Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial

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ABSTRACT **The purpose of this work was to determine the effects of varying levels of dietary protein on body composition and muscle protein synthesis during energy deficit (ED). A randomized controlled trial of 39 adults assigned the subjects diets providing protein at 0.8 (recommended dietary allowance; RDA), 1.6 (2×-RDA), and 2.4 (3×-RDA) g kg^{-1} d^{-1} for 31 d. A 10-d weight-maintenance (WM) period was followed by a 21 d, 40% ED. Body composition and postabsorptive and postprandial muscle protein synthesis were assessed during WM (d 9–10) and ED (d 30–31). Volunteers lost ($P<0.05$) 3.2 ± 0.2 kg body weight during ED regardless of dietary protein. The proportion of weight loss due to reductions in fat-free mass was lower ($P<0.05$) and the loss of fat mass was higher ($P<0.05$) in those receiving 2×-RDA and 3×-RDA compared to RDA. The anabolic muscle response to a protein-rich meal during ED was not different ($P>0.05$) from WM for 2×-RDA and 3×-RDA, but was lower during ED than WM for those consuming RDA levels of protein (energy × protein interaction, $P<0.05$). To assess muscle protein metabolic responses to varied protein intakes during ED, RDA served as the study control. In summary, we determined that consuming dietary protein at levels exceeding the RDA may protect fat-free mass during short-term weight loss.—Pasiakos, S. M., Cao, J. J., Margolis, L. M., Sauter, E. R., Whigham, L. D., McClung, J. P., Rood, J. C., Carbone, J. W., Combs, G. F., Jr., Young, A. J. Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial. *FASEB J.* 27, 3837–3847 (2013). www.fasebj.org**

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Energy deficit (ED) is generally associated with a decline in body weight. Although health-related outcomes are often improved with the loss of body weight due to declines in body fat, skeletal muscle mass is also lost and may account for 25% or more of the total body weight loss (1). In overweight and obese individuals, reductions in muscle mass may impede further weight loss and compromise weight management by downregulating metabolic processes, including protein turnover and basal metabolic rate (2–4). In addition, in individuals such as athletes and military personnel undergoing unavoidable EDs resulting from dietary energy restriction, increased energy expenditure, or both, diminished muscle mass may degrade physical performance and increase susceptibility to injury (5–7).

Dietary interventions that promote protein consumption at levels above the recommended dietary allowance (RDA; 0.8 g kg^{-1} d^{-1}) are becoming increasingly popular weight-loss strategies, as several studies have demonstrated that consuming high-protein (~1.5 g kg^{-1} d^{-1}) diets during ED can attenuate the loss of

Abbreviations: Akt, protein kinase B; ATF4, activating transcription factor 4; BMI, body mass index; DXA, dual-energy X-ray absorptiometry; ED, energy deficit; eEF2, eukaryotic elongation factor 2; eIF4E, eukaryotic initiation factor 4E; FFM, fat-free mass; FM, fat mass; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; hVps34, human vacuolar protein sorting-34; LAT1, L-type amino acid transporter; MAP4K3, mitogen-activated protein 4 kinase 3; $p70^{S6K}$, 70-kDa S6 kinase; RDA, recommended dietary allowance; REDD1, DNA-damage-inducible transcript 4; REDD2, DNA-damage-inducible transcript 4-like; rpS6, ribosomal protein S6; SNAT2, sodium-coupled neutral amino acid transporter 2; WM, weight maintenance

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fat-free mass (FFM) (8–13). The mechanism for this purported FFM-sparing effect may relate to dietary protein-induced alterations in protein turnover, particularly muscle protein synthesis (14). Only 3 studies have investigated muscle protein synthetic responses to ED, and the findings from these studies are highly discordant. Differences in dietary interventions, the methodologies employed to assess protein synthesis, the conditions during which protein synthesis measurements were obtained, and the physiological characteristics of study populations may have contributed to the discordant findings (15–17). Nevertheless, Pasiakos *et al.* (16) demonstrated that an acute ED in normal-weight adults down-regulates postabsorptive muscle protein synthesis, suggesting a unique adaptive mechanism to conserve energy and protein reserves. However, these findings have not been confirmed, and no studies in normal-weight adults have characterized both postabsorptive and postprandial skeletal muscle metabolic responses to ED. Although a recent report by Bray *et al.* (18) assessed body composition responses to high-protein diets during energy surplus, no investigations have compared body composition and muscle anabolic responses to varying levels of dietary protein intake during ED. Thus, the optimal level of dietary protein necessary to protect FFM during weight loss and the possible mechanism by which this may occur have not been elucidated.

The current study assessed body composition and muscle protein synthesis responses to controlled diets manipulating protein intake over a range that spans the current acceptable macronutrient distribution range (19) during short-term ED. We hypothesized that short-term ED would cause a loss of FFM with concomitant reductions in protein synthesis and that high-protein diets would spare FFM and sustain muscle anabolic responses during ED.

MATERIALS AND METHODS

Subjects

The Human Use Review Committee at the U.S. Army Research Institute of Environmental Medicine (Natick, MA,. USA) and the Institutional Review Board at the University of North Dakota (Grand Forks, ND, USA) approved this study. Human research volunteers provided informed voluntary written consent and were medically cleared for participation. Investigators adhered to U.S. Army Regulation 70–25 and U.S. Army Medical Research and Material Command regulation 70–25 on the participation of volunteers in research.

Volunteers were military personnel from the U.S. Army Natick Research, Development and Engineering Center, Human Research Volunteer recruit platoon, and civilians from the Grand Forks, ND, area. Individuals were screened for eligibility after attending a study information brief and providing informed voluntary consent. Volunteers were required to be physically active (physical activity 3–4 d/wk), weight stable (±2 kg) for a minimum of 2 mo before the study, with a body mass index (BMI) between 22 and 29 kg/m^2, and recreationally fit as indicated by baseline-study screening (Vo_{2peak} 40–60 ml kg^{-1} min^{-1}). Prospective volunteers reporting metabolic or cardiovascular abnormalities, musculoskeletal injuries, or the use of medications known to influence protein metabolism were excluded from participation. Details regarding volunteer recruitment, randomization, and study design are presented in **Fig. 1**. In total, 42 volunteers were randomized, but 39 volunteers, 32 men (11 military, 21 civilians) and 7 women (7 civilians), completed the randomized controlled study (**Table 1**).

Experimental design

This study was designed to assess the effects of dietary protein intake on body composition and postabsorptive and postprandial muscle protein synthesis responses to short-term ED. Using a randomized block design, volunteers were assigned to diets providing protein at 0.8 RDA, 1.6 g kg^{-1} d^{-1} (2×-RDA), and 2.4 g kg^{-1} d^{-1} (3×-RDA) for 31 d. A 10-d weight maintenance (WM) period was immediately followed by 21 d of ED, during which energy intake was restricted by 30% and physical activity was increased 10% above total daily energy expenditure to elicit a 40% ED. Body composition and muscle protein synthesis were assessed at the conclusion of WM (d 9–10) and ED (d 30–31). Volunteers were admitted to the metabolic ward at the U.S. Department of Agriculture Grand Forks Human Nutrition Research Center for the duration of the study to ensure experimental control. Volunteers were required to abstain from nutritional supplements, alcohol, smoking, and all medications, unless acetaminophen-containing products were provided by the investigator or study physician. Volunteers were also required to be in their assigned rooms with lights out by 11 P.M. to ensure adequate and similar levels of sleep. No protocol deviations occurred.

Diet

Energy intake was individualized for volunteers using prestudy indirect calorimetry assessments of resting metabolic rate (TrueOne 2400 Metabolic Measurement System; ParvoMedics, Sandy, UT, USA), a factor of 1.3 to account for activities of daily living and diet-induced thermogenesis, and 7-d physical activity records to estimate energy expended during physical activity (20). Estimates of total daily energy expenditure were averaged with calculated energy requirements derived from the Harris-Benedict equation (21) to ensure appropriate energy prescription during WM. Habitual dietary intake prior to initiating the study was assessed using 3-d dietary records to estimate prestudy protein intake.

Dietary protein intake remained constant during WM and ED, and was provided as mixed, high-quality proteins (*e.g.*, dairy, lean meats, and vegetable-based proteins). Dietary fat accounted for ≤30% of total energy, and carbohydrate provided the remainder of the prescribed energy. Volunteers consumed a daily multivitamin and mineral supplement (One A Day Maximum; Bayer Healthcare, Morristown, NJ, USA) to ensure that micronutrient requirements were met. At the conclusion of the 10-d WM period, volunteers consumed the 21-d ED diet, which reduced energy intake by 30%. Research dietitians prepared individualized 3-d WM and ED menus that were chemically analyzed for nutrient accuracy, administered meals in a metabolic kitchen, and supervised meal consumption to ensure compliance. Energy and macronutrient intakes were recorded and analyzed (**Table 2**).

Physical activity

To isolate the effects of the diet and minimize the potential of an exercise training stimulus, physical activity during WM was prescribed at levels comparable to those reported in prestudy

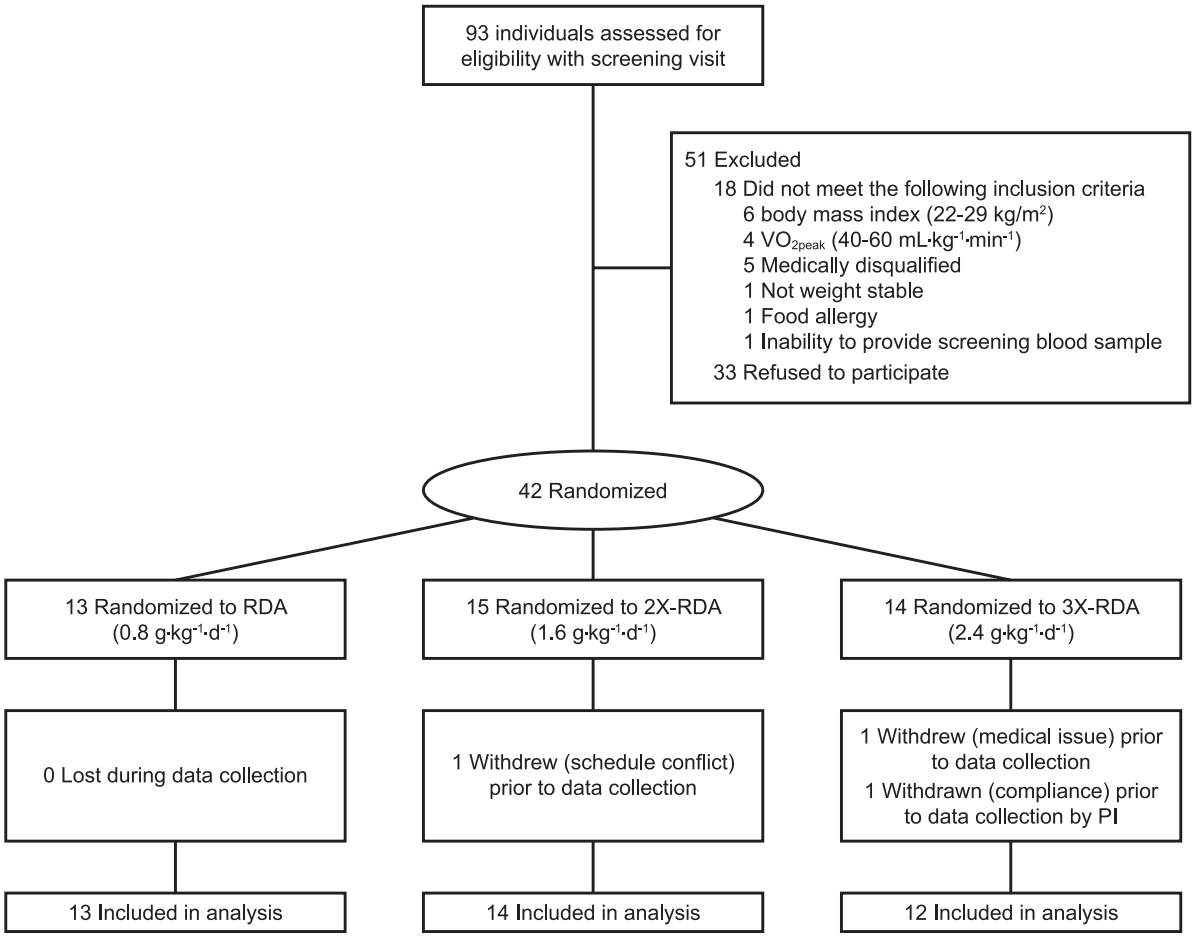

Figure 1. Schematic of volunteer recruitment and random assignment. Ninety-three individuals were screened for participation; 51 were excluded, 42 were randomized into dietary groups, and 39 completed the intervention.

7-d physical activity records. Volunteers performed low-to-moderate-intensity (40–60% Vo_{2peak}) treadmill and cycle ergometry steady-state physical activity sessions daily. Intensity was based on prestudy Vo_{2peak} measurements obtained during a progressive intensity treadmill test and verified during familiarization trials using indirect calorimetry (ParvoMedics) and corresponding heart rate (22). Workloads during steady-state physical activity sessions were adjusted accordingly to ensure accuracy using the heart rate reserve method and portable heart rate monitors.

Volunteers also performed resistive-type physical activity 3 d/wk to maintain prestudy muscular fitness levels. However, to minimize the potential of an unaccustomed, anabolic stimulus influencing study outcome measures, the intensity and volume of the resistive-type exercise was low. Specifically, volunteers performed one single-joint movement per major muscle group (3 sets of 15 repetitions) using workloads determined during the prestudy period. Frequency, intensity, mode, and volume of resistive-type activities did not change during the 31-d study. Research staff who were blinded from dietary assignment supervised all physical activity sessions for safety and accuracy.

Beginning on d 12, the duration of steady-state endurance type physical activity was increased to expend 10% more energy than predicted energy requirements estimated for WM. This was done to elicit a 40% ED when the increased exercise energy expenditure was combined with the 30% reduction in energy intake. Exercise-induced energy expenditure during ED was 485 ± 24 kcal for RDA, 498 ± 28 kcal for 2×-RDA, and 480 ± 31 kcal for 3×-RDA. The ED achieved was 39.5 ± 0.002%.

TABLE 1. *Prestudy volunteer characteristics*

Characteristic	RDA	2×-RDA	3×-RDA
n	13	14	12
Age (yr)	21 ± 1	21 ± 1	21 ± 1
Height (cm)	178 ± 2	176 ± 2	176 ± 2
Weight (kg)	78 ± 3	76 ± 3	77 ± 2
BMI (kg/m^2)	25 ± 1	25 ± 1	25 ± 1
Vo_{2peak} (ml kg^{-1} min^{-1})	47 ± 1	48 ± 2	49 ± 2
Protein (g kg^{-1} d^{-1})	1.43 ± 0.14	1.36 ± 0.12	1.54 ± 0.14

Values are means ± SEM, block randomized according to sex, BMI, fitness level (Vo_{2peak}, peak oxygen uptake), and prestudy dietary protein intake. Homogeneity was determined using analysis of variance.

Anthropometrics and body composition

Height was measured to the nearest 0.1 cm using an anthropometer (Seca model 770; Seca, Hanover, MD, USA). Body weight was assessed to the nearest 0.1 kg using a calibrated digital scale (Ultegra Scale SCB-R9000 HS; Fairbanks Scales, Kansas City, MO, USA). Body weight was measured daily after an overnight fast and morning void to ensure either WM

TABLE 2. *Energy and macronutrient distribution of WM and ED controlled diets*

Component	RDA		2×-RDA		3×-RDA	
	WM	ED	WM	ED	WM	ED
Energy (kcal/d)	2642 ± 120	1883 ± 88	2583 ± 97	1820 ± 75	2511 ± 86	1766 ± 62
Absolute (g/d)						
Protein	64 ± 2	64 ± 3	123 ± 4	122 ± 4	187 ± 5	185 ± 5
Carbohydrate	408 ± 20	272 ± 14	335 ± 14	199 ± 10	254 ± 12	121 ± 8
Fat	90 ± 4	64 ± 3	87 ± 3	61 ± 9	84 ± 3	58 ± 2
Relative (g kg^{-1} d^{-1})						
Protein	0.82 ± 0.01	0.85 ± 0.01	1.61 ± 0.01	1.63 (0.01)	2.43 ± 0.01	2.47 ± 0.02
Carbohydrate	5.24 ± 0.18	3.62 ± 0.15	4.40 ± 0.11	2.65 (0.07)	3.28 ± 0.12	1.61 ± 0.09
Fat	1.15 ± 0.04	0.85 ± 0.03	1.14 ± 0.02	0.81 (0.01)	1.09 ± 0.02	0.78 ± 0.02

Values are means ± SEM; $n = 13$ (RDA), 14 (2×-RDA), 12 (3×-RDA). Nutrient intake determined from the U.S. Department of Agriculture National Nutrient Database for Standard Reference, Release 22. Relative nutrient intake determined using mean body weight during WM and ED dietary interventions.

(energy intake was adjusted if WM was not achieved by adding carbohydrate) or weight loss. Body composition was measured after an overnight fast by the same trained technician using dual-energy X-ray absorptiometry (DXA; Delphi QDR, Hologic, Bedford, MA, USA) during WM (d 9) and ED (d 30).

Stable isotope infusion protocol

Stable isotope infusion and muscle biopsy studies to assess protein synthesis were completed at the conclusion of WM (d 10) and ED (d 31) after the volunteers fasted overnight. Volunteers refrained from any physical activity for 2 d before each infusion study to minimize the potential effects physical activity on muscle protein measures. After intravenous catheters were placed, and baseline blood samples were obtained, a primed, constant infusion of L-[$^2H_5$]-phenylalanine (2.8 mmol/kg; 0.07 mmol kg^{-1} min^{-1}) was initiated and maintained for the next 480 min. Isotopes were commercially available (Cambridge Isotope Laboratories, Andover, MA, USA) and certified sterile and pyrogen free.

To assess postabsorptive muscle protein synthesis, muscle biopsies were obtained from a single incision at 120 min (T_{120}) and 300 min (T_{300}) of the 480-min infusion period as described previously (23). Immediately after the T_{300} muscle biopsy, volunteers consumed a commercially available nutrition supplement (Boost; Nestle HealthCare Nutrition, Florham Park, NJ, USA). Supplements were enriched with L-[$^2H_5$]-phenylalanine to 4% of the measured phenylalanine content to minimize disturbances in isotopic equilibrium (24). Supplements were chemically analyzed for nutrient accuracy (Covance Laboratories, Madison, WI, USA), and provided 480 kcal, 20 g of protein, 8 g of fat, and 82 g of carbohydrate. The quantity of protein provided was chosen based on studies demonstrating that acute muscle protein synthetic responses to feeding appear to be maximally stimulated at rest after consuming 20 g of high-quality protein (25, 26). To assess postprandial muscle protein synthesis, a final muscle biopsy from the same incision was obtained at 480 min (T_{480}).

Visible blood and connective tissue were removed from biopsy specimens, and samples were immediately frozen in liquid nitrogen. Blood samples were obtained throughout the infusion period and used to measure isotopic enrichments by gas chromatography mass spectroscopy. To minimize risk, muscle samples were obtained from the opposite leg during the ED infusion experiment (d 31). Two nonserious, expected adverse events (*i.e.*, postbiopsy soreness) occurred after the WM biopsy procedures; these volunteers completed the study without further complications.

Determination of muscle protein synthesis

Mixed muscle protein synthesis was calculated using the single-pool precursor-product model to determine the rate of tracer incorporation from the muscle-free amino acid intracellular pool into bound muscle protein (27). Bound and muscle-free intracellular [$^2H_5$]-phenylalanine enrichments obtained from T_{120} and T_{300} were used to determine postabsorptive muscle protein synthesis, and T_{300} and T_{480} were used to calculate postprandial muscle protein synthesis.

Anabolic intracellular signaling and gene expression

Immunoblotting was used to quantify the phosphorylation status proteins associated with the intracellular regulation of muscle protein synthesis during WM and ED using muscle biopsy samples obtained under postabsorptive (T_0) and postprandial (T_{480}) conditions as previously reported (23, 28). After homogenization and protein content analysis (BCA Protein Assay; Thermo Scientific, Waltham, MA, USA), equal amounts of total protein (20 μg) were subjected to SDS-PAGE using precast Tris·HCl gels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were then transferred to polyvinylidene fluoride membranes, followed by exposure to commercially available phospho-specific [protein kinase B (Akt; Ser^{473}), 70-kDa S6 kinase ($p70^{S6K1}$; Ser^{424}/Thr^{421}), $p70^{S6K1}$ (Thr^{389}), eukaryotic initiation factor 4E (eIF4E; Ser^{209}), eukaryotic elongation factor 2 (eEF2; Thr^{56}), and ribosomal protein S6 (rpS6; $Ser^{235}/^{236}$); Cell Signaling Technology, Danvers, MA, USA] primary antibodies produced in rabbits.

Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was immunoblotted and confirmed equal protein loading (data not shown). Labeling was performed using an anti-rabbit IgG conjugate with horseradish peroxidase (Cell Signaling Technology), and chemiluminescent reagent was applied (Super Signal, West Pico Kit; Pierce Biotechnology, Rockford, IL, USA). Optical density was measured using a phosphoimager (ChemiDoc XRS; Bio-Rad), and densitometry analysis was performed using Image Lab software (Bio-Rad). Immunoblot data were normalized to an internal control (loaded onto every gel) for comparison across gels and expressed relative to GAPDH. Phosphorylation status is presented as fold change compared to WM, postabsorptive RDA as the appropriate study control.

Changes in mRNA expression of proteins implicated in the

intracellular regulation of muscle protein synthesis, particularly proteins associated with amino acid sensing and transport, were determined in muscle biopsy samples obtained under postabsorptive (T_{120}) conditions during WM and ED using quantitative real-time PCR, as described previously (23). Gene expression measures under postprandial conditions were not possible due to limited muscle sample availability. Total RNA was isolated in ~40 mg muscle samples using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and RNA quantity and 260/280 ratios were measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total RNA (2 μg) were synthesized into cDNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Carlsbad, CA, USA), followed by amplification using standardized TaqMan Gene Expression Assays for genes of interest [L-type amino acid transporter (LAT1), Hs00185826; sodium-coupled neutral amino acid transporter 2 (SNAT2), Hs01089954; activating transcription factor 4 (ATF4), Hs00909569; human vacuolar protein sorting-34 (hVps34), Hs00176908; mitogen-activated protein 4 kinase 3 (MAP4K3), Hs200269284; DNA-damage-inducible transcript 4 (REDD1), Hs01111686; DNA-damage-inducible transcript 4-like (REDD2), Hs00293956; and GAPDH, Hs03929097] on a StepOnePlus Real-Time PCR System (Applied Biosystems). Expression data are presented as fold change compared to WM, postabsorptive RDA using the $2^{-\Delta\Delta C_t}$ method (29).

Nitrogen balance and resting metabolic rate

Total nitrogen content of the urine was determined from a single pooled 24-h urine sample using pyrochemiluminescence (Antek 9000; Antek Instruments, Houston, TX, USA) to assess nitrogen responses to varying levels of protein intake during WM (d 9) and ED (d 14, 17, 24, and 30). Apparent nitrogen balance was calculated as the difference of nitrogen intake minus urinary nitrogen excretion plus miscellaneous (estimated at 5 mg/kg) and fecal (estimated at 2 g/d) losses (30). Urinary creatinine was measured using the Jaffe reaction to verify complete urine collections (UniCel DxC 600 Pro; Beckman Coulter, Brea, CA, USA). Corresponding resting metabolic rate measurements were determined using open circuit indirect calorimetry (ParvoMedics) following an overnight fast. Volunteers rested in the supine position for ~30 min prior to each measurement, and tests were stopped when 20 min of steady-state oxygen kinetics were recorded.

Statistical analysis

Power analyses indicated that 12 volunteers/group would provide >85% power to detect differences in muscle protein synthesis between energy and feeding states across dietary protein groups (15, 16). Although 2 participants withdrew and 1 was withdrawn prior to data collection (Fig. 1), a modified intention-to-treat principle was followed, as not all randomized volunteers were included in the analysis.

An independent statistician block randomized volunteers by sex (male and female), BMI (22–25 and 25.1–29 kg/m^2), Vo_{2peak} (40–50 and 50.1–60 ml kg^{-1} min^{-1}), and prestudy dietary protein intake (0.8–1.6 and 1.61–2.4 g kg^{-1} d^{-1}) using Taves's minimization method of treatment assignment (31). Prestudy volunteer characteristics are described using common descriptive statistics, and a 1-way ANOVA was used to confirm homogeneity between diet groups. Changes in body composition and muscle protein synthesis responses to feeding were calculated to determine if changes in response to the ED varied between dietary protein levels. All outcome variables were analyzed using a mixed-model repeated measures ANOVA including within-subjects factors for energy (WM and ED), state (postabsorptive and postprandial), and time (study days), with a between-subjects factor of protein (RDA, 2×-RDA, and 3×-RDA). Compound symmetry was determined as the appropriate covariance model based on the Akaike's information criterion. Following the observation of a significant interaction, *post hoc* pairwise analyses were conducted using Bonferroni adjustments for multiple comparisons. The α level for significance was set at $P < 0.05$. Data were analyzed using the Proc Glimmix in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and are expressed as means ± SEM.

RESULTS

Changes in body weight and composition

Body weight during WM was similar between dietary treatment groups and remained stable from d 1 (group mean, 77.5±1.5 kg) through d 10 (77.1±1.5 kg). Overall, volunteers lost 3.2 ± 0.2 kg during the 21-d ED; 3.5 ± 0.3 kg for RDA, 2.7 ± 0.2 kg for 2×-RDA, and 3.3 ± 0.3 kg for 3×-RDA ($P<0.05$; **Fig. 2*A***).

Independent of dietary protein, percentage body fat decreased ($P<0.05$) from 19.8 ± 1% during WM to

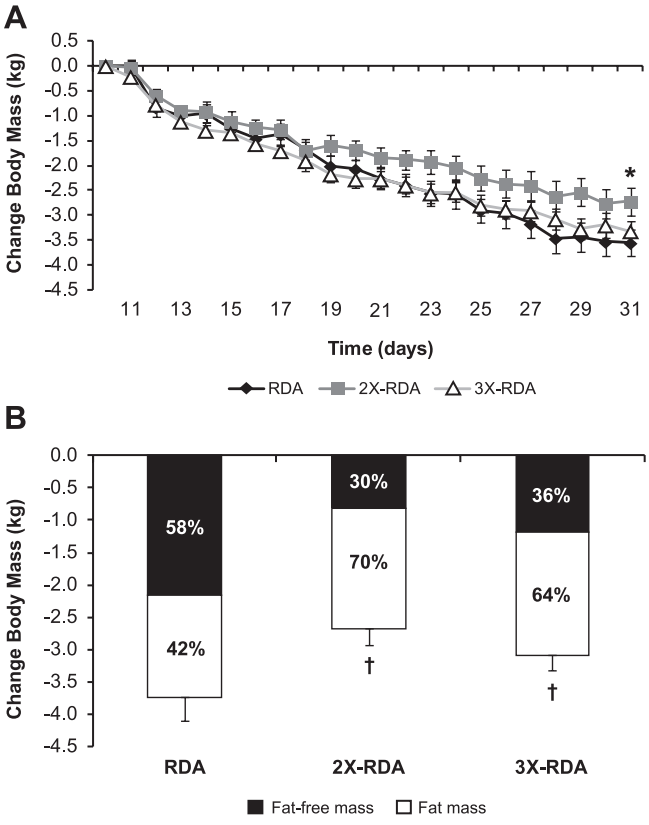

Figure 2. Mean ± SEM [n=13 (RDA), 14 (2×-RDA), 12 (3×-RDA)] changes in body weight (*A*) and the proportion (%) of fat mass and fat-free mass loss (*B*) during the 21-d ED. Data were analyzed using mixed-model repeated measures ANOVA with Bonferroni corrections to determine main effects and interactions for energy (WM *vs.* ED) and diet (RDA *vs.* 2×-RDA *vs.* 3×-RDA). *$P < 0.05$ *vs.* d 10; †$P < 0.05$ *vs.* RDA.

18.1 ± 1% during ED, and the change in percentage body fat was similar between RDA (1.3±0.3%), 2×-RDA (1.8±0.4%), and 3×-RDA (1.9±0.3%). However, the proportion of total weight loss due to changes in fat mass (FM) and FFM differed across dietary protein levels (Fig. 2*B*). Specifically, the percentage of total weight loss attributed to reductions in FM was higher ($P<0.05$) for 2×-RDA (70.1±7%; 1.9±0.3 kg) and 3×-RDA (63.6±5%; 1.9±0.2 kg) than for RDA (41.8±5%; 1.6±0.2 kg). The percentage of total weight loss due to a loss of FFM was lower for 2×-RDA (29.8±7%; 0.8±0.2 kg) and 3×-RDA (36.4±5%; 1.2±0.3 kg) as compared to RDA (58.2±5%; 2.3±0.3 kg). The proportion of weight loss due to reductions in FM and FFM was not different between 2×-RDA and 3×-RDA.

Phenylalanine enrichments and changes in muscle protein synthesis

Fluctuations in postabsorptive and postprandial plasma and muscle free intracellular L-[$^2H_5$]-phenylalanine did not occur, suggesting that isotopic equilibrium was achieved (**Fig. 3*A, B***). An energy × state × protein interaction ($P<0.05$) was observed for muscle protein synthesis, as consuming a mixed-meal providing 20 g of protein during WM increased ($P<0.05$) protein synthesis for RDA and 2×-RDA, but not for 3×-RDA (Fig. 3*C, D*). However, postprandial muscle protein synthesis increased ($P<0.05$) relative to postabsorptive muscle protein synthesis for only those consuming 3×-RDA during ED. Postabsorptive muscle protein synthesis was not different between energy states and dietary protein levels.

An energy × protein interaction ($P<0.05$) was observed for the change in muscle protein synthesis in response to feeding (*i.e.,* postprandial-postabsorptive) during WM and ED. Specifically, the anabolic response to feeding protein was maintained during ED and was not different from WM for volunteers consuming 2×-RDA (WM *vs.* ED: 0.03±0.01 *vs.* 0.01±0.01%/h) and 3×-RDA (WM *vs.* ED: 0.01±0.01 *vs.* 0.03±0.01%/h). Interestingly, the muscle protein synthetic response to feeding was inhibited ($P<0.05$) during ED for volunteers consuming the RDA (WM *vs.* ED: 0.05±0.01 *vs.* −0.001 ± 0.01%/h).

Anabolic intracellular signaling and gene expression

Intracellular signaling was up-regulated 3 h after consuming a protein-containing meal, as demonstrated by a main effect of state (postabsorptive *vs.* postprandial) for all proteins of interest ($P<0.05$). Specifically, postprandial Akt (Ser^{473}; **Fig. 4*A***) phosphorylation was 1.4-fold higher ($P<0.05$) compared to postabsorptive levels. Similarly, postprandial p70^{S6K1} (Thr^{389}), eIF4E

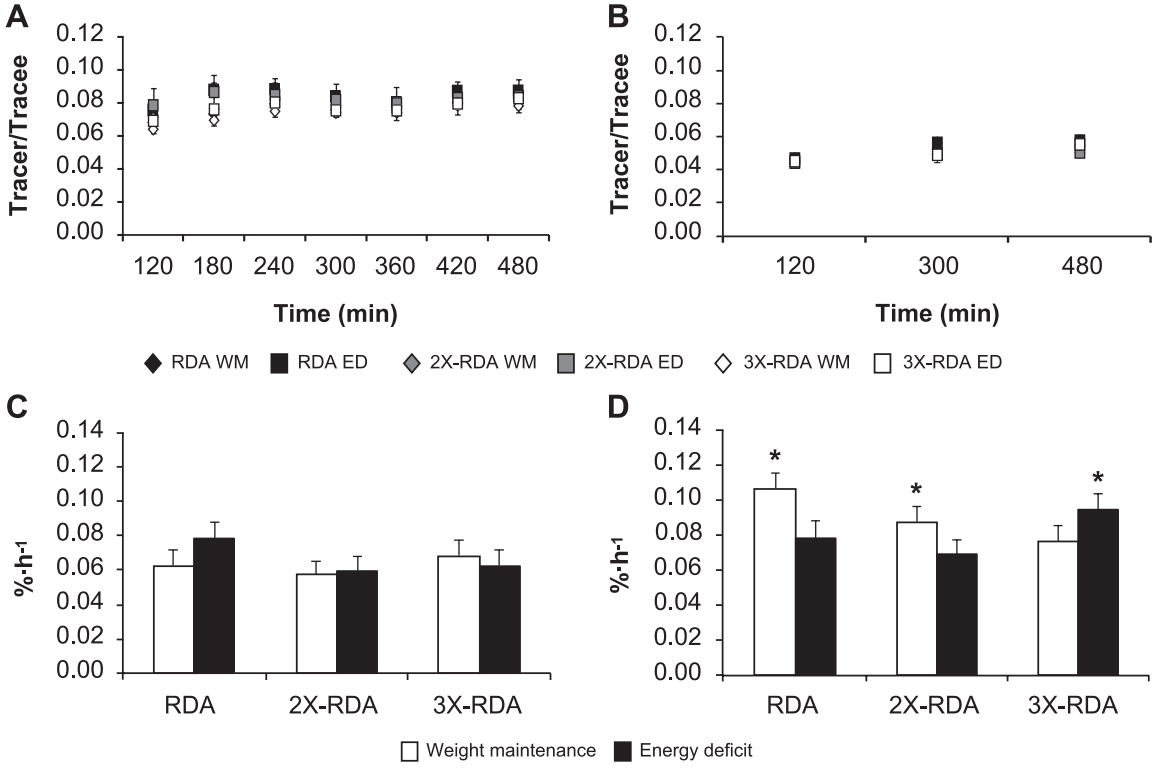

Figure 3. Mean ± SEM [n=13 (RDA), 14 (2×-RDA), 12 (3×-RDA)] plasma (*A*) and muscle intracellular (*B*) L-[$^2H_5$]-phenylalanine enrichments during WM and ED stable isotope infusion trials; and postabsorptive muscle protein synthesis (*C*) and postprandial muscle protein synthesis (*D*) responses to varying levels of dietary protein intake during ED. Data were analyzed using mixed-model repeated measures ANOVA with Bonferroni corrections to determine main effects and interactions for energy (WM *vs.* ED), state (postabsorptive *vs.* postprandial), and diet (RDA *vs.* 2×-RDA *vs.* 3×-RDA). *$P < 0.05$ *vs.* corresponding WM or ED postabsorptive value.

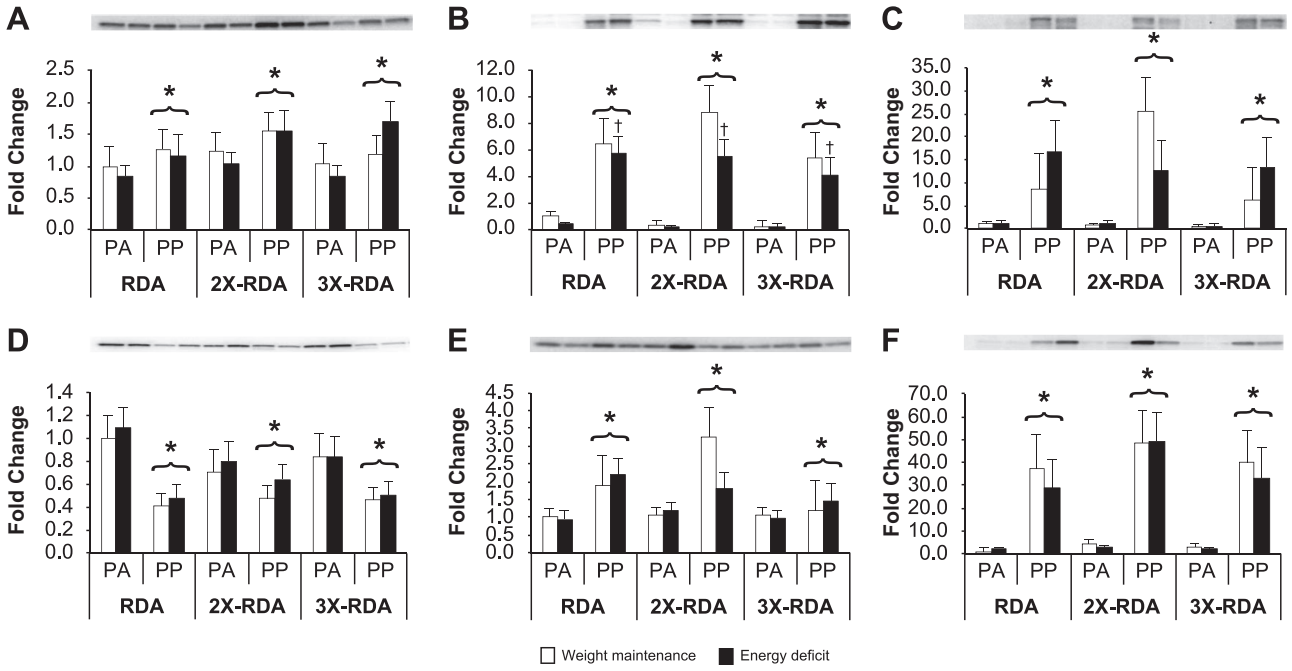

Figure 4. Mean ± SEM [n=13 (RDA), 14 (2×-RDA), 12 (3×-RDA)] Akt (Ser^{473}; *A*), $p70^{S6K}$ (Ser^{424}/Thr^{421}; *B*), $p70^{S6K}$ (Thr^{389}; *C*), eEF2 (Thr^{56}; *D*), eIF4E (Ser^{209}; *E*), and rpS6 ($Ser^{235/236}$; *F*) phosphorylation status expressed relative to GAPDH, and presented as fold change as compared to WM, postabsorptive RDA. Data were analyzed using mixed-model repeated measures ANOVA with Bonferroni corrections to determine main effects and interactions for energy (WM *vs.* ED), state (postabsorptive *vs.* postprandial), and protein (RDA *vs.* 2×-RDA *vs.* 3×-RDA). *$P < 0.05$ *vs.* postabsorptive; †$P < 0.05$ *vs.* WM postprandial.

(Ser^{209}), and rpS6 ($Ser^{235/236}$) phosphorylation status was 16, 1.9, and 15.5-fold higher ($P<0.05$), respectively, compared to postabsorptive phosphorylation levels (Fig. 4*C, E, F*). Phosphorylation status of eEF2 (Thr^{56}), however, was lower ($P<0.05$) after feeding (Fig. 4*D*). Energy state and feeding influenced the phosphorylation of $p70^{S6K1}$ (Ser^{424}/Thr^{421}), as postprandial phosphorylation during ED was lower than phosphorylation levels observed during WM (energy × state interaction, $P<0.05$; Fig. 4*B*). Neither energy status nor level of dietary protein intake further influenced anabolic intracellular signaling.

ED increased mRNA expression of proteins implicated in the intracellular regulation of muscle protein synthesis (**Table 3**). Transcription of Vps34, a protein involved in amino acid sensing and amino acid-mediated stimulation of mammalian target of rapamycin (mTORC1) signaling, was 1.2-fold higher ($P<0.05$), while expression of mTORC1 inhibitors REDD1 and REDD2 were both 1.3-fold higher ($P<0.05$) after ED compared to WM. Increasing dietary protein intake increased Vps34 mRNA expression, with 1.2-fold higher levels for 3×-RDA than RDA ($P<0.05$). MAP4K3, LAT1, and SNAT2 mRNA levels were not influenced by energy and dietary protein manipulations.

Nitrogen balance and resting metabolic rate

Nitrogen balance was similar between dietary protein groups during WM (d 9; overall mean −10±5 mgN kg^{-1} d^{-1}; $P>0.05$). The reduction in nitrogen balance at the initiation of ED (d 14) did not differ between

TABLE 3. *Postabsorptive muscle protein synthesis-associated mRNA expression levels during WM and ED*

	RDA		2×-RDA		3×-RDA	
mRNA	WM	ED	WM	ED	WM	ED
Vps34	1.03 ± 0.08	1.06 ± 0.11*	1.03 ± 0.07	1.28 ± 0.10*	1.20 ± 0.08#	1.41 ± 0.11
MAP4K3	1.07 ± 0.10	0.93 ± 0.12	0.85 ± 0.10	1.11 ± 0.11	0.92 ± 0.10	1.13 ± 0.11
REDD1	1.19 ± 0.20	1.19 ± 0.27	1.20 ± 0.18	1.70 ± 0.25*	0.99 ± 0.19	1.55 ± 0.26*
REDD2	1.06 ± 0.13	1.23 ± 0.21	1.21 ± 0.12	1.49 ± 0.19*	1.25 ± 0.12	1.97 ± 0.20*
LAT1	1.31 ± 0.30	1.32 ± 0.53	1.18 ± 0.28	1.21 ± 0.48	1.16 ± 0.29	1.82 ± 0.50
SNAT2	1.14 ± 0.13	0.78 ± 0.17	0.89 ± 0.12	1.11 ± 0.16	1.11 ± 0.12	1.20 ± 0.17
ATF4	1.02 ± 0.08	1.00 ± 0.09	1.19 ± 0.07	1.23 ± 0.09	1.13 ± 0.08	1.26 ± 0.09

Values are means ± SEM, n = 13 (RDA), 14 (2×-RDA), 12 (3×-RDA). Postabsorptive mRNA data during WM and ED are expressed as fold change relative to weight maintenance RDA using the $2^{-\Delta\Delta Ct}$ method. Data were analyzed using mixed-model repeated measures ANOVA with Bonferroni corrections to determine main effects and interactions for energy (weight maintenance *vs.* energy deficit) and protein (RDA *vs.* 2×-RDA *vs.* 3×-RDA). *$P < 0.05$ *vs.* WM, energy main effect; #$P < 0.05$ *vs.* RDA, protein main effect.

groups (overall mean −40±5 mgN kg^{-1} d^{-1}; $P>0.05$). Nitrogen balance remained negative throughout the 21-d ED for RDA; however, it returned to levels comparable to WM on d 17 for 2×-RDA and d 30 for 3×-RDA (protein × time interaction, $P<0.05$; **Fig. 5*A***). Nitrogen balance was not different between 2×-RDA and 3×-RDA on d 30. Resting metabolic rate was not influenced by energy and dietary protein manipulations (Fig. 5*B*).

DISCUSSION

The major finding from this controlled, human trial was that consuming dietary protein at levels above the RDA spared FFM while promoting the loss of body fat in response to short-term, moderate ED. Perhaps as important, this study demonstrated that consuming dietary protein at levels beyond twice the current RDA fail to confer further FFM protection during short-term weight loss. Furthermore, this study demonstrated that high-protein diets preserve skeletal muscle anabolic sensitivity to protein-containing meals during ED. To the best of our knowledge, this is the first study to assess body composition and postabsorptive and postprandial muscle protein synthetic responses to varying levels of dietary protein intake during short-term ED.

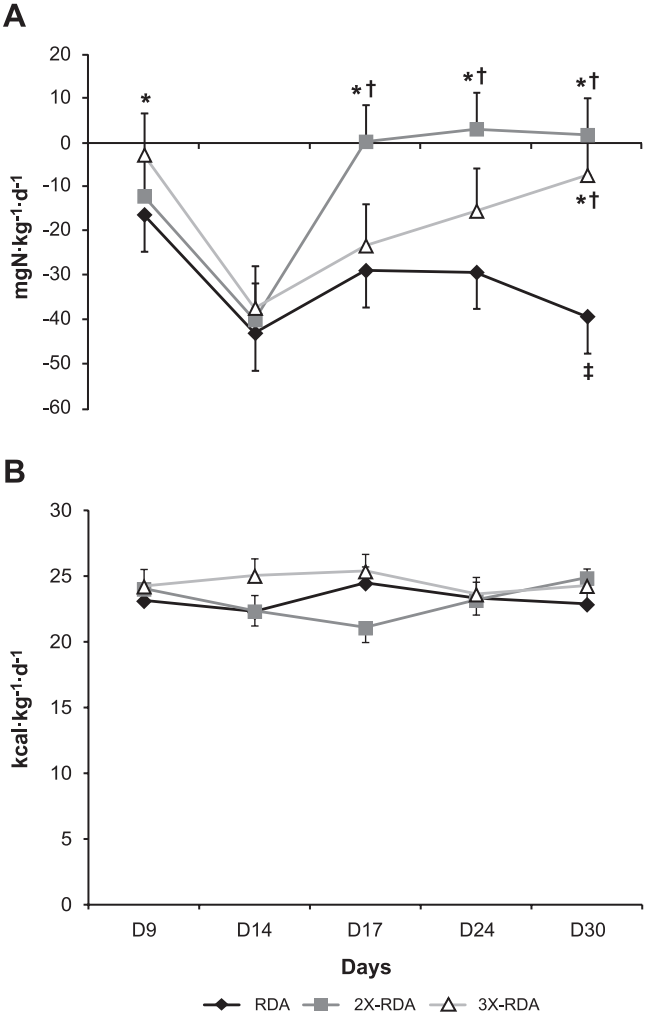

Figure 5. Mean ± SEM [n=13 (RDA), 14 (2×-RDA), 12 (3×-RDA)] nitrogen balance (*A*) and resting metabolic rate (*B*) during WM and ED. Data were analyzed using mixed-model repeated measures ANOVA with Bonferroni adjustments to determine the main effects and interaction for time (d 9, 14, 17, 24, and 30) and protein (RDA *vs.* 2×-RDA *vs.* 3×-RDA). *$P < 0.05$ *vs.* d 14, time × diet interaction; †$P < 0.05$ *vs.* RDA, time × protein interaction; ‡$P < 0.05$ *vs.* d 9, time × protein interaction.

Given the prevalence of obesity (32), and the proportion of adults attempting weight loss (33, 34), studies investigating the effects of high-protein diets during ED have focused primarily on body composition adaptations in obese adults (9, 11, 35–37). In those studies, consuming dietary protein at levels comparable to that provided in the 2×-RDA diet attenuated the loss of FFM during ED (9, 11, 13, 36, 37). However, direct comparisons between the current study and previous studies may not be appropriate, given differences in the populations studied and the duration and severity of the ED. Classical studies have clearly demonstrated that normal-weight adults conserve muscle mass during prolonged semistarvation, but the initial loss of lean body mass during ED can be rapid and severe, depending on the degree of energy insufficiency (38, 39). Considering normal and moderately overweight adults possess less body fat than obese individuals, declines in lean mass may be more pronounced than those experienced by obese adults during similar periods of weight loss (40), suggesting that normal and moderately overweight individuals may benefit from consuming more dietary protein than current recommendations for healthy weight loss (~1.5 g kg^{-1} d^{-1}) during periods of unavoidable or planned negative energy balance (12, 14). Mettler *et al.* (41) recently demonstrated that consuming dietary protein at 2.3 g kg^{-1} d^{-1} was superior to 1.0 g kg^{-1} d^{-1} for the maintenance of FFM in response to a 2-wk, 40% ED in young athletes. In contrast, consuming protein at 2.4 g kg^{-1} d^{-1} in the current study failed to elicit greater lean mass protection as compared to 1.6 g kg^{-1} d^{-1}, suggesting that levels as high as 2.4 g kg^{-1} d^{-1} are likely unnecessary and that a plateau may exist above which consuming more dietary protein confers no additional benefit in the context of short-term weight loss. Subjects consuming 1.0 g kg^{-1} d^{-1} in the Mettler *et al.* (41) study did not appear to achieve that plateau.

The maintenance of FFM after consuming high-protein diets during short-term ED may be attributed to alterations in postprandial muscle protein synthesis. Our laboratory recently demonstrated that muscle protein synthetic responses to acute protein consumption are dictated by the metabolic demand for energy and amino acids, as increasing the leucine content of a "maximal" (25, 26) dose of protein limits the dependence on endogenous protein stores and enhances protein synthesis in recovery from steady-state exercise (28). Our current findings are consistent with previous studies indicating that the level of protein habitually

consumed during ED may modulate the muscle anabolic responses to acute protein ingestion. Specifically, we observed that muscle protein synthesis increased in response to consuming a fixed level of protein above postabsorptive levels for those consuming RDA and 2×-RDA, but not for those consuming 3×-RDA, during WM. Although direct measures of proteolysis were not measured, these findings suggest that increased endogenous amino acid levels consequent to consuming the 3×-RDA diet attenuates endogenous protein breakdown, resulting in a diminished stimulus to synthesize new protein. Interestingly, postprandial muscle protein synthesis during ED was increased only for those assigned to the 3×-RDA diet, suggesting that elevated amino acid levels resulting from habitual high protein intakes may have been sufficient to saturate basal energy and whole-body protein requirements during increased metabolic demand, thereby sparing exogenous amino acids for muscle protein synthesis. Maintaining the change in muscle protein synthesis from postabsorptive to postprandial conditions during ED for volunteers consuming both the 2×-RDA and 3×-RDA further demonstrates this, as exogenous amino acids consumed by those in the RDA group were likely utilized for energy and whole-body protein requiring processes and not muscle synthesis. The linear increase in extracellular amino acid levels (Supplemental Table S1) with increasing levels of dietary protein intakes supports these findings, as habitual consumption of high-protein diets likely expanded extracellular amino acid pools affecting muscle anabolic responses to exogenous protein (42). Certainly, data quantifying muscle intracellular amino acid concentrations would strengthen this hypothesis, yet these data are not available. Nevertheless, Bolster *et al.* (43) demonstrated that habitual consumption of protein at 3.6 g kg^{-1} d^{-1} enhanced amino acid concentrations, resulting in lower post-steady-state exercise muscle protein synthesis when compared to dietary protein intakes similar to the RDA and 2×-RDA provided in the current study, further suggesting that dietary protein-induced alterations in extracellular amino acids may dictate the response to potent effectors of muscle protein synthesis, and potentially highlight a condition-specific metabolic benefit for protein intakes beyond twice the RDA.

In the current study, neither short-term ED nor dietary protein influenced postabsorptive muscle protein synthesis. These findings conflict with a recent report that demonstrated that a 10-d moderate ED (−500 kcal/d) resulted in a 19% reduction in postabsorptive muscle protein synthesis in a similar group of healthy, normal-weight adults (16). We suspect that, as with whole-body nitrogen adaptations observed during sustained ED (44, 45), down-regulations in postabsorptive muscle protein synthesis in the early stages of ED are reversed as the body adapts to prolonged energy insufficiency. Evidence from Villareal *et al.* (17) supports this hypothesis, as no changes in postabsorptive muscle protein synthesis were observed in older adults following a 3-mo weight-loss diet. Unfortunately, there are no comparable reports examining the effects of varying levels of dietary protein intake on either postabsorptive or postprandial muscle protein synthesis during ED. Consistent with the nitrogen adaptation hypothesis, we suspect that energy cost of protein metabolism may have also contributed to the preservation of FFM consequent to consuming high-protein diets. Elevated urinary nitrogen excretion (Supplemental Table S2) and preservation of nitrogen balance at the conclusion of the ED for those consuming the 2×-RDA and 3×-RDA and but not the RDA supports the nutrient partitioning hypothesis, suggesting that nitrogen and FFM may have been spared at the expense of body fat due to the metabolic cost of exogenous protein catabolism (18, 46, 47). Interestingly, we did not observe further protein metabolic advantages from consuming protein beyond twice the RDA, despite the difference in nitrogen intake and excretion for those consuming the 3×-RDA diet, as fat loss and nitrogen retention was not different between the 3×-RDA and 2×-RDA diets, and resting metabolic rate remained unchanged regardless of energy status and dietary protein. Nevertheless, these data suggest that the metabolic advantage gleaned from consuming dietary protein at levels above the RDA during weight loss on FFM are attributed to the energetics of protein metabolism, and perhaps because of protein-induced modulations in postprandial muscle protein synthesis (14).

This investigation is the latest from our laboratory attempting to define optimal dietary conditions that promote muscle health in adults in response to a variety of physiological stressors (28, 48, 49). Although the magnitude of FFM protection, the limitations associated with the assumptions of a 3-compartment model to assess body composition (*i.e.*, DXA), characteristics of the study population, and sample size may limit the generalizability of this study, we contend that our FFM data are valid and that adults who may undergo short-term periods of ED for a variety of reasons would benefit from consuming a high (twice the RDA) protein diet (32, 33, 50). Of note, the sample size (n=39) exceeds those used in similar studies, (15–17) and the data are strengthened by an experimental design that allows for a dose-dependent analysis of protein synthesis and body composition responses to ED across a wide range of dietary protein intakes. But we must recognize that our inability to identify an intracellular mechanism accounting for the muscle protein synthetic responses to the dietary and acute feeding interventions limits our interpretation of the muscle protein synthesis data. However, intracellular signaling is not necessarily a definitive predictor of muscle protein synthesis, as studies have clearly demonstrated time course discordance between cumulative muscle protein synthetic rates and static anabolic intracellular measures in response to exogenous protein (51, 52). It is possible that intracellular signaling would have aligned more closely with muscle protein synthesis if additional muscle biopsy samples were obtained. To minimize risk, however,

additional muscle biopsy procedures were not conducted.

In summary, consuming twice the amount of dietary protein than current recommendations measurably protects FFM and promotes the loss of body fat during short-term weight loss, likely through the maintenance of muscle anabolic sensitivity to protein ingestion. However, consuming dietary protein at 3 times the RDA does not appear to confer any additional protective advantage. Future studies are required to determine body composition and muscle anabolic responses to high-protein diets that vary in high-quality protein sources. Nevertheless, our findings indicate that consuming dietary protein at 1.6 g kg^{-1} d^{-1} may be an effective nutritional strategy to protect lean mass in response to unavoidable or planned dietary- and/or physical activity-induced weight loss. FJ

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