 3 g EAA) in addition to dissociations
between anabolic signaling and MPS (2, 22), this is relatively uncontrollable. To conclude,
our findings show low dose leucine enriched EAA-supplements have potential alone, or
combined with exercise, as strategies for older women to enhance muscle maintenance.
ACKNOWLEDGMENTS We would like to thank Margaret Baker and Amanda Gates for expert technical assistance. This work was supported, by funding from Ajinomoto Co., Inc. The authors declare no other conflicts of interest.

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### TABLE 1: Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>LEAA (3 g)</th>
<th>WP (20 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>66±1</td>
<td>66±1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29±1</td>
<td>29±2</td>
</tr>
<tr>
<td>Lean Body Mass (LBM; kg)</td>
<td>41±2</td>
<td>40±2</td>
</tr>
<tr>
<td>EAA dose (g/kg LBM)</td>
<td>0.075±0.003</td>
<td>0.25±0.01***</td>
</tr>
<tr>
<td>Appendicular Lean Mass (kg)</td>
<td>17±1</td>
<td>17±1</td>
</tr>
<tr>
<td>Skeletal Muscle Index (SMI)</td>
<td>25±1</td>
<td>26±2</td>
</tr>
</tbody>
</table>

*** P<0.001 vs. LEAA

### TABLE 2: Essential amino acid composition of both types of feed

<table>
<thead>
<tr>
<th></th>
<th>LEAA (3 g)</th>
<th>WP (20 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucine</td>
<td>1.2g</td>
<td>2g</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.32g</td>
<td>1.4g</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.33g</td>
<td>1.2g</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.28g</td>
<td>1.4g</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.5g</td>
<td>1.8g</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.1g</td>
<td>0.4g</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.05g</td>
<td>0.4g</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.2g</td>
<td>0.6g</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.02g</td>
<td>0.4g</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

**Figure 1** Study protocol: effects of LEAA and WP at rest and after RE in older women.

**Figure 2** Time-course effects of 3 g LEAA or 20 g WP on plasma amino acids (AA) and insulin concentrations A) sum AA, B) sum essential AA (EAA), C) sum non-essential AA (NEAA), D) sum Branched chain AA (BCAA), E) Leucine (LEU), F) insulin- a) greater than basal (P<0.05); b) greater than other group at time-point (P<0.05). Main effects of feeding, P<0.001; effects of feed-type, P<0.001; interaction P<0.001.

**Figure 3** The effect of 3 g LEAA or 20 g WP in skeletal muscle of older women on leg blood flow (LBF) and muscle blood flow (MBF) responses to A, B) FED or C, D) FED-EX. *P<0.05 vs. basal. A, Main effects of feeding, P<0.01; effects of feed-type, P>0.05; interaction P>0.05; B, Main effects of feeding, P<0.05; effects of feed-type, P>0.05; interaction P>0.05.

**Figure 4** The effect of 3 g LEAA or 20 g WP on APS in old women. *P<0.05, # P<0.1 vs. basal. Main effects of feeding, P<0.001; effects of feed-type, P>0.05; interaction P<0.05.

**Figure 5** The effect of 3 g LEAA or 20 g WP in skeletal muscle of older women on muscle protein synthesis (MPS) responses to A) FED and B) FED-EX. *P<0.05, vs. basal; **P<0.001 vs. men. A, Main effects of feeding, P<0.01; effects of feed-type, P>0.05; Interaction P>0.05; B, Main effects of feeding, P<0.05; effects of feed-type, P>0.05; Interaction P>0.05.
Figure 6 The effect of 3 g LEAA or 20 g WP in skeletal muscle of older women on muscle signaling responses to A) FED, B) FED-EX. *$P<0.05$, vs. basal. Main effects of feeding, $P<0.001$; effects of feed-type, $P>0.05$; Interaction $P>0.05$.

Figure 7 The impact of 3 g LEAA or 20 g WP in skeletal muscle of older women on muscle signaling responses with representative blots for signaling proteins.
FIGURE 1

Primed (0.3 mg.kg⁻¹), constant (0.6 mg.kg⁻¹) infusion of ¹³C₆ Phenylalanine

Postabsorptive

Unilateral Knee-extension:
6 x 8 at 75% of 1RM
Whey or Leu-EAA (+ 6% ¹³C₆ Phe)

Arterialised Bloods
Leg Blood Flow (Doppler)
Muscle Nutritive Flow (CEUS)
Muscle Biopsies - Rest Leg
Exercise Leg
FIGURE 2

A

Total AA (µM)

LEAA (3 g)

WP (20 g)

Time (min)

B

EAA (µM)

LEAA (3 g)

WP (20 g)

Time (min)

C

NEAA (µM)

LEAA (3 g)

WP (20 g)

Time (min)

D

BCAA (µM)

LEAA (3 g)

Whey (20 g)

Time (min)

E

Leucine (µM)

LEAA (3 g)

Whey (20 g)

Time (min)

F

Insulin (mU/L)

LEAA (3 g)

Whey (20 g)

Time (min)
FIGURE 5

A

Mycobrillar FSR (% h⁻¹)

LEAA (3 g)
WP (20 g)

B

Mycobrillar FSR (% h⁻¹)

LEAA (3 g) + RE
WP (20 g) + RE

* indicates significant difference.
FIGURE 6

A

B

C

D

E

F

G

H

- LEAA (3g)
- Whey (20g)

- LEAA (3g) + RE
- Whey (20g) + RE

- LEAA (3g)
- Whey (20g)

- LEAA (3g) + RE
- Whey (20g) + RE

- LEAA (3g)
- Whey (20g)

- LEAA (3g) + RE
- Whey (20g) + RE

- LEAA (3g)
- Whey (20g)

- LEAA (3g) + RE
- Whey (20g) + RE

- LEAA (3g)
- Whey (20g)

- LEAA (3g) + RE
- Whey (20g) + RE

Time (h)

Time (h)

Time (h)

Time (h)

Time (h)

Time (h)
FIGURE 7

- **p-Akt<sup>Thr<sub>473</sub></sup>**
  - LEAA
  - Coomassie Whey
  - Coomassie

- **p-p70S6K<sup>Thr<sub>389</sub></sup>**
  - LEAA
  - Coomassie Whey
  - Coomassie

- **p-4EBP1<sup>Thr<sub>37/46</sub></sup>**
  - LEAA
  - Coomassie Whey
  - Coomassie

- **p-eEF2<sup>Thr<sub>56</sub></sup>**
  - LEAA
  - Coomassie Whey
  - Coomassie