Protein Supplementation Does Not Affect Myogenic Adaptations to Resistance Training

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1Division of Rehabilitation Sciences, University of Texas Medical Branch, Galveston, TX; 2Department of Nutrition and Metabolism, University of Texas Medical Branch, Galveston, TX; 3School of Medicine, University of Texas Medical Branch, Galveston, TX; 4Sealy Center on Aging, University of Texas Medical Branch, Galveston, TX; 5Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX; 6DuPont Nutrition and Health, St. Louis, MO

ABSTRACT

REIDY, P. T., C. S. FRY, S. IGBINIGIE, R. R. DEER, K. JENNINGS, M. B. COPE, R. MUKHERJEA, E. VOLPI, and B. B. RASMUSSEN. Protein Supplementation Does Not Affect Myogenic Adaptations to Resistance Training. Med. Sci. Sports Exerc., Vol. 49, No. 6, pp. 1197–1208, 2017. It has been proposed that protein supplementation during resistance exercise training enhances muscle hypertrophy. The degree of hypertrophy during training is controlled in part through the activation of satellite cells and myonuclear accretion. Pro-tein supplementation during training has been shown to enhance myofiber hypertrophy, satellite cell content, and myonuclear addition. In this study, we investigated the efficacy of protein supplementation and the type of protein during resistance training in young healthy men. We propose that as long as protein intake is adequate during muscle overload, the adaptations in muscle growth and function will not be influenced by protein supplementation. Key Words: MUSCLE, HYPERTROPHY, MYOFIBER, PROTEIN TYPE, WHEY, SOY, RESISTANCE, EXERCISE, TRAINING

It has been reported that the amount of protein in the diet does not influence muscle hypertrophy after resistance exercise (RE) training (45). Despite this information, the notion that protein supplements can enhance muscle growth at rest or during exercise training is very popular (39). One reason for this popularity is that several acute studies have shown protein or amino acid ingestion after a bout of RE (see Document, Supplemental Digital Content 1, List of abbreviations, http://links.lww.com/MSS/A866) can enhance muscle protein synthesis (39). On the other hand, there are a limited number of longitudinal studies that have examined whether protein supplementation during RE training (RET) enhances muscle growth and strength as compared with RET without protein supplementation (8,31,35,41).

The prevailing theory for contraction-induced myofiber growth posits that acute and periodic increases in protein synthesis lead to the accumulation of protein and expansion of myofiber volume. Expansion of the myofiber strains the myonuclear domain, the volume of a myofiber maintained by one myonucleus to regulate essential cell function. The addition of myonuclei to a growing myofiber occurs by inducing satellite cell (SC) proliferation and migration to target myofibers. These proliferating SC may then undergo terminal differentiation and fuse to current myofibers as myonuclei (i.e., myonuclear addition) to facilitate additional hypertrophy (16). Additional functions of SC include the maintenance of the myofiber environment and the repair/remodeling of myofibers. Because muscle hypertrophy includes the addition of...
new proteins and myofiber expansion, the idea of myonuclear addition during exercise training to support muscle growth seems logical; however, the exact role and necessity for SC to support hypertrophy through myonuclear addition is controversial (14).

Olsen et al. (32) first demonstrated that RET with protein supplementation may provide enhancement of the SC pool compared with RET alone. Recently, Farup et al. have expanded on these findings by demonstrating that this effect is muscle fiber–type specific, as reported in results from both acute (11) and chronic studies (12). These findings in human skeletal muscle have been supported by basic and preclinical approaches demonstrating that nutrient provision (in particular the leucine metabolite β-hydroxy-β-methylbutyrate) enhances myogenic proliferation via mTORC1 signaling (27,40). Farup et al. (12) also conducted studies to assess the separate effects of contraction mode (concentric vs eccentric) and protein supplementation on myofiber growth and expansion of the SC and the myonuclear pools. However, no study has determined and/or reported the effect of protein supplementation during traditional RET, with concurrent concentric and eccentric muscle action, on expansion of the SC pool and myonuclear addition at the fiber-type–specific level. Using a large cohort of young men, our goal was to determine the role of protein supplementation (and the type of protein used as supplementation) during RET on fiber-type–specific myofiber growth, SC content, and myonuclear addition. We hypothesized that protein supplementation (independent of the type protein used) would enhance myofiber growth and SC and myonuclear content during RET.

**METHODS**

**Participants.** We recruited healthy male participants for this double-blind, randomized clinical trial. Participants were of similar age (protein blend [PB], 24.1 ± 0.6; whey protein [WP], 24.6 ± 1.0; maltodextrin placebo [MDP], 25.2 ± 1.1), height (PB, 176.6 ± 1.5; WP, 180.0 ± 2.1; MDP, 176.0 ± 1.6), weight (PB, 77.5 ± 2.3; WP, 83.5 ± 3.4; MDP, 76.3 ± 1.3), body mass index (PB, 24.3 ± 0.6; WP, 25.7 ± 0.9; MDP, 24.5 ± 0.8), whole-body lean mass (PB, 56.2 ± 1.3; WP, 58.9 ± 2.3; MDP, 55.4 ± 1.7), and leg lean mass (PB, 19.0 ± 0.4; WP, 20.7 ± 1.0; MDP, 18.7 ± 0.8) at pretraining. The subjects included in this study were a subset of a larger clinical trial (38), where screening and enrollment details can be found alongside information concerning treatment compliance, dietary intake, and strength testing. There were no differences between treatments at pretraining for any of the descriptive characteristics (P > 0.10). As our previous report indicated (38), the habitual protein intake for participants was ~1.3 g kg⁻¹ d⁻¹, and the participants increased protein intake in both protein supplementation groups above this level.

The participants were healthy and recreationally active but were not engaged in any regular exercise training program (less than two sessions of high-intensity aerobic or RE/week) at the time of enrollment. All participants gave written informed consent before enrollment in the study. The study was approved by the University of Texas Medical Branch (UTMB) Institutional Review Board and is in compliance with the Declaration of Helsinki as revised in 1983. Of the 70 participants who underwent pretesting, 2 withdrew before undergoing exercise training (WP, n = 1; PB, n = 1), 4 withdrew during the first 6 wk (MDP, n = 3; WP, n = 1), and 6 withdrew during the last 6 wk (MDP, n = 4; WP, n = 2). Of the 58 study completers, snap frozen muscle samples necessary for immunohistochemical analysis could not be obtained for four participants. Thus, all the data provided herein are from the 54 completers for whom we have data on the primary immunohistochemical outcomes (PB, n = 22; WP, n = 15; MDP, n = 17).

**Study design.** The study design can be found in more detail elsewhere (38), briefly, after enrollment, completion of a run-in period consisted of the pretraining study day at UTMB, and then three nonconsecutive days of exercise familiarization and before one-repetition maximum (1-RM) strength testing at the UTMB Alumni Field House. The pretraining study day included the assessment of body composition, a muscle biopsy, and an isometric and isokinetic strength test of the thigh as we have previously described in the initial clinical trial (38). Soon after, the participants reported to the UTMB Alumni Field House for familiarization/testing before beginning 12 wk of training. After 12 wk of training, participants were retested exactly 3 d after the final exercise session of the training program. For the posttesting, participants reported to the Institute for Translational Science Clinical Research Center at the same time in the morning as for the pretraining study day to repeat the same laboratory tests and sample collection.

**Pre- and posttesting study days.** Participants reported to the Institute for Translational Science Clinical Research Center at UTMB in the morning after an overnight fast. They were instructed to refrain from any medication that affects muscle metabolism, and also caffeine, supplements, and alcohol for several days before testing. After arrival on the unit, anthropometric tests and a dual-energy x-ray absorptiometry (DXA) scan were completed (Hologic ADR 4500W, Bedford, MA).

All the pretesting exercise familiarization and the 1-RM testing were conducted after the muscle biopsy. A percutaneous biopsy sample of the VL muscle was performed using a 5-mm Bergström biopsy needle with suction, under sterile procedure and local anesthesia (1% lidocaine). The sample was aliquoted and snap frozen in liquid nitrogen and stored at −80°C for future analysis. Suitable muscle samples where orientation was apparent were carefully laid on Tissue Tek Optimal Cutting Temperature (OCT; Thermo Fisher Scientific, Rockford, IL) affixed to cork, submerged in liquid nitrogen-cooled isopentane and then placed on dry ice until they could be stored at −80°C until subsequent immunohistochemical analysis. After isometric and isokinetic knee extension and flexion strength testing on a dynamometer, participants were fed a meal before leaving the unit. All testing was repeated on the posttesting day in the same order.
RET. After familiarization and 1-RM strength testing, participants began a 12-wk whole-body progressive RET program as we have previously described in the initial clinical trial (38). All exercise training sessions were performed at the UTMB Alumni Field House. Exercise sessions were performed on nonconsecutive days, three times weekly, with four rest days per week, under supervision of qualified personal trainers. Participants were allowed to maintain their recreational physical activity but were instructed not to do any other strength training outside of the study. To allow for unforeseen life events, participants were given 13 wk after the familiarization period to complete 36 exercise sessions. This allowed for 100% exercise compliance.

Supplementation. Participants were randomized (20 per group) to the placebo (MDP), whey (WP), or blend (PB) isocaloric treatments as previously described (38). The PB and the WP treatments were isonitrogenous and pooled to reflect protein supplementation (PRO) overall. Immediately after each workout, under direct observation of the study personnel, the participants ingested either the placebo beverage or one of the protein supplements to which they were assigned. On the four resting (nonexercise) days each week, the participants ingested the placebo or supplement one time between meals. Participants were instructed to refrain from any other food or macronutrient-containing beverage for 2 h before or after exercise or supplementation.

WP and PB samples were provided by DuPont Nutrition and Health (St. Louis, MO) and were independently tested for amino acid profile. The soy–dairy blend (PB) was composed of 25% soy protein isolate, 25% WP isolate, and 50% sodium caseinate. The whey (WP) treatment consisted of 100% WP isolate, and carbohydrate placebo (MDP) was an isocaloric maltodextrin mixture. The dose for the two protein nutritional supplements was ~22 g protein per day; thus, the leucine content was 2.00 g for the PB and 2.31 g for the WP. Supplements were separated into individual ready-made packets for daily consumption, and participants were given a 2-wk supply. The personal trainer collected the empty supplement packets from each subject every 2 wk. Supplements and placebo were given in powdered form and dissolved in 300 mL water to ensure a rapid and predictable absorption.

RNA concentration. Total RNA was isolated by homogenizing 10–20 mg of tissue with a handheld homogenizing dispenser (T10 Basic Ultra Turrax, IKA, Wilmington, NC) in 1 mL of Tri reagent. The RNA was separated into an aqueous phase using 0.5 mL of 75% ethanol, air-dried, and suspended in a total volume of the aqueous phase. RNA was washed twice with 1 mL of 75% ethanol, air-dried, and suspended in a known amount of nuclease-free water. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Immunohistochemistry. Immunohistochemical techniques were conducted as previously described (15). Samples were removed from the cork at −25°C in a ThermoFisher Cryostat (Fisher Scientific HM 525X) where they were cut in 7-μm cross sections. Pre- and postsamples for the same subject were placed on the same slides Fisherbrand Superfrost®/Plus microscope slides (Fisher Scientific, USA). Two slides were generated per subject, one for analysis of myofiber myosin heavy chain (MHC) typing and cross-sectional area (CSA) and the other for fiber-type–specific SC and myonuclear content. After cutting, a hydrophobic marker (Vector, H-4000, Burlingame, CA) separated the sections, which were dried at room temperature (RT) and then stored at −20°C until analysis.

Myofiber MHC type and CSA were determined as follows. Sections were rehydrated in phosphate-buffered saline (PBS) for 2 × 5 min at RT. Slides were incubated for at least 1 h at RT and then overnight at 4°C with primary antibodies, mouse anti-MHC type I (BA.D5 IgG2b, 1:50, Developmental Studies Hybridoma Bank, Iowa City, IA) in a 1:1 ratio of supernatant with mouse anti-MHC Ila (SC.71 IgG1, Developmental Studies Hybridoma Bank) and mouse anti-MHC IIX (6H1 IgM, Developmental Studies Hybridoma Bank). Slides were rinsed three times for 5 min each with PBS followed by 1 h incubation with secondary antibodies diluted in PBS, Alexa Fluor 488 conjugated goat antimouse IgG1 (for MHC Ila: 1:500, no. A21121; Invitrogen, Carlsbad, CA), Alexa Fluor 647 conjugated goat antimouse IgG2b (for MHC I: 1:250, no. A21242; Invitrogen), and Alexa Fluor 594 goat antimouse IgM (for MHC IIx: 1:250, no. A21044; Invitrogen) at RT in the dark. Slides were rinsed 3 × 5 min each with PBS, before and after a 5-min postfix in methanol. Slides were mounted with fluorescent mounting media (Vector, H-4000) and dried before imaging. Staining procedures resulted in MHC Ila staining green, MHC I staining purple, and MHC type IIx staining red (Fig. 1). Images for fiber typing were captured at 100 × magnification using a fluorescence microscope (Axio Imager.M1m, Carl Zeiss, Toronto, Ontario, Canada) and AxioCam MRm camera (Carl Zeiss). Image processing and analysis was done using AxioVision 4.8.2 software. For each image, the number of muscle fibers for pure MHC types I, Ila, and IIx and hybrid types I, I/IIa, I/Ila, IIa/Ila/IIX, and I/Ila/IIX fibers was counted, and the CSA for MHC types I, Ila, IIa/Ila/IIX, and I/Ila/IIX fibers was measured. Fibers with frequencies less than 1%–2% (pure IIx and hybrid I/Ila and I/IIX) were removed from further analysis because of insufficient data for analysis. Hybrid denotes all hybrid groups combined. T2 represents all MHC II (Ila + Ila/IIX) isoforms pooled together. Approximately 250 muscle fibers were analyzed for fiber-type distribution and 200 for CSA in each sample.

Fiber-type–specific SC and myonuclei were determined as follows. Sections were fixed in ice cold acetone for 3 min followed by three 3-min rinses in PBS. Sections were incubated for at least an hour at RT and then overnight at 4°C with primary antibodies against MHC I (BA.D5 IgG2b, 1:50, Developmental Studies Hybridoma Bank) and Laminin (L9393, 1:200; Sigma-Aldrich, St. Louis, MO). On day 2, three 5-min washes in PBS preceded a 7-min H2O2 treatment (3% in PBS) to block endogenous peroxidases. After three 3-min rinses in PBS,
sections were incubated for 1 h with secondary antibodies: Alexa Fluor 647 conjugated goat antimouse IgG2b (for MHC I: 1:250, no. A21242; Invitrogen) and Alexa Fluor 594 goat antirat IgG1 (for laminin: 1:500, no. A11034; Invitrogen) diluted in PBS at RT in the dark. After three 3-min rinses in PBS, sections were blocked for 1 h at RT and then overnight at 4°C with a primary antibody against mouse anti-Pax7 (1:100, Developmental Studies Hybridoma Bank). On day 3 of staining, sections were rinsed 4 × 5 min with PBS before and after 1 h incubation with goat antimouse IgG biotin–SP-conjugated (1:1000) (Jackson Immuno Research, cat no. 115-065-205) in 2.5% normal horse serum (for Pax7) at RT. Sections were exposed to a 1-h incubation of Streptavidin–horseradish peroxidase conjugate (1:100) in PBS, washed, 3 × 5 min in PBS, and incubated for 20 min in Alexa Fluor 488 (1:200, Tyramide signal amplification kit, no. T20932; Invitrogen) in amplification diluents. After three 5-min washes in PBS, sections were mounted in 4',6-diamidino-2-phenylindole (DAPI) containing medium mounting media (Vector, H-1200) and allowed to air dry. This staining protocol of muscle fiber-type–specific identification resulted in DAPI-positive nuclei (staining blue), Pax7+ cells (staining yellow), MHC I (staining purple), MHC II (black, negative staining), and laminin basement membrane (staining red) (Fig. 2).
Myonuclei were manually counted in images captured at 100× magnification using AxioVision 4.8.2 software to determine the number of myonuclei per fiber. A nucleus was identified as a myonucleus (DAPI+/Pax7+) if it met one of the following criteria: 1) it was clearly located within the laminin boundary, 2) it was on the boundary facing inside the fiber, or 3) greater than 50% of the area fell inside the laminin boundary. Rapid, repeated manual switching back and forth between single channel laminin images and merged laminin/DAPI images was used to determine the location of a nucleus as inside or outside of the laminin boundary. After counting of myonuclei within an image, fiber number was quantified manually to express the number of myonuclei per fiber specific to each fiber type (MHC I or II). Pax7+ nuclei/myofiber, % SC, myonuclei per fiber, and myonuclear domain (fiber area per myonucleus) were determined from >200 cross-sectional muscle fibers at each time point.

Statistical analysis. Values are the raw values or change scores expressed as mean ± SEM or mean ± 95% confidence interval as indicated. Primary outcome data were evaluated for equal variances and normality, and no major violations of model assumptions were found. For each outcome, a mixed model ANOVA with fixed effects of treatment, time, and a treatment-by-time interaction was conducted. Subject was treated as a random effect, and time points (e.g., before and after) were incorporated into each outcome's mixed model. ANCOVA was conducted on posttraining with treatment as the main effect and pretraining as the covariate. To test the effect of protein supplementation, we pooled the protein treatments WP and PB as PRO. An additional model was conducted with treatment effects of PRO and MDP only. Alpha was set to 0.05 for significance, but P values between 0.05 and 0.1 were considered indicative of a trend. When interactions were found to be significant (P < 0.05) or a trend (P < 0.1), Tukey HSD pairwise comparisons were conducted to compare time points and treatments broken down by treatment level and time point, respectively. All analyses were conducted with R 3.1.1. (Vienna, Austria), except Pearson correlations, which were calculated with Graph Pad Prism 6.0f for Mac (La Jolla, CA). Effect size and sample size estimations for significant effects of PRO supplementation were calculated as previously described (39). All figures were generated with the same program.

RESULTS

Lean mass and strength. Muscle hypertrophy was observed at the whole muscle level. Percent change in DXA
whole-body lean mass was increased with all treatments (PB, 5.23% ± 0.52%; WP, 4.08% ± 0.52%; MDP, 3.40% ± 0.80%) ($P < 0.05$), and there was a trend with PB supplementation showing a greater increase in lean mass than MDP ($P = 0.057$). Combined results from pooling treatments with the two protein supplements (PRO) also showed a significant treatment effect ($P = 0.050$) compared with MDP. Percent change in DXA leg lean mass significantly increased across all treatments (PB, 6.03 ± 0.95; WP, 4.67 ± 0.89; MDP, 4.64 ± 0.88) ($P < 0.05$) and was not different by treatment ($P = 0.444$). Thigh circumference (cm) in PB (pretraining, 50.15 ± 0.78; posttraining, 52.43 ± 0.66), WP (pretraining, 51.1 ± 1.15; posttraining, 52.60 ± 1.07), and MDP (pretraining, 51.9 ± 0.52; posttraining, 53.26 ± 1.29) were increased ($P < 0.05$) and were not different across treatment ($P = 0.454$).

At the pretraining time point, isometric and isokinetic peak torque (relative to body weight) and power for flexion and extension were not different ($P > 0.10$) between treatments (data not shown). Isometric knee extension torque (N·m) increased (PB, 34.8 ± 9.8; WP, 44.9 ± 13.6; MDP, 34.0 ± 10.3) across time ($P < 0.001$), but there were no treatment effects ($P > 0.10$) or interactions ($P > 0.10$). Isometric (PB, 8.9 ± 7.0; WP, 9.4 ± 7.6; MDP, 14.6 ± 6.0) and isokinetic (PB, 3.8 ± 6.0; WP, 13.5 ± 9.6; MDP, 7.4 ± 6.6) knee flexion torque did not change across time or demonstrate a treatment effect or an interaction ($P > 0.10$). Isokinetic knee extension torque differed over time ($P < 0.05$) and demonstrated an interaction and a treatment effect ($P < 0.05$). Thus, isokinetic knee extension torque did not change in subjects treated with MDP (1.0 ± 5.8), but treatment with WP (15.9 ± 6.5) and WP (28.8 ± 7.5) similarly resulted in an effect of protein (PRO) that was present compared with MDP for torque ($P < 0.05$). Also, for isokinetic knee extension torque, the change in subjects treated with WP was greater than the change after treatment with MDP ($P < 0.05$).

**Muscle RNA concentration.** A proxy for translational capacity, vastus lateralis RNA concentration was not different at baseline (PB, 0.559 ± 0.013; WP, 0.570 ± 0.015; MDP, 0.592 ± 0.014 μg RNA per milligram of muscle) and was increased ($P < 0.05$) with RET in each treatment (PB, 0.055 ± 0.018; WP, 0.059 ± 0.019; MDP, 0.072 ± 0.019 μg RNA per milligram of muscle), but did not differ by treatment or demonstrate an interaction ($P > 0.10$).

**Vastus lateralis MHC fiber-type composition.** MHC II and MHC hybrid fiber-type compositions were different by time ($P < 0.05$) with no effect of treatment or an interaction ($P > 0.10$). The pre- and posttraining MHC fiber-type composition (Fig. 3, Table 1) changes showed reduction in hybrid fibers, in all treatments ($P < 0.05$). The reduction in hybrid fibers resulted in a shift toward more pure MHC IIa fibers that was significant for PB and PRO treatments ($P < 0.05$). Myofiber MHC type I frequency remained unchanged ($P > 0.10$) and did not differ by treatment ($P > 0.10$).

**Vastus lateralis myofiber CSA.** Vastus lateralis myofiber CSA means (Fig. 1, Table 1) were increased after RET as

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<td>PRO</td>
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Boldface indicates $P < 0.05$, underlined $P < 0.10$ vs pretraining for that treatment or comparison. TRT, treatment.

*Data are presented as mean ± 95% confidence interval or SEM, n = 115 (WP), 22 (PB), and 17 (MDP).
revealed by difference by time (P < 0.05) with no treatment or interaction effects (P > 0.10). Mean fiber area of all fiber types was increased ~800–900 μm² after RET (P < 0.05). However, there was no effect of treatment (P = 0.967). Mean MHC I CSA was increased ~500 μm² after WP and PB treatment and ~750 μm² after supplementation of MDP after RET (P < 0.05). There was also no effect of treatment (P = 0.721). Individual treatment changes revealed significant increases after treatment with PB and MDP (P < 0.05) and a trend for an increase (P = 0.083) after treatment with WP. Mean MHC Ila CSA was increased ~900–1000 μm² after RET (P < 0.05) with no ANCOVA effect of treatment (P = 0.921). Mean fiber area (MFA) of all hybrid fiber types increased ~1000–1100 μm² after RET (P < 0.05) with no effect of treatment (P = 0.906). PRO (PB + WP) treatment displayed significant increases in all fiber types (P < 0.05). No significant effect of PRO vs MDP treatment was observed in any fiber type (P > 0.423).

Analysis of CSA relative frequency distribution demonstrated that all treatments displayed myofiber growth (rightward shift) (see Figure, Supplemental Digital Content 2, Relative frequency of vastus lateralis MHC I, II, and all myofibers pooled by select CSA bins, http://links.lww.com/MSS/A867). However, few papers report that MHC II fiber types are responsive to protein supplementation (12,19) (see Table, Supplemental Digital Content 3, Summary of all protein supplement studies with a placebo group directly assessing muscle mass during RET in young adults, http://links.lww.com/MSS/A868). Thus, we explored the frequency distributions of MHC II myofibers to determine whether slight patterns for differences between treatments not observed with CSA means were evident. CSA bins were expanded to reflect changes in large myofibers (CSA bins in myofibers larger than 6000, 7000, 7500, and 8000 μm²) and also smaller fibers (CSA bins in myofibers sized 1000 to 5000 μm²) after RET (see Figure, Supplemental Digital Content 4, Change in the relative frequency of larger vastus lateralis MHC II myofibers by select cross-sectional area bins, http://links.lww.com/MSS/A869). Similar to CSA means, this analysis revealed time differences (P < 0.001) with no treatment or interaction effects (P > 0.10), except the smaller bin (myofibers sized 1000 to 5000 μm²), which had a treatment effect (P = 0.031). This treatment difference was shown as a greater absolute frequency of smaller myofibers at pretraining (P = 0.020) and posttraining (P = 0.095) for MDP vs PRO treatment. All treatments demonstrated clear decreases (P < 0.001) in the frequency of smaller MHC Ila myofibers (P < 0.007). Treatment with (PRO: pooled PB and WP) resulted in clear increases (P < 0.001) in the frequency of larger MHC Ila myofibers, whereas increases after treatment with MDP were less evident (P = 0.064 to P = 0.535). When examining these larger myofiber CSA bins, only tendencies approaching statistical significance (P = 0.098–0.194) were observed for an effect of protein (PRO) treatments vs MDP treatment.

Effect size and sample size estimations for significant effects of PRO supplementation during RET on body composition, strength, and muscle mass are shown in Supplemental Digital Content (see Table, Supplemental Digital Content 5, Effect size and sample size estimations for significant effects of PRO supplementation, http://links.lww.com/MSS/A870).

**Vastus lateralis SC content.** Vastus lateralis myofiber Pax7+ SC content displayed a main effect of time (P < 0.05), which was evident by a ~50% increase in abundance after RET (Fig. 2, Table 2). There were no interactions or effects of treatment with any of the SC outcomes. Because the responses in the PB and WP treatments were identical, the treatments were pooled as PRO and tested against changes in MDP. Mean fiber SC content (SC/fiber), proportion (% SC/myonuclei), and domain (SC/mm²) increased after RET (P < 0.05) with no effect of treatment (P > 0.588). This increase was driven primarily by changes in MHC II myofibers. MHC II SC content (SC/fiber), proportion (% SC/myonuclei), and domain (SC/mm²) increased after RET (P < 0.05), and there was no effect of treatment (P > 0.575). MHC I SC content (SC/fiber) was not globally altered across time (P < 0.05), but there was a trend for an effect of PRO treatment versus MDP treatment (P = 0.073). MHC I SC proportion (% SC/myonuclei) and domain (SC/mm²) were unchanged after RET (P > 0.10). SC domain (SC/mm²) displayed a trend for an effect of PRO treatment vs MDP treatment (P = 0.072). MHC I SC proportion

### Table 2. Pre- to posttraining absolute change for Pax7+ SC immunohistochemical analysis.

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<th>TRT</th>
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<th>ANCOVA Estimates</th>
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Boldface indicates P < 0.05, underlined P = 0.10 vs pretraining for that treatment or comparison. TRT: P < 0.05 for an overall change over time. TRT, treatment.

aData are presented as mean ± 95% confidence interval, n = 115 (WP), 22 (PB), and 17 (MDP).
(\% SC/myonuclei) displayed a weak trend for an effect of PRO treatment vs MDP treatment (P = 0.099).

**Vastus lateralis myonuclei.** Vastus lateralis myofiber myonuclei content and domain (Fig. 2, Table 3) were altered by RET. Pre- to postchanges in all treatments pooled together demonstrated an increase (P < 0.05) with RET. Thus, a main effect of time was seen for MHC I, MHC Ia, and mean myonuclei content (P < 0.05). There were no interactions or effects of treatment with any of the myonuclei outcomes (P > 0.10). Because the responses in the PB and WP treatments were identical, the treatments were pooled as PRO and tested against changes in MDP Mean myonuclei content (MyoN/ fiber) increased after RET (P < 0.05) with no effect of PRO vs MDP treatment (P = 0.602). The changes in MHC II fibers exerted the greatest influence on the mean myofiber MyoN response. The effect of time was observed as a trend for an overall increase from pre- to posttraining (P = 0.096) in MHC II fibers, which was seen as an increased change score (P < 0.05) with no PRO vs MDP treatment effect (P = 0.378). MHC I myonuclei content did not show changes from pre- to posttraining, except with PRO (P < 0.05) and a PRO treatment effect vs MDP was not evident (P = 0.143).

Myonuclear domain (Fig. 2, Table 3) (MHC II myofibers and all myofibers pooled) demonstrated a slight increase after RET (P < 0.05), whereas MHC I myofibers were not significant (P > 0.10). There were no interactions or treatment effects (P > 0.10). Overall increases from pre- to postraining were evident in MHC II myofibers (P < 0.05) and demonstrated as a trend (P = 0.086) when all myofiber types were pooled. Both PRO and MDP treatments increased myonuclear domain (P < 0.05) in MHC II myofibers. When pooling all fiber types, a significant increase was indicated in treatment with PRO (P < 0.05) and a trend after treatment with MDP (P = 0.084). This increase was likely due to greater statistical power by grouping a greater number of myofibers with PRO. No changes were observed in MHC I fiber myonuclear domain (P > 0.10). There was no significant effect in the change of PRO over MDP in all fibers pooled (P = 0.746), MHC I (P = 0.880), or MHC II (P = 0.379) myofibers.

**Correlational analysis.** Associations and statistical results between measures of muscle hypertrophy (lean mass, myofiber CSA, and muscle thickness) and SC, myonuclei, and myonuclear domain are shown in Supplemental Digital Content (see Tables, Supplemental Digital Content 6, Correlation analysis between myofiber cross-sectional area and myonuclear number, http://links.lww.com/MSS/A871; and Supplemental Digital Content 7, secondary correlation analysis, http://links.lww.com/MSS/A872). Myonuclei number per fiber was highly correlated with fiber size at each time point and in all fiber types and with the increase in myofiber size. Myofiber number per fiber change was well correlated to CSA change and also to the change in SC per fiber. Those who had smaller myonuclear domains at pretraining experienced expansion of their myonuclear domain at postraining. Also, those participants with the largest CSA changes also experienced the greatest expansion of their myonuclear domain. Absolute values of lean mass but not change values correlated with myofiber size. There was a moderate association for MFA change to positively correlate with all SC per fiber change, which was prominent in MHC I, but not MHC II fibers.

**DISCUSSION**

This is the first study reporting the role of protein supplementation and protein supplementation type on fiber-type-specific adaptations of myofiber growth, SC, and myonuclei during traditional progressive resistance training using shortening and lengthening contractions. We demonstrated a similar increase in myofiber CSA, SC content, and myonuclear addition for all three treatment groups. This study was a follow-up analysis to our initial clinical trial (38), where we demonstrate minimal trends for group differences in whole-body and arm-specific lean mass (PB > MDP), yet no differences in the increases in leg lean mass or vastus lateralis muscle thickness. Collectively, these data suggest that the additional lean mass in the PB group was accrued in other locations (arms or trunk) and/or that leg hypertrophy had peaked for all treatments after 3 months of RET with our protocol. This indicates that protein supplementation during RET did not enhance muscle-specific adaptations in the lower limb.

Unfortunately, most of the studies claiming an effect of protein to enhance muscle adaptations to RET rely on whole-body lean mass. Indeed, in examination of the literature, we have previously highlighted that ~62 to ~140 participants (depending on the clinical trial, effect size = 0.24–0.67) would be needed to find a statistical effect of protein supplementation on

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**TABLE 3. Pre- to postraining absolute change for myonuclei immunohistochemical analysis.**

<table>
<thead>
<tr>
<th>TRT</th>
<th>MyoN/fiber</th>
<th>Change</th>
<th>ANCOVA Estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myonuclear domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>107 (63 to 276)</td>
<td>54 (193 to 301)</td>
<td>26 (120 to 173)</td>
</tr>
<tr>
<td>II</td>
<td>187 (45 to 329)</td>
<td>266 (18 to 434)</td>
<td>4 (178 to 186)</td>
</tr>
</tbody>
</table>

Boldface indicates P < 0.05, underlined P < 0.10 vs pretraining for that treatment or comparison. TRT, treatment.

Data are presented as mean ± 95% confidence interval, n = 115 (WP), 22 (PB), 17 (MDP), and 37 (PRO).
whole-body lean mass or fat-free mass (39). Similar to these studies, we report an effect for protein supplementation to increases whole-body lean mass as compared with placebo (effect size = 0.565), yet we found similar changes in more direct measures of muscle hypertrophy (ultrasound muscle thickness (38), leg lean mass, and myofiber-type–specific CSA) after RET. As we previously highlighted (38,39), the use and interpretation of whole-body lean mass DXA data to act as the sole measure of muscle hypertrophy is suspect and would likely have limited functional relevance on force production.

Our WP treatment demonstrated similar adaptations when compared with MDP. Contrary to popular dogma, it is not unusual to observe no effect of protein supplementation, in particular WP, over placebo on lean mass or myofiber CSA (39). A meta-analysis determined that protein supplementation during RET in young adults will produce greater increases in vastus lateralis CSA, ~250 μm², yet that analysis only included data from four studies and is in conflict with results from another meta-analysis (41). We are aware of only three studies demonstrating greater changes in vastus lateralis myofiber CSA (2,12,19) and two studies with magnetic resonance imaging (13,21) comparing protein versus carbohydrate placebo supplementation during RET. In one of the vastus lateralis myofiber CSA studies, the placebo group started with higher CSA and did not experience hypertrophy after RET (2), whereas the other two studies demonstrated this effect only in MHC II fibers (12,19). In comparison, five other studies demonstrated equivalent increases in vastus lateralis myofiber CSA in protein-supplemented treatments (WP, n = 13; milk, n = 11; EAA, n = 11) and carbohydrate placebo treatments (6,9,23,31,32). In addition, studies using magnetic resonance imaging of the biceps (10) or latissimus dorsi (33) and ultrasound (3,4,22,23,48) of the thigh muscles have clearly shown the same pattern: no effect of protein supplementation (whey) to enhance vastus lateralis muscle hypertrophy. Given these findings, it is no surprise that only one study on protein supplementation showed an enhancement of strength, although myofiber CSA was not different with protein supplementation (9). The remainder of the studies demonstrate identical increases in strength with protein supplementation compared with carbohydrate placebo (2,6,10,12,13,19,21–23,31–33), similar to our observations, in part. Indeed, in examination of the carbohydrate placebo (2,6,10,12,13,19,21–23,31–33), similar in strength with protein supplementation compared with carbohydrate placebo supplementation during RET. However, we stress that this effect is minimal, and given the low statistical confidence seen in these examples, we believe this effect is limited to a subpopulation of myofibers/individuals that is likely an example of responder/nonresponder clustering. The functional relevance of this finding is unknown. However, a minimal effect of protein supplementation to increase whole-body lean mass (not limb/appendicular lean mass) after RET does exist, which we have speculated (39) may likely include nonforce producing lean mass (e.g., trunk muscle). This would result in increased body weight with similar changes in muscle mass. Thus, to maintain the mass-to-strength ratio, this pattern with MHC II myofibers may provide support for increased muscle force to serve as a compensatory mechanism and offset the increased weight. In partial support of this concept, we demonstrated improved isokinetic torque in the protein-supplemented treatments only, suggesting a possible role for the changes in these MHC II fibers with protein supplementation.

Although, SC is not necessary to support hypertrophy through myonuclear addition (14), they are involved in the magnitude of muscle growth (5,14,37). Given that protein supplementation was also thought to influence the magnitude of muscle growth, we sought to examine the role between SC and protein supplementation. Very little research has examined the acute effects of protein/amino acids on the enhancement of SC content after RE. We were aware of one study that used a severe 4-d protein restriction protocol to compare normal (~90 g) versus very low (~11 g) of protein per day, to find no effect on skeletal muscle SC content during the 3-d recovery period after RE (43). It is hard to find relevance in that study design to our findings other than an overall lack of effect of protein to enhance myogenic adaptations. Olsen et al. (32) first demonstrated that chronic RET with protein supplementation may provide a slight enhancement
of the SC pool compared with RET alone. On the basis of basic science and preclinical findings, we anticipated that protein supplementation would enhance SC activity and content through mTORC1 (17,40) and particularly on MHC II fibers (1,11). Instead, we demonstrated similar increases in SC content between treatments, which were driven primarily through increases in MHC II fibers. However, we did demonstrate a significant increase in SC number per fiber for MHC I fibers with protein supplementation but not with an MDP. This resulted in a trend for an effect of protein (P = 0.073) over MDP, which was also seen when expressed as SC per square millimeter and proportion of SC/MyoN. Interestingly, MHC I, but not MHC II, SC number per myofiber change was correlated with CSA change. Farup et al. demonstrated similar findings, to ours, after 3 months of RET with protein supplementation in MHC I, but not MHC II fibers, suggesting that protein supplementation may provide greater expansion of the SC pool in this fiber type to regulate myofiber growth. Taken together, these findings are somewhat contradictory, although they may be explained as differences between preclinical and clinical research. MHC II fibers are thought to be most responsive to heavy strength training (47), yet the training program we utilized was whole-body, high-intensity training, which likely recruited all fiber types. We also discovered that those who had lower initial SC content in MHC I fibers experienced the greatest change in MHC I SC per fiber (r = −0.529, P < 0.001) and MHC I myonuclei per fiber (r = −0.383, P = 0.006). However, this effect was absent in MHC II fibers. These data suggest that myonuclear addition was a primary fate of SC in MHC I fibers. Our data are in agreement with Bellany et al. (5) but in contrast with a previous report (37) in the literature, suggesting that a higher pretraining SC content is a characteristic of high-responders to RET. We are unsure as to why this difference exists in the literature, but we suspect that the differences could be explained by the use of different markers of SC (NCAM for Petrella et al. and Pax7 in this manuscript and with Bellany et al.)

Myonuclear accretion occurred with RET, as has been previously demonstrated (36), but was not different by treatment as has been demonstrated elsewhere (33,44). A significant increase was seen with PB and WP but not MDP treatment. Others have suggested that CSA changes greater than ~15% are needed before changes in myonuclear number occur (25,36). Here we demonstrated 15%–20%, ~20%, and 20%–30% increases in CSA of MHC I, II, and hybrid fibers, respectively, suggesting that our larger sample size included enough participants with substantial changes in CSA to detect changes in myonuclear number with RET. Myonuclear number was highly correlated with fiber size at each time point and in all fiber types (r = 0.724–0.826, P < 0.001), illustrating remarkable control of the myonuclear domain, as others have shown (20,24,26,29,30).

Even with such tight coupling of myonuclear number to myofiber size, we observed a slight but significant expansion of the myonuclear domain, -150 μm² per myonucleus, after 3 months of RET. In fact, a significant, inverse relationship (r = −0.634, P < 0.001) was demonstrated, indicating that those with smaller initial myonuclear domains experienced the greatest change in myonuclear domain over the course of the training. This effect was most evident in MHC II fibers, highlighting their remarkable plasticity to this contractile stimulus. Maintenance of this expanded domain was likely assisted by increased total RNA content (translational capacity), and through increases in myonuclear size, as demonstrated by Cabric et al. (7) in human skeletal muscle after 3 wk of electrical stimulation. This would suggest enhanced transcriptional capacity per myonucleus.

Certainly, many studies, including those from our laboratory, have clearly demonstrated a robust effect of protein/amino acids to stimulate the early metabolic response of muscle growth (i.e., muscle protein synthesis) (39). The question persists as to why these effects are not as readily discovered in physiological outcomes after chronic exposure to such a stimulus (35). Our hypothesis is that physiological adaptation may best explain the insensitivity to protein supplementation typically seen in chronic exercise studies. Farup et al. (11) demonstrated that WP supplementation after eccentric exercise accelerated the SC pool expansion compared with consumption of carbohydrate placebo. However, by 168 h postexercise (11) and after 12 wk of training (12), the SC pool was identical between treatments. For novice exercisers, peak SC activity occurs after 2 wk of RET (18). Also, some evidence suggests that the majority of the SC pool expansion occurs early, ~4 wk into RET, during dietary supplementation (32). These data suggest that protein supplementation may provide an enhancement early during exercise training, but additional protein is unlikely to confer added benefit to further promote muscle growth as adaptation occurs. Interestingly, this timeframe is also when most myofiber damage and remodeling is likely to occur. Although attractive, this hypothesis has not yet been clearly proven (34,35). Protein metabolism also becomes more efficient after resistance training (80, 81), which provides further support that in the presence of a well-balanced diet, muscle hypertrophy, and strength are not further augmented by protein supplementation (35).

Limitations. A limitation to this study is that several samples from the WP group were not suitable for immuno-histochemical analysis and as a result the sample size of that group was smaller than the size of the other treatments. It is possible that we were slightly underpowered in our ability to determine certain exercise effects (myonuclear domain or number); however, statistical analysis clearly demonstrated an absence of treatment differences in most outcomes, suggesting that sample size was not an issue in delineating treatment effects. It was not feasible for us to sample at earlier time points throughout the training, although this may have provided greater insight into the effect of protein supplementation. This would have allowed a preferential examination of SC content, myonuclear domain, and myonuclear addition during RET. Also, although many of the inferences were made using correlational analyses, a major strength of this study is that a cohort of this size makes correlational analysis possible and generates additional research questions.
The majority of similar studies were collected after biopsies at 24–48 h after the last exercise session; however, we took our samples at 72 h postexercise. It could be hypothesized that this 72-h time point was examining the acute effects of the last exercise session. We found only one paper that examined the acute response (in the trained state) demonstrating an increase in SC content at 72 h after exercise and a return to basal–pretraining values at 96 h (4 d) posttraining. This recent study may suggest that our postbiopsy effects could be due to the acute exercise response. However, there are several studies in the literature with conflicting results showing that increases in SC content are detected 4 d post-RET (28,42,46) and after 10 d of detraining (25). These conflicting data suggest that the timing of sampling for SC studies should be an important consideration in designing these studies.

CONCLUSIONS

Daily supplementation of protein during RET did not enhance muscle adaptations in the vastus lateralis as demonstrated by the nearly identical increases in muscle strength, hypertrophy (whole muscle and myofiber-type specific), MHC II SC content, and overall myonuclear addition. When results from the soy–dairy PB and WP treatments were pooled, very modest effects of protein supplementation existed to enhance MHC I SC content, isokinetic torque, and a slight expansion of a greater proportion of larger MHC II fibers over placebo after RET. We conclude that protein supplementation during RET has a modest effect on promoting a larger gain in whole-body lean mass as compared with exercise training without protein supplementation. However, protein supplementation does not enhance RE-induced increases in myofiber hypertrophy, SC content or myonuclear addition in young healthy men. We propose that as long as protein intake is adequate during muscle overload the adaptations in muscle growth and function will not be influenced by protein supplementation. Future work should focus on the effectiveness of protein supplementation or increasing daily protein intake in clinical populations undergoing significant muscle atrophy.

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B. B. R., E. V., P. T. R., R. R. D., M. B. C., and R. M. designed the research; P. T. R., C. S. F., B. B. R., S. I., M. S. B., and R. R. D. conducted research; B. B. R., E. V., P. T. R., S. H. H., R. R. D., C. S. F., K. J., M. B. C., and R. M. reviewed the manuscript; P. T. R., R. R. D., M. S. B., K. J., and B. B. R. analyzed data; and P. T. R. and B. B. R. wrote the manuscript and had primary responsibility for final content. M. B. C. and R. M. were not involved with conducted research or laboratory analysis. All authors read and approved the final manuscript.

The authors declare that this study was funded by Dupont Nutrition and Health. Representatives from Dupont Nutrition and Health were not involved with data collection and laboratory analysis.

The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

REFERENCES


2. Andersen LL, Tufekovic G, Zebis MK, et al. The effect of resistance training combined with timed ingestion of protein on muscle hypertrophy and function will not be influenced by protein supplementation. Future work should focus on the effectiveness of protein supplementation or increasing daily protein intake in clinical populations undergoing significant muscle atrophy.

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