Even effect of milk protein and carbohydrate intake but no further effect of heavy resistance exercise on myofibrillar protein synthesis in older men

Reitelseder, Søren; Dideriksen, Kasper; Agergaard, Jakob; Malmgaard-Clausen, Nikolaj M; Bechshoeft, Rasmus L; Petersen, Rasmus K; Serena, Anja; Mikkelsen, Ulla R; Holm, Lars

DOI:
10.1007/s00394-018-1641-1

Even effect of milk protein and carbohydrate intake but no further effect of heavy resistance exercise on myofibrillar protein synthesis in older men

Reitelseder, Søren; Dideriksen, Kasper; Agergaard, Jakob; Malmgaard-Clausen, Nikolaj M; Bechshoeft, Rasmus L; Petersen, Rasmus K; Serena, Anja; Mikkelsen, Ulla R; Holm, Lars

DOI:
10.1007/s00394-018-1641-1

Document Version
Peer reviewed version

Citation for published version (Harvard):
Reitelseder, S, Dideriksen, K, Agergaard, J, Malmgaard-Clausen, NM, Bechshoeft, RL, Petersen, RK, Serena, A, Mikkelsen, UR & Holm, L 2018, 'Even effect of milk protein and carbohydrate intake but no further effect of heavy resistance exercise on myofibrillar protein synthesis in older men' European Journal of Nutrition. DOI: 10.1007/s00394-018-1641-1

Even effect of milk protein and carbohydrate intake but no further effect of heavy resistance exercise on myofibrillar protein synthesis in older men

Søren Reitelseder, [1]✉

Phone +45 38635398
Email soeren.reitelseder@regionh.dk

Kasper Dideriksen, [1]

Jakob Agergaard, [1]

Nikolaj M. Malmgaard-Clausen, [1]

Rasmus L. Bechshoeft, [1]

Rasmus K. Petersen, [3]

Anja Serena, [4]

Ulla R. Mikkelsen, [5]

Lars Holm, [1,2]

[1] Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery M, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Bispebjerg Bakke 23, building 8, 1st floor, 2400 Copenhagen NV, Denmark

[2] School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, UK

[3] Department of Biology, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

[4] Arla Foods amba, Viby J, Denmark

[5] Arla Foods Ingredients Group P/S, Viby J, Denmark

Received: 14 August 2017 / Accepted: 17 February 2018

Abstract

Purpose

The responsiveness of older individuals' skeletal muscle to anabolic strategies may be impaired. However, direct comparisons within the same experimental setting are sparse. The aim of this study was to assess the resting and post-resistance exercise muscle protein synthesis rates in response to two types of milk protein and carbohydrate using a unilateral exercise leg model.

AQ1

Methods

Twenty-seven older (69 ± 1 year, mean ± SE) men were randomly assigned one of three groups: Whey hydrolysate (WH), caseinate (CAS), or carbohydrate (CHO). By applying stable isotope tracer techniques (L-[^{15}N]phenylalanine), the fasted-rested (basal) myofibrillar fractional synthesis rate (FSR) was measured. Hereafter, FSR was measured in the postprandial phase (0.45 g nutrient/kg LBM) in both legs, one rested (fed-rest) and one exercised (10×8 reps at 70% 1RM; fed-exercise). In addition, the activity of p70S6K and venous plasma insulin, phenylalanine, and leucine concentrations were measured.

Results

Insulin, phenylalanine, and leucine concentrations differed markedly after intake of the different study drinks. The basal FSR in WH, CAS, and CHO were 0.027 ± 0.003, 0.030 ± 0.003, and 0.030 ± 0.004%/h, the

fed-rested FSR were 0.043 ± 0.004, 0.045 ± 0.003, and 0.035 ± 0.004%/h, and the fed-exercised FSR were 0.041 ± 0.004, 0.043 ± 0.004, and 0.034 ± 0.004%/h, respectively. No significant differences were observed at any state between the groups. Fed-rested- and fed-exercised FSR were higher than basal ($P < 0.001$). 3 h after exercise and feeding, no significant group differences were detected in the activity of p70S6K. **AQ2**

Conclusions

Milk protein and carbohydrate supplementation stimulate myofibrillar protein synthesis in older men, with no further effect of heavy resistance exercise within 0–3 h post exercise.

Keywords

Whey hydrolysate
Caseinate
Anabolic resistance
Muscle protein
Sarcopenia

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00394-018-1641-1) contains supplementary material, which is available to authorized users.

Introduction

It is generally accepted that older individuals display a reduced responsiveness to classic anabolic stimuli such as protein intake [1, 2, 3] and exercise [4, 5, 6]. These observations have been termed ‘anabolic resistance’ and are associated with the aging process [7, 8] and partly responsible for the sarcopenic process [9, 10]. Still, protein intake and exercise are repeatedly suggested to be promising strategies to counteract the sarcopenic development [11]. Therefore, investigations to compare

responses and to reveal the actual potency of these classic stimuli remain to be a target of interest.

Protein quality determined by amino acid composition and digestibility, is known to be a major regulating factor on whole-body protein turnover in the rested postprandial state [12, 13]. However, while the essential amino acids are primarily responsible for stimulating muscle protein synthesis (MPS) [14], the impact of protein digestibility, and hence postprandial peak and pattern of hyperaminoacidemia are more unclear. The comparisons of the two milk proteins, whey and casein, are examples of natural proteins of high quality with distinct digestion and amino acid absorption kinetics where whey is digested ‘fast’ and casein is digested ‘slow’ [13]. In older, the impact of these two milk proteins are not clearly [15, 16, 17, 18, 19] supported also by results from a study where amino acids were provided in two different ingestion patterns to mimic different absorption patterns [20]. Here, 15 g of essential amino acids (corresponding to approximately 30 g of a good quality protein, like milk proteins) were provided either in one bolus or as 4 × 3.75 g at 45-min intervals resulting in very distinct insulin and amino acid concentrations during the total 4-h period. Despite marked differences in the p70S6K-Thr389 phosphorylation, the MPS rates were evenly enhanced over the entire period, though with a tendency to a more immediate and prolonged stimulation by the ‘slow delivery’ pattern [20]. Therefore, which characteristic of protein-feed derived amino acid delivery profile that exerts the most anabolic stimulus in aging muscle is still unclear: is it the fast availability and high peak concentration or is it the more moderate, though prolonged, delivery?

Muscle contractions are known to stimulate MPS independently of amino acid availability [5, 6] and some indications exist of an additive effect with hyperaminoacidemia [15, 21, 22, 23]. Therefore, resistance exercise seems to enhance the stimulatory effect of hyperaminoacidemia in older muscle, while the interaction with protein digestibility is less investigated [15, 23].

The aim of this study is to compare the immediate 3-h myofibrillar protein synthesis rate responses to a single, isocaloric bolus drink of fast whey hydrolysate, slow calcium caseinate, or carbohydrate at rest and following

a single, unilateral bout of heavy resistance exercise as compared to an overnight fasted and rested basal state in healthy, older men. The carbohydrate group is chosen as an isocaloric control group providing energy and a marked insulin response, but without amino acids. The hypothesis is that the MPS response following nutrient intake will be gradually higher as compared to the basal state in the rest leg and in the heavy resistance exercised leg. Whey hydrolysate is expected to be superior to caseinate and carbohydrate, and caseinate is expected to be superior to carbohydrate.

Methods

Subjects and groups

Twenty-seven older men were recruited and randomly assigned to one of three groups. All participants were initially screened and considered eligible for the experiment by standard laboratory blood tests and by a health, exercise and nutrition questionnaire. The recruitment was done together with another part of the project, which had an inclusion criteria of low-grade systemic inflammation determined as plasma C-reactive protein (CRP) above 2 mg/L [22]. Participants with CRP values below 2 mg/L were included and randomized to this non-low-grade inflamed part of the project or to the control group in the low-grade inflamed part [22]. Common inclusion criteria were: age > 60 years, body mass index > 18.5 kg/m^2, Western diet (protein content > 0.8 g/kg/day), moderately active but with no regular participation in heavy resistance exercise within last 6 months, and no metabolic disorders including diabetes. Each participant was given written and oral information about the study design, purpose, and possible risks before submitting written consent to participate. The project adhered to the Helsinki Declaration and was approved by the Ethics Committee of the Capital Region of Denmark (H-4-2011-028). The experiments in all three groups were identical except for the protein or carbohydrate supplementations, which were whey hydrolysate (WH), calcium caseinate (CAS), or carbohydrate as maltodextrin (CHO). Subject characteristics are listed in Table 1.

AQ3

Table 1

Subject characteristics

	Whey hydrolysate	Caseinate (*N*	Carbohydrate (*N*
Values are means ± SE. Blood glucose data were analysed by a one factor non-repeated measure ANOVA. No significant differences were found between any of the groups			

Pre-experiment tests

All participants were scanned by dual-energy X-ray absorbance (DXA, Lunar DPX-IQ; GE Healthcare, Chalfont St. Giles, UK) for determination of body soft tissue composition, which included determination of lean body mass (LBM). The exercise leg was randomly chosen so that equally many performed the exercise with the dominant leg as with the non-dominant leg. The unilateral one repetition maximum (1RM) leg-extensor strength was determined in the leg extension machine (Technogym, Gambettola, Italy). This was done after a 5–10 min warm-up on a cycle ergometer, familiarization to the leg extension machine, and by gradually increasing the weight until the heaviest load that could be lifted once was reached.

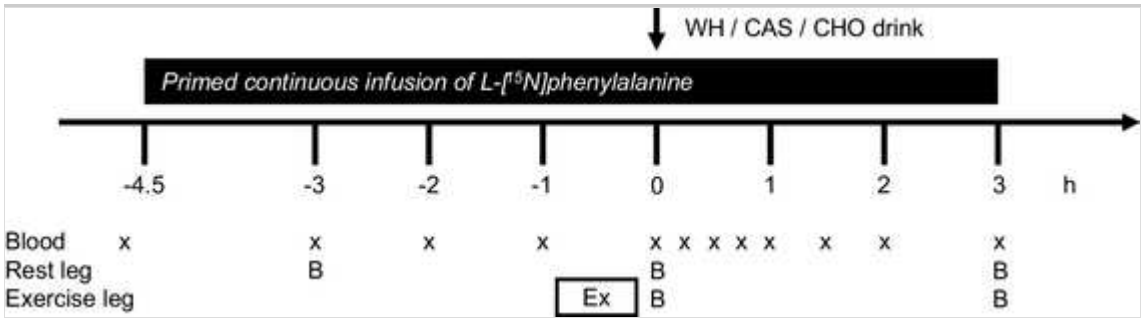
Experimental protocol

All participants were asked to refrain from any strenuous physical activity 3 days prior to the trial day, and, furthermore, to refrain from alcohol and caffeine intake 1 day prior to the trial day. Subjects fasted from 10 p.m. the day before and arrived by car to the laboratory. The experiments started at 7 or 8 a.m. Participants were placed in beds and had an antecubital catheter inserted into a vein in both arms. One catheter was used for infusion of L-[^{15}N]phenylalanine (99% enriched, Cambridge Isotopes Laboratories, Tewksbury, MA) and the other for blood sampling. After collection of a background blood sample, the primed (8 μmol/kg LBM), continuous (8 μmol/kgLBM/h) infusion of L-[^{15}N]phenylalanine was initiated and maintained for 7.5 h. The primed, continuous infusion of the phenylalanine tracer was calculated to produce a tracer-to-tracee ratio (TTR) of around 10% at isotopic steady state in the muscle-free pool. Thereafter, blood samples were collected at − 3, − 2, − 1, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, and 3 h.

M. vastus lateralis biopsies were obtained in the rested leg at − 3, 0, and 3 h, and at 0 and 3 h in the exercise leg. The biopsies were taken in the mid-part of m. vastus lateralis with random proximal–distal location and approximately 4 cm apart with 4-mm Bergström needles (Stille, Stockholm, Sweden) with manual suction. Muscle biopsy samples were quickly washed free of blood on ice-cold saline swabs, cut free of visible connective tissue, frozen in liquid nitrogen, and stored at − 80 °C. 20 mg, muscle tissue was allocated to protein-bound phenylalanine enrichment measurements, another 10 mg was allocated to muscle-free phenylalanine enrichment measurements, and the remainder of the muscle tissue was allocated to the Western Blot analyses. The experimental protocol is illustrated in Fig. 1.

Fig. 1

The experiment protocol. All participants arrived at the laboratory in the morning after an overnight fast from 10 p.m.. Venous blood samples were collected prior to infusion of L-[^{15}N]phenylalanine and during the protocol as marked by x. Muscle biopsies were obtained in each leg at time points marked by B. A single bout of unilateral heavy resistance exercise (leg extension) was conducted from − 1 h and consisted of ten sets of eight repetitions at a predetermined load corresponding to 70% of 1 repetition maximum with duration of approximately 45 min. The study drink was given at time point 0 h, and consisted of milk proteins (whey hydrolysate, WH or caseinate, CAS) or carbohydrate (CHO) in amounts adjusted to 0.45 g per kilo soft tissue lean body mass of each participant. The specific trial proteins were PEPTIGEN® IF-3090 (WH) and MIPRODAN® 40 (CAS), and the CHO was maltodextrin. This study protocol provides a 3-h basal state, a 3-h fed-rest state, and a 3-h fed-exercise state for measurements of myofibrillar protein fractional synthesis rates



Unilateral exercise protocol

A single, unilateral heavy resistance exercise bout was started at − 1 h according to the study protocol and took approximately 45 min. The exercise was leg extension in the leg extension machine (Technogym). The exercise protocol consisted of ten sets with eight repetitions at a predetermined load of 70% of 1RM with 2 min of rest between each set. Each repetition was performed with approximately a 1 s concentric and a 2 s eccentric phase with no muscle rest between the eight repetitions.

Protein and carbohydrate supplementations

The milk protein and carbohydrate supplementation drinks were ingested at 0 h after the exercise bout was completed. The supplements were isoenergetic and contained 0.45 g of milk protein or carbohydrate per kg lean body mass (LBM). The whey hydrolysate was PEPTIGEN® IF-3090 (Arla Foods Ingredients P/S, Viby J., Denmark), the calcium caseinate was MIPRODAN® 40 (Arla Foods amba, Viby J., Denmark), and the carbohydrate was in the form of maltodextrin. The amino acid compositions of the two different types of milk protein are listed in Table 2. All supplements were dissolved in 300 mL of water and ingested within 5 min. Free L-[^{15}N]phenylalanine was added to the whey hydrolysate drink in WH trials to minimize tracer dilution from the rapid endogenous phenylalanine uptake following intake, and in CAS trials the infusion rate of L-[^{15}N]phenylalanine was increased from 8 to 12 µmol/kg LBM/h in the 3-h post caseinate intake period also to minimize tracer dilution from the more moderate but prolonged endogenous phenylalanine uptake.

Table 2

Milk protein amino acid compositions

Typical amino acid	Whey hydrolysate	Caseinate
Values are provided by Arla Foods Ingredients P/S and Arla Foods amba		
BCAA branched-chain amino acid; *TAA* total amino acid		

Venous plasma analyses

Insulin concentration was analysed by commercially available enzyme-linked immunosorbent assay kits (cat. no. K6219; Dako Denmark A/S, Glostrup, Denmark) with an intra-assay CV ≤ 7.5% and an inter-assay CV < 10%.

Venous phenylalanine enrichment and phenylalanine and leucine concentration were analysed with the use of 200 μL plasma-added known amounts of [ring-$^{13}C_6$]phenylalanine and [U-$^{13}C_6$]leucine as internal standards (Cambridge Isotope Laboratories). Derivatives were separated by gas chromatography and measured on a triple-stage quadrupole-mass spectrometer (GC-MS/MS; TSQ Quantum, Thermo Scientific, San Jose, CA). Details of the analysis protocol have been described elsewhere [24, 25].

Muscle tissue analyses

10 mg of muscle tissue was used for measurement of muscle-free [^{15}N]phenylalanine enrichment. The muscle tissue was homogenized in 2% perchloric acid and centrifuged at 1615 g for 10 min where after the supernatant was collected. This homogenization procedure was repeated twice on the pellet. The muscle-free amino acids in the supernatant were purified on cation exchange resin columns (AG 50W-X8 resin; Bio-Rad Laboratories, Hercules, CA) and derivatized and analysed by GC-MS/MS as described above for the venous plasma [^{15}N]phenylalanine enrichment measurements.

20 mg of muscle tissue was used for measurement of [^{15}N]phenylalanine incorporation in myofibrillar proteins. The muscle tissue was

homogenized, myofibrillar proteins isolated and hydrolysed, liberated amino acids purified, and *N*-acetyl *n*-propyl (NAP) derivatized as previously described [26]. Finally, [^{15}N]phenylalanine myofibrillar protein-bound enrichment was measured by gas chromatography–combustion–isotope ratio mass spectrometer (GC-C-IRMS; Delta Plus XL; Thermo Finnigan, Bremen, Germany) with the use of a CP-Sil 19 CB capillary column (60 m × ID 0.25 mm, coating 1.5 µm; ChromPack; Varian, Palo Alto, CA). Carbon monoxide (CO) was trapped in the column between analyte combustion and the IRMS inlet with the use of liquid nitrogen to avoid CO interference on mass 28 since CO and $^{14}N_2$ both have mass 28.

The phosphorylated p70S6K (Thr389)/total p70S6K ratio was measured by Western Blot technique. Muscle tissue samples were homogenized under liquid nitrogen and solubilized in SDS sample buffer (2.5% SDS, 10% glycerol, 50 mM of Tris–HCl (pH 6.8), 10 mM of dithioerythritol, 10 mM of β-glycerophosphate, 10 mM of NaF, 0.1 mM of sodium orthovanadate, and SIGMAFASTTM protease inhibitor cocktail (Sigma–Aldrich Denmark, Copenhagen, Denmark)). A total of 50 µg of protein per lane was separated on Criterion 10% gels (Bio-Rad Laboratories) and blotted to PVDF membranes. Equal loading and transfer was verified by amidoblack staining. Membranes were blocked with 5% dry milk in tris-buffered saline (TBS) with 0.1% Tween 20. Primary antibodies (Phospho-p70S6K (Thr389, #9234) and p70S6K (#9202) from Cell Signaling Technology, Danvers, MA) and HRP-coupled secondary antibody (Dako) were applied in 5% dry milk in TBS. Membrane-bound antibody was visualized using the Chemiluminescence Detection Kit for HRP (Biological Industries, Cromwell, CT) and the Fusion FX5 Chemiluminescence imaging system (Vilber Lourmat, Eberhardzell, Germany). Obtained images were quantified using the associated Bio-1D software (Vilber Lourmat). Each gel included samples belonging to all experimental groups, and to normalize for differences between incubation efficiency and exposure time among membranes, the intensity of individual bands representing phosphorylated or total p70S6K were set relative to the total sum of band intensities representing phosphorylated or total p70S6K on the membrane. Finally, the intensity of a sample from the control group was arbitrarily set to 1 and the remaining samples expressed accordingly. Due to the need of

sufficient muscle tissue material, only a subset of participants could be analysed for p70S6K (WH, $N = 4$; CAS, $N = 5$; CHO, $N = 4$) as some muscle biopsies were too small for both tracer and Western Blot analyses.

Calculations

Myofibrillar protein FSR was calculated on the basis of [^{15}N]phenylalanine incorporation as FSR (%/h) = $((E_{p2} - E_{p1})/(E_{precursor} \times (T_2 - T_1))) \times 100$, where $E_{p2} - E_{p1}$ is the myofibrillar protein-bound phenylalanine enrichment difference between two biopsies, $E_{precursor}$ is the weighted mean venous plasma, [^{15}N]phenylalanine enrichment in the specific FSR period, and T_2–T_1 is the time in h between two biopsies [27]. In the exercise leg FSR calculations, the precursor weighted mean venous plasma phenylalanine enrichment was adjusted by the muscle-free phenylalanine difference ratio between the rest and exercise leg.

Statistics

Subject characteristics and delta FSR values were compared with one-factor non-repeated ANOVA. All other measurements were compared by two-factor (group-factor: non-repeated, time-factor: repeated) ANOVA. In case of main significant effects, Student–Newman–Keuls post hoc tests were performed. Western blot data on p70S6K activation were log-transformed before statistical analyses to avoid skewed data, and results are presented as geometric means ± back-transformed SE. All other values are means ± SE, and the specific P values are displayed in the figure legends. Statistical significance was set at $P < 0.05$, and all statistical analyses were carried out using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA).

Results

Subject characteristics

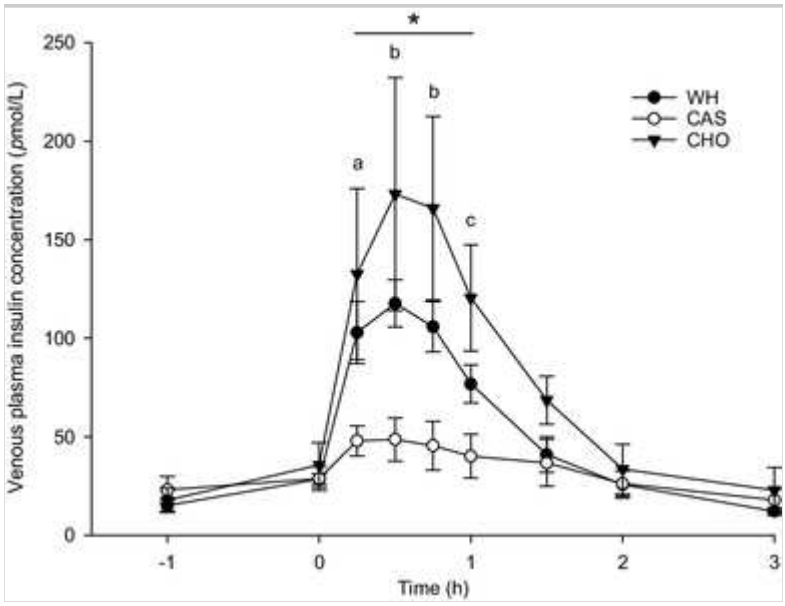
Subject characteristics are displayed in Table 1. There were no differences between the three groups in any parameters, which include age, body stature, body composition, muscle strength, and blood cholesterol and CRP levels.

Venous plasma insulin concentration

Venous plasma insulin concentrations showed a significant interaction after the study drink intake and were significantly increased from 15 min to 1 h in CHO and WH, but not in CAS (Fig. 2). At time point 15 min the insulin concentration in CHO and WH was significantly higher as compared to CAS. At time point 30 and 45 min the insulin concentration was significantly different between all three groups, so that CHO had higher concentration as compared to WH and CAS, and also WH had higher concentration as compared to CAS. At time point 1 h the insulin concentration was significantly higher in CHO only as compared to CAS.

Fig. 2

Mean (± SE) venous plasma insulin concentrations at fasted and rested state (− 1 h), fasted but after exercise (0 h), and following intake of whey hydrolysate (WH), caseinate (CAS), or carbohydrate (CHO) drinks. Data were analysed with a two-factor ANOVA (group non-repeated, time repeated) and the Student–Newman–Keuls post tests. Interaction was observed ($P < 0.001$), and post tests revealed: *within WH and CHO 15 min to 1 h higher than all other time points ($P < 0.05$); a—WH and CHO higher than CAS ($P < 0.05$); b—all groups are different ($P < 0.05$); c—CHO higher than CAS ($P < 0.05$)

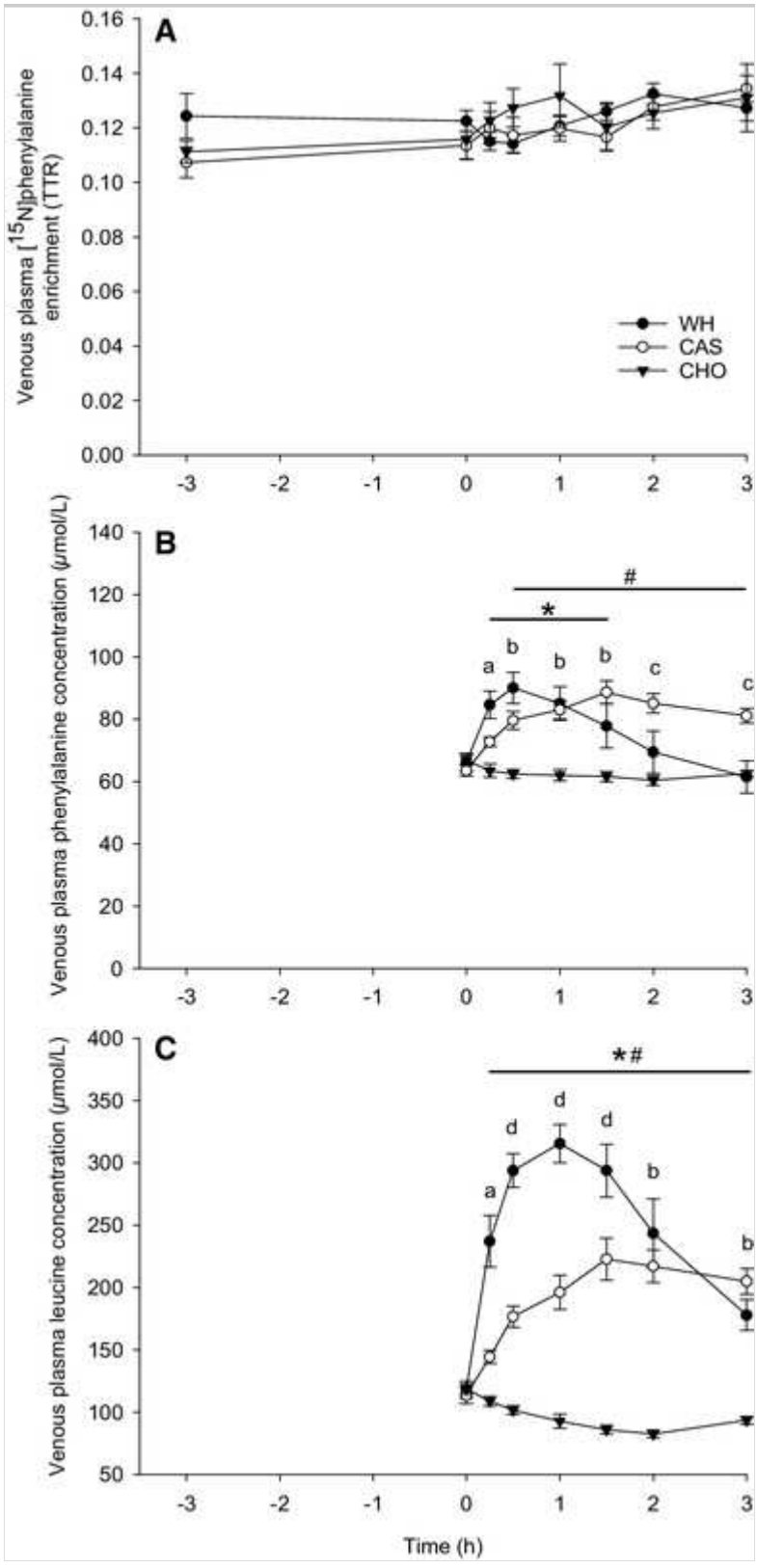


Venous plasma phenylalanine enrichment and phenylalanine and leucine concentration

Venous plasma [^{15}N]phenylalanine enrichment was in steady state in the tracer incorporation period for FSR measurements in all three groups (Fig. 3a). Venous plasma phenylalanine and leucine concentration showed a significant interaction after intake of the study drinks and were significantly increased in WH and CAS (Fig. 3b-c). For phenylalanine concentration, the significant increase was transient from 15 min to 1.5 h in WH and prolonged from 30 min to 3 h in CAS, WH was significantly higher than CAS at 15 min, and CAS was significantly higher than WH at 2 and 3 h. For leucine concentration, the significant increase was evident in both WH and CAS from 15 min to 3 h, and WH was significantly higher than CAS from 15 min to 1.5 h. No significant amino acid concentration increase was observed after intake in CHO.

Fig. 3

Mean (± SE) venous plasma [^{15}N]phenylalanine enrichment (**a**), phenylalanine concentration (**b**), and leucine concentration (**c**) at fasted and rested state (− 3 h), fasted but after exercise (0 h), and following intake of whey hydrolysate (WH), caseinate (CAS), or carbohydrate (CHO) drinks. Data were analysed with a two-factor ANOVA (group non-repeated, time repeated) and the Student–Newman–Keuls post tests. Phenylalanine enrichments showed no differences. Phenylalanine and leucine concentrations showed interaction ($P < 0.001$), and post tests revealed: *time points within WH higher than 0 h, and $^{\#}$time points within CAS higher than 0 h (both $P < 0.05$); **a** WH higher than CAS and CHO, **b** WH and CAS higher than CHO, **c** CAS higher than WH and CHO, and **d** all groups are different (all $P < 0.05$)



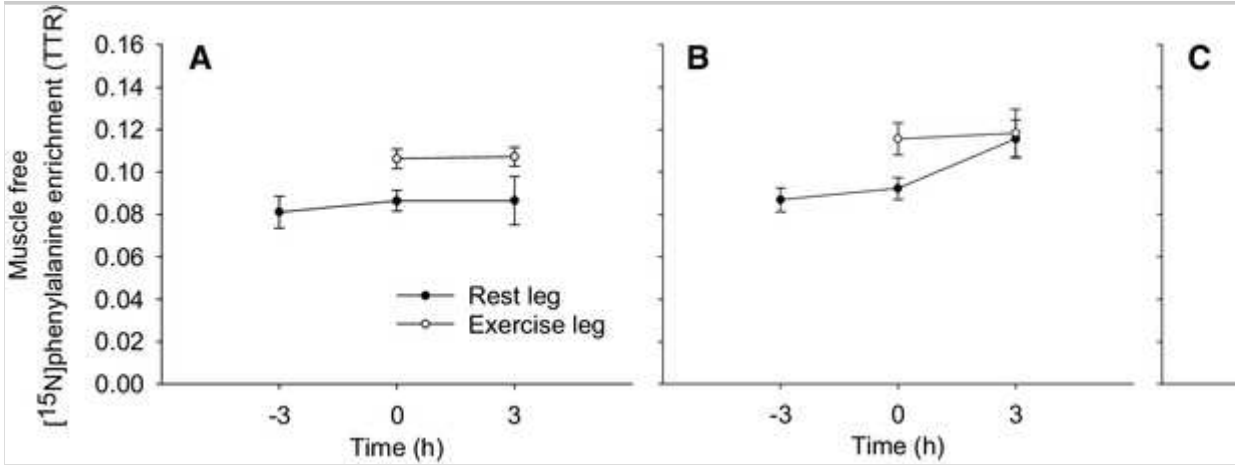
Muscle tissue-free phenylalanine enrichment

Muscle tissue-free [^{15}N]phenylalanine enrichments are displayed in Fig. 4.

The enrichment was clearly higher in the exercised leg as compared to the rest leg immediately after exercise at 0 h, and this difference was not evident in CAS and CHO at 3 h. The mean enrichment ratio in the postprandial and post exercise period (exercise leg/rest leg 0–3 h) was used to adjust the weighted mean venous plasma [^{15}N]phenylalanine enrichment.

Fig. 4

Mean (± SE) muscle-free [^{15}N]phenylalanine enrichments for whey hydrolysate (**a**), caseinate (**b**), and carbohydrate (**c**) in rest and exercise legs



Muscle protein synthesis

Myofibrillar protein FSR revealed a significant effect of the study drink intake alone (fed rest) and of the study drink intake and heavy resistance exercise (fed exercise) as compared to the basal-fasted rest state (Fig. 5). There was no significant difference between the fed-rest- and fed-exercise states, and, furthermore, no significant differences were observed between the three groups in any state. The individual myofibrillar FSR response values are displayed in Fig. 6. Within the fed-rest- and fed-exercise states, one-factor ANOVA tests revealed no significant differences between the three groups. The 27 basal myofibrillar FSR values did not correlate to any of the subject characteristics listed in Table 1 except a weak correlation with age (Supplementary Fig. 1). The fed-rest state and fed-exercised state responses did correlate ($P < 0.01$, $r^2 = 0.26$, Supplementary Fig. 2).

Fig. 5

Mean (± SE) myofibrillar protein fractional synthesis rates (FSR) with mean plasma [^{15}N]phenylalanine enrichment corrected for leg state-dependent muscle-free [^{15}N]phenylalanine enrichment as precursor in whey hydrolysate (WH), caseinate (CAS), and carbohydrate (CHO) groups. Data were analysed with a two-factor ANOVA (group non-repeated, state repeated) and the Student–Newman–Keuls post tests. Effect of state was observed ($P < 0.001$), and post tests revealed: *fed-rest- and fed-exercise states were higher than basal ($P < 0.001$). No group differences were observed

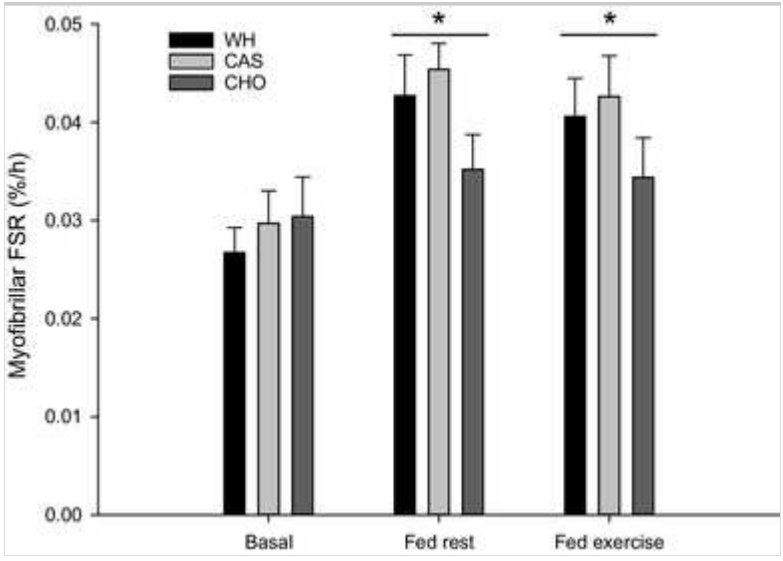
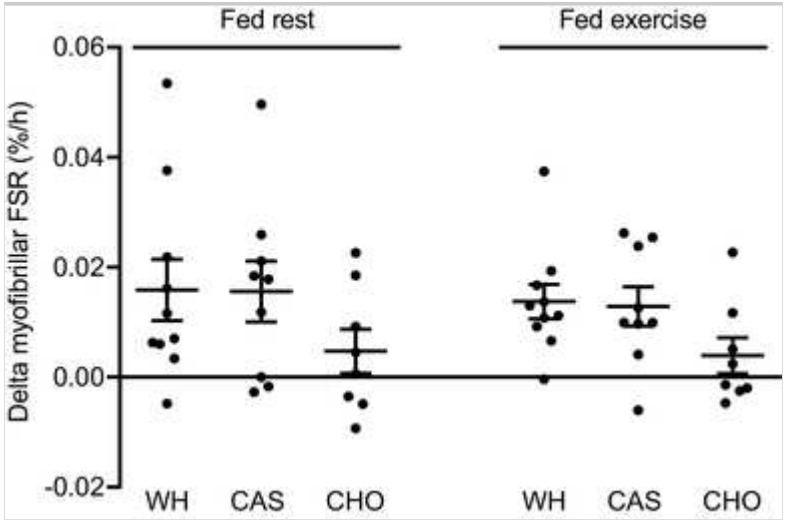


Fig. 6

Delta (fed-rest—basal and fed-exercise—basal) mean (± SE) and individual values of myofibrillar protein fractional synthesis rates (FSR) with mean plasma [^{15}N]phenylalanine enrichment corrected for leg state-dependent muscle-free [^{15}N]phenylalanine enrichment as precursor in whey hydrolysate (WH), caseinate (CAS), and carbohydrate (CHO) groups. Delta data were analysed with a one-factor ANOVA (group non-repeated) within each state. No group differences were observed (fed rest $P = 0.268$; fed exercise $P = 0.102$)

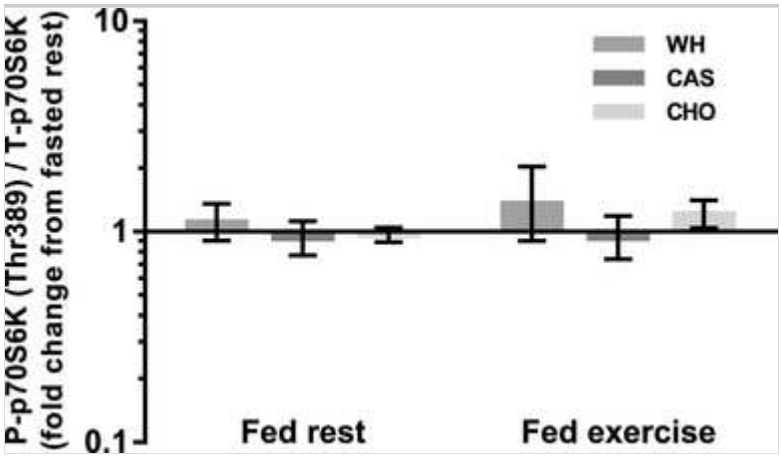


Muscle protein synthesis signalling

The phosphorylated p70S6K (Thr389)/total p70S6K ratios expressed as fold changes in the fed-rest and fed-exercise states from the fasted rest biopsy time point 0 h in the rest leg are presented in Fig. 7. There was no significant interaction or main effects of state or group.

Fig. 7

Muscle protein synthesis signalling, phosphorylated 70-kDa ribosomal protein S6 kinase (p70S6K) phosphorylation Thr389/total p70S6K. Values are presented for the fed-rest and fed-exercise states muscle biopsy time point 3 h as fold changes to fasted rest state time point 0 h as geometric means ± back-transformed SE in whey hydrolysate (WH), caseinate (CAS), and carbohydrate (CHO) groups. No interaction and no effects of state or group were detected. Due to limited muscle tissue, only a subset of participants could be included in Western Blot analysis of p70S6K (WH, $N = 4$; CAS, $N = 5$; CHO, $N = 4$)



Discussion

The present study compared the impact of different postprandial hyperaminoacidemia patterns on the resting and post-exercise MPS response in aging skeletal muscle. The main findings were that energy intake with or without hyperaminoacidemia induced a similar increase in MPS rate and with no additional effect of a single, unilateral bout of heavy resistance exercise when measured 0–3 h after exercise.

Hyperaminoacidemia pattern

In a recent review, Reidy and Rasmussen [28] concluded that protein intake results in a superior stimulatory effect on young adult MPS as compared to carbohydrate, although they noted that also in the younger muscle the effect size of protein supplementation when compared to a carbohydrate control was small and with high variability [28]. The present finding of an even stimulatory effect of hyperaminoacidemia and hyperinsulinemia on myofibrillar protein synthesis rate supports the existence of an impaired responsiveness to hyperaminoacidemia in the ageing muscle. Furthermore, the pattern of the postprandial hyperaminoacidemia was markedly different between the two protein types during the 3-h period, but it had no impact on the response in the ageing muscle. When measured over a 6-h postprandial period, Koopman and colleagues could not detect any significant change in the MPS response after provision of intact vs. hydrolysed casein to older men despite marked differences in amino acid delivery pattern [29]. Similarly, over a 5-h

postprandial period, Dideriksen and colleagues showed no differences on the myofibrillar protein FSR in older men when changing timing (caseinate either before or after a session of resistance exercise), or type (caseinate or whey protein after exercise) [18]. An even MPS response over such extended (5–6 h) postprandial periods are in accordance with findings in young people, where the tendency is that acute differences between whey and caseinate are averaged due to the fluctuating nature of hyperaminoacidemia [26]. In the present study, we measured the acute 3-h postprandial response only. Evidence to support that older muscle is insensitive to amino acid delivery profile was shown in a study by Mitchell and colleagues in which essential amino acids were provided either in one bolus (15 g) or in four 3.75 g pulses every 45th minute to mimic different protein digestibility and amino acid appearance patterns [20]. They divided the 4-h postprandial period into several shorter intervals of MPS but found no impact of amino acid provision pattern despite marked differences on intramuscular signalling favouring the large-bolus intake [20]. Similarly, Agergaard et al. supplemented older men with whey protein either in a large bolus or in repeated smaller pulses over a prolonged period [23]. Of special interest for the current study, neither any impact of delivery profile—nor amount (total of 28 g whey in bolus vs. 12 g whey in pulse) was found on the myofibrillar protein FSR during the initial 3-h period in the older men [23]. Over several hours (7 and 10 h), no differences in MPS were apparent between protein provision strategies and hyperaminoacidemia pattern [23], which is in contrast to the finding in skeletal muscle of young men [30]. In summary, results suggest that the pattern of hyperaminoacidemia and hence, protein digestibility is not a major determinant for the postprandial stimulation of MPS in older adults.

Hyperinsulinemia pattern and effect of carbohydrate

While we and others previously have used a control group ingesting nothing or just water [18, 26, 31, 32, 33], or only comparing different protein sources [15, 16, 34, 35, 36], we here applied an isocaloric carbohydrate comparison. The impact of carbohydrate (glucose) intake and insulin secretion on muscle protein turnover rates remains rather unexplored. In rats, a 12-h fasting-induced reduction in resting MPS rate was restored to fed-state rates by insulin infusion [37], and intake of

carbohydrate enhanced the phosphorylation state of key enzymes critical for protein translation to the same extent as protein in the post-exercise period [38]. In healthy as well as in insulin-resistant humans, insulin infusion in the resting condition deprived intracellular amino acids [39], lowered circulating amino acid concentrations by 30–60% [40], enhanced inward transport of e.g., leucine, lysine and alanine, and importantly enhanced MPS rate [39, 41]. Some studies have compared the effects of carbohydrate together with protein [42, 43, 44, 45, 46] and carbohydrates together with protein and additional leucine [42, 47] to a carbohydrate-only intake on MPS in relation to exercise. In general, the results favour the intake of protein together with carbohydrate (and leucine in some studies) as compared to carbohydrate only. However, the results are equivocal. For example, the study by Koopman et al. [42] compared three different and not isocaloric post-exercise supplements given as small boluses every 30 min (carbohydrate; carbohydrate and whey protein hydrolysate; carbohydrate and whey protein hydrolysate and leucine) and only detected a significant difference in FSR of mixed muscle proteins between the carbohydrate intake as compared to the carbohydrate and protein and leucine intake in young. No difference was found in FSR between carbohydrate and carbohydrate and whey protein [42]. However, whole-body turnover clearly favoured the two different beverages including protein. Later, this group showed favourable effects of the carbohydrate, protein, and leucine intake as compared to carbohydrate only in both young and old [47]. A recent study by Rahbek et al. [45] could not detect any differences in young following both concentric and eccentric exercise regimens on myofibrillar FSR with a post-exercise intake of isocaloric carbohydrate (0.6 g/kg BW) or a mix of carbohydrate (0.3 g/kg BW) and whey protein hydrolysate (0.3 g/kg BW). Drawbacks of these studies are that no basal (i.e., fasted and rested state) FSRs were measured, and, therefore, it cannot be ruled out whether supplements actually induced an increase in FSR, and further that carbohydrate are not compared to a protein-only intake [42, 43, 44, 45, 46, 47]. A few studies have utilised the indirect 2-pool model measurements and found superior effects on local leg protein turnover after intake of carbohydrate together with protein and AA [48] and carbohydrate together with AA [49] as compared to carbohydrate intake only. Interestingly, in the latter study, there was no

difference between intake of the carbohydrate only and AA only [49]. Furthermore, in a study by Roy et al. [50] post-exercise supplements of carbohydrates together with protein and fat, isocaloric carbohydrate only, and a non-caloric placebo were compared on measures of whole-body protein turnover. This study found even effects of the two nutrient-containing supplements as compared to the placebo on nonoxidative leucine disposal as an indirect measure of whole-body protein synthesis [50]. In our present study, the test drinks were isocaloric, and a major difference compared to the previous studies was that the carbohydrate intake was compared to a protein-only intake. Furthermore, we had basal measurements of MPS. The findings in a very recent study in older men illustrate that a basal FSR measurement can be important since the study document a positive response to a standard breakfast meal, which contained 1567 kJ in the form of 60 g carbohydrates, 11 g fat, and only 6 g of protein [51]. However, in this study, the standard breakfast meal was compared to intake of the breakfast with the addition of a vitamin D and leucine-enriched whey protein medical nutrition drink (containing 21 g of protein including 3 g of leucine). The supplement and breakfast intake had superior effects on postprandial FSR as compared to the breakfast [51]. The point here is that without the basal FSR measurement, one could tend to see no effect of the breakfast-only meal.

In older peoples' skeletal muscle, we here demonstrate that carbohydrate intake inducing a moderate secretion of insulin resulted in increased myofibrillar protein synthesis rate similar to that gained after protein intake (Figs. 5, 6). In further support of the findings, Agergaard et al. [23] could not detect any difference in the myofibrillar protein fractional synthesis rate in older men after intake of 3 × 4 g of maltodextrin and moderate insulin secretion over a 3-h period compared to 3 × 4 or 28 g of whey protein intake. However, the numerical change in the present study appears somewhat smaller in the CHO group than in the protein groups (Figs. 5, 6). While no statistical difference was apparent when including all three groups in a statistical analysis (two-way ANOVA, Fig. 5 and one-way ANOVA on delta differences, Fig. 6), we also performed one-way repeated measures ANOVA within each of the groups separately (comparing basal-, fed-rest-, and fed-exercised states). With these tests, the CHO group did not reach statistical significance ($P = 0.365$), hence, the

strength of the stimulatory capacity of carbohydrate seemed inferior to milk proteins (WH $P = 0.008$ and CAS $P = 0.011$). In summary, carbohydrate intake elicits an inferior stimulatory impact on MPS in older men's skeletal muscle as compared to milk protein intake.

Are the older men resistant to anabolic stimuli?

Except from a power-issue, the finding of no difference between conditions with hyperaminoacidemia and hyperinsulinemia on myofibrillar protein synthesis rate in older men could be caused by other means: either by provision of a suboptimal amount of protein, hence submaximal stimulation or by an overall blunted response to hyperaminoacidemia—i.e., presence of anabolic resistance.

It is well accepted that the dose–response relation for protein intake and enhanced MPS rate is right-shifted with increasing age [52], whereas some controversy remains as to whether the maximal ability to increase MPS is blunted in older muscle [1, 53, 54, 55, 56]. In a recent report, Moore and colleagues found that the older on average need 0.61 g protein per kg LBM as compared to 0.25 g protein per kg LBM in young adults to obtain the maximal stimulatory effect of intake of a high-quality protein [57]. We provided 0.45 g protein per kg LBM, hence a suboptimal dose for most of our subjects. It is, therefore, likely, that we could have seen a larger stimulatory effect in the protein groups had we increased the amounts. However, Agergaard et al. could not detect differences between 28 g in one bolus and 3 × 4 g over 2 h in a 3-h FSR period [23]. Hence, as obvious from the previous report by Moore et al., the inter-individual variation increases with advancing age [57], and it appears individually dependent how well older persons respond to a given quantity of protein.

Another determining factor for the stimulatory effect of a protein intake is the quantity of leucine [58]. In accordance, addition of leucine to a moderate amount of whole protein can stimulate the MPS rate above that of the protein amount alone both in young [59, 60] and in older subjects [61]. The leucine content of a given protein or amino acid-based supplement is considered important, and a threshold of 2 g of leucine per dose has been suggested for young adults [28]. Due to the right shift of the

dose–response relationship, this threshold is expected to be higher for older adults [61, 62]. In the provided protein sources, the leucine content was similar (although slightly higher in CAS than in WH, Table 2), but due to the faster digestibility of the WH protein, venous plasma peak leucine concentration was higher after the WH (Fig. 3c). In this study, the protein dose was adjusted to the individual's total lean body mass, therefore, the leucine intake was slightly different from subject to subject but averaged 2.3 g in WH and 2.5 g in CAS. Despite provision of these amounts of leucine and the marked concomitant increases in circulating plasma leucine concentrations (Fig. 3c) no differences in MPS were observed between the protein types. This finding definitely points toward some degree of resistance to the known anabolic stimuli provided by full amino acid and leucine provision, and likely, the threshold level for leucine has not been reached in all participants [63].

A recent systematic review concluded that evidence of anabolic resistance in older skeletal muscle appeared in 18 study arms out of 48, which were collected from 24 studies, and it was discussed that to overcome anabolic resistance, muscle contractile activity (i.e., intensity and volume) could be applied [11]. However, completion of the heavy resistance exercises in our study (10 × 8 repetitions at 70% 1RM), was neither capable of adding an effect on the MPS or making the muscle more responsive to hyperaminoacidemia. It may be though, that the volume and rather strenuous nature of the protocol may have postponed the responsiveness till beyond the 3-h window in which we measured the response [64]. However, impaired mTOR-responsiveness to heavy resistance exercise has been demonstrated to be part of the aging muscle [5], and the phosphorylation state of key enzyme p70S6K downstream of mTOR do not suggest that the translational capacity is to be enhanced shortly beyond 3 h. The strenuous nature of the exercises may also have abolished the acute sensitizing impact of contractions, as Agergaard et al. after completion of light-load contractions found an additive increase in myofibrillar protein synthesis, though irrespective of protein/carbohydrate provision [23]. Such a sensitizing impact of moderate contractile activity in the older muscle has previously been demonstrated after treadmill walking [65]. This suggests the anabolic potency of such contractile modalities in older muscle as compared to younger where no additive

effect was seen after light-load contractions [66], whereas this has been shown after more heavy exercises [67]. Long-term studies need to be conducted to determine whether exercise of more moderate intensity is favourable for maintaining muscle mass at old age.

Study and method limitations

True tracer steady state in muscle tissue-free enrichment is difficult to achieve in exercise models, and the evaluation of eventual steady state enrichment will always be incomplete due to the limited number of muscle biopsies in a human trial setup. In this unilateral exercise study, there is a marked difference between the rested and exercised state in muscle-free phenylalanine enrichment at the two post-exercise time points (0 and 3 h, see Fig. 4). Therefore, the weighted mean venous plasma enrichments were corrected for this difference, and the adjusted value was used as precursor in the calculations of myofibrillar FSR. This adjustment was applied to the plasma-enrichment values, which are measured frequently throughout the 3-h postprandial and post-exercise period to catch small fluctuations that cannot be detected by only measuring mean muscle-free enrichment between the muscle biopsy time points 0 and 3 h.

The inability to detect a difference between the milk protein supplementations and the carbohydrate is probably due to a higher than expected variation in individual responses, and, therefore, also a lower than expected power for estimating the true response to the supplements. Although increasing the number of investigated participants would enhance power, the best way to study such questions would be to apply a cross-over design, which would eliminate the inter-individual variations shown to be higher among older participants [57]. However, with the correlation between the fed-rest response and the fed-exercised response, it is evident that the individual variation or the degree of responsiveness is even between rested and exercised muscle (see Supplementary Fig. 2). Further, we only measured protein synthesis in the hours immediately following the intervention, and therefore, a later than three-hour response was not included in the comparison. Other studies have previously demonstrated a delayed response to anabolic stimuli especially in the protein fed and exercised state [23, 66, 68, 69]. As the leucine

concentration is not back to baseline at 3 h in the protein groups, it could be speculated that the total response is not covered. Therefore, this 3-h protocol may underestimate the myofibrillar FSR response, especially after CAS intake [26]. It must also be recognised that the fasted measure of FSR is in the rested leg both before but also during the exercise protocol in the contralateral leg. Some cross-leg effects cannot be ruled out both in the fasted state and also in the 3-h fed state; however, we believe these effects are minor if any.

Other limitations of the study protocol were that the timing of muscle tissue sampling was limited to the fasted state at − 3 h and to 0 and 3 h post intervention. Therefore, potential fast and temporal activation of p70SK6 cannot be detected. Finally, we did not have any measures of muscle protein breakdown, and, therefore, effects on muscle protein balance or the net anabolic response to the different supplementations remain unknown.

Conclusion

In conclusion, two high-quality types of milk protein with very distinct digestibility elicited even increases in myofibrillar protein fractional synthesis rate within the first 3 hours after consumption. However, the stimulatory effect of the milk proteins was not significantly superior to intake of an isocaloric carbohydrate drink, which induced a higher insulin response. Furthermore, older men did not gain an additional effect of heavy resistance exercise together with the milk protein or carbohydrate intake in the immediate 3-h post intervention period.

Acknowledgements

We thank our voluntary participants for their time and effort, and Ann-Marie Sedstrøm and Ann-Christina Reimann for technical assistance and analyses. Arla Foods Ingredients P/S supported this work.

Author contributions

SR, KD, JA, NMM-C, RLB conducted the experimental work; SR, KD, JA, RKP, and LH analysed and interpreted data; SR, KD, AS, URM, and LH designed study; SR drafted the manuscript; all authors edited and

revised the manuscript. All authors approved the final content and this version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest, financial or otherwise. Anja Serena is employed at Arla Foods amba, and Ulla R. Mikkelsen is employed at Arla Foods Ingredients P/S.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary material 1 (DOCX 1583 KB)

References

1. Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor PM, Rennie MJ (2005) Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. FASEB J 19:422–424

2. Guillet C, Prod'homme M, Balage M, Gachon P, Giraudet C, Morin L, Grizard J, Boirie Y (2004) Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans. FASEB J 18:1586–1587

3. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR (2005) Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. Am J Clin Nutr 82:1065–1073

4. Durham WJ, Casperson SL, Dillon EL, Keske MA, Paddon-Jones D, Sanford AP, Hickner RC, Grady JJ, Sheffield-Moore M (2010) Age-related anabolic resistance after endurance-type exercise in healthy humans. FASEB J 24:4117–4127

5. Fry CS, Drummond MJ, Glynn EL, Dickinson JM, Gundermann DM, Timmerman KL, Walker DK, Dhanani S, Volpi E, Rasmussen BB (2011) Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis. Skelet Muscle 1:11

6. Kumar V, Selby A, Rankin D, Patel R, Atherton P, Hildebrandt W, Williams J, Smith K, Seynnes O, Hiscock N, Rennie MJ (2009) Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men. J Physiol 587:211–217

7. Phillips BE, Hill DS, Atherton PJ (2012) Regulation of muscle protein synthesis in humans. Curr Opin Clin Nutr Metab Care 15:58–63

8. Rennie MJ (2009) Anabolic resistance: the effects of aging, sexual dimorphism, and immobilization on human muscle protein turnover. Appl Physiol Nutr Metab 34:377–381

9. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel J-P, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M (2010) Sarcopenia: European consensus on definition and diagnosis. Age Ageing 39:412–423

10. Morley JE, Abbatecola AM, Argiles JM et al (2011) Sarcopenia with limited mobility: an international consensus. J Am Med Dir Assoc 12:403–409

11. Shad BJ, Thompson JL, Breen L (2016) Does the muscle protein synthetic response to exercise and amino acid-based nutrition diminish with advancing age? A systematic review. Am J Physiol Endocrinol Metab 311:E803–E817
AQ4

12. Dangin M, Boirie Y, Garcia-Rodenas C, Gachon P, Fauquant J, Callier P, Ballèvre O, Beaufrère B (2001) The digestion rate of protein is an independent regulating factor of postprandial protein retention. Am J Physiol Endocrinol Metab 280:E340–E348

13. Boirie Y, Dangin M, Gachon P, Vasson M-P, Maubois J-L, Beaufrère B (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. Proc Natl Acad Sci USA 94:14930–14935

14. Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B, Wolfe RR (2003) Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. Am J Clin Nutr 78:250–258

15. Burd NA, Yang Y, Moore DR, Tang JE, Tarnopolsky MA, Phillips SM (2012) Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men. Br J Nutr 108:958–962

16. Pennings B, Boirie Y, Senden JMG, Gijsen AP, Kuipers H, van Loon LJC (2011) Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. Am J Clin Nutr 93:997–1005

17. Gorissen SHM, Horstman AMH, Franssen R, Crombag JJR, Langer H, Bierau J, Respondek F, van Loon LJC (2016) Ingestion of wheat protein increases in vivo muscle protein synthesis rates in healthy older men in a randomized trial. J Nutr 146:1651–1659

18. Dideriksen KJ, Reitelseder S, Petersen SG, Hjort M, Helmark IC, Kjaer M, Holm L (2011) Stimulation of muscle protein synthesis by whey and caseinate ingestion after resistance exercise in elderly individuals. Scand J Med Sci Sports 21:e372–e383

19. Soop M, Nehra V, Henderson GC, Boirie Y, Ford GC, Nair KS (2012) Coingestion of whey protein and casein in a mixed meal: demonstration of a more sustained anabolic effect of casein. Am J Physiol Endocrinol Metab 303:E152–E162

20. Mitchell WK, Phillips BE, Williams JP, Rankin D, Lund JN, Wilkinson DJ, Smith K, Atherton PJ (2015) The impact of delivery

profile of essential amino acids upon skeletal muscle protein synthesis in older men: clinical efficacy of pulse vs. bolus supply. Am J Physiol Endocrinol Metab 309:E450–E457

21. Timmerman KL, Dhanani S, Glynn EL, Fry CS, Drummond MJ, Jennings K, Rasmussen BB, Volpi E (2012) A moderate acute increase in physical activity enhances nutritive flow and the muscle protein anabolic response to mixed nutrient intake in older adults. Am J Clin Nutr 95:1403–1412

22. Dideriksen K, Reitelseder S, Malmgaard-Clausen NM, Bechshoeft R, Petersen RK, Mikkelsen UR, Holm L (2016) No effect of anti-inflammatory medication on postprandial and postexercise muscle protein synthesis in elderly men with slightly elevated systemic inflammation. Exp Gerontol 83:120–129

23. Agergaard J, Bülow J, Jensen JK, Reitelseder S, Drummond MJ, Schjerling P, Scheike T, Serena A, Holm L (2017) Light-load resistance exercise increases muscle protein synthesis and hypertrophy signaling in elderly men. Am J Physiol Endocrinol Metab 312:E326–E338

24. Bülow J, Agergaard J, Kjaer M, Holm L, Reitelseder S (2016) No additional effect of different types of physical activity on 10-hour muscle protein synthesis in elderly men on a controlled energy- and protein-sufficient diet. Exp Gerontol 79:16–25

25. Holm L, Reitelseder S, Dideriksen K, Nielsen RH, Bülow J, Kjaer M (2014) The single-biopsy approach in determining protein synthesis in human slow-turning-over tissue: use of flood-primed, continuous infusion of amino acid tracers. Am J Physiol Endocrinol Metab 306:E1330–E1339

26. Reitelseder S, Agergaard J, Doessing S, Helmark IC, Lund P, Kristensen NB, Frystyk J, Flyvbjerg A, Schjerling P, van Hall G, Kjaer M, Holm L (2011) Whey and casein labeled with L-[1-^{13}C]leucine and muscle protein synthesis: effect of resistance exercise and protein ingestion. Am J Physiol Endocrinol Metab 300:E231–E242

27. Wolfe RR, Chinkes DL (2005) Isotope tracers in metabolic research: principles and practice of kinetic analysis, 2nd edn. John Wiley, Hoboken

28. Reidy PT, Rasmussen BB (2016) Role of ingested amino acids and protein in the promotion of resistance exercise-induced muscle protein anabolism. J Nutr 146:155–183

29. Koopman R, Crombach N, Gijsen AP, Walrand S, Fauquant J, Kies AK, Lemosquet S, Saris WHM, Boirie Y, van Loon LJC (2009) Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein. Am J Clin Nutr 90:106–115

30. Areta JL, Burke LM, Ross ML, Camera DM, West DWD, Broad EM, Jeacocke NA, Moore DR, Stellingwerff T, Phillips SM, Hawley JA, Coffey VG (2013) Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. J Physiol 591:2319–2331

31. Fujita S, Dreyer HC, Drummond MJ, Glynn EL, Cadenas JG, Yoshizawa F, Volpi E, Rasmussen BB (2007) Nutrient signalling in the regulation of human muscle protein synthesis. J Physiol 582:813–823

32. Tipton KD, Elliott TA, Cree MG, Wolf SE, Sanford AP, Wolfe RR (2004) Ingestion of casein and whey prosteins result in muscle anabolism after resistance exercise. Med Sci Sports Exerc 36:2073–2081

33. Groen BBL, Res PT, Pennings B, Hertle E, Senden JMG, Saris WHM, van Loon LJC (2012) Intragastric protein administration stimulates overnight muscle protein synthesis in elderly men. Am J Physiol Endocrinol Metab 302:E52–E60

34. Tang JE, Moore DR, Kujbida GW, Tarnopolsky MA, Phillips SM (2009) Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following

resistance exercise in young men. J Appl Physiol 107:987–992

35. Wilkinson SB, Tarnopolsky MA, MacDonald MJ, MacDonald JR, Armstron D, Phillips SM (2007) Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. Am J Clin Nutr 85:1031–1040

36. Burd NA, Gorissen SH, van Vliet S, Snijders T, van Loon LJC (2015) Differences in postprandial protein handling after beef compared with milk ingestion during postexercise recovery: a randomized controlled trial. Am J Clin Nutr 102:828–836

37. Baillie AGS, Garlick PJ (1991) Responses of protein synthesis in different skeletal muscles to fasting and insulin in rats. Am J Physiol Endocrinol Metab 260:E891–E896

38. Morrison PJ, Hara D, Ding Z, Ivy JL (2008) Adding protein to a carbohydrate supplement provided after endurance exercise enhances 4E-BP1 and RPS6 signaling in skeletal muscle. J Appl Physiol 104:1029–1036

39. Biolo G, Fleming RYD, Wolfe RR (1995) Physiologic hyperinsulinemia stimulates protein synthesis and enhances transport of selected amino acids in human skeletal muscle. J Clin Invest 95:811–819

40. Drummond MJ, Bell JA, Fujita S, Dreyer HC, Glynn EL, Volpi E, Rasmussen BB (2008) Amino acids are necessary for the insulin-induced activation of mTOR/S6K1 signaling and protein synthesis in healthy and insulin resistant human skeletal muscle. Clin Nutr 27:447–456

41. Biolo G, Williams BD, Fleming RYD, Wolfe RR (1999) Insulin action on muscle protein kinetics and amino acid transport during recovery after resistance exercise. Diabetes 48:949–957

42. Koopman R, Wagenmakers AJM, Manders RJF, Zorenc AHG, Senden JMG, Gorselink M, Keizer HA, van Loon LJC (2005) Combined ingestion of protein and free leucine with carbohydrate increases postexercise muscle protein synthesis in vivo in male subjects. Am J Physiol Endocrinol Metab 288:E645–E653

43. Tang JE, Manolakos JJ, Kujbida GW, Lysecki PJ, Moore DR, Phillips SM (2007) Minimal whey protein with carbohydrate stimulates muscle protein synthesis following resistance exercise in trained young men. Appl Physiol Nutr Metab 32:1132–1138

44. Beelen M, Koopman R, Gijsen AP, Vandereyt H, Kies AK, Kuipers H, Saris WHM, van Loon LJC (2008) Protein coingestion stimulates muscle protein synthesis during resistance-type exercise. Am J Physiol Endocrinol Metab 295:E70–E77

45. Rahbek SK, Farup J, Møller AB, Vendelbo MH, Holm L, Jessen N, Vissing K (2014) Effects of divergent resistance exercise contraction mode and dietary supplementation type on anabolic signalling, muscle protein synthesis and muscle hypertrophy. Amino Acids 46:2377–2392

46. Howarth KR, Moreau NA, Phillips SM, Gibala MJ (2009) Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. J Appl Physiol 106:1394–1402

47. Koopman R, Verdijk L, Manders RJF, Gijsen AP, Gorselink M, Pijpers E, Wagenmakers AJM, van Loon LJC (2006) Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly lean men. Am J Clin Nutr 84:623–632

48. Børsheim E, Aarsland A, Wolfe RR (2004) Effect of an amino acid, protein, and carbohydrate mixture on net muscle protein balance after resistance exercise. Int J Sport Nutr Exerc Metab 14:255–271

49. Miller SL, Tipton KD, Chinkes DL, Wolf SE, Wolfe RR (2003) Independent and combined effects of amino acids and glucose after

resistance exercise. Med Sci Sports Exerc 35:449–455

50. Roy BD, Fowles JR, Hill R, Tarnopolsky MA (2000) Macronutrient intake and whole body protein metabolism following resistance exercise. Med Sci Sports Exerc 32:1412–1418

51. Chanet A, Verlaan S, Salles J, Giraudet C, Patrac V, Pidou V, Pouyet C, Hafnaoui N, Blot A, Cano N, Farigon N, Bongers A, Jourdan M, Luiking Y, Walrand S, Boirie Y (2017) Supplementing breakfast with a vitamin D and leucine-enriched whey protein medical nutrition drink enhances postprandial muscle protein synthesis and muscle mass in healthy older men. J Nutr 147:2262–2271

52. Pennings B, Groen B, de Lange A, Gijsen AP, Zorenc AH, Senden JMG, van Loon LJC (2012) Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. Am J Physiol Endocrinol Metab 302:E992–E999

53. Pennings B, Koopman R, Beelen M, Senden JMG, Saris WHM, van Loon LJC (2011) Exercising before protein intake allows for greater use of dietary protein-derived amino acids for de novo muscle protein synthesis in both young and elderly men. Am J Clin Nutr 93:322–331

54. Symons TB, Sheffield-Moore M, Mamerow MM, Wolfe RR, Paddon-Jones D (2011) The anabolic response to resistance exercise and a protein-rich meal is not diminished by age. J Nutr Health Aging 15:376–381

55. Symons TB, Sheffield-Moore M, Wolfe RR, Paddon-Jones D (2009) A moderate serving of high-quality protein maximally stimulates skeletal muscle protein synthesis in young and elderly subjects. J Am Diet Assoc 109:1582–1586

56. Symons TB, Schutzler SE, Cocke TL, Chinkes DL, Wolfe RR, Paddon-Jones D (2007) Aging does not impair the anabolic response to a protein-rich meal. Am J Clin Nutr 86:451–456

57. Moore DR, Churchward-Venne TA, Witard O, Breen L, Burd NA, Tipton KD, Phillips SM (2015) Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. J Gerontol A Biol Sci Med Sci 70:57–62

58. Drummond MJ, Rasmussen BB (2008) Leucine-enriched nutrients and the regulation of mammalian target of rapamycin signalling and human skeletal muscle protein synthesis. Curr Opin Clin Nutr Metab Care 11:222–226

59. Churchward-Venne TA, Burd NA, Mitchell CJ, West DWD, Philp A, Marcotte GR, Baker SK, Baar K, Phillips SM (2012) Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men. J Physiol 590:2751–2765

60. Churchward-Venne TA, Breen L, Di Donato DM, Hector AJ, Mitchell CJ, Moore DR, Stellingwerff T, Breuille D, Offord EA, Baker SK, Phillips SM (2014) Leucine supplementation of a low-protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double-blind, randomized trial. Am J Clin Nutr 99:276–286

61. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR (2006) A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. Am J Physiol Endocrinol Metab 291:E381–E387

62. Koopman R, Verdijk LB, Beelen M, Gorselink M, Kruseman AN, Wagenmakers AJM, Kuipers H, van Loon LJC (2008) Co-ingestion of leucine with protein does not further augment post-exercise muscle protein synthesis rates in elderly men. Br J Nutr 99:571–580

63. Phillips SM (2014) A brief review of critical processes in exercise-induced muscular hypertrophy. Sports Med 44:S71–S77

64. Drummond MJ, Dreyer HC, Pennings B, Fry CS, Dhanani S, Dillon EL, Sheffield-Moore M, Volpi E, Rasmussen BB (2008) Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging. J Appl Physiol 104:1452–1461

65. Sheffield-Moore M, Yeckel CW, Volpi E, Wolf SE, Morio B, Chinkes DL, Paddon-Jones D, Wolfe RR (2004) Postexercise protein metabolism in older and younger men following moderate-intensity aerobic exercise. Am J Physiol Endocrinol Metab 287:E513–E522

66. Bechshoeft R, Dideriksen KJ, Reitelseder S, Scheike T, Kjaer M, Holm L (2013) The anabolic potential of dietary protein intake on skeletal muscle is prolonged by prior light-load exercise. Clin Nutr 32:236–244

67. Burd NA, West DWD, Moore DR, Atherton PJ, Staples AW, Prior T, Tang JE, Rennie MJ, Baker SK, Phillips SM (2011) Enhanced amino acid sensitivity of myofibrillar protein synthesis persists for up to 24 h after resistance exercise in young men. J Nutr 141:568–573

68. Holm L, van Hall G, Rose AJ, Miller BF, Doessing S, Richter EA, Kjaer M (2010) Contraction intensity and feeding affect collagen and myofibrillar protein synthesis rates differently in human skeletal muscle. Am J Physiol Endocrinol Metab 298:E257–E269

69. Moore DR, Tang JE, Burd NA, Rerecich T, Tarnopolsky MA, Phillips SM (2009) Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise. J Physiol 587:897–904