The Degree of Aminoacidemia after Dairy Protein Ingestion Does Not Modulate the Postexercise Anabolic Response in Young Men: A Randomized Controlled Trial

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ABSTRACT

Background: Resistance exercise and dietary protein stimulate muscle protein synthesis (MPS). The rate at which proteins are digested and absorbed into circulation alters peak plasma amino acid concentrations and may modulate postexercise MPS. A novel mineral modified milk protein concentrate (mMPC), with identical amino acid composition to standard milk protein concentrate (MPC), was formulated to induce rapid aminoacidemia.

Objectives: The aim of this study was to determine whether rapid aminoacidemia and greater peak essential amino acid (EAA) concentrations induced by mMPC would stimulate greater postresistance exercise MPS, anabolic signaling, and ribosome biogenesis compared to standard dairy proteins, which induce a small but sustained plasma essential aminoacidemia.

Methods: Thirty healthy young men (22.5 ± 3.0 y; BMI 23.8 ± 2.7 kg/m²) received primed constant infusions of L-[ring-13C6]-phenylalanine and completed 3 sets of leg presses and leg extensions at 80% of 1 repetition. Afterwards, participants were randomly assigned in a double-blind fashion to consume 25 g mMPC, MPC, or calcium caseinate (CAS). Vastus lateralis biopsies were collected at rest, and 2 and 4 h post exercise.

Results: Plasma EAA concentrations, including leucine, were 19.2–26.6% greater in the mMPC group 45–90 min post ingestion than in MPC and CAS groups (P < 0.001). Myofibrillar fractional synthetic rate from baseline to 4 h was increased by 82.6 ± 64.8%, 137.8 ± 72.1%, and 140.6 ± 52.4% in the MPC, mMPC, and CAS groups, respectively, with no difference between groups (P = 0.548). Phosphorylation of anabolic signaling targets (P70S6K Thr389, P70S6KThr421/Ser424, RPS6Ser235/236, RPS6Ser240/244, P90RSKSer380, 4EBP1) were elevated by <3-fold at both 2 and 4 h post exercise in all groups (P < 0.05).

Conclusions: The amplitude of plasma leucine and EAA concentrations does not modulate the anabolic response to resistance exercise after ingestion of 25 g dairy protein in young men. This trial was registered at http://www.anzctr.org.au as ACTRN12617000393358. J Nutr 2019;00:1–12.

Keywords: human, resistance exercise, milk protein, amino acids, fractional synthetic rate, anabolic signaling, ribosome biogenesis

Introduction

Muscle mass begins to decline in middle age, which accelerates in later life, leading to physical frailty and a higher risk of both mortality and morbidity (1, 2). Achieving a high peak muscle mass through resistance training in young adulthood and middle age may help to maintain physical function and metabolic health in later life (3). Both protein intake and exercise are important for muscle mass maintenance, primarily by increasing muscle protein synthesis (MPS) rates (4). The efficiency of MPS is primarily regulated by the mechanistic target of rapamycin (mTOR) pathway (5). mTOR is a central protein target that integrates signals from muscle contraction and nutrition to coordinate 2 main processes: translational capacity (the amount of ribosomes available) and translation efficiency (the amount of protein produced per mRNA) (6, 7). Most research has focused on translational efficiency as the main regulator of MPS in humans (8). However, it is possible...
that in some situations translational capacity could limit MPS or muscle hypertrophy (9) as ribosomal content increases proportionally with muscle size after resistance training (10, 11) and in animal models of overload (6). Pre-ribosomal RNA (pre-rRNA) has also been shown to respond to postexercise protein feeding in older men (12).

Milk protein primarily comprises 2 distinct types of protein; whey protein accounts for 20% of milk proteins and is characterized by a rapid rate of digestion and a rapid but transient peak aminoacidemia (13). The remaining 80% of milk protein exists as casein, which is slowly digested, and results in a more sustained but lower peak aminoacidemia (13). Previous studies have shown that whey protein was more effective than casein for increasing MPS after a single bout of resistance exercise in young and older adults (14, 15). Based on these studies and a body of similar work (16–18), it has been suggested that magnitude and rate of aminoacidemia (particularly of leucine) is the primary nutritional stimulator of postexercise MPS. This has been termed the leucine trigger hypothesis (4), which is supported by further studies that isolated the role of aminoacidemia by manipulating feeding patterns of the same dose of protein, suggesting that the magnitude of peak aminoacidemia is an important determinant of postexercise MPS (16, 19). However, a number of postexercise protein ingestion studies in which participants have ingested proteins that vary slightly in amino acids composition but vary greatly in the magnitude of aminoacidemia induced, have failed to support a role of aminoacidemia distinct from protein dose in regulating postexercise MPS (20–24). Recently, technology has become available that allows the rate and magnitude of aminoacidemia to be manipulated while controlling for amino acid content by creating a rapidly digested milk protein concentrate with modified mineral content to disrupt the formation of casein micelles (mMPC).

Interpretation of the role of aminoacidemia in regulation of postexercise MPS is complicated by conflicting information from studies manipulating feeding patterns (16, 25) and studies of proteins that vary in both amino acid composition and rate of appearance of dietary protein-derived amino acids into circulation during the postprandial period (14, 23). Therefore, the primary aim of this study was to determine whether consumption of a protein that induces a large but transient aminoacidemia (mMPC) would result in a larger postexercise MPS response than a protein with an identical amino acid composition but which induced a small but sustained aminoacidemia [milk protein concentrate (MPC)] or a control [calcium caseinate (CAS)]. The secondary aim was to measure the effects of these dietary protein manipulations on upstream regulators of MPS, including mTOR pathway targets and markers of ribosome biogenesis during recovery from resistance exercise. It is hypothesized that the more rapid and higher magnitude of aminoacidemia induced by mMPC will result in higher postexercise MPS than that observed after MPC or CAS ingestion. It is also hypothesized that this will be mediated by greater mTOR pathway signaling and increased pre-rRNA expression in the mMPC group.

Methods

Participant recruitment

Healthy young men (n = 30) between the ages of 18 and 35 y with no lower body resistance training in the previous 6 months were recruited using social media. Participants were eligible if they had a BMI between 18 and 30 kg/m², were nonsmokers, free of cardiovascular, musculoskeletal, or metabolic conditions, and were not taking any medications or supplements of any kind. Subjects were randomly assigned according to a 1:1:1 ratio using www.random.org. Participant flow through the trial is shown in Figure 1. All participants gave their informed consent for inclusion before they participated in the study. The study received ethical approval from the Health and Disability Committee Ethics Committee (New Zealand), reference 16/CEN/206. This study was registered with the Australia New Zealand Clinical Trials as ACTRN1261700393358.

Experimental protocol

A parallel single-dose randomized design was used. Participants were asked to refrain from any strenuous exercise and alcohol consumption for at least 72 h before the trial day. At least 72 h before the trial day, participants underwent a DXA scan (Lunar Prodigy) to quantitate lean, fat, and total body mass. Participants were familiarized with the leg presses and leg extensions, and performed a single set of each exercise where failure was achieved in ≤6 repetitions. The Brazycki equation (26) was then used to estimate 1 repetition maximum (1RM) for each exercise. On the day before the test, participants were provided a standard evening meal (60% energy carbohydrate, 20% protein, 20% fat) containing ~40% of their daily energy requirements as estimated by the Harris–Benedict equation (27). The meal was consumed before 2200 the night before the trial day. After an overnight fast, participants arrived at the clinic at 0700 and a 20-gauge plastic catheter was inserted into an antecubital vein and used to collect a baseline blood sample. A slow saline drip was provided to control hydration-state and keep the catheter patent. A muscle biopsy (<100 mg) was collected from the vastus lateralis using a Bergström needle modified for manual suction under local anesthesia (2% xylocaine). Visible fat and connective tissue were dissected away, and the biopsy was immediately frozen in liquid nitrogen. After the biopsy, another catheter (22-gauge) was inserted into the contralateral antecubital vein and a primed constant infusion of [2-13C]-phenylalanine (prime: 2 μmol/kg; infusion: 0.05 μmol·kg⁻¹·min⁻¹) was commenced and maintained for the remainder of the trial. After 3 h of rest, a second biopsy was obtained from the contralateral leg. Participants then performed 2 warmup sets (10 repetitions) of leg presses at 50% and 70% of 1RM, followed by 3 sets (10 repetitions) at 80% 1RM of leg presses and leg extensions, with 2 min of rest between sets, and the final set of each exercise performed until failure. Immediately afterwards, participants consumed 1 of the 3 dairy protein beverages within 5 min of the conclusion of exercise. Participants rested quietly in a supine position with further biopsies...
being collected at 2 and 4 h after exercise. Blood samples were collected at 180, 120, 60, and 0 min pre intervention, and 15, 30, 45, 60, 90, 120, 180, and 240 min post intervention.

**Study beverages**
Participants were randomly assigned to consume 25 g MPC (Fonterra Co-operative Group Ltd.), mMPC (Fonterra Co-operative Group Ltd.), or CAS (Fonterra Co-operative Group Ltd.) in a double-blind fashion. The mMPC was manufactured from fresh skim milk using ultrafiltration, and underwent proprietary processes modifying the mineral profile, and disrupting the casein micelle, which results in a more rapid aminoacidemia without altering amino acid composition or hydrolyzing proteins. Each ingredient was dissolved in 350 mL of water and mixed with 7.8 mg free l-[ring-13C6]-phenylalanine to enrich the beverage to ∼6% of total phenylalanine. The macronutrient and amino acid compositions of the beverages are displayed in Table 1.

**Blood biochemistry**
Baseline blood biochemistry and time course concentrations of plasma glucose were measured as previously described (28). Briefly, plasma HDL cholesterol, LDL cholesterol, TGs, and total cholesterol were measured with the Roche Cobas c311 autoanalyzer (Roche). Time course plasma concentrations of insulin were measured with the Roche Cobas e411 autoanalyzer (Roche) using electrochemiluminescence immune-assay.

**Plasma amino acid concentrations**
Plasma free amino acids were assessed by ultra-performance liquid chromatography as previously described (29) with l-norvaline as an internal standard. The ultra-performance liquid chromatography was organized with a Thermo Scientific Dionex Ultimate 3000 pump, auto sampler, column oven, fluorescence detector (ThermoFisher Scientific), and a Kinetex C18 column headed by a Krudkatcher inline filter (Phenomenex). Chromelon 7.1 software (ThermoFisher Scientific) was used to capture data.

**Muscle fractional synthesis rate**
Protein extraction was conducted similarly to a previous study (30). Briefly, frozen muscle samples between 30 and 60 mg were homogenized for 40 s at 20 Hz using a TissueLyser (Qiagen) in buffer (10 μL/mg 25 mM Tris 0.5% v/v Triton X-100) and protease/phosphatase inhibitor cocktail with 5 mmol/L EDTA solution [Halt Protease and Phosphatase Inhibitor Cocktail (100X), Thermo Scientific, #78,446]. The samples were centrifuged at 4500 × g for 15 min at 4°C to isolate the cytosolic fraction. The supernatant was collected in a fresh tube for western blot analysis and frozen at −80°C, while the pellet was used to calculate the rate of protein synthesis for a myofibrillar-enriched fraction.

Myofibrillar protein-bound and plasma-free l-[ring-13C6]-phenylalanine enrichments were quantified as previously described (31). Briefly, the protein pellets were hydrolyzed overnight in 6 M HCl at 110°C. Free amino acids were purified using cation exchange chromatography and dried under vacuum, followed by resuspension in 60% methanol. Liquid chromatography separation was performed on a Thermo Hypercarb column (4.6 × 100 mm, 5 μm) with mobile phase TABLE 1

<table>
<thead>
<tr>
<th>Protein, g/25 g</th>
<th>MPC</th>
<th>mMPC</th>
<th>CAS</th>
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<tr>
<td>Energy, kJ/25 g</td>
<td>535</td>
<td>556</td>
<td>439</td>
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<tr>
<td>Protein, g/25 g</td>
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<td>0.91</td>
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<td>0.95</td>
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<td>0.73</td>
<td>0.70</td>
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<td>Valine, g/25 g</td>
<td>1.61</td>
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1 Values are displayed as 25-g doses of protein, n = 10/group. CAS, calcium caseinate; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate.

Aminoacidemia and anabolic response 3
A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). Samples were analyzed using a 5300 QTRAP liquid chromatography-tandem mass spectrometer and the mass spectra were acquired under positive electrospray ionization with the ion spray voltage at +5500 V. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 65, and 53 pounds/square inch, respectively. The l-[ring-13C6]-phenylalanine enrichments were determined by multiply reaction monitoring at m/z 166.0 → 103.0 and 172.0 → 109.0 for unlabeled and labelled l-[ring-13C6]-phenylalanine, respectively. Software Analyst 1.6.2 (Agilent) was used for data acquisition and analysis. l-[ring-13C6]-phenylalanine enrichments in plasma were determined by GC-MS analysis using electron impact ionization by ion monitoring at m/z of 336 (m + 0) and 342 (m + 6) for unlabeled and labeled phenylalanine, respectively.

Myofibrillar fractional synthetic rate (FSR) was calculated using the standard equation of precursor to product divided by the increment in tracer enrichment into the myofibrillar proteins by the enrichment of the plasma free precursor pool over time.

\[
FSR \left( \%h^{-1} \right) = \left[ \frac{E_{m2} - E_{m1}}{E_{\text{plasma}} \cdot t} \right] \cdot 100 \tag{1}
\]

FSR is the fractional synthetic rate measure; \(E_{m2}\) and \(E_{m1}\) are the protein-bond enrichments from the muscle biopsies, and so the difference between these determines the change in bound protein enrichment between the 2 time points; \(E_{\text{plasma}}\) is the time weight mean of phenylalanine enrichment in plasma during the incorporation period; and \(t\) is the isotope incorporation time.

### Western blotting

Protein content in the homogenate was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were prepared to contain 20 \(\mu\)g protein, suspended in Laemmli buffer and boiled at 95°C for 10 min. Protocol for sample separation via SDS-PAGE, protein transfer, antibody binding, and imaging were performed as previously described (30). Primary antibodies used to measure all protein targets are found in Supplemental Table 1. Densitometry analysis of protein bands was performed using Image J software. Abundance of proteins of interest was normalized for protein loading by stripping and re-probing membranes for GAPDH. Sample western blots are displayed in Supplemental Figure 1.

### Real-time qPCR

Total RNA and DNA were extracted from ~20 mg muscle tissue using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN GmbH). Total RNA concentration, DNA concentration, and purity were measured using a NanoDrop 1000 running 3.1.2 NanoDrop software (Biolab). cDNA was synthesized using High-Capacity RNA-to-cDNA kit (Life Technologies).

Real-time qPCR was conducted on a LightCycler 480 II (Roche Applied Science), using SYBR Green I master mix (Roche Applied Science). A total of 8 reference genes were run on all samples, and the 3 most stable genes across all time points were selected for normalization (valosin containing protein, chromosome 1 open reading frame 43, endoplasmic membrane protein 7). Target genes were, CMYC, eukaryote initiation factor 4E (EIF4E), 60S ribosome subunit biogenesis protein NIP7 homolog (NIP7), RNA polymerase I subunit B (POLR1B), transcription initiation factor 1A (TIF1A), and upstream binding factor (UBF). The primer sequences for all gene targets, including all housekeeper genes, are listed in Supplemental Table 2.

Target rRNAs were 45S pre-rRNA, 28S, 18S, 5.8S, and 5S. The primers were all designed by QIAGEN, using RT² Profiler PCR Arrays. The product details are all displayed in Supplemental Table 3.

### Statistical analysis

Statistical analysis was performed on SigmaPlot 13.0 (Systat Software, Inc.). Normality was assessed with the Shapiro-Wilk test, log transformations were used for data that was not normally distributed. Differences in gene expression, anabolic signaling, MPS and amino acid concentrations were assessed with 2-factor repeated measures ANOVA with time as within-subject factor and group as a between-subject factor. Between- and within-group differences were assessed with Holm-Šidak post hoc analysis when statistically significant interactions were observed. AUC was calculated in 2 ways as a measure of amino acid availability, pAUC was calculated as the total positive area above baseline whereas iAUC was the integrated sum of the area both above and below baseline. One-factor ANOVA was used to assess between group differences in AUC. A priori sample size calculations were conducted to detect a postexercise between-group difference in FSR of 0.0225%/h with 80% power, \(\alpha\) set at 5% and SD of 0.013 based on our previous work (32). This calculation yielded a sample size of 10 participants/group. All data are reported as means ± SDs in the text and tables. Significance is set for \(\alpha\) ≤ 0.05.

### Results

#### Participant characteristics

Participant baseline characteristics are displayed in Table 2. There were no differences between supplement groups for age \((P = 0.197)\), body mass characteristics \((P ≥ 0.417)\), leg strength \((P ≥ 0.732)\), and plasma biochemistry \((P ≥ 0.311)\).

#### Plasma insulin and glucose

There was a significant group \(\times\) time interaction for plasma insulin \((P < 0.001, Figure 2A)\), such that the mMPC group had a greater concentration at 60 min when compared with both MPC \((P = 0.011)\) and CAS \((P < 0.001)\) groups. Insulin concentrations were elevated above baseline at 30 min in all groups \((P < 0.001)\), at 60 min for the mMPC \((P < 0.001)\) and MPC \((P = 0.005)\) groups, and at 90 min for the mMPC group only \((P = 0.028)\).

There was no group \(\times\) time interaction for plasma glucose \((P = 0.975, Figure 2B)\). There was a main effect of time for a decrease in glucose concentrations below baseline at 60 and 90 min \((P < 0.001)\) and at 240 min \((P = 0.037)\).

#### Plasma amino acids

There was a group \(\times\) time interaction for changes in plasma total amino acid (TAA) concentrations \((P = 0.034, Figure 3A)\). TAA concentrations were elevated above baseline at 45 min for all groups \((P ≤ 0.022)\), at 60 min for mMPC and MPC groups \((P ≤ 0.012)\), and at 90 min for mMPC group only \((P < 0.001)\). There were no differences in pAUC or iAUC between groups for TAA over the 240 min period \((P = 0.158\) and \(P = 0.732\), respectively).

Plasma nonessential amino acids (NEAAs) showed a trend for group \(\times\) time interaction \((P = 0.069, Figure 3B)\). There was a main effect of time for an increase in NEAA concentrations above baseline from 15 to 120 min \((P ≤ 0.044)\). There were no differences in pAUC or iAUC between groups for NEAAs over the 240 min period \((P = 0.598\) and \(P = 0.975\), respectively).

There was a group \(\times\) time interaction for changes in plasma essential amino acid (EAA) concentrations \((P = 0.005, Figure 3C)\), such that the mMPC group had a greater concentration than both the MPC and CAS groups at 60 and 90 min \((P ≤ 0.023)\). EAA concentrations were elevated above baseline from 45 to 120 min for the mMPC group only.
(P ≤ 0.029), only at 180 min for the CAS group (P = 0.030), and from 180 to 240 min for the MPC group (P ≤ 0.031). pAUC for EAAs was greater in the mMPC group when compared to both the MPC and CAS groups (48% compared with MPC, P = 0.036; 69% compared with CAS, P = 0.003), whereas there was a trend for differences in iAUC between groups (P = 0.057).

There was a group × time interaction for plasma leucine concentration (P < 0.001, Figure 3D), such that the mMPC group had a greater concentration than both the MPC and CAS groups from 45 to 90 min (P ≤ 0.001). Leucine concentrations were above baseline from 45 to 180 min (P ≤ 0.006) for the mMPC group, only at 180 min for the CAS group (P = 0.044), and from 180 to 240 min for the MPC group (P ≤ 0.031). pAUC for leucine was greater for the mMPC group than in both MPC and CAS groups (54% compared with MPC, P = 0.010; 58% compared with CAS, P = 0.007), whereas there was a trend for differences in iAUC between groups (P = 0.061).

Ribosomal RNA

There was no group × time interaction observed for total RNA concentration (P = 0.958), 45S pre-rRNA (P = 0.759), 28S (P = 0.986), 18S (P = 0.253), 5.8S (P = 0.739), and 5S (P = 0.245). There was no increase above baseline at any time for all RNAs (Supplemental Table 4). However, there was a trend for an increase in 45S pre-rRNA concentration above baseline at 4 h (P = 0.088).

There was no group × time interaction observed for the expression of CMYC (P = 0.601, Figure 4A), EIF4E (P = 0.531, Figure 4B), NIP7 (P = 0.998, Figure 4C), TIFA (P = 0.764, Figure 4D), POLR1B (P = 0.948, Figure 4E), and UBF (P = 0.987, Figure 4F). There was a main effect of time for increased expression above baseline at 2 and 4 h for CMYC (P < 0.001), EIF4E (P < 0.001), and NIP7 (P < 0.002), and above baseline at 4 h only for TIFA (P ≤ 0.001) and POLR1B (P ≤ 0.005). In addition, the expression of EIF4E (P = 0.001), NIP7 (P = 0.037), TIFA (P = 0.001) and POLR1B (P = 0.005) was higher at 4 h than 2 h. There was a main effect of time for decreased expression of UBF below baseline at 2 and 4 h (P ≤ 0.003). In addition, UBF expression at 4 h was lower than expression at 2 h (P = 0.026).

Anabolic signaling

There was no group × time interaction of either phosphorylation site of AKT (AKTThr308, P = 0.509, Figure 5A; AKTSer473, P = 0.973, Figure 5B). There was a main effect of time for increased phosphorylation above baseline at 2 h for AKTThr308 (P < 0.001), followed by decreased expression at 4 h below that seen at 2 h (P = 0.002). There was a main effect of time for decreased phosphorylation at 4 h below baseline and 2 h for AKTSer473 (P ≤ 0.002).

There was no group × time interaction of either phosphorylation site of P70S6K (P70S6KThr389, P = 0.600, Figure 3C; P70S6KThr421/Ser424, P = 0.200, Figure 3D). There was a main effect of time for increased phosphorylation above baseline for P70S6KThr389 and P70S6KThr421/Ser424 at 2 h (P < 0.001 and P < 0.001, respectively) and 4 h (P < 0.001 and P < 0.001, respectively). Phosphorylation of P70S6KThr421/Ser424 at 4 h had decreased from 2 h (P = 0.023).

There was no group × time interaction of either phosphorylation site of RPS6 (RPS6Ser235/236, P = 0.780, Figure 5E; RPS6Ser240/244, P = 0.825, Figure 5F). There was a main effect of time for increased phosphorylation above baseline for RPS6Ser235/236 and RPS6Ser240/244 at 2 h (P < 0.001 and P < 0.001, respectively) and 4 h (P < 0.001 and P < 0.001, respectively). Phosphorylation of RPS6Ser235/236 and RPS6Ser240/244 at 4 h had decreased from 2 h (P = 0.012 and P = 0.010, respectively).

Phosphorylation of 4EBP1 was measured by a mobility shift of the electrophoretic bands as described by Kimball et al. (33) and us (34). There was no group × time interaction for 4EBP1α (P = 0.443, Figure 6A), 4EBP1β (P = 0.485, Figure 6B), 4EBP1γ (P = 0.143, Figure 6C), and 4EBP1δ (P = 0.283, Figure 6D). There was a main effect of time for increased phosphorylation above baseline at 2 h for 4EBP1α, 4EBP1β, 4EBP1γ, and 4EBP1δ (P = 0.008; P < 0.001; P < 0.001; P < 0.001, respectively) and at 4 h for 4EBP1γ and 4EBP1δ (P = 0.041 and P = 0.001, respectively). Phosphorylation of 4EBP1δ at 4 h had decreased from 2 h (P = 0.030).

Aminoacidemia and anabolic response
FIGURE 2 Plasma insulin (A) and glucose (B) concentrations after consumption of mMPC, CAS, or MPC protein beverages over 240 min in healthy young men. Values represent means ± SEMs, n = 10/group. Data were analyzed with 2-factor repeated measures ANOVA. Labelled means without a common letter differ at that time point. ∗Different from respective group’s baseline; a horizontal bar indicates a main effect of time for all 3 groups. CAS, calcium caseinate; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate.

P38 MAPKThr180/182, MNK, and P90RSKSer380 (P < 0.001; P < 0.001; P = 0.022, respectively), and at 4 h for P38 MAPKThr180/182, MNK, and P90RSKSer380 (P = 0.001; P < 0.001; P = 0.040, respectively).

Muscle fractional synthesis rate
Resting myofibrillar fractional synthesis rate (FSR) was calculated over a 3 h baseline incorporation period and there was no group × time interaction (P = 0.560, Figure 8A). The FSR for the first 2 h after consumption of the protein beverage was elevated above baseline (P = 0.006) with no difference between groups. The FSR between 2 and 4 h remained elevated above baseline (P < 0.001), and not different to that during the first 2 h (P = 0.132). FSR was elevated above baseline even when expressed as an aggregate rate from 0 to 4 h (0.062 ± 0.027%, P < 0.001, Figure 8B). There was no group × time interaction for translation efficiency (P = 0.575, Figure 8C). However, this was elevated above baseline at 2 and 4 h (P = 0.028 and P = 0.002, respectively).

FIGURE 3 Plasma concentrations of TAA (A), NEAA (B), EAA (C), and leucine (D) after consumption of mMPC, CAS, or MPC protein beverages over 240 min in healthy young men. Values represent means ± SEMs, n = 10/group. Data were analyzed with 2-factor repeated measures ANOVA. Labelled means without a common letter differ at that time point. ∗Different from respective group’s baseline; a horizontal bar indicates a main effect of time for all 3 groups. CAS, calcium caseinate; EAA, essential amino acids; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate; NEAA, nonessential amino acids; TAA, total amino acids.
FIGURE 4 Tukey’s box and whisker plots illustrate the changes in ribosome biogenesis regulatory genes CMYC (A), EIF4E (B), NIP7 (C), TIF1A (D), POLR1B (E), and UBF (F) after consumption of mMPC, CAS, or MPC protein beverages at 2 and 4 h in healthy young men. Middle horizontal line represents the median, values displayed as 95% CI, n = 10/group. Data were analyzed with 2-factor repeated measures ANOVA. * indicates significant difference to baseline (P ≤ 0.05). # indicates when 4 h is significantly different to 2 h (P ≤ 0.05). A horizontal bar indicates a main effect of time for all 3 groups. CAS, calcium caseinate; EIF4E, eukaryote initiation factor 4E; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate; NIP7, 60S ribosome subunit biogenesis protein NIP7 homolog; POLR1B, RNA polymerase I subunit B; TIF1A, transcription initiation factor IA; UBF, upstream binding factor; 3HK, the 3 most stable housekeeper genes.

There was a group x time interaction for plasma isotope enrichment (P = 0.031, Figure 9), such that at 60 min mMPC was less than MPC (P = 0.027) and CAS (P = 0.048). Plasma phenylalanine enrichment was above baseline at 30, 60, 90, and 120 min in MPC (P ≤ 0.016) and CAS (P ≤ 0.037).

Discussion

The importance of large increases in blood leucine and essential amino acid concentrations in regulation of postexercise anabolic potential has not been clearly defined in response to bolus protein ingestion. The present study characterized anabolic signaling mechanisms and postexercise MPS in response to a dairy protein source specifically processed to result in a more pronounced aminoacidemia when compared with its native state. We showed that the ingestion of 25 g mMPC resulted in a much larger, but more transient, plasma EAA response despite being matched for AA content with standard MPC. However, these overt differences in plasma aminoacidemia, net exposure of amino acids (leucine and EAAs), and insulin responses induced by the mMPC condition did not enhance postexercise MPS, anabolic signaling mechanisms, or markers of ribosome biogenesis when compared with MPC or CAS conditions. As such, the magnitude or timing of peak amplitude of aminoacidemia does not regulate the anabolic response when a maximal dose of high-quality dairy protein is consumed immediately after resistance exercise in untrained young men.

Previous studies have shown the importance of manipulating blood EAA concentrations within close temporal proximity of exercise bouts to maximize anabolic action on muscles. For example, the postexercise MPS response to quickly and slowly digesting proteins, such as whey and casein, respectively, have been previously examined. It has been shown that whey protein ingestion resulted in a superior postexercise MPS response when compared with the ingestion of casein (14, 17), whereas other groups observed no differences (21, 22). The ability of these studies to isolate the effects of the degree of aminoacidemia on muscle anabolic potential is confounded by the differences in AA composition between whey and casein. To overcome this limitation, studies have been conducted in which postexercise peak aminoacidemia has been manipulated by feeding the same dose of protein as a single bolus or a number of pulsed feeds. These studies show that bolus feeding gives a larger but more transient aminoacidemia resulting in greater postexercise MPS (16, 19). However, the pulsed feeding pattern is a possible confounder. The present study is unique as mMPC was specifically manufactured to manipulate aminoacidemia while matching both AA composition and feeding pattern, in conjunction with resistance exercise to sensitize the muscle to feeding. Our results do not support a role of the pattern of plasma aminoacidemia in enhancing mTOR signaling pathways or MPS when a 25 g bolus dose of protein is consumed during recovery from resistance exercise in young men.

According to the leucine trigger hypothesis, blood leucine concentrations, as a proxy for intramuscular concentrations, have been suggested to reflect the magnitude of the MPS response (14, 35). However, the results from the current study did not reflect this hypothesis as the peak leucine concentration from mMPC was much greater than MPC and CAS, but the stimulation of postexercise MPS was the same.
Figure 5: Tukey's box and whisker plots illustrate the changes in anabolic signaling targets via the mTOR pathway, AKT\textsuperscript{Thr308} (A), AKT\textsuperscript{Ser473} (B), P70S6K\textsuperscript{Thr389} (C), P70S6K\textsuperscript{Thr421/Ser424} (D), RPS6\textsuperscript{Ser235/236} (E), and RPS6\textsuperscript{Ser240/244} (F) after consumption of mMPC, CAS, or MPC protein beverages at 2 and 4 h in healthy young men. Middle horizontal line represents the median, values displayed at 95\% CI, \( n = 10 \) /group. Data were analyzed with 2-factor repeated measures ANOVA. * indicates significant difference to baseline \( (P \leq 0.05) \). # indicates when 4 h is significantly different to 2 h \( (P \leq 0.05) \). A horizontal bar indicates a main effect of time for all 3 groups. AKT, protein kinase B; CAS, calcium caseinate; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate; mTOR, mechanistic target of rapamycin; P70S6K, ribosomal protein S6 kinase beta 1; RPS6, ribosomal protein S6.

between all 3 protein supplements. Indeed, it is likely that the minimal stimulatory leucine threshold was achieved in all ingested protein conditions to stimulate a robust MPS response. However, our current finding is in agreement with multiple recent studies that have shown that peak blood leucine concentrations do not appear to be a primary determinant of the magnitude of the MPS response both at rest and after exercise in middle-aged and older adults (23, 24, 30, 36). These findings add further support to other work showing (21, 24, 37) that once a maximal dose of higher-quality protein is ingested, additional modifications to change the intrinsic qualities of the protein being ingested have minimal bearing on the postexercise MPS response. Churchward-Venne et al. have shown that whey protein was able to sustain the MPS response for a longer duration than a smaller dose of whey protein supplemented with EAA and leucine during recovery from resistance exercise (38). Hence, it is possible that whey protein may contain anabolic components, other than amino acids, which could be responsible for some of its previously observed benefits (39). However, it is likely that in most cases when a sufficient dose of protein is provided, whey and MPC would induce similar anabolic responses even after exercise.

Recent evidence has suggested resistance training-induced muscle hypertrophy may be related to increased translational capacity through the process of ribosome biogenesis (10). Ribosome biogenesis is the process of synthesizing new ribosomes from ribosomal RNA. However, few studies have examined the role of nutrition in regulating postexercise ribosome biogenesis, yet it has been suggested that dairy protein might augment this response (12). The current study did not find any changes in the rRNA markers (45S pre-rRNA, 28S, 18S, 5.8S, and 5S) associated with ribosome biogenesis up to 4 h after protein ingestion and resistance exercise. Previous studies have reported that 43S pre-rRNA does not change up to 3 h post exercise (10, 40, 41), but reported a \( \sim 2 \)-fold increase at 4 h post exercise (42) and a \( \sim 1.5 \)-fold increase at 24 h post exercise (41). In the current study, 45S pre-rRNA did not change up to 4 h post exercise, which disagrees with the findings from Nader et al. (42). The difference may be a result of the muscle group studied, where Nader et al. (42) exercised the biceps brachii, and the current study exercised the vastus lateralis. Previous work in the vastus lateralis did not observe an increase in 45S pre-rRNA in the early hours after exercise (10, 41). Despite this, in the current study, a trend for an increase for 45S pre-rRNA was observed at 4 h, which may indicate initiation of ribosome biogenesis. Genes including \textit{CMYC} and \textit{NIP7}, which regulate ribosome biogenesis, had increased expression at 4 h post exercise, suggesting that a later measurement time point would have been more appropriate to capture the ribosome biogenesis response.

The presumed difference in rates of phenylalanine appearance, based on the blood EAA concentrations, induced by the mMPC and the other 2 proteins resulted in differences in plasma precursor availability within the early postprandial phase between the conditions. We enriched the study beverages with a tracer to minimize disturbances in plasma tracer enrichment from the appearance of unlabeled dietary phenylalanine into circulation (43). Specifically, \( l-([\text{ring-13C}_6]) \)-phenylalanine...
**FIGURE 6** Tukey's box and whisker plots illustrate the changes in phosphorylation of 4EBP1α (A), 4EBP1β (B), 4EBP1γ (C), and 4EBP1δ (D) after consumption of mMPC, CAS, or MPC protein beverages at 2 and 4 h in healthy young men. Middle horizontal line represents the median, values displayed at 95% CI, n = 10/group. Data were analyzed with 2-factor repeated measures ANOVA. *Indicates significant difference to baseline (P ≤ 0.05). # indicates when 4 h is significantly different to 2 h (P ≤ 0.05). A horizontal bar indicates a main effect of time for all 3 groups. CAS, calcium caseinate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate; 4EBP1, eukaryotic translation initiation factor 4E binding protein 1.

**FIGURE 7** Tukey's box and whisker plots illustrate the changes in MAPK signaling cascade, P38 MAPKThr180/Tyr182 (A), MNKThr197/Tyr202 (B), ERK1/2Thr202/Tyr204 (C), and P90RSKSer380 (D) after consumption of mMPC, CAS, or MPC protein beverages at 2 and 4 h in healthy young men. Middle horizontal line represents the median, values displayed at 95% CI, n = 10/group. Data were analyzed with 2-factor repeated measures ANOVA. *Indicates significant difference to baseline (P ≤ 0.05). A horizontal bar indicates a main effect of time for all 3 groups. CAS, calcium caseinate; ERK1/2, extracellular signal regulated kinase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mMPC, mineral modified milk protein concentrate; MNK, mitogen activated protein kinase interacting protein kinase; MPC, milk protein concentrate; P38 MAPK, P38 mitogen activated protein kinase; P90RSK, P90 ribosomal S6 kinase.
Tukey’s box and whisker plots illustrate the changes in myofibrillar fractional synthesis rate (A), aggregated myofibrillar fractional synthesis rate (B), and translation efficiency of FSR relative to RNA concentration per mg of tissue (C) after consumption of mMPC, CAS, or MPC protein beverages between baseline and 4 h in healthy young men. Middle horizontal line represents the median, values displayed at 95% CI, n = 10/group. Data were analyzed with 2-factor repeated measures ANOVA.

\[ \text{Time (h)} \]

\[ \text{Time (min)} \]

\[ \text{Time: } P<0.001 \]

\[ \text{Interaction: } P=0.031 \]

A horizontal bar indicates a main effect of time for all 3 groups. CAS, calcium caseinate; mMPC, mineral modified milk protein concentrate; MPC, milk protein concentrate.

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