

Pronounced energy restriction with elevated protein intake results in no change in proteolysis and reductions in skeletal muscle protein synthesis that are mitigated by resistance exercise

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ABSTRACT: Preservation of lean body mass (LBM) may be important during dietary energy restriction (ER) and requires equal rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Currently, the relative contribution of MPS and MPB to the loss of LBM during ER in humans is unknown. We aimed to determine the impact of dietary protein intake and resistance exercise on MPS and MPB during a controlled short-term energy deficit. Adult men (body mass index, 28.6 ± 0.6 kg/m²; age 22 ± 1 yr) underwent 10 d of 40%-reduced energy intake while performing unilateral resistance exercise and consuming lower protein (1.2 g/kg/d, $n = 12$) or higher protein (2.4 g/kg/d, $n = 12$). Pre- and postintervention testing included dual-energy X-ray absorptiometry, primed constant infusion of *ring*-[¹³C₆]phenylalanine, and [¹⁵N]phenylalanine to measure acute postabsorptive MPS and MPB; D₂O to measure integrated MPS; and gene and protein expression. There was a decrease in acute MPS after ER (higher protein, 0.059 ± 0.006 to $0.051 \pm 0.009\%/h$; lower protein, 0.061 ± 0.005 to $0.045 \pm 0.006\%/h$; $P < 0.05$) that was attenuated with resistance exercise (higher protein, $0.067 \pm 0.01\%/h$; lower protein, $0.061 \pm 0.006\%/h$), and integrated MPS followed a similar pattern. There was no change in MPB (energy balance, $0.080 \pm 0.01\%/hr$; ER rested legs, $0.078 \pm 0.008\%/hr$; ER exercised legs, $0.079 \pm 0.006\%/hr$). We conclude that a reduction in MPS is the main mechanism that underpins LBM loss early in ER in adult men.—Hector, A. J., McGlory, C., Damas, F., Mazara, N., Baker, S. K., Phillips, S. M. Pronounced energy restriction with elevated protein intake results in no change in proteolysis and reductions in skeletal muscle protein synthesis that are mitigated by resistance exercise. FASEB J. 32, 265–275 (2018). www.fasebj.org

KEY WORDS: muscle protein turnover • weight loss • dietary protein

Dietary energy restriction (ER) is commonly used to reduce total body mass; however, a potentially unfavorable consequence of ER is loss of lean body mass (LBM) (1) that can comprise up to 25% of lost body mass (2). Given that skeletal muscle is the largest component in LBM and a highly metabolically active tissue, such loss could have an important impact on mobility and aspects of metabolic health (2). Thus, strategies that

promote the retention of LBM during ER are of clinical importance.

The maintenance of skeletal muscle mass is dependent on the balance between fasted and fed state changes in rates of skeletal muscle protein synthesis (MPS) and skeletal muscle protein breakdown (MPB). In energy balance (EB), periods of positive and negative protein balance are equal, which results in a net neutral protein balance and stable muscle mass (3). During ER, rates of MPS are reduced in the fasted and fed states (4–7), which promotes an overall decline in net protein balance that could, in part, underpin a reduction in LBM. However, higher protein intake and resistance exercise attenuate LBM losses and can even result in gains in LBM during ER (8). Indeed, we have shown that even during a marked—40% below energy requirements—energy deficit, young men who consume 2.4 g/kg/d of protein and who perform resistance exercise demonstrated significant increases in LBM (8). Resistance exercise and protein ingestion are known to attenuate the energy

ABBREVIATIONS: ABR, absolute breakdown rate; ASR, absolute synthesis rate; BMI, body mass index; DXA, dual-energy X-ray absorptiometry; EB, energy balance; ER, energy restriction; FFM, fat-free mass; FGR, fractional growth rate; FSR, fractional synthetic rate; LBM, lean body mass; LC3, light chain 3; MPB, muscle protein breakdown; MPS, muscle protein synthesis; myo-PS, myofibrillar protein synthesis; PCA, perchloric acid; RDA, recommended dietary allowance; RM, repetition maximum

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deficit-induced declines in resting MPS (6), which may explain why LBM is spared. In addition, consuming 2 or 3 times the recommended dietary allowance (RDA; 1.6 and 2.4 g/kg/d, respectively) of protein during ER preserved MPS response to a 20-g serving of milk protein compared with protein consumed at the RDA (0.8 g/kg/d) (4). The practical implications for studying the 40% reduction in energy intake are relevant when we consider athletic populations who may be trying to cut weight quickly (9) or situations when energy deficit might be unpreventable, such as during military training or operations (10).

Whereas a significant amount of research exists that has characterized changes in the rates of MPS during ER, relatively little is known about the effect of ER on the rates of MPB. Studies of molecular markers of MPB, such as changes in the gene and protein expression of targets that are involved in the ubiquitin-proteasome and autophagic-lysosomal pathways, have yielded inconsistent results (11–13). Few direct measurements of the rates of MPB during ER have been made; however, a single report has shown that after a 10-d 20% energy deficit in healthy adults (consuming 1.5 g/kg/d of protein), there was a 60% increase in the rates of postabsorptive MPB (12). Such a large increase in MPB during a 20% energy deficit (12) is difficult to reconcile when considered together with a reported ER-induced decline in MPS (4, 7). If MPS were to decline and MPB to increase, then the loss of muscle mass would be much greater than what has been reported. Currently, to our knowledge, there is no study that has made simultaneous measurements of both MPS and MPB to yield net protein balance during a marked 40% ER.

The primary aim of this study was to examine the impact of short-term (10 d) ER—40% reduction in energy intake from that required for weight maintenance—on acute (hourly) rates of mixed MPS and MPB, and integrated myofibrillar protein synthesis (myo-PS; daily). We used unilateral resistance exercise to examine the impact of loading exercise on the same variables. We also examined how differing dietary protein intakes affected MPS and MPB responses. Participants were randomly assigned to consume a higher (2.4 g protein/kg/d) or lower (1.2 g protein/kg/d) protein intake. To provide mechanistic insight, we examined the expression of genes and proteins that are involved in the ubiquitin-proteasome and autophagic-lysosomal pathways. We hypothesized, given the known rates of skeletal muscle loss during ER, that mixed MPB and MPS would change in parallel—that is, MPS would be decreased and MPB would not be significantly elevated or would be adaptively reduced—and that integrated myo-PS would be reduced after ER, but to a lesser extent in the resistance-exercised leg. We also hypothesized, given the body composition differences from Longland *et al.* (8), that the higher (2.4 g protein/kg/d) protein intake would act synergistically with exercise to stimulate mixed and myofibrillar MPS greater than that observed in participants with lower (1.2 g protein/kg/d) protein intake.

MATERIALS AND METHODS

Participants

A Consolidated Standards of Reporting Trials (CONSORT; <http://www.consort-statement.org/>) diagram of the participant flow-through of this parallel group randomized trial is shown in Supplemental Fig. 1. A total of 25 men were recruited *via* posters and gave their written, informed consent after being screened for eligibility. Inclusion criteria were as follows: body mass index (BMI) of 25–33 kg/m², age 18–30 yr, nonsmoker, and non-diabetic. Participants were not undertaking any form of ER or exercise program at the time of enrollment and were asked to maintain their habitual physical activity level throughout the study. Participants were informed of the purpose of the study and all experimental procedures and risks before providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and was conducted in accordance with standards set by the Canadian Tri-Council Policy on the use of human participants in research (14). This trial was registered at ClinicalTrials.gov (NCT02406040).

Before dietary intervention, participants' height and body mass were measured (Rice Lake Weighing Systems, Rice Lake, WI, USA). Participants were instructed not to consume any vitamin or mineral supplements or alcohol for the duration of the study.

Study design

A schematic of the overall study is shown in Fig. 1A. In a single-blind investigation, 24 men completed a 10-d ER diet that contained either 1.2 or 2.4 g/kg/d of protein. Groups were matched on the basis of age, BMI, body fat, LBM, 10-repetition maximum (RM) unilateral leg press (Maxam Fitness, Hamilton, ON, Canada) and 10-RM unilateral leg extension (Atlantis, Laval, QC, Canada; Table 1), with matched pairs for BMI, 10-RM leg press, and 10-RM leg extension. Participants' energy requirements were determined by using indirect calorimetry to establish a resting metabolic rate (Moxus Metabolic System; Aei Technologies, Pittsburgh, PA, USA) with an activity factor (15) assigned by using participants' activity logs. Exercised legs were randomly selected and counterbalanced for dominance on the basis of strength in each group.

Before beginning any dietary control (no longer than 1 wk), a muscle biopsy was obtained to assess baseline deuterium enrichment levels. Five days before the first infusion protocol, all participants consumed 100 ml of 70% D₂O (Cambridge Isotope Laboratories, Tewksbury, MA, USA), began collecting daily saliva samples in the morning before consuming any food/water or brushing teeth, and began an EB diet that was designed to provide 100% of energy requirements and a protein intake of 1.2 g/kg/d. Participants were provided with all food for the study, which consisted of frozen meals (Heart to Home Meals, Brampton, ON, Canada) and prepackaged snacks. After the EB diet, participants underwent a stable isotope infusion trial, shown in Fig. 1B, to measure acute mixed MPS and MPB. In brief, after an overnight fast, participants arrived to the laboratory, and a 20-gauge catheter was inserted into a vein in the hand/arm for blood sampling and infusion. We initiated a primed continuous filtered (0.2 µm) infusion of ring-¹³C₆]phenylalanine (2.0 µM/kg, 0.05 µM/kg/min) and ¹⁵[N]phenylalanine (2 µM/kg, 0.05 µM/kg/min; Cambridge Isotope Laboratories). After 150 min of infusion, the ¹⁵[N]phenylalanine tracer was discontinued to calculate MPB by using the tracee-release method (16). At 40 and 60 min after the cessation of the ¹⁵[N]phenylalanine tracer, we obtained muscle biopsies from the *vastus lateralis*. In addition to measuring acute mixed MPS and MPB, ~45 mg of muscle was collected at the 190-min biopsy for

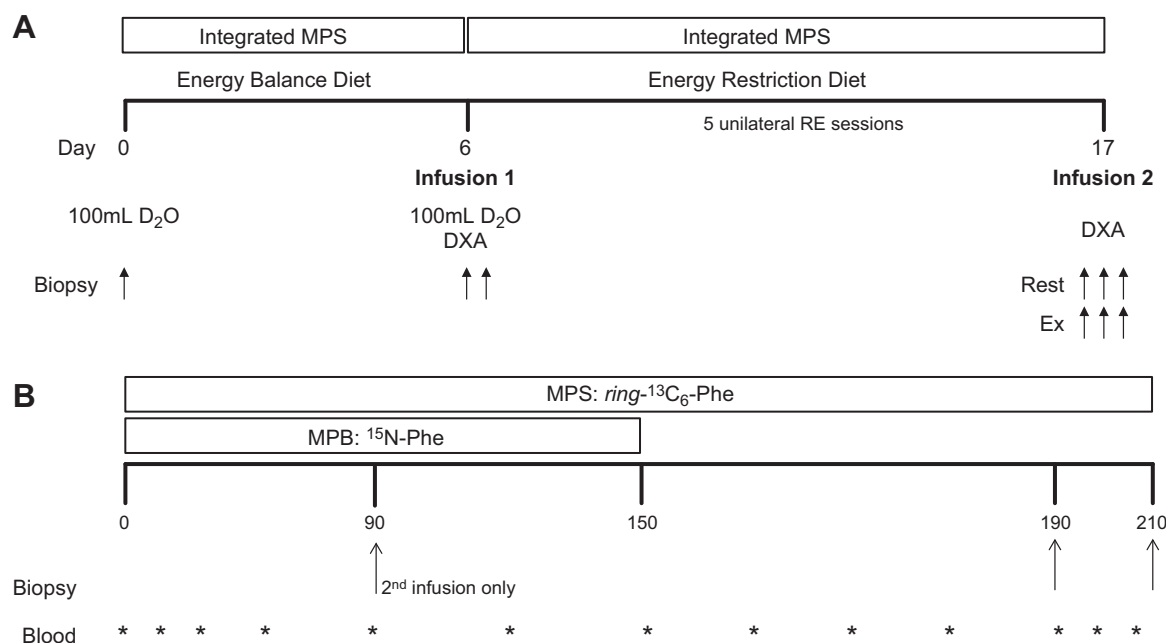


Figure 1. A) Timeline of study. Before any dietary control, a baseline muscle biopsy was obtained. Five days before the first infusion protocol, participants ingested a bolus of D₂O to measure basal integrated myo-PS and consumed an EB diet. Participants then completed an acute infusion protocol to measure postabsorptive rates of mixed MPS and MPB, as well as a DXA scan to measure body composition. Participants then consumed a second bolus of D₂O and completed the ER diet (40% energy deficit, 2.4 or 1.2 g/kg/d of protein) with 5 unilateral resistance exercise sessions. After 10 d of this intervention, a second infusion protocol was conducted to assess changes in acute MPS and MPB, and body composition was measured by DXA. B) Infusion protocol. After an overnight fast, participants arrived to the laboratory, and a 20-gauge catheter was inserted into the vein of one arm, and a baseline blood sample was obtained before a 0.9% saline drip was started to keep the catheter patent for repeated blood sampling. A second catheter was placed in the contralateral arm for a primed continuous infusion of ring-[¹³C₆]phenylalanine (2.0 μM/kg, 0.05 μM/kg/min) and [¹⁵N]phenylalanine (2 μM/kg, 0.05 μM/kg/min). After 150 min of infusion, the [¹⁵N]phenylalanine tracer was discontinued to calculate MPB. During the first infusion (EB) only one leg (rested) was analyzed; however, after weight loss, both the rested and exercised legs were assessed.

the measurement of integrated myo-PS by using ingested D₂O, gene, and protein expression. Muscle biopsy samples were cleared of visible blood and connective tissue and rapidly frozen in liquid nitrogen.

The day after the first infusion protocol, participants consumed another 100-ml bolus of D₂O with daily saliva samples—obtained in the morning before consuming any food/water or brushing teeth—and began a 10-d ER diet, which placed each participant in a 40% energy deficit from their individual calculated energy requirements. In addition to meals, participants also consumed 2 chocolate-flavored protein supplements per day, which were included in caloric requirements. The higher-protein

group consumed macronutrients in a ratio of 35:50:15% (protein:carbohydrate:fat) that included, in a blinded manner, 2 high-protein supplements per day that contained 35 g of whey protein isolate in 250 ml of skimmed (<1% fat) milk. The lower-protein group consumed macronutrients in a ratio of 15:50:35% (protein:carbohydrate:fat), which included 1 high-protein supplement (35 g of whey protein isolate in 250 ml of 2% milk) and 1 placebo (250 ml of 2% milk; Table 2). During the ER diet, participants arrived to the laboratory on 5 separate occasions for the performance of unilateral resistance exercise sessions spread as evenly as possible over the 10-d ER diet, with no more than 2 d of resistance exercise in a row. Hydration/water consumption was *ad libitum* for every exercise session. Exercise sessions 1–4 were performed at any time of day, but not before eating breakfast. The fifth exercise set was performed 48 h before the last infusion trial. Unilateral exercise sessions consisted of 3 sets of 10 repetitions at 85% of predetermined 10 RM, with the last set performed to volitional failure on both the leg press and leg extension. The repetition load for each participant was adjusted to maintain an 8- to 12-repetition range on the last set. Immediately after the exercise sessions, participants consumed 1 high-protein supplement.

After 10 d of the ER diet, participants arrived to the laboratory after an overnight fast for a second infusion protocol. This protocol was identical to the pre-ER infusion protocol to measure acute mixed MPS and MPB, myo-PS, and gene/protein expression, with the exception that a biopsy at 90 min was also obtained to account for new baseline isotope enrichment levels of ring-[¹³C₆]phenylalanine from the previous

TABLE 1. Participants' baseline characteristics

Characteristic	Group		P
	Lower-protein	Higher-protein	
Age (yr)	22 ± 4	22 ± 3	0.78
BMI (kg/m ²)	28 ± 3	29 ± 3	0.83
Body mass (kg)	89.5 ± 10	90.1 ± 16	0.92
Body fat (%)	28 ± 6	30 ± 6	0.47
LBM (kg)	62 ± 7	60 ± 8	0.64
Leg press strength, 10 RM (kg)	111 ± 44	110 ± 19	0.93
Leg extension strength, 10 RM (kg)	41 ± 14	38 ± 9	0.62

Data are presented as means ± SD.

TABLE 2. Diet composition

Diet	Group		<i>P</i>
	Lower-protein	Higher-protein	
5-d EB diet			
Protein (g/kg/d)	1.29 ± 0.09	1.27 ± 0.09	0.56
Protein (g)	117 ± 4	114 ± 5	0.70
Fat (g)	142 ± 18	136 ± 17	0.39
Cho (g)	454 ± 64	429 ± 58	0.34
Energy (kcal/d)	3565 ± 448	3521 ± 486	0.30
Pro:Cho:Fat (% energy)	13:51:36	13:51:36	0.48:0.81:0.29
10-d ER diet			
Protein (g/kg/d)	1.20 ± 0.05	2.35 ± 0.06*	<0.001
Protein (g)	108 ± 11	212 ± 10	<0.001
Fat (g)	82 ± 12	33 ± 5*	<0.001
Cho (g)	267 ± 40	249 ± 34	0.26
Energy (kcal/d)	2215 ± 280	2148 ± 256	0.56
Pro (% energy)	20 ± 3	42 ± 5*	<0.001
Fat (% energy)	33 ± 2	14 ± 1*	<0.001
Cho (% energy)	48 ± 2	44 ± 4*	0.02

Data are presented as means ± SD. Cho, cholesterol; pro, protein. * $P < 0.05$ vs. lower-protein group.

infusion protocol, and biopsies were obtained from both rested and exercised legs.

Body composition and body mass

Body composition and body mass were determined at the same time of day under the same nutritional conditions after each infusion day (pre- and post-ER). A dual-energy X-ray absorptiometry (DXA) scan (GE Lunar iDXA; GE Healthcare Life Sciences, Mississauga, ON, Canada) was performed with a standardized protocol with participants lying similarly on the bed. The same research technician performed all body compartment analyses to minimize variability.

Integrated myo-PS

Approximately 50 mg of wet muscle was homogenized on ice in buffer [500 μ l of 25 mM Tris 0.5% (v:v) Triton X-100 and protease/phosphatase inhibitor cocktail tablets; Complete Protease Inhibitor Mini-Tabs, PhosStop; Roche Diagnostics, Laval, QC, Canada] and centrifuged at 1500 g for 10 min at 4°C to separate supernatant (sarcoplasmic) and pellet (myofibrillar) fractions. To determine myofibrillar protein-bound enrichments, the myofibrillar fraction (pellet) was washed with distilled deionized water, then purified of collagen in NaOH (17). The myofibrillar fraction was then hydrolyzed for 72 h in 1 M HCl and Dowex (50WX8-200 resin; Sigma-Aldrich, Oakville, ON, Canada) at 110°C and mixed on a vortex every 24 h. Free amino acids were purified by using Dowex ion exchange chromatography. *N*-acetyl-*n*-propyl ester of alanine was analyzed by gas chromatography pyrolysis isotope ratio mass spectrometry (Metabolic Solutions, Nashua, NH, USA). The fractional synthetic rate (FSR) of myofibrillar protein in percentage per day was calculated by using the standard precursor-product equation as described previously (18). Saliva samples were analyzed for ^2H enrichment by cavity ring-down spectroscopy by Metabolic Solutions using a Liquid Water Isotope Analyzer with automated injection system (Los Gatos Research, Mountain View, CA, USA) as described previously (19).

Leg absolute synthetic rate (ASR) and absolute breakdown rate (ABR) were calculated as previously described (20). In brief, ~15 mg of frozen muscle tissue was freeze dried and weighed, then homogenized in 0.2 M perchloric acid (PCA) and spun to form a pellet. Supernatant was discarded, and this process was repeated twice. NaOH (0.3 M, 800 μ l) was then added to the pellet, which was dissolved for 30 min at 37°C. Supernatant was used to quantify the protein at A280 on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). By using the rates of integrated myo-PS, leg lean tissue mass that was derived from DXA, and average alkali soluble protein over the 10-d period, ASR and ABR were calculated using the following equations:

$$\text{ASR(g}\cdot\text{d)} = \frac{\text{FSR}}{100} \times \text{LFFM} \times \frac{\text{ASP}}{100}$$

where FSR is the integrated myo-PS rate in percentage per day, LFFM (leg fat-free mass) is the amount of FFM in the leg obtained by DXA corrected for the amount of water in the muscle (wet weight – dry weight), and ASP (alkali soluble protein) is the average alkali soluble protein concentration;

$$\text{ABR(g}\cdot\text{d)} = \frac{\text{FBR}}{100} \times \text{LFFM} \times \frac{\text{ASP}}{100}$$

where fractional breakdown rate (FBR) = FSR – fractional growth rate (FGR), and FGR is the percentage change in leg lean mass per day.

Plasma enrichments

Plasma (50 μ l) was deproteinized in 500 μ l of acetonitrile. The heptafluorobutyl derivative was prepared and the enrichment of ring- $^{13}\text{C}_6$ phenylalanine and ^{15}N phenylalanine was determined by GC-MS (Hewlett Packard 6890, MSD model 5973 Network; Agilent Technologies, Santa Clara, CA, USA; Supplemental Fig. 3).

Intracellular enrichments

Approximately 25 mg of frozen muscle was first washed in 1× PBS to remove blood. Muscle was homogenized with a Teflon pestle in 500 μ l PCA to precipitate the muscle proteins. The

sample was then centrifuged at 10,000 g at 4°C for 5 min. Supernatant was collected, and the procedure was repeated twice for a total volume of 1.5 ml of supernatant. PCA supernatant was purified by using Dowex ion exchange chromatography and the heptafluorobutyl derivative was prepared. Enrichment of *ring*-[¹³C₆]phenylalanine and [¹⁵N]phenylalanine was determined by GC-MS (Hewlett Packard 6890, MSD model 5973 Network). To determine the intracellular phenylalanine concentration, we added 0.225 μl/mg of wet muscle of 0.001 μM D8-phenylalanine (Cambridge Isotope Laboratories) internal standard to 1 sample from each participant. The concentration was calculated as previously described (21). Mixed MPB was calculated by using the tracee-release method (22), where the dilution of the enrichment of [¹⁵N]phenylalanine in the intracellular-free amino acid pool is a function of the release of unlabeled phenylalanine from the protein-bound fraction of the muscle (breakdown), the decay of [¹⁵N]phenylalanine in the blood after cessation of i.v. infusion, and the concentration of unlabeled intracellular-free and protein-bound phenylalanine. Thus, the equation to calculate MPB is:

$$\text{FBR}(\% \cdot \text{h}) = \frac{E_M(t_2) - E_M(t_1)}{P \int_{t_1}^{t_2} E_A(t) dt - (1 + P) \int_{t_1}^{t_2} E_M(t) dt} \times \left(\frac{Q_M}{T} \right)$$

where $E_M(t_2) - E_M(t_1)$ is the change in enrichment in the muscle intracellular fraction from t_1 (40 min) to t_2 (60 min) when isotope infusion is discontinued, the area under the arterialized blood enrichment decay curve is:

$$\int_{t_1}^{t_2} E_A(t) dt$$

the area under the intracellular muscle enrichment decay curve is:

$$\int_{t_1}^{t_2} E_M(t) dt$$

and Q_M/T is the ratio of intracellular-free tracee concentration and protein-bound tracee concentration, $P = E_M/(E_M - E_M)$ at isotope plateau.

Mixed muscle protein synthesis

The precipitated mixed protein pellet that was obtained from homogenization in PCA—from intracellular enrichments—was washed in distilled deionized water, washed 3 times in absolute ethanol, and then placed at 50°C to dry. The dried pellet was weighed, then hydrolyzed for 72 h in 0.1 M HCl and Dowex (50WX8–200 resin) at 110°C and mixed on a vortex every 24 h. Free amino acids were purified by using Dowex ion exchange chromatography, and the *N*-acetyl-*n*-propyl derivative was prepared and analyzed by isotope ratio mass spectrometry to measure bound enrichment of *ring*-[¹³C₆]phenylalanine as previously described (16). Mixed muscle protein synthesis in percentage per hour was calculated by using the standard precursor-product equation as previously described (23). To determine the protein-bound concentration of phenylalanine in the mixed muscle fraction, we added 0.225 μl/mg of dry muscle of 0.001 μM D8-phenylalanine (Cambridge Isotope Laboratories) internal standard. The concentration of phenylalanine was calculated as previously described (24).

Western blotting

Expression of intracellular signaling proteins was assessed by using SDS-PAGE and Western blotting. In brief, after homogenization for

integrated myo-PS, total protein concentration of the sarcoplasmic fraction was determined by using the bicinchoninic acid assay (Thermo Fisher Scientific). Working samples of equal concentration were prepared in Laemmli buffer. Equal amounts of protein (20 μg) from each sample were subjected to SDS-PAGE. Proteins were then transferred to PVDF membranes and exposed overnight at 4°C to primary antibodies after blocking for 2 h in 5% bovine serum albumin. Membranes were washed in Tris-buffered saline and Tween 20, and incubated in anti-rabbit IgG conjugates with horseradish peroxidase secondary Ab (GE Healthcare Life Sciences) for 1 h at room temperature. Signals were detected by using chemiluminescence Super-SignalWest Dura Extended Duration Substrate (Thermo Fisher Scientific) on a FluorChem SP Imaging system (Alpha Innotech, Santa Clara, CA, USA), bands were quantified by using ImageJ scanning densitometry (National Institutes of Health, Bethesda, MD, USA), and data were normalized relative to α-tubulin loading control. The following antibodies used were purchased from Cell Signaling Technology (Danvers, MA, USA): microtubule-associated protein 1A/1B-light chain 3 (LC3) (1:500), caspase-3 (1:500), ubiquitin (1:1000), p-Foxo3a (Ser253; 1:1000), TRAF6 (1:1000), p-TSC2 (Thr1462; 1:1000), and α-tubulin (1:2000).

RNA extraction and RT-PCR

Approximately 20 mg of skeletal muscle was used to isolate RNA using the Trizol phenol-chloroform procedure, as previously described (25). Reverse transcription was performed by using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase was used as housekeeping gene. Relative amounts of mRNA were calculated using the $2^{-\Delta\Delta C_t}$ method (26). Primer sequences for atrogen-1, MuRF-1, Foxo3a, LC3, Gabarap11, Ulk2, Beclin1, BNIP3, and TRAF6 have been previously published (27, 28).

Statistical analyses

Statistical analyses were performed by using SPSS (version 19; IBM, Armonk, NY, USA). A mixed model ANOVA was conducted on body composition, integrated myo-PS, mixed protein turnover, absolute protein turnover, plasma enrichment, protein expression, and gene expression (group × time). Linear regression was performed on plasma and intracellular-free phenylalanine enrichment. Diet analysis, baseline characteristics, and unilateral resistance exercise were analyzed by independent sample Student's *t* test. Significance was set at $P < 0.05$. Data are presented as box and whisker plots, where the box represents the interquartile range, the line in the box represents the median, the cross represents the mean, and the whiskers are the maximum and minimum. Data in tables are all presented as means ± SD.

RESULTS

Participant characteristics

Baseline anthropometric characteristics of participants are provided in Table 1. There were no significant differences between groups for any of the variables at study entry (all $P > 0.05$).

TABLE 3. Body mass and composition

Variable	Lower-protein group			Higher-protein group		
	Pre-weight loss	Post-weight loss	Change	Pre-weight loss	Post-weight loss	Change
Total body mass (kg)	89.5 ± 10.0	87.7 ± 9.8*	−1.8 ± 1.0	90.1 ± 15.9	88.8 ± 16.3*	−1.3 ± 0.8
LBM (kg)	61.8 ± 6.6	60.8 ± 6.8*	−1.0 ± 0.8	60.4 ± 8.5	59.8 ± 8.9*	−0.6 ± 1.0
Fat mass (kg)	24.2 ± 6.7	23.5 ± 9.2*	−0.7 ± 0.6	26.3 ± 9.1	25.5 ± 9.2*	−0.8 ± 0.8
Fat mass, %	27.9 ± 6.0	27.7 ± 6.1	−0.3 ± 0.5	29.7 ± 6.0	29.2 ± 6.2*	−0.6 ± 1.0
Lean mass, exercised leg (kg)	11.3 ± 1.4	11.1 ± 1.4	−0.2 ± 0.4 ^a	10.8 ± 1.5	10.8 ± 1.8	−0.1 ± 0.4 ^a
Lean mass, rested leg (kg)	11.2 ± 1.4	10.8 ± 1.4	−0.4 ± 0.3 ^b	10.8 ± 1.6	10.5 ± 1.8	−0.3 ± 0.4 ^b

Data are presented as means ± SD. ^{a,b}Values without a common letter differ within that group. The change in the rested and exercised legs are significantly different within that group. * $P < 0.05$ vs. pre-weight loss mean within that group.

Dietary manipulation

The composition of the 5-d EB diets is provided in Table 2. There were no significant differences between groups for any of the variables. The composition of the 10-d ER diet is shown in Table 2. The higher-protein group consumed significantly more protein and significantly less fat than the lower-protein group ($P < 0.05$). There were no significant differences in energy intake per day or average energy deficit between groups (all $P > 0.05$). *Post hoc* surveys revealed that 7 of 12 participants in the higher-protein group and 7 of 11 participants in the lower-protein group guessed their weight loss diet—higher- or lower-protein group—correctly.

Unilateral resistance exercise

Total mass lifted (mass/repetition × repetitions/set × sets) (kg) for unilateral leg press was the same in the lower-protein (18,675 ± 6121) and higher-protein (19,188 ± 5155) groups ($P = 0.83$). Total mass lifted (mass/repetition × repetitions/set × sets) (kg) for unilateral leg extension was the same in the lower-protein (6051 ± 1999) and higher-protein (5744 ± 1250) groups ($P = 0.66$).

Body composition

Body composition changes are shown in Table 3. There was a main effect of ER on body mass ($P < 0.001$), fat mass ($P < 0.001$), and total LBM ($P < 0.001$), with no interaction ($P > 0.05$) for each measurement. The change in appendicular LBM displayed a main effect of exercise ($P = 0.003$), but no interaction ($P = 0.29$).

Integrated myo-PS

Integrated myo-PS (%/d) values are shown in Fig. 2. Integrated myo-PS (Fig. 2A) demonstrated a main effect of time ($P < 0.001$) and an interaction ($P = 0.03$). Integrated myo-PS in the exercised leg was preserved compared with EB in the higher-protein group ($P = 0.21$), but only partially preserved in the lower-protein group after ER ($P = 0.03$ vs. EB and $P < 0.001$ vs. energy deficit, rested leg). Body water enrichment (Supplemental Fig. 2) demonstrated a linear decline after dose administration (a linear regression

equation explained a significant proportion of the variance; $r^2 = 0.99$). The change in integrated myo-PS (Fig. 2B) was significantly greater in the rested leg than in the exercised leg in both the higher-protein ($P < 0.001$) and lower-protein ($P < 0.001$) groups, with no difference between protein groups ($P = 0.44$).

Absolute synthesis and breakdown

Calculation of absolute synthesis and breakdown (g/d) is shown in Fig. 3. The FGR of leg lean tissue (%/d) demonstrated a main effect of time ($P < 0.01$), but no interaction ($P = 0.34$; Fig. 3A). FGR was higher in the higher-protein exercised leg compared with the corresponding rested leg ($P = 0.01$; Fig. 3A). ASR (g/d) demonstrated a main effect of time ($P < 0.001$; Fig. 3B), but no interaction ($P = 0.87$). The

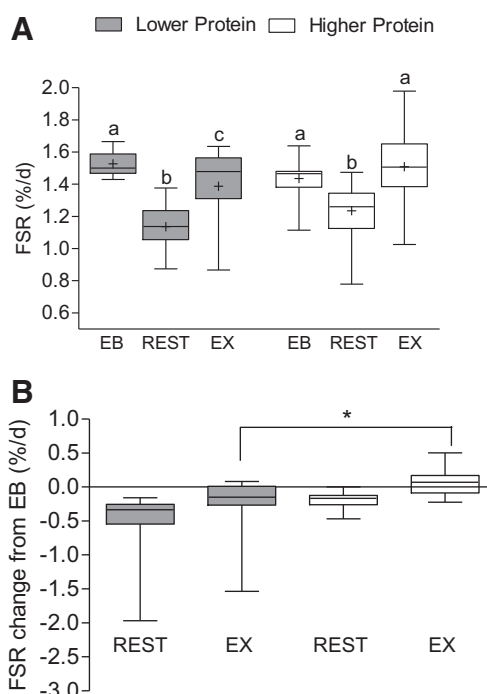


Figure 2. A) Integrated myo-PS. Letters represent within-group statistics, and means without a common letter differ (i.e., a, b, c are significantly different within group). B) Change in myo-PS from energy balance. EX, ER exercised leg. * $P < 0.05$ vs. ER rested leg (Rest).

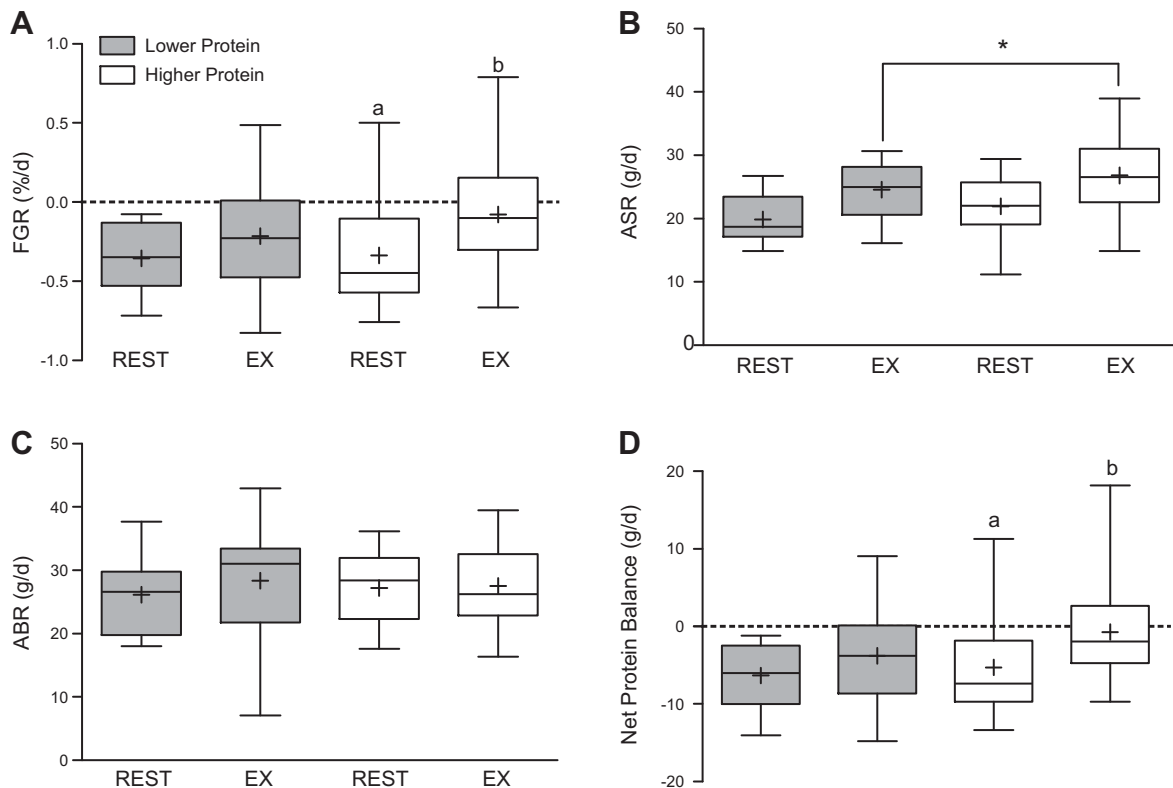


Figure 3. FGR (A), ASR (B), ABR (C), and absolute net protein balance (D). Letters (a, b) represent within-group statistics, and means without a common letter differ (*i.e.*, a and b are significantly different within group). EX, ER exercised leg; * $P < 0.05$ vs. ER rested leg (Rest).

ASR was significantly lower in rested legs compared with exercised legs of both groups ($P = 0.001$; Fig. 3B). ABR (Fig. 3C) was not different between groups or legs (legs, $P = 0.39$; group $\times$ legs, $P = 0.53$). Net protein balance (g/d; ASR – ABR; Fig. 3D) had a main effect for exercise ($P = 0.01$), but no interaction ($P = 0.38$). Net protein balance was significantly higher in the exercised leg of the higher-protein group compared with the corresponding rested leg ($P = 0.01$).

Mixed muscle protein turnover

Postabsorptive mixed MPS (%/h), mixed MPB (%/h), and net protein balance change from EB (%/h) are shown in Fig. 4. There was a main effect of time ($P < 0.001$), but no interaction ($P = 0.07$; Fig. 4A). Mixed MPS was significantly reduced from EB in the rested leg in both groups after ER (higher-protein group, $P = 0.01$; lower-protein group, $P < 0.001$; Fig. 4A). In the lower-protein group, mixed MPS in the exercised leg was no different from EB ($P = 0.97$). In the higher-protein group, mixed MPS was elevated above EB in the exercised leg 48 h after the last exercise bout ($P = 0.01$). Mixed MPB was not different between EB and ER time points in either group (time, $P = 0.17$; interaction, $P = 0.57$; Fig. 4B). The exercised leg had a significantly smaller change in net protein balance from EB than did the rested leg in both groups ($P < 0.001$; Fig. 4C), and there was a strong trend for between-group difference in exercised legs ($P = 0.054$).

Western blots

Total protein ubiquitination, caspase-3 expression, LC3II/LC3I ratio, p-Foxo3a (Ser253), TRAF6, and p-TSC2 (Thr1462) did not change with ER in either group ($P > 0.05$; Fig. 5).

Gene expression

Expression of all genes (Fig. 6), except TRAF6, was not different between groups or across time: atrogin-1 (time, $P = 0.91$; group $\times$ time, $P = 0.97$), MuRF1 (time, $P = 0.09$; group $\times$ time, $P = 0.64$), FoxO3a (time, $P = 0.44$; group $\times$ time, $P = 0.80$), LC3 (time, $P = 0.26$ group $\times$ time, $P = 0.67$), Gabarap (time, $P = 0.07$; group $\times$ time, $P = 0.26$), Ulk2 (time, $P = 0.84$; group $\times$ time, $P = 0.80$), Beclin1 (time, $P = 0.49$; group $\times$ time, $P = 0.36$), BNIP3 (time, $P = 0.06$; time $\times$ group, $P = 0.25$), and TRAF6 (time, $P = 0.01$; group $\times$ time, $P = 0.94$). In the rested leg of the lower-protein group, expression of TRAF6 was significantly greater than in the exercised leg ($P = 0.04$).

DISCUSSION

We ascertained that ER resulted in a significant reduction in integrated (daily) myo-PS, which was alleviated by resistance exercise. In addition, ER resulted in a significant reduction in acute (hourly) postabsorptive mixed MPS, which was also mitigated by resistance exercise that was performed in the preceding 48 h. Consistent with changes in MPS, we observed a significantly greater reduction in

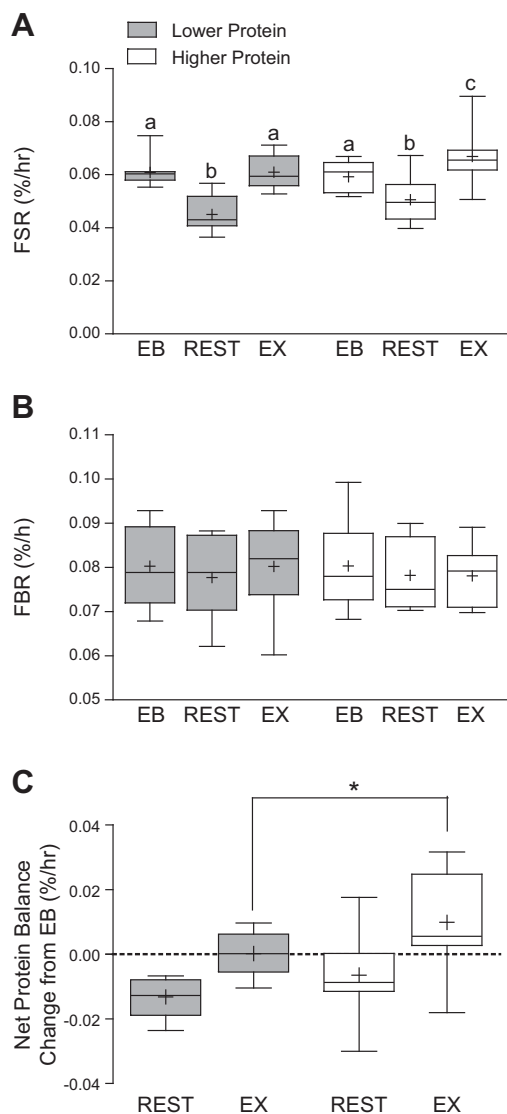


Figure 4. A) Mixed MPS (FSR, %/h). B) Mixed MPB (fractional breakdown rate, %/h). C) Change from EB (%/h). Letters represent within-group statistics, and means without a common letter differ within each group (*i.e.*, *a*, *b*, *c* are significantly different within group). EX, ER exercised leg. * $P < 0.05$ vs. ER rested leg (Rest).

LBM in the rested *vs.* exercised legs—regardless of protein intake—after ER in both groups. Of importance, muscle proteolysis—measured as rates of MPB, ABR, and gene and the protein expression of markers in the ubiquitin-proteasome and autophagic-lysosomal pathways—were unchanged throughout the diet and exercise intervention. These results demonstrate that the main driver of LBM loss during a marked energy deficit in young overweight men is a significant decline in MPS with little to no change in MPB or consummate markers of proteolysis.

MPS data in the current study are consistent with several other studies that have investigated the impact of ER, protein intake, and exercise on the rate of MPS (4–6). For example, Pasiakos *et al.* (5) reported a 19% reduction in postabsorptive mixed MPS in response to 10 d of a 20% energy deficit when participants consumed 1.5 g/kg/d of protein. Murphy and Phillips (29) reported a significant

reduction in integrated myo-PS in overweight older men after 2 wk of ER, which was increased after a second 2-wk period of ER with resistance exercise. The current study provides novel data on the effect of ER, protein, and resistance exercise on integrated day-to-day myo-PS in young men, which are in general agreement with data in older men (29). In addition, we demonstrated that even in ER, resistance exercise has the capacity to stimulate postabsorptive mixed MPS up to 48 h after an exercise bout, which is consistent with work from situations of EB (16) and further demonstrates the potency of resistance exercise in muscle protein synthesis (6), providing a fundamentally anabolic signal to skeletal muscle.

In contrast to data that show that postabsorptive MPB increased by 60% after 10 d of a 20% energy deficit (with participants consuming 1.5 g protein/kg/d) (12), we did not detect any measurable changes in the rates of postabsorptive MPB, despite our participants being in a 40% energy deficit. These disparate findings could be a result of differences in participant characteristics [normal weight (12) compared with overweight in the current study]. Differences in participant characteristics may be important when considering findings from Heymsfield *et al.* (30), where the fraction of weight lost as LBM during semistarvation in young men was plotted against baseline adiposity. Heymsfield *et al.* (30) reported that men in the lowest quintile of baseline percentage fat lost more LBM than did men in the higher quintiles of percentage fat; therefore, individuals who are leaner may be more susceptible to LBM loss. However, whether LBM loss in lean individuals is a result of a large increase in MPB as observed by Carbone *et al.* (12) requires additional investigation, especially when considering previously reported declines in MPS in the same participants (5), which would contribute to LBM loss. An important consideration is that individuals spend most of their waking hours in the postprandial state, assuming they do not skip meals. Use of ingested D_2O allowed an integrated measure that encompassed postprandial, postabsorptive, and fasting (sleeping) periods. In the current study, the average decline of integrated myo-PS in rested legs was $-0.36 \pm 0.08\%/d$, which was close to the average change in leg LBM per day of $-0.34 \pm 0.06\%/d$. Thus, even if we assume that only 10 h/d were spent in the fasted state, given that the changes in integrated MPS align with the loss of LBM, it is difficult to reconcile that increases in postabsorptive MPB are a significant mechanism that underpins the loss of LBM during ER in overweight young men.

By simultaneously assessing the rates of MPS and MPB, we were able to calculate the net protein balance, which, to our knowledge, has not been done during ER in humans. Consistent with our other data, changes in net protein balance were mainly driven by changes in mixed MPS (Fig. 4A). The reduction in the net protein balance—change from energy balance—was significantly greater in the rested leg than in the exercised leg in both groups, and it is of interest to note the strong trend for a group effect in exercised legs, which tended to be elevated from EB in the higher-protein group ($P = 0.054$). To further support our acute protein turnover data, we calculated absolute

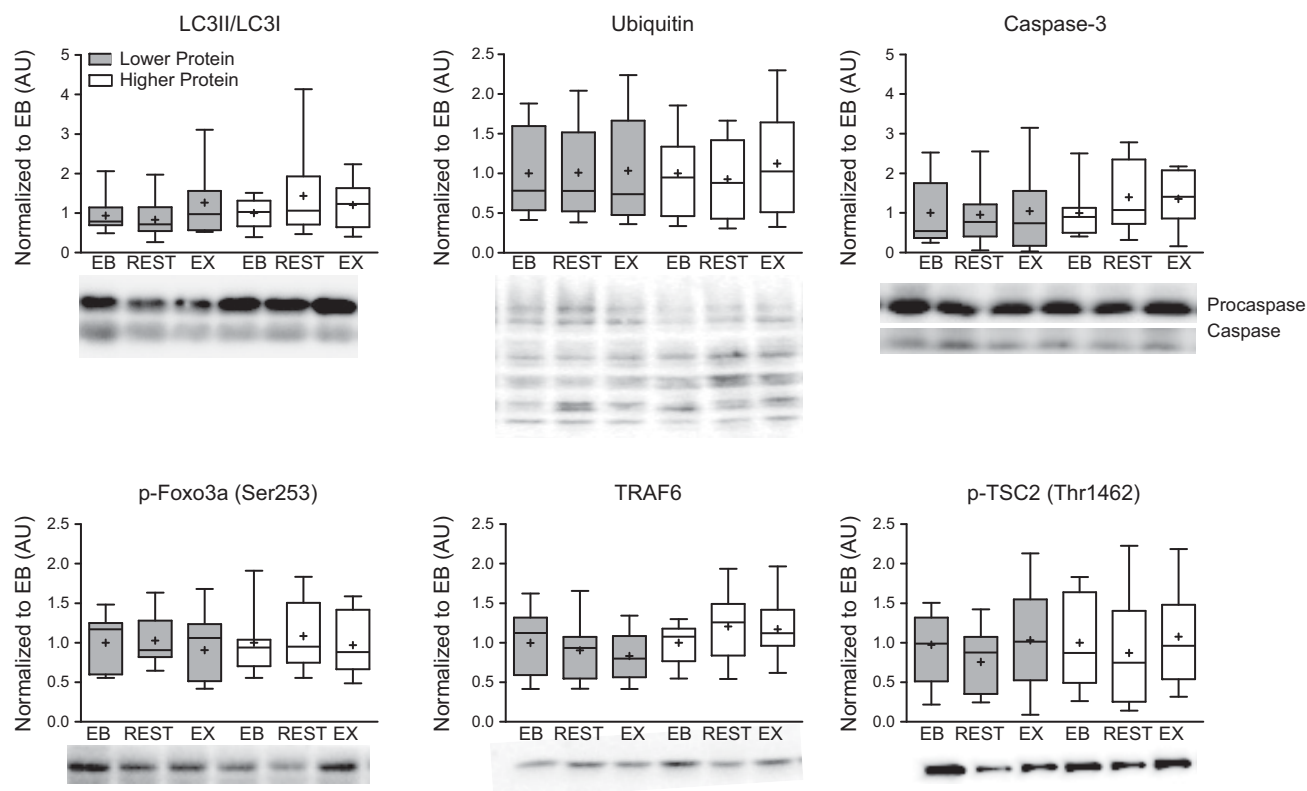


Figure 5. Western blot quantification with representative images. Data are normalized to the average weight maintenance value. AU, arbitrary units; EX, ER exercised leg; LC3, microtubule-associated protein 1A/1B light chain 3; Foxo3a, forkhead box O3; Rest, ER rested leg; TRAF6, TNF receptor-associated factor 6; TSC2, tuberous sclerosis complex 2.

(grams/day) myo-PS, breakdown, and net protein balance. Similar to our acute data, ABR remained essentially unchanged across time, and ASR was reduced in the rested leg compared with the exercised leg. Absolute net protein balance was elevated in the exercised leg compared with the rested leg of the higher-protein group, which, again, is in agreement with the fundamentally anabolic nature of resistance exercise. In keeping with the study design from Longland *et al.*, we opted to keep carbohydrate intake constant between the higher- and lower-protein groups because of the known effects that carbohydrates have on exercise performance (31). Thus, we cannot attribute our observed effects on muscle protein turnover solely to protein intake because there were also differences in fat intake between these two groups; however, there is nothing to suggest that differences in daily dietary fat intake that do not exceed the acceptable macronutrient distribution range would induce changes in muscle protein turnover. Hammond *et al.* (32) reported that high-fat feeding (3.5 g/kg) postexercise suppresses p70S6K1 activity; however, the protein supplement that was provided to all participants postexercise in the current study contained a maximum of 5 g of fat (from 2% milk), and we thus would not expect the same effect to occur (32).

In agreement with the acute rates of MPB in the current study, we did not observe any changes in gene or protein expression of targets in the ubiquitin-proteasome and autophagic-lysosomal pathways, which is consistent with some short-term studies in humans (6, 12, 13). Other ER studies have observed only small increases in these

proteolytic genes and proteins. For example, Carbone *et al.* (12) observed no change in 26S proteasome proteolytic activity, and an 11% increase in caspase-3 protein expression, despite reporting a 60% increase in MPB. After 21 d of participants in a 40% energy deficit, increases of 20 and 30% in the genes that encode MuRF-1 and Atrogin-1 were reported, but there was no increase in the activities of the subunits of the 26S proteasome or caspase-3, and dietary protein intake did not affect the expression of these atrogenes (11). Thus, it seems entirely equivocal that these pathways are up-regulated with ER in young men. In the current study, we observed a slightly elevated (1.4-fold) level of TRAF6 [a regulator of both the ubiquitin-proteasome and autophagic-lysosomal pathways (33)] mRNA in the rested leg of the lower-protein group; however, this was not significantly elevated above the expression observed in EB and the protein content of TRAF6 was unchanged.

Despite the novelty of our data, we acknowledge some limitations of the current study. First, the short-term nature of the study resulted in small changes in body composition; however, we were still able to detect an exercise-mediated effect and hypothesize that an effect of dietary protein would take more time and, potentially, a larger sample size to detect when outcomes were measured by DXA (8). A second limitation of the current study is a result of the timing of the biopsies that were used to examine gene and protein expression, which were taken in the basal, fasted state pre- and post-ER. The timing of these biopsies was strategic in that the most likely time to detect changes in MPB and its associated markers is the fasted

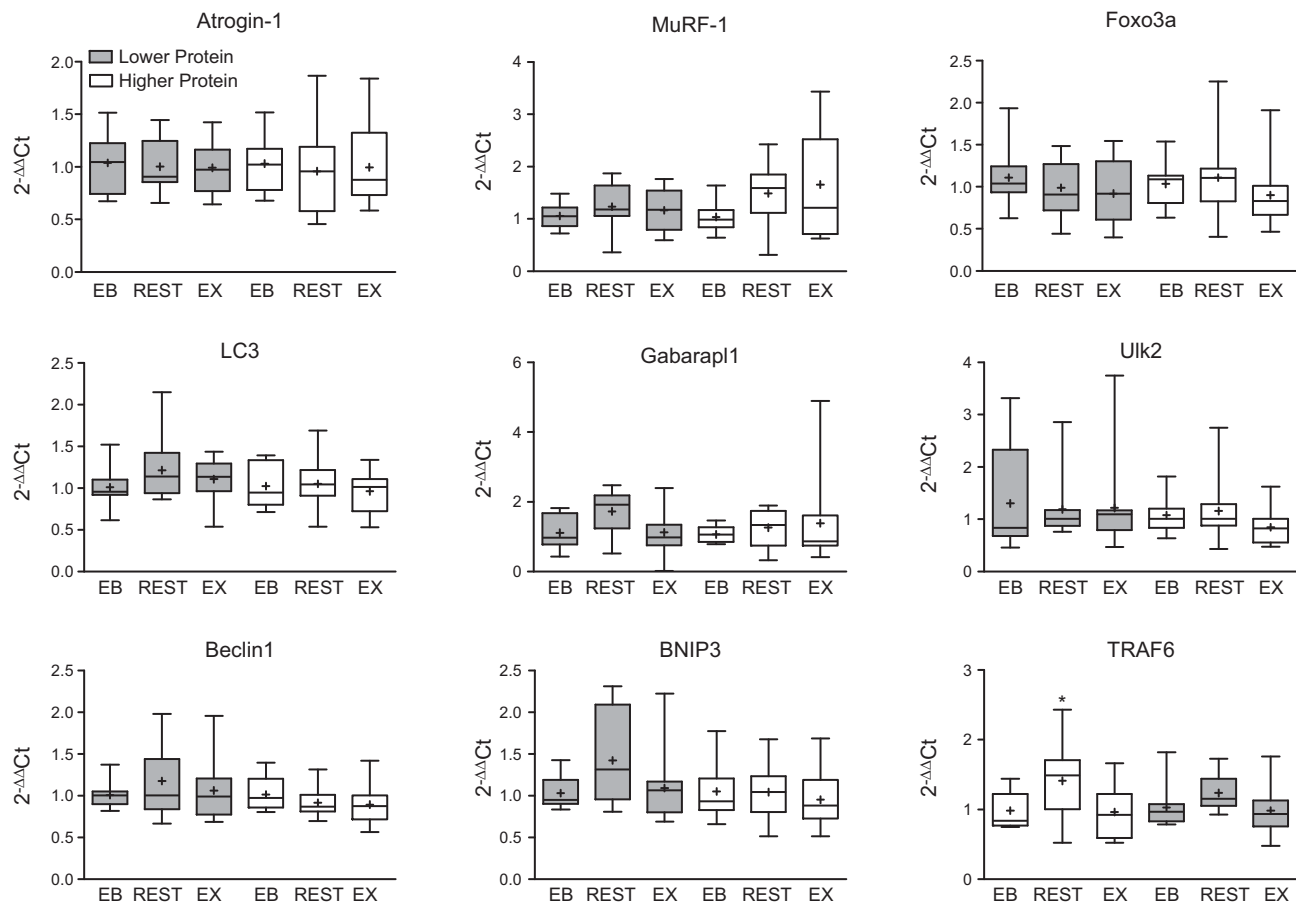


Figure 6. Gene expression of targets in the ubiquitin-proteasome and autophagic-lysosomal pathways. BNIP3, BCL2 interacting protein 3; Foxo3a, forkhead box O3; Gabarapl1, GABA_A receptor-associated protein-like 1; LC3, microtubule-associated protein 1A/1B light chain 3; MuRF-1, muscle RING-finger protein-1; Rest, ER rested leg; TRAF6, TNF receptor-associated factor 6; Ulk2, Unc-51-like autophagy activating kinase 2. * $P < 0.05$ vs. ER exercised leg (EX) within that group.

state (34). Nonetheless, the one target related to MPS that we did analyze—p-TSC2 (Thr1462)—did not change pre- to post-ER. Because of the numerous muscle biopsies already being performed on participants, we did not attempt to characterize a time course or effect of feeding on changes in gene and protein expression, which may have allowed us to detect differences in signaling related to MPS if an effect were present. Nonetheless, we acknowledge that there are data that show a reduction in the phosphorylation of Akt and 4EBP-1 after 10 d of ER (5), as well as a reduction in the MPS response to protein ingestion during ER (4, 7). Data from rodents suggest that ER results in the down-regulation of the phosphorylation of protein synthesis-related proteins (Akt, mammalian target of rapamycin, rps6, and p70S6K) (35) and a reduction in active ribosomes (36). Thus, strategies that target the mammalian target of rapamycin complex 1 pathway, such as resistance exercise and increased protein intake (3), are important for the preservation of MPS and LBM during ER. Despite our best efforts to reduce the inherent variability in human data, which included providing all food to participants, supervising all resistance exercise sessions, performing all measures of MPS and MPB at the same time of day, and matching groups as closely as possible, we acknowledge the variability of the data.

In summary, we show that in young overweight men, ER results in a decrease in MPS, but resistance exercise

with protein intake that is 3 times higher than the RDA can mitigate this decline. Furthermore, we demonstrate that MPB does not change, despite marked ER in overweight young men. Finally, we provide a comprehensive assessment of the processes that govern changes in skeletal muscle mass during ER in overweight young men. Our findings may be of importance for designing weight loss programs that wish to preserve skeletal muscle and promote the loss of fat mass. **[F]**

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AUTHOR CONTRIBUTIONS

A. J. Hector and S. M. Phillips designed the research; A. J. Hector, C. McGlory, F. Damas, N. Mazara, S. K. Baker, and S. M. Phillips performed the data collection; A. J. Hector performed the laboratory analyses; A. J. Hector, C. McGlory,

and S. M. Phillips analyzed the data; A. J. Hector drafted the manuscript; and all authors edited and approved the final version.

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