Increased protein intake derived from leucine-enriched protein enhances the integrated myofibrillar protein synthetic response to short-term resistance training in untrained men and women: a 4-day randomized controlled trial

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Running Head: Leucine-enriched protein and MyoPS

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Abstract

Leucine is a critical amino acid stimulating myofibrillar protein synthesis (MyoPS). The consumption of higher leucine-containing drinks stimulates MyoPS, but we know less about higher leucine solid foods. Here we examined the effect of short-term resistance exercise training (STRT) combined with supplementation of a protein and leucine-enriched bar, compared with STRT alone, on integrated (%/d) rates of MyoPS and anabolic protein signaling. In a non-blinded, randomized crossover trial, eight young adults performed four sessions of STRT without or while consuming the study bar (STRT+Leu, 16g of protein containing ~3g of leucine) for two 4d phases, separated by 2d non-exercise (Rest) washout. In combination with serial muscle biopsies, deuterated water permitted the measurement of myofibrillar protein synthesis and protein signaling phosphorylation. MyoPS during STRT (1.43 ± 0.06 %/d) and STRT+Leu (1.53 ± 0.06 %/d) were greater than Rest (1.31 ± 0.05 %/d), and MyoPS during STRT+Leu (1.53 ± 0.06 %/d) was greater than STRT alone (1.43 ± 0.06 %/d). STRT+Leu increased the ratio of phosphorylated to total mTOR and 4EBP1 compared to Rest. Engaging in STRT increased integrated MyoPS and protein signaling in young adults and was enhanced with increased protein intake derived from a leucine-enriched protein bar. This study was registered at clinicaltrials.gov as NCT03796897.

Keywords

Amino acids, milk protein, skeletal muscle, protein bar, leucine, protein synthesis

Introduction

Resistance training (RT) stimulates pathways that 'sensitize' skeletal muscle to the anabolic responses of protein ingestion (Burd et al. 2011). Ingestion of an adequate dose of protein results in subsequent hyperaminoacidemia and stimulation of myofibrillar protein synthesis (MyoPS) in humans (Rennie et al. 2004). In addition, supplementation of fast and slow digestible proteins (e.g., whey and casein) enriched with crystalline leucine results in a rapid and sustained increase in circulating essential amino acids and effectively stimulates MyoPS in young and old adults (Churchward-Venne et al. 2012; Churchward-Venne et al. 2014; Kramer et al. 2015; Kramer et al. 2017). Milk-based proteins have the highest protein quality scores, whether measured as protein digestibility-corrected amino acid score or digestible indispensable amino acid score (FAO 2013). Thus, milk-derived proteins represent the most effective (leucine and essential amino acids per g of total protein) protein source to achieve an anabolic stimulation of MyoPS.

Leucine is an essential amino acid necessary for activating MyoPS via the mechanistic target of rapamycin (mTOR) signaling pathway in human skeletal muscle (Wolfson and Sabatini 2017; Wolfson et al. 2016). Recently, we reported that consuming a protein and leucine-enriched bar – formulated with 16 g of a blend of micellar casein, whey protein, and whey protein hydrolysate – induced aminoacidemia comparable to consuming a single higher-protein-containing meal (0.5g of protein/kg) (Traylor et al. 2021). Previous studies also reported that higher leucine-containing proteins enhance MyoPS and whole-body protein synthesis in young and older adults (Glynn et al. 2010; Zaromskyte et al. 2021). The effect of leucine on daily MyoPS can be independent of daily protein intake (Devries et al. 2018a, 2018b; Murphy et al. 2016). Thus, further studies that evaluate the effect of protein-rich whole food — notably higher quality protein (i.e., higher leucine) — intake on protein synthesis in untrained young adults are needed.

The potential for short-term resistance training (STRT, 2 to 6 sessions) has applications in sports settings for athletic trainers and physical therapists who develop programs to help athletes return to practice and competition. Short resistance training may also provide clinicians with scientific justifications for recommendations regarding the design of resistance training programs, including the number of rehabilitative sessions necessary to obtain a specific strength outcome. Most STRT studies have examined strength, power, and neuromuscular adaptations in humans (Beck et al. 2007; Brown and Whitehurst 2003; Coburn et al. 2006). There is little information about the effect of STRT on integrated daily MyoPS. Some previous studies reported that early exercise-induced increases in MyoPS do not align with hypertrophic outcomes (Damas et al. 2016). Muscle hypertrophy, however, is known to result from accumulated increases in MyoPS after resistance exercise.

The present study aimed to examine the effect of STRT combined with high-leucine-containing protein bar supplementation (STRT+Leu), compared with STRT alone, on integrated (%/day) rates of MyoPS and purported anabolic signaling proteins. We hypothesized that STRT and STRT+Leu would increase MyoPS over resting values and that STRT+Leu would increase the MyoPS response more than STRT alone. Moreover, the acute phosphorylation of mTOR complex 1 (mTORC1)-related signaling proteins would be commensurate with the MyoPS responses.

Materials and methods

Participants. Eight young adults (five men and three women; Table 1) were recruited and provided written consent after being informed of the purpose, protocol, and risks of the study (Figure 1). Participants were recreationally active but had not been involved in a resistance training program for at least the previous three months and did not meet Canada's Physical Activity Guidelines (150 minutes of moderate-intensity exercise/week). Inclusion criteria

were as follows: men and women 18-29 years of age and generally healthy according to a standard health screening questionnaire. Exclusion criteria were the regular use of analgesic or anti-inflammatory drugs, history of neuromuscular problems, musculoskeletal disease, any acute or chronic illness, tobacco use or smoking, metabolic disorders, the use of corticosteroids, and not being able to perform lower-body resistance training. The Hamilton Integrated Research Ethics Board approved all experimental procedures (HIREB Project 5706). This study was registered at clinicaltrials.gov as NCT03796897. Due to time constraints, this study did not achieve full recruitment.

Experimental design. The present study was a nonblinded, controlled randomized crossover design, consisting of STRT and STRT combined with protein and leucine-enriched bar supplementation (STRT+Leu). The participants visited the laboratory on eleven separate occasions, and specific details of the study design appear in Figure 2. At least one week before the oral deuterium oxide intake sessions for determining MyoPS and the underpinning mechanisms, study participants underwent familiarization with the study protocol and a whole-body dual-energy X-ray absorptiometry (DXA) scan (GE-Lunar iDXA; Aymes Medical, Newmarket, ON) to measure body composition. Additionally, participants performed unilateral/contralateral strength testing, which involved a five repetition maximum (RM) test of standard seated leg press and seated leg extension exercise in the same manner as previously described (Churchward-Venne et al. 2012; Churchward-Venne et al. 2014; Oikawa et al. 2020). Following baseline testing, participants began an 11 day controlled diet designed to meet energy requirements and to provide moderate daily protein intake (1.3 g/kg/day) with all food provided by the study investigators. To monitor body-water deuterium enrichment, fasted state saliva samples were collected each morning of the study duration. During the visits for exercise training, the participants underwent unilateral/contralateral training sessions of the leg extensors with/without consumption of the

protein and leucine-enriched bars, respectively. During the Pre-STRT visits (day 1 and 7), muscle biopsies were taken from the *vastus lateralis* before a training session. During the post-test visits (day 4 and 10), muscle biopsies were taken 1 hour after the training session (STRT phase) or 1 hour after the training session and bar consumption (STRT+Leu phase) to determine the integrated MyoPS during each test phase and select molecular responses to acute resistance exercise. The leg for muscle biopsies were randomized for each treatment phase. There was a washout period for the crossover on days 5 and 6. Although it was impossible to blind the participants or investigators, all samples were analyzed, and data collated in a blinded fashion until statistical analysis was complete.

Diets. Each participant's energy requirement to maintain energy balance was determined using the Harris-Benedict equation and adjusted using a moderate activity factor of 1.7 for accounting for participants' reported physical activity patterns (Roza and Shizgal 1984). The study participants refrained from physical exercise 72 hours before testing and consumed their evening meal no later than 22.00 hours. The diet provided a protein intake in line with what Canadians generally consume (Auclair and Burgos 2021), which reflected a high acceptable protein distribution range and per the recent recommendations from several expert committees and researchers for optimal protein intakes (1.3 g/kg/day) (Auclair and Burgos 2021; Moore et al. 2015; Traylor et al. 2018). To enhance compliance, investigators designed the study diets by meeting with each participant individually to customize meal plans according to each participant’s personal food preferences. Participants were supplied with all of the required food, which consisted of pre-packaged, frozen meals (Heart-to-Home Meals, Hamilton, ON, Canada) containing animal/plant-based proteins and other items that required minimal preparation (i.e., granola bars, fruit cups, and juices). The participants consumed breakfast within 30 minutes of waking up and were instructed to space meals ~4-6 hours apart.

Short-term resistance training. The participants performed four separate unilateral resistance exercise sessions counterbalanced for their dominant leg and non-dominant leg. Twenty-four hours of rest were allowed between all training sessions. Each training session began with a leg press exercise and a warm-up of ten submaximal muscle contractions at ~50% of 1RM. Following a two-minute rest period, the participants performed four sets of eight to ten leg press and leg extension corresponding to 75% of 1RM values. The participants were verbally encouraged to produce maximal effort during the muscle action for each repetition. After completing each concentric muscle action, the participants were instructed to return their leg to full flexion in a controlled fashion. Two minutes of rest were allowed between each set. After the leg press exercise and two minutes of rest, the participants performed the leg extension exercise in the same fashion.

Protein and leucine-enriched bar. During the STRT+Leu phase, participants were administered the high-leucine-containing protein bars immediately before and after training (two bars in total per training session). The protein and leucine-enriched bar supplement contained ~16 g of a blend of micellar casein (AMCO, Burlington, NJ, USA), whey protein (Hilmar, Hilmar, CA, USA), whey protein hydrolysate (Hilmar, Hilmar, CA, USA), 1.5 g of free-leucine (Ajinomoto, Raleigh, NC, USA), 22 g low-glycemic carbohydrates (Ciranda, Hudson, WI, USA), and 11 g monounsaturated fat (Golden Barrel, Honey Brook, PA, USA). The total leucine content of the bar was ~3 g, which results in a robust elevation in aminoacidemia (particularly leucinemia) and previous studies indicate that leucinemia induces stimulation of MyoPS at the *vastus lateralis* (Churchward-Venne et al. 2014; Devries et al. 2018a; Traylor et al. 2021). Covance (Eurofins) produced the bars; their nutrient content is given in Table 2.

Oral deuterated water protocol. As previously described, deuterium can be labelled to non-essential amino acids (e.g., alanine) by intermediary reactions, so we utilized oral

consumption of deuterium oxide ([D_2O]; 70% enriched; Cambridge Isotope Laboratories) to track the incorporation of newly labeled synthesized myofibrillar proteins (MacDonald et al. 2013; Wilkinson et al. 2014). Per previous protocols, the present study determined body-water deuterium enrichment from saliva samples (MacDonald et al. 2013; Wilkinson et al. 2014).

Participants reported to the laboratory in the fasted state on visit one (day 0 = D_2O loading phase) and gave a pre-enrichment saliva sample, followed by ingestion of eight doses (0.8 ml/kg of lean mass) of 70% D_2O evenly spaced every hour through the day. Participants consumed one dose of D_2O every morning to maintain deuterium enrichment in the circulation of body water at a steady state throughout the study. Participants' saliva samples were collected in the fasted state to measure deuterium enrichment in body water. Samples were stored at -20 ℃ before analysis (MacDonald et al. 2013; Wilkinson et al. 2014).

Saliva sample analysis. Each saliva sample was centrifuged at 1500 *g* for 10 minutes at 4 ℃, and the supernatant (water phase) was collected for further analysis. The sample was then diluted using double-distilled water to a ratio of 1:35 and analyzed for deuterium-enrichment utilizing a cavity ring-down spectroscopy and liquid isotope analyzer equipped with an automated injection system (Picarro L2130-I, Santa Clara, CA). The sample was injected six times per run, and data generated utilized the average deuterium isotopic concentrations of the last three injections resulting in a coefficient of variation of ≤ 0.8%. The deuterium enrichments were initially expressed as change in deuterium % relative to Vienna Standard Mean Ocean Water and were converted to APE using standard equations (Gasier et al. 2010).

Muscle biopsy protocol. Each participant reported to the laboratory after an overnight fast (> 10 h) on each biopsy testing day. Investigators created a sterile field over the biopsy site and injected ~5 ml of a local anesthetic (2% xylocaine) under the skin and fascia. Muscle samples

were cleaned of visible blood, fat, and connective tissue before snap-freezing in liquid nitrogen and stored at -80℃ for further analysis.

Analytical methods. Each muscle sample (~30-50 mg) was homogenized on ice in a buffer [10 µl of buffer per mg of muscle, 25 mM Tris-HCl (pH 7.4), 0.5% v/v Triton X-100 (Sigma Aldrich, Oakville, ON), and a complete protease/phosphatase inhibitor cocktail tablet (Complete Protease Inhibitor Mini-Tabs, Roche)]. The sample was then centrifuged at 700 *g* for 10 minutes at 4 ℃, and the supernatant (sarcoplasmic fraction) and pellet were separated for western blotting and MyoPS analysis, respectively. The pellet containing muscle-bound alanine was solubilized and centrifuged as previously described (Dufner et al. 2005). Samples (myofibrillar proteins) were precipitated in 1 ml of 1 M perchloric acid and centrifuged at 700 *g* for 10 minutes at 4 ℃. The sample was washed twice with 70% ethanol and then hydrolyzed in 0.5 M HCL and Dowex (50WX8-200 resin; Sigma-Aldrich) at 110 ℃ for 72 hours to liberate the muscle-bound amino acids. The free amino acids were then purified using Dowex ion-exchange chromatography, and myofibrillar ^{2}H-alanine enrichments were analyzed as previously described (MacDonald et al. 2013).

Determination of myofibrillar protein synthesis. The integrated rate of MyoPS was calculated via the precursor-product approach; the incorporation of deuterium-labeled alanine into the contractile proteins using body water enrichment (corrected for the average number of deuterium moieties incorporated per alanine) as the surrogate precursor labeling between subsequent biopsies as described previously (McGlory et al. 2018; Murphy et al. 2016).

Western blotting. The ratio of phosphorylation to total protein content was assessed by Western blotting. To standardize the protein concentration, 2 µg/µl of the sample (sarcoplasmic fraction) was mixed with double-distilled water in 4× Laemmli buffer containing 0.25 M Tris at 4% SDS, 20% glycerol, 0.015% bromophenol blue, and 10% 2-mercaptoethanol. Then each sample and a protein-ladder (Precision Plus Protein Standard,

Bio-Rad, Hercules, CA) were loaded into wells on a 26-well 4%-15% TGX Stain-Free Precast Gel (Bio-Rad, Hercules, CA). Four internal standards loaded in the last four wells on every gel and stain-free image on membranes were used as calibration curves to normalize the protein content (Mollica et al. 2009). To ensure a complete transfer, investigators examined membranes using ultraviolet activation of the gel pre-and post-transfer (ChemiDoc MP Imagining System, Bio-Rad). Membranes were then blocked with 5% bovine serum albumin for 90 minutes at room temperature before exposure to primary antibodies [1:1000; total form: mTOR (2972S), p70S6K (9202L), S6K (2217L), and 4EBP1 (9644S); phosphorylation form: mTOR ^{Ser2448} (5536S), p70S6K ^{Thr389} (9205L), S6K$^{ser235/236}$ (2211S), and 4EBP1 $^{Thr37/46}$ (2855C); Cell Signaling Technology] for 12 hours at 4˚C. Additionally, membranes were then washed for 3 x 5 minutes in Tris-buffered saline and Tween 20 (TBS-T, Millipore Sigma) and incubated in secondary antibody (1:3000, anti-rabbit IgG conjugated with horseradish peroxidase, 7074S; Cell Signaling Technology) for 90 minutes at room temperature. After the secondary incubation, the membranes were washed for 3 x 5 minutes using TBS-T. Membrane bands (protein expression) were detected using a chemiluminescence solution (Clarity Western ECL substrate, Bio-Rad), and the bands were quantified using Image Lab 6.0.1 (Image Lab Software for Mac Version 6.0.1).

Statistical analyses. Normality for the distribution of data was assessed using the Shapiro-Wilk test. Non-normally distributed data (the ratio of phosphorylated to total p70S6K, S6K, and 4EBP1) were converted by log transformation (Curran-Everett 2018), and all the values were normally distributed. For comparing estimated 1RM and performed training volume between conditions, *t*-tests were used. One-way analysis of variance (ANOVA; intervention: REST, STRT, and STRT+Leu) was used to assess integrated MyoPS. Two-way repeated-measures ANOVA (condition by time) were applied to assess protein signaling. Follow-up analyses included *t*-tests with Bonferroni correction for multiple comparisons. An alpha level

of $P \leq 0.05$ was considered statistically significant for all comparisons. Statistical analyses were completed using the SPSS Statistics Software package (Version 26.0 for Windows; IBM Corp., Chicago, IL).

Results

Participant Characteristics. Participants' anthropometric measures and body composition are presented in Table 1.

Short-term resistance exercise training. Participants' estimated 1RM and performed training volume are presented in Table 3. There were no differences in 1RM for leg press (P = 0.92; 95% CI, 39.62-43.59) and leg extension (P = 0.72; 95% CI, 11.11-15.65) between STRT and STRT+Leu phases. Participants adhered to 100% of the STRT sessions. There were no differences in leg press (P = 0.92; 95% CI, 282.07-257.34) and leg extension (P = 0.87; 95% CI, 68.51-80.18) training volumes between STRT and STRT+Leu phases.

Dietary intake. Nutritional profiles for dietary intake and bar are presented in Table 2. There were no significant differences in the consumed energy (P = 0.33; 95% CI, 15.30-6.10), carbohydrate (P = 0.75; 95% CI, 2.74-2.07), and fat (P = 0.16; 95% CI, 0.41-0.05) between STRT and STRT+Leu phases. Protein intake during STRT+Leu was greater than STRT phase ($P < 0.001$; 95% CI, 0.39-0.24).

Integrated myofibrillar protein synthesis. Integrated daily MyoPS during STRT (1.43 ± 0.06 %/d) and STRT+Leu (1.53 ± 0.06 %/d) phase were greater than Rest (1.31 ± 0.05 %/d) by 8% ± 5% (P = 0.001; 95% CI, 0.05-0.19) and 14% ± 5% ($P < 0.001$; 95% CI, 0.15-0.29), respectively (Figure 3). Protein and leucine-enriched bar consumption with resistance exercise (STRT+Leu) increased integrated MyoPS above STRT by 7% ± 5% (P = 0.004; 95% CI, 0.03-0.17; Figure 3).

Phosphorylation status of proteins in the mTORC1 signaling pathway. There was an interaction between condition and time for P/T mTOR (P=0.039; ηp^2 = 0.48) and 4EBP1 (P=0.046, ηp^2 = 0.26). An acute session of resistance exercise and the ingestion of study bars (STRT+Leu) elevated the ratio of phosphorylated to total (P/T) mTOR (P = 0.030; 95% CI, 0.39-0.03) and 4EBP1 (P = 0.008; 95% CI, 0.24-0.04) compared to Pre-STRT by 13% ± 18% and 13% ± 12%, respectively, while STRT alone did not (Figure 4A and D). There were main effects for P/T p70S6K (P=0.002; ηp^2 = 0.50) and S6K (P=0.016; ηp^2 = 0.35). The ratio of P/T p70S6K was 14% ± 16% greater (P = 0.002; 95% CI, 0.31-0.08) at Post-STRT compared to Pre-STRT with no difference between conditions (P = 0.25; 95% CI, 0.57-0.16; Figure 3B). The ratio of P/T S6K was 20% ± 38% greater (P = 0.016; 95% CI, 0.47-0.06) at Post-STRT compared to Pre-STRT with no difference conditions (P = 0.054; 95% CI, 0.72-0.01 Figure 4C).

Discussion

The present study results indicate that STRT increased MyoPS and protein intake derived from twice-daily ingestion of a protein and leucine-enriched bar potentiated integrated rates of MyoPS. Corroborating the MyoPS data, the acute phosphorylation of mTOR and 4EBP1 one hour after the STRT+Leu condition was more significant than Pre-STRT only in the STRT+Leu condition. These findings highlight that the consumption of the protein and leucine-enriched bars during four RE sessions led to an additional enhancement in the myofibrillar remodelling of RE.

In a previous study, we reported that ingestion of the study bar containing the isolated milk protein whey protein, and micellar casein resulted in a peak plasma leucine concentration of 593 ± 176 μM (range: 210-790 μM) when consumed as a snack two hours after consuming a low-protein breakfast (0.2 g protein/kg) in older adults (Traylor et al.

2021). The peak leucine concentration exceeded levels (~450 μM) reported to increase MyoPS in older adults (Murphy et al. 2016) and after consuming 40 g of whey protein-containing beverage (~320 μM) in younger adults (Yang et al. 2012). The protein and leucine-enriched study bar elevated EAA and BCAA concentrations comparable to consuming a higher-protein meal (0.5 g protein/kg) (Moore et al. 2015; Traylor et al. 2021). In concordance with the aminoacidemia and leucinemia in our previous work (Traylor et al. 2021), the present study discovered that increasing protein intake through supplemental protein and leucine-enriched bar ingestion induced an additional increase in integrated rates of MyoPS during a unilateral lower-body resistance training protocol. We note that within a normal eating pattern a food matrix containing protein is consumed that blends whole food sources, and attenuates a rapid aminoacidemia/leucinemia response (Burd et al. 2019). Optimal stimulation of MyoPS, therefore, may not require hyperaminoacidemia (Mitchell et al. 2015). Thus, the results presented here align with previous studies indicating that increasing protein intake and periodically inducing hyperaminoacidemia, particularly leucinemia, induces further stimulation of MyoPS during STRT (Devries et al. 2018a, 2018b; Pennings et al. 2012; Yang et al. 2012).

In the present study, we controlled daily protein intake at 1.3 g of protein/kg/d, which is 62% higher than RDA for daily protein intake in healthy adults (Rand et al. 2003) and is within estimates of an optimal protein intake to potentiate RT-induced gains in lean mass (Morton et al. 2018). Our results indicated that while STRT induced a rise in MyoPS above resting, twice daily consumption of the study bar increased protein intake to 1.6 g/kg/d potentiating the anabolic effect of resistance exercise on the muscle (Figure 3). Although all essential amino acids are necessary to support MyoPS, leucine is the anabolic amino acid that activates MyoPS via the mTOR signalling pathway (Devries et al. 2018b; Phillips 2014). Our lab previously reported, despite low-protein consumption (6.25 g), that co-consumption of a

high amount of leucine (~5 g) increased acute MyoPS to the same extent as 25 g of protein in young men (Churchward-Venne et al. 2014). Also, higher leucine supplements (~4.5 g) or diets (~5 g) effectively enhanced acute (Devries et al. 2018a) and integrated MyoPS (Devries et al. 2018b; Murphy et al. 2016) in free-living older adults, regardless of their total daily protein intake. On the other hand, protein dose-response studies have shown an increase in MyoPS plateaus at approximately 0.3-0.35 g of protein/kg in young adults (Cuthbertson et al. 2005; Moore et al. 2009; Yang et al. 2012). The present results indicated that twice-daily consumption of the study bar, which contained 6 g of leucine, potentiated day-to-day integrated MyoPS in young adults even when consuming a diet containing 1.3 g of protein/kg/d. We note that 6 g of leucine stimulates MyoPS (Churchward-Venne et al. 2012; Churchward-Venne et al. 2014; Devries et al. 2018a, 2018b). Given the previous leucine and protein dose-response studies (Devries et al. 2018a, 2018b), we speculated that the potentiated MyoPS response in the STRT+Leu condition was supported in part by an increased intake of leucine and essential amino acid as components of overall increased protein intake.

We investigated the mTORC1 signaling pathway in response to a resistance exercise session during STRT and STRT+Leu. Acute STRT+Leu resulted in a significant increase in phosphorylation of mTORC1 and 4EBP1 one hour following exercise and was not observed in the STRT condition (Figure 4). Furthermore, STRT with consumption of the study bars led to higher phosphorylation of 4EBP1 compared to STRT only (Figure 4D). Peri-workout protein ingestion results in MyoPS stimulation (Cermak et al. 2012; Schoenfeld et al. 2017). Pennings et al. showed that protein intake after exercise resulted in greater use of dietary-derived amino acids for MyoPS in young and older men (Pennings et al. 2011). Fujita et al. (Fujita et al. 2009) reported that protein consumption before resistance exercise increased MyoPS and phosphorylation of the mTORC1 signalling pathway post-exercise. Immediately

following resistance exercise, protein ingestion results in an additional increase in signalling and MyoPS (Dreyer et al. 2008). Thus, it is perhaps not surprising that consuming the study bar before and after resistance exercise in our study potentiated additional anabolic effects by activating the mTORC1 signalling pathway proteins.

Our findings highlight that the anabolic responses to STRT and a moderate daily protein intake over four days improved by ingesting a protein and leucine-enriched bar twice daily. Also, we analysed the acute phosphorylation of mTORC1 signalling proteins one hour following STRT or STRT+Leu. However, because this was a short-term measure of MyoPS and an acute measurement of protein signalling, we are limited in our ability to translate our findings to longer-term resistance training interventions. Due to the untrained nature of the study participants, subsequent 'damage-repair' was likely prevalent but did not necessarily align with functional benefits (Davies et al. 2020; Waskiw-Ford et al. 2020). We did not match total protein intake between conditions and did not directly measure plasma aminoacidemia after consuming the protein and leucine-enriched bar. Our previous study did show pronounced hyperaminoacidemia and leucinemia using the present study bar. (Traylor et al. 2021).

In conclusion, we showed that four lower-body resistance training sessions increased the daily rate of MyoPS. Increased protein intake derived from ingestion of the protein and leucine-enriched bar during STRT potentiated the integrated MyoPS response and stimulated mTOR signalling pathway protein phosphorylation. These findings highlight the effect of the protein and leucine-enriched bar to promote an anabolic response to resistance training and rationale for future research in developing enriched protein formulations for muscle recovery and growth with RT programs.

Acknowledgments

SMP thanks the National Science and Engineering Research Council of Canada and the Canada Research Chairs program, and the Canadian Institutes of Health Research for support. EAN is a Tier 2 Productivity Fellow for the Brazilian National Council for Scientific and Technological Development (CNPq), grant number 308584/2019-8. JCM was supported by an Ontario Graduate Scholarship.

Competing interests statement

Stuart M. Phillips reports having received competitive research funding from the US National Dairy Council (NDC) and Dairy Farmers of Canada during the time of this work. Stuart M. Phillips is listed as an inventor on Canadian patent 3052324 issued to Exerkine and a patent (US) 16/182891 pending to Exerkine (but reports no financial gains). Stuart M. Phillips reports receiving product from Enhanced Recovery but no payment outside the submitted work. CL, DAT, CM, SJ, JM, TG, JCM, TP, EAN and ML declare no conflicts of interest.

Funding statement

This research was supported by a Canadian Institutes of Health Research Catalyst Grant for Musculoskeletal Health and Arthritis (grant No. #384214).

Contributors' statement

CL and DAT contributed equally to this study; Conceptualization, CL, DAT, and SMP; Conducted the research, CL, DAT, CM, SJ, and JM; formal analysis, CL, DAT, JCM, and TP; Writing – original draft preparation, CL, DAT, EAN, and SMP; Writing – review and editing, all authors; Funding acquisition, DAT and SMP.

Data availability

The data underpinning the research are available from the corresponding author on reasonable request.

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535 Tables

536 **Table 1. Characteristics of participants.**

	Participants (n=8)
Sex (M/W)	5/3
Age (yrs.)	22 ± 1
Height (cm)	181 ± 8
Body mass (kg)	87 ± 17
BMI (kg/m^2)	27.1 ± 4.6
LM (kg)	61 ± 8
Body fat (%)	29 ± 11

537 Value are means ± SD, BMI, body mass index; LM, (fat- and bone-free) lean mass; M, men;
538 W, women.

539

540 **Table 2. Dietary intake during intervention.**

	STRT	**STRT+Leu**	**Bar (2/d)**	***P***
Energy (kcal/kg/d)	30±4	33±5	5±1	0.301
Protein (g/kg/d)	1.3±0.1	1.6±0.1*	0.4±0.1	< 0.001
Carbohydrate (g/kg/d)	5±1	5±1	1±0	0.438
Fat (g/kg/d)	1±0	1±0	0±0	0.163

541 Values are means ± SD. * $P < 0.05$, significant difference from STRT. STRT, short-term
542 resistance training; STRT+Leu, STRT with protein and leucine-enriched bar
543 supplementation.

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549 **Table 3. Estimated 1RM and performed training volume for each test.**

	STRT	STRT+Leu	*P*
Estimated 1RM			
Leg press (kg)	93±39	91±39	0.920
Leg extension (kg)	41±39	39±13	0.722
Training volume			
Leg press (kg/set)	663±250	675±251	0.923
Leg extension (kg/set)	244±71	238±68	0.869

550 Values are means ± SD (n=8). STRT, short-term resistance training; STRT+Leu, short-term
551 resistance training combined with protein and leucine-enriched bar supplementation; 1RM,
552 one-repetition maximum.

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555 **Figure captions**

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557 **Figure 1.** Consort 2010 standards of reporting randomized human trials flow diagram of
558 participant enrollment and random assignment to and analysis of study interventions.

559

560 **Figure 2. Schematic overview of study design.** STRT, short-term resistance training;
561 STRT+Leu, short-term resistance training combined with high-leucine-containing protein bar
562 supplementation; RE, resistance exercise; MB, muscle biopsy; PB, protein bar consumption;
563 DXA, dual-energy X-ray absorptiometry; D_2O, deuterium oxide.

564

565 **Figure 3. Integrated fractional synthesis rate (%/d) of vastus lateralis during Rest,**
566 **STRT, and STRT+Leu.** Data are plotted as individual points (closed circles, men; open
567 circles, women) with the mean ± SD (n=8). * $P < 0.05$, significantly different from Rest, # P
568 < 0.05, significantly different from STRT. STRT, short-term resistance training; STRT+Leu,
569 STRT with high-leucine-containing protein bar supplementation.

570

571 **Figure 4. Changes in phosphorylation status of mTOR signaling pathway proteins**
572 **during STRT and STRT+Leu study phases.** The ratio of phosphorylated to total (A)
573 mTOR, (B) p70S6K, (C) S6K, (D) 4EBP1, and (E) representative western blot bands. Data
574 are expressed as mean ± SD with individual data plots (circles, men; squares, women; n=8). *
575 $P < 0.05$, significantly different from pre-test within conditions, # $P < 0.05$, main effect for
576 time. STRT, short-term resistance training; STRT+Leu, STRT with high-leucine-containing
577 protein bar supplementation; Ex. exercise; P/T, the ratio of phosphorylated to total protein
578 expression.

Enrollment of Eligible Participants