REVIEW

Recent Advances in the Characterization of Skeletal Muscle and Whole-Body Protein Responses to Dietary Protein and Exercise during Negative Energy Balance

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ABSTRACT

In a review published in 2012, we concluded that higher-protein diets preserve muscle mass during energy deficit via stimulated mammalian target of rapamycin complex 1 signaling, coincident increased muscle protein synthesis (PS), inhibited ubiquitin-mediated proteolysis, and suppressed muscle protein breakdown (PB). Since then, there have been significant advances in understanding the fundamental effects of higher-protein diets, with or without exercise training, on muscle and whole-body protein homeostasis during negative energy balance. Therefore, an update on the evolution of this field of research is warranted to better inform recommendations on best practices for healthy weight loss and muscle preservation. We will review the most recent studies examining the effects of higher-protein diets and negative energy balance on body composition, muscle PS, muscle PB, associated intracellular regulatory pathway activities, and whole-body protein homeostasis. In addition to critically analyzing contemporary findings, knowledge gaps and opportunities for continued research will be identified. Overall, the newest research confirms that consuming higher-protein diets, particularly when coupled with resistance exercise, preserves muscle mass and maintains whole-body protein homeostasis during moderate energy deficits (i.e., normal weight loss). However, these newer findings also indicate that as the magnitude of energy deficit increases, the efficacy of higher-protein diets for mitigating losses of fat-free mass is diminished. Further, recent results suggest that alterations in muscle PS, more so than muscle PB, may be primarily responsible for changes in muscle mass that occur in response to negative energy balance. *Adv Nutr* 2019;10:70–79.

Keywords: energy deficit, dietary protein, body composition, protein synthesis, net protein balance

Introduction

In 2012, we reviewed the existing evidence regarding the fundamental mechanisms by which skeletal muscle responds to dietary protein intakes above the RDA (0.8 g · kg body weight^{-1} · d^{-1}) during negative energy balance (i.e., weight loss) (1). At the time, our conclusions were limited by a relative paucity of studies assessing the effects of energy deficit (the percentage of energy required to achieve energy balance based on the difference between expenditure and intake) and dietary protein intake on the regulation of muscle protein synthesis (PS) and muscle protein breakdown (PB). Those conclusions, that consuming higher-protein diets during sustained periods of energy deficit would stimulate muscle PS, suppress muscle PB, and spare muscle mass, were based on only 3 available studies evaluating muscle PS (2–4) and muscle PB (5–7) during weight loss.

Since that 2012 review was published, investigations of the mechanisms regulating muscle mass and their responses to negative energy balance and dietary protein interventions have continued at an accelerated pace. Within the past decade, a considerable amount of new information has been generated by various prominent laboratories (**Table 1**). As a result, we feel it warranted to revisit this topic and consider how our understanding of muscle and whole-body

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Abbreviations used: Akt, protein kinase B; Atg5, autophagy-related gene 5; c-miR, circulating micro-RNA; miRNA, micro-RNA; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; PB, protein breakdown; PS, protein synthesis; p70S6K, 70kDa S6 kinase; rpS6, ribosomal protein S6.

TABLE 1 Effects of negative energy balance, dietary protein, exercise, and environment on body composition and muscle and whole-body protein turnover[1]

Study category; author (ref)	Volunteers	Energy deficit	Intervention	Primary findings
Energy deficit and protein intake				
Carbone et al. (8)	39 recreationally active men ($n = 32$) and women ($n = 7$)	21 d, 40% (~1000 kcal/d)	Dietary protein intake at 0.8, 1.6, or 2.4 g · kg^{-1} · d^{-1}; postabsorptive and postprandial (mixed-meal; 20 g protein) measures	ED did not significantly alter ubiquitin-mediated proteolysis, but it was suppressed by acute feeding regardless of chronic dietary protein intake.
Hector et al. (9)	40 overweight and obese men ($n = 19$) and women ($n = 21$)	2 wk, 28–34% (range: 680–860 kcal/d)	Twice-daily isonitrogenous whey or soy or isoenergetic carbohydrate supplementation, resulting in dietary protein intakes of 1.3, 1.3, or 0.7 g · kg^{-1} · d^{-1}, respectively	ED suppressed postabsorptive muscle PS. Whey supplementation attenuated postabsorptive, and enhanced postprandial, muscle PS response, relative to soy or carbohydrate.
Pasiakos et al. (10)	39 recreationally active men ($n = 32$) and women ($n = 7$)	21 d, 40% (~1000 kcal/d)	Dietary protein intake at 0.8, 1.6, or 2.4 g · kg^{-1} · d^{-1}; postabsorptive and postprandial (mixed-meal; 20 g protein) measures	Consuming higher-protein diets (double and triple the RDA) maintained the anabolic response to feeding during ED and preserved LBM during weight loss, compared with protein intake at the RDA.
Pasiakos et al. (11)	39 recreationally active men ($n = 32$) and women ($n = 7$)	21 d, 40% (~1000 kcal/d)	Dietary protein intake at 0.8, 1.6, or 2.4 g · kg^{-1} · d^{-1}; postabsorptive and postprandial (mixed-meal; 20 g protein) measures	ED attenuated whole-body protein flux, PS, and PB, regardless of dietary protein intake. Acute feeding increased whole-body protein flux and PS, regardless of dietary protein intake. Higher-protein intakes increased protein oxidation and decreased net protein balance.
Energy deficit and exercise				
Carbone et al. (12)	10 recreationally active men ($n = 6$) and women ($n = 4$)	10 d, 20% (500 kcal/d)	Postabsorptive measures taken after rest or after a 45-min moderate-intensity run	ED increased muscle PB and caspase-3 activation without altering ubiquitin ligase expression or proteasome activity.
Karl et al. (13)	23 male ($n = 17$) and female ($n = 6$) US soldiers	2 d, 93% (~3680 kcal/d)	Postabsorptive measures taken before and after significant exercise-induced energy expenditure (~1300 kcal/d) during 2-d energy balance and subsequent 2-d ED	High exercise-induced energy expenditure suppressed whole-body protein flux, PS, and PB. Subsequent ED increased PB without altering flux and PS, driving net protein balance more negative.
Margolis et al. (14)	21 male Norwegian soldiers	4 d, 50% (3390 kcal/d)	Postabsorptive measures after a 4-d, 54-km cross-country ski march	Ski march and associated ED increased postabsorptive whole-body protein flux, PS, and PB.
Margolis et al. (15)	73 male ($n = 71$) and female ($n = 2$) Norwegian soldiers	4 d, 50–60% (3000–3600 kcal/d)	24-h measures after a 4-d, 51-km cross-country ski march	Ski march and associated ED suppressed 24-h whole-body protein flux, PS, and PB.
Moberg et al. (16)	24 male ($n = 17$) and female ($n = 7$) Swedish soldiers	8 d, 29% (1690 kcal/d) for men and 15% (750 kcal/d) for women	Postabsorptive measures before and after an 8-d military training exercise	8-d military training increased autophagy-related proteolysis without eliciting impactful changes in ubiquitin-mediated proteolysis.
Energy deficit, exercise, and protein intake				
Areta et al. (17)	15 resistance-trained men ($n = 8$) and women ($n = 7$)	5 d, 33% (−15 kcal · kg FFM^{-1} · d^{-1}; ~900 kcal/d)	REX, postexercise placebo or 15 or 30 g whey protein	ED suppressed muscle PS, but REX and postexercise protein increased muscle PS during ED.

(*Continued*)

TABLE 1 (*Continued*)

Study category; author (ref)	Volunteers	Energy deficit	Intervention	Primary findings
Berryman et al. (18)	63 male US Marines	7 d, ∼4200 kcal/d	7-d intensive military training with accompanying ED, followed by 27-d refeeding period providing supplemental protein (7, 84, or 133 g/d) to ad libitum diet	Training and concomitant ED increased whole-body PB while lowering whole-body PS and net protein balance. Refeeding increased postabsorptive whole-body PS, PB, and net protein balance. Protein supplementation of a higher-protein diet (∼2.0 g · kg^{-1} · d^{-1}) did not alter whole-body protein turnover.
Hector et al. (19)	24 untrained men	10 d, 40% (1360 kcal/d)	Dietary protein intake at 1.20 or 2.35 g · kg^{-1} · d^{-1}; 5 REX sessions during 10-d ED	ED suppressed muscle PS, but inclusion of REX abolished this decrease. Muscle PB was not significantly influenced by ED or REX.
Josse et al. (20)	90 premenopausal overweight and obese women	16 wk, ∼25% (470 kcal/d)	AEX and REX; high (30% kcal) or adequate (15% kcal) dietary protein intake	High protein consumption during exercise- and diet-induced weight loss resulted in more favorable body composition changes than adequate protein, including greater LBM gain and fat mass loss.
Longland et al. (21)	40 recreationally active, overweight men	4 wk, 40% (∼1560 kcal/d)	HIIT and REX; high (35% kcal) or adequate (15% kcal) dietary protein intake	Combining higher protein consumption with HIIT and REX induced greater LBM gain and fat mass loss than observed with adequate protein intake.
Murphy et al. (22)	20 overweight and obese older men	4 wk, 300 kcal/d	2-wk ED followed by 2-wk ED with 3-d/wk REX; dietary protein provided at 1.3 g · kg^{-1} · d^{-1} either evenly distributed (25%/meal × 4 meals) or skewed (7%, 17%, 72%, 4%) over 4 meals	ED suppressed myofibrillar PS. REX increased myofibrillar PS during ED. Evenly distributed protein intake increased postprandial myofibrillar PS, relative to skewed intake. Combining REX with evenly distributed protein intake, but not skewed intake, during ED restored myofibrillar PS rates to those observed during energy balance.
Smiles et al. (23)	15 resistance-trained men ($n = 8$) and women ($n = 7$)	5-d, 33% (−15 kcal · kg FFM^{-1} · d^{-1}; ∼900 kcal/d)	REX, postexercise placebo or 30 g whey protein	ED suppressed autophagy-related protein expression, but REX with postexercise protein consumption during ED enhanced the autophagy response.
Energy deficit, exercise, environment, and protein intake				
Berryman et al. (24)	17 recreationally active men	21-d, 70% (∼1840 kcal/d)	21-d acclimatization to HA (4300 m) with accompanying ED; dietary protein intake at 1.0 or 2.0 g · kg^{-1} · d^{-1}	HA and accompanying ED induced significant loss of body weight and LBM, independent of dietary protein intake, and suppressed postabsorptive whole-body PS and PB. Higher-protein intake increased postabsorptive protein oxidation and drove whole-body protein balance more negative.

(*Continued*)

TABLE 1 (*Continued*)

Study category; author (ref)	Volunteers	Energy deficit	Intervention	Primary findings
Margolis et al. (25)	8 recreationally active men	21 d, 70% (∼1840 kcal/d)	21-d acclimatization to HA (4300 m) with accompanying ED; dietary protein intake at 1.0 or 2.0 g · kg^{-1} · d^{-1}; acute aerobic exercise and postexercise recovery protein (25 g whey) ingestion	HA exposure suppressed postexercise and recovery mTORC1 stimulation, an effect that was compounded by ED. Ubiquitin-mediated proteolysis was largely unaffected by HA.
Energy deficit and miRNA				
Margolis et al. (26)	16 sedentary, overweight, older men	28 d, 30% (800 kcal/d)	Dietary protein at 1.0 g · kg^{-1} · d^{-1}; plasma miRNA and 24-h whole-body protein turnover measures pre- and post-ED	Expression of protein synthetic inhibitory miRNA increased after ED; this increase was inversely associated with whole-body PS.
Parr et al. (27)	40 overweight and obese men and women	16 wk, ∼25% (500 kcal/d)	Volunteers classified as either high- or low-responders based on body mass lost during ED (>10% vs. <5%)	Specific miRNAs associated with exercise, dietary intervention, and/or weight loss were higher pre- and post-ED in hyper-responders, or increased post-ED regardless of weight-loss response.

[1] AEX, aerobic exercise; ED, energy deficit; FFM, fat-free mass; HA, high altitude; HIIT, high-intensity interval training; LBM, lean body mass; miRNA, micro-RNA; mTORC1, mammalian target of rapamycin complex 1; PB, protein breakdown; PS, protein synthesis; ref, reference; REX, resistance exercise.

protein homeostatic responses to dietary protein interventions during negative energy balance has evolved. Doing so will establish a contemporary foundation from which new research directions and informed recommendations on best practices for healthy weight loss and muscle preservation can be made.

Accordingly, the effects of exercise and the impact of other potential metabolic stressors on protein balance during energy deficit will be discussed, with particular emphasis on the findings published since 2012. Studies examining effects of negative energy balance, dietary protein, exercise, and metabolic stress on the intracellular regulators of muscle PS and muscle PB will be highlighted to illustrate advancements in the field. We will also identify knowledge gaps and opportunities for continued investigation, such that recent fundamental advances in our understanding of this discipline can be continued and targeted to those areas which could benefit most from additional study.

Current Status of Knowledge

The protective effects of dietary protein on muscle mass

Sustained periods of negative energy balance present a major challenge to muscle maintenance because muscle, which serves as an amino acid reservoir, can be readily catabolized during negative energy balance to provide precursors for gluconeogenesis and oxidative energy metabolism (28). It is generally accepted that consuming higher-protein diets can spare muscle mass in response to negative energy balance (1). Thus, the higher-than-RDA protein intakes (∼1.2 g · kg body $weight^{-1}$ · d^{-1}) typical of the Western diet (29) may offer muscle-protective advantages during weight loss. Recent studies have advanced our understanding of this protective role by examining changes in muscle mass produced by manipulating the degree of energy deficit and the relative amount of dietary protein consumed, as well as differentiating the influence of aerobic and resistance exercise on changes in muscle mass. In healthy young men, fat-free mass loss was attenuated during a 3-wk, 40% energy deficit (∼1000 kcal/d) when protein intakes were double or triple the RDA, compared with consuming only the RDA for protein (10). More specifically, although the 3 protein groups lost the same amount of total body mass, those participants consuming double and triple the RDA lost proportionately more fat and less fat-free mass than the participants consuming the RDA for protein.

Coupling exercise training with higher-protein diets during negative energy balance potentiates these benefits. Longland et al. (21) demonstrated that healthy young men lost significantly more fat mass when consuming higher-protein diets (2.4 compared with 1.2 g · kg^{-1} · d^{-1}) and actually gained lean mass during a 4-wk, 40% energy deficit (∼1560 kcal/d), concomitant with a 6-d/wk resistance-based, high-intensity, high-volume interval training program. In that study (21), however, those participants adhering to the lower-protein study diet still consumed 1.5 times the RDA for protein, and their lean mass was completely preserved during the 4-wk underfeeding period. In contrast, the participants in our study (10), who engaged in primarily aerobic-type exercise only, exhibited attenuation, not complete prevention, of fat-free mass loss during a 3-wk, 40% energy deficit (10). In addition, we did not observe a further

fat-free mass protective advantage of consuming a triple- rather than double-RDA protein diet, whereas Longland et al. (21) reported a clear benefit of consuming protein at triple compared with 1.5 times the RDA. Together, these findings illustrate the significant anabolic stimulus produced by coupling optimization of dietary protein intake with resistance exercise training. Similar benefits of higher-dietary protein in conjunction with exercise during negative energy balance have also been observed in healthy, premenopausal overweight and obese women performing combined aerobic- and resistance-type training (20).

The efficacy of consuming higher-protein diets during negative energy balance must, however, be considered in the context of the magnitude of the overall energy deficit. In the studies discussed above (10, 21), the energy deficit was relatively modest (40%). In contrast, supplementing ad libitum, higher-protein diets with additional high-quality protein did not alter body composition or whole-body net protein balance in US Marines in recovery from a 7-d, severe energy deficit (∼4200 kcal/d) (18). Similarly, doubling dietary protein intake (2.0 compared with 1.0 g · kg^{-1} · d^{-1}) did not attenuate fat-free mass loss in healthy young men exposed to a ∼70% energy deficit (∼1840 kcal/d) for 21 d while acclimatizing to high altitude (4300 m) (24). Thus, it appears that consuming protein at amounts in excess of the RDA can be beneficial for supporting muscle mass during modest energy deficit (e.g., ≤40%), but that a plateau may exist (e.g., >40%) after which increases in dietary protein intake may not confer greater fat-free mass protection. Likewise, the magnitude of the energy deficit dictates dietary protein utilization, as underfeeding upregulates the targeting of amino acids to energy-yielding pathways, mitigating the potential for dietary amino acids to support muscle PS. Currently, the energy-deficit threshold at which higher-protein intakes no longer offer net benefit, in terms of muscle mass preservation, is unknown and presents a clear research opportunity. Identifying this potentially advantageous protein metabolic breakpoint could allow for more informed development of nutritional countermeasures to offset muscle loss during energy deficit.

Muscle protein turnover responses to negative energy balance and dietary protein

Negative energy balance downregulates muscle PS (2). This reduction in muscle PS can be influenced not just by the quantity of dietary protein but by the quality of protein consumed and the timing of protein consumption over the course of the day. In overweight and obese older men, both postabsorptive and postprandial myofibrillar PS were significantly suppressed after a 2-wk, 300-kcal/d energy deficit as compared with energy balance (22). Regardless of energy status, postprandial myofibrillar PS was significantly greater when protein intake was evenly distributed over the course of the day (25% per meal × 4 meals) compared with a skewed intake that provided 7%, 17%, 72%, and 4% of daily protein over 4 meals, respectively. In another study (9), overweight and obese men and women exhibited suppressed postabsorptive muscle PS after a 2-wk, 28–34% energy deficit (range: 680–860 kcal/d), but the magnitude of muscle PS downregulation was mitigated to the greatest extent after consuming 27 g supplemental whey protein, as compared with isonitrogenous soy protein or isoenergetic carbohydrate. This effect mirrors the greater stimulation of postprandial muscle PS observed after whey protein ingestion, relative to soy and carbohydrate, during energy balance (9). It is reasonable to conclude that the anabolic stimulus achieved by regular ingestion of high-quality dietary protein underlies the preservation of fat-free mass observed with the adherence to a higher-protein, energy-deficient diet. This is similar to what we observed in healthy young adults after a 21-d, 40% energy deficit (∼1000 kcal/d) (10). Those consuming double and triple the RDA for protein maintained the muscle anabolic response to a protein-containing mixed-meal during the energy deficit, whereas the postprandial muscle PS response to the same protein-containing mixed-meal was blunted during the energy deficit for those consuming the RDA for protein.

Structured exercise training, particularly resistance exercise, can also mitigate the decrements in muscle PS observed during energy deficit. After a 10-d, 40% energy deficit (1360 kcal/d), the addition of resistance training abolished the declines in muscle PS observed without exercise training (19). Murphy et al. (22) reported that engaging in resistance exercise training (3 d/wk) during a modest 14-d energy deficit (300 kcal/d) increased myofibrillar PS compared with energy deficit alone. In the same study (22), participants performing resistance training during the energy deficit and consuming evenly distributed daily protein intake (25% per meal × 4 meals) exhibited the same rate of myofibrillar PS as observed during energy balance, whereas in participants skewing their daily protein intake (7%:17%:72%:4%), myofibrillar PS during the energy deficit remained lower than observed during energy balance, even with the additional anabolic stimulus of resistance training.

It would seem logical then that combining resistance exercise with postexercise protein ingestion could further mitigate the decline in muscle PS observed during energy deficit. Indeed, postabsorptive muscle PS in rested healthy young adults was significantly lower than during energy balance after a 5-d, 33% energy deficit (−15 kcal · kg FFM^{-1} · d^{-1}; ∼900 kcal/d), but postabsorptive muscle PS measured immediately after a resistance exercise bout at the conclusion of the energy deficit was comparable to rates observed during energy balance, with no difference between men and women (17). Furthermore, ingesting high-quality protein (15 compared with 30 g of whey) immediately postexercise increased muscle PS, surpassing in a dose-dependent manner not just postabsorptive muscle PS rates observed during energy deficit but also postabsorptive muscle PS rates measured during energy balance.

While the independent and collective effects of dietary protein intake and exercise on muscle PS during energy deficit have been better delineated, the maintenance of muscle mass, regardless of energy status, is dictated by

the balance between muscle PS and muscle PB. However, factors affecting muscle PB during periods of energy deficit have received very limited attention. We previously showed a 60% increase in the postabsorptive muscle PB rate in healthy young adults after a 10-d, 20% energy deficit (500 kcal/d), an effect not exacerbated by an aerobic exercise bout (12). In contrast to our findings, Hector et al. (19) showed no change in muscle PB after a 10-d, 40% energy deficit (~1360 kcal/d), with no influence of resistance training on muscle PB measures. These discordant observations may be related to baseline volunteer characteristics, as illustrated by higher postabsorptive muscle PB for Hector's overweight volunteers (19) relative to our lean group (12), even under eucaloric conditions, and/or the primary exercise approach (aerobic compared with resistance), as previous reports showed higher muscle PB after 4 wk of aerobic training (30) but no difference from baseline 48 h after resistance exercise (31). It is also important to note that muscle protein fractional breakdown rate using labeled amino acid tracers can be an inherently difficult measure to obtain (32), and that the required modeling assumptions are essentially impossible to achieve in feeding studies where protein ingestion would disrupt the amino acid enrichments needed to calculate the breakdown rate. Recent developments in stable isotope modeling for postprandial measures of plasma protein turnover (33) may offer insight for assessing postprandial muscle PS and PB.

It must also be acknowledged that muscle protein turnover measures are typically assessed within finite periods of time and thus do not reflect shifts in net protein balance that occur in response to feeding, fasting, and exercise during the overall energy deficit period, and therefore should not be construed as representative of net alterations in muscle mass. Advances in assessing muscle PS and turnover using deuterated water and muscle biopsies separated by weeks rather than hours provide an opportunity to gain a better understanding of longer-term changes resulting from energy deficit and nutritional interventions (34–36). However, we continue to stress the importance of assessing both muscle PS and muscle PB in response to nutrition and metabolic stress in order to achieve a holistic understanding of muscle protein turnover responses to these interventions.

Molecular regulation of protein turnover in response to dietary protein and negative energy balance

The observed changes in muscle PS and muscle PB in response to energy deficit, exercise, and feeding are likely mediated by changes in the expression and activation of key intracellular molecular regulators. Intracellular regulation of muscle PS is typically ascribed to the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and its associated factors, which upregulate and expedite mRNA translation. The key contributors to this regulation, as well as the influence of exercise and amino acid availability, have been reviewed previously (37), but research concerning the impact of energy deficit on these intracellular regulatory processes has not been reviewed.

In 12-wk-old rats, a 16-wk, 40% energy restriction led to decreased phosphorylation and total expression of Akt (protein kinase B), mTOR, transcriptional regulator 70kDa S6 kinase (p70S6K), and its target ribosomal protein S6 (rpS6) (38). These changes were independent of differences in dietary protein intake (10% compared with 32% of kilocalories). In contrast, muscle samples collected from healthy humans after a 21-d, 40% energy deficit (~1000 kcal/d) exhibited increased mRNA expression of phosphoinositide 3-kinase *vacuolar protein sorting-34* (*Vps34*), purportedly involved in amino acid sensing and amino acid–mediated mTORC1 signaling, and of the mTORC1 inhibitors Regulated in development and DNA damage responses 1 and 2 (*REDD1* and *REDD2*) (10). However, energy deficit had no significant effect on phosphorylation of Akt, p70S6K, rpS6, eukaryotic elongation factor 2, or eukaryotic translation initiation factor 4E.

These inconsistent findings highlight the need to evaluate and consider the overall changes in expression and activation of molecular regulators of muscle PS in response to energy deficit and the need, when designing experiments, to also consider the timing of muscle biopsies relative to the established interventions. Often, biopsies are timed specifically for kinetic measures of muscle protein turnover over a finite period of time, and the time points chosen may not represent the best windows to evaluate changes in intracellular regulators, since the time courses for gene expression, protein translation, and enzyme activation differ (39).

Given that protein ingestion upregulates muscle PS during energy deficit, it is reasonable to expect that those changes would be modulated by the activation of protein synthetic mTORC1 signaling. After a 21-d, 40% energy deficit (~1000 kcal/d), we observed significant activation of Akt, p70S6K, rpS6, and eukaryotic elongation factor 2 subsequent to consuming a protein-containing mixed-meal, yet the activation of these factors was no different than that observed during energy balance (10). Only eukaryotic translation initiation factor 4E, required for mRNA translation, exhibited lower postprandial phosphorylation during energy deficit than during energy balance. Areta et al. (17) documented comparable activation of Akt, mTOR, p70S6K, and rpS6 during energy balance and a 33% energy deficit (−15 kcal · kg FFM^{-1} · d^{-1}; ~900 kcal/d), which was higher after acute resistance exercise and whey protein ingestion. That protein ingestion and resistance exercise acutely stimulated mTORC1 signaling confirms that the intracellular regulation of muscle PS follows a similar pattern as previously described for kinetic measures of muscle PS.

Traditionally, investigations on the regulation of muscle PB in response to energy deficit have focused on the ubiquitin–proteasome pathway and its associated components (1). We observed an increase in caspase-3 activation after a 10-d, 20% energy deficit (500 kcal/d) and higher proteasome subunit alpha type-2 (PSMA2) protein expression after acute aerobic exercise during energy deficit, but no effect of energy deficit or exercise on 26S proteasome

β_5 enzyme activity or ubiquitin ligase atrogin-1 expression (12). This is in contrast to our earlier reporting of increased atrogin-1 and Muscle RING-Finger protein-1 (MuRF1) expression after a 21-d, 40% energy deficit (~1000 kcal/d) (8). Whereas the 21-d energy deficit did not significantly influence protein ubiquitylation or 26S proteasome β_1, β_2, and β_5 enzymatic activities, consuming a protein-containing mixed-meal suppressed ubiquitylation and the activity of each proteasome subunit relative to the postabsorptive state, again illustrating the net anabolic benefits of consuming high-quality dietary protein.

We recently reported on the effects of a 21-d, severe ~70% energy deficit (~1840 kcal/d) while acclimatizing to high altitude (4300 m) on regulators of both muscle PS and muscle PB (25). Basal phosphorylation of mTOR, p70S6K, and rpS6 was similar at sea level and after chronic (21-d) high-altitude exposure. However, phosphorylation of these synthetic factors after aerobic exercise (80-min treadmill march and subsequent 2-mile time trial) and 3 h into recovery after ingesting 25 g whey protein was significantly suppressed at chronic high altitude. After exercise, β_1 and β_5 proteasome activities were lower than basal (i.e., resting) or recovery values, but there was no effect of energy deficit and high-altitude exposure on proteasome activity, transcription factor Forkhead Box Protein O1 phosphorylation, protein ubiquitylation, or atrogin-1 mRNA expression. Thus, the significant total body mass (7.6 kg) and fat-free mass (4.3 kg) lost by these study participants over the 21-d intervention suggests that the negative muscle protein balance observed during this energy deficit resulted primarily from inhibition of protein synthetic pathways, rather than stimulation of ubiquitin-mediated proteolysis. Similarly, there were no observed differences in any measures between those volunteers consuming 2.0 or 1.0 g protein $\cdot$ kg^{-1} $\cdot$ d^{-1}, once more highlighting the importance of the overall magnitude of the energy deficit in modulating the benefits, or lack thereof, of higher-protein diets. With such a severe energy deficit, the doubling of protein intake (2.0 compared with 1.0 g protein $\cdot$ kg^{-1} $\cdot$ d^{-1}) resulted in a parallel increase in protein oxidation (24), thereby limiting amino acid availability to support muscle protein turnover purposes.

Moberg et al. (16) observed no change in 26S proteasome β_1, β_2, and β_5 enzymatic activities and only a minor reduction in MuRF1 protein expression in military personnel completing a strenuous, 8-d military field-training exercise that produced an energy deficit (15%, 750 kcal/d for women; 29%, 1675 kcal/d for men). Although changes in ubiquitin-mediated proteolysis could not explain the losses in fat-free mass experienced by these study participants during energy deficit, Moberg et al. (16) did observe an upregulation in the activation of autophagy-related pathways, with a 3-fold increase in microtubule-associated protein 1A/1B-light chain 3β (LC3b)-II/I protein ratio after the training exercise, indicative of greater autophagosome activity. In addition, expression and phosphorylation of the autophagy activator Unc-51–like autophagy activating kinase 1 (ULK1) were significantly higher after the completion of the training, potentially signifying a role for autophagy in regulating net proteolysis during energy deficit. In contrast, Smiles et al. (23) demonstrated lower postabsorptive protein expression of autophagy-related gene 5 (Atg5) and conjugated Atg5 (cAtg12) at rest after a 5-d, 33% energy deficit (−15 kcal $\cdot$ kg FFM^{-1} $\cdot$ d^{-1}; ~900 kcal/d), with no difference between men and women. Consuming 30 g whey protein after an acute bout of resistance exercise during the energy deficit increased Atg5 and cAtg12 expression to levels observed in energy balance, while also increasing ULK1 phosphorylation status above initial energy balance values. These discrepant results make it difficult to ascribe muscle loss during underfeeding to either ubiquitin- or autophagy-mediated proteolysis and present a clear opportunity for continued investigation of both proteolytic systems in human volunteers with controlled feeding and activity. Future studies should carefully plan the timing of muscle biopsies for proteolytic assessment, such that they align with anticipated changes, given the time needed to adequately account for alterations in mRNA and protein expression and enzyme activation. Although dietary protein content and subsequent amino acid availability may acutely influence these proteolytic responses, alterations in muscle PS and amino acid availability resulting from changes in whole-body PS and PB likely underlie the observed changes in muscle mass during energy deficit.

Micro-RNAs (miRNAs) also deserve attention when evaluating the molecular regulation of changes in muscle mass during negative energy balance. miRNA inhibition of mTORC1 gene translation may depress muscle PS (40, 41). Rat skeletal muscle miRNAs known to regulate the mTORC1 pathway were not altered by 40% energy restriction or varied protein intake (10% compared with 32% of kilocalories) (38). In overweight and sedentary older men, individual (miR-133a-3p and -133b) and median fold-change (sum of miR-1, -133a-3p, -133b, and -206) expressions of miRNA associated with pathophysiological muscle dysfunction were increased after a 28-d, 30% energy deficit (800 kcal/d) (26). In addition, a significant inverse association was noted between the circulating muscle-specific miRNA and whole-body PS ($r^2 = -0.530$). Alternatively, Parr et al. (27) evaluated circulating miRNA after 16 wk of a ~25% energy deficit (500 kcal/d) in overweight and obese volunteers classified as either high- or low-responders according to their relative amount of body mass lost during the intervention (>10% compared with <5%). Of 13 miRNAs (selected based on past studies suggesting responsiveness to exercise, dietary intervention, and/or weight loss), circulating miRNA-935 (c-miR-935) abundance was greater in high responders before and after the energy deficit. c-miR-221-3p and c-miR-223-3p increased in response to the energy deficit but were not significantly different between high- and low-responders at either time point.

The potential for miRNA to differentially regulate intracellular responses to energy balance and substrate availability is an avenue for future research. The discrepant observations regarding effects of energy deficit on molecular regulation

of PS and PB in rodents compared with humans should be considered when designing such future studies. Any influence on known constituents of ubiquitin- and autophagy-mediated proteolysis deserves future investigation as a possible explanation for the disparate results observed in the response of these markers to diet and exercise.

Whole-body protein turnover responses to negative energy balance and dietary protein

In the postabsorptive state, whole-body PB provides amino acid substrates for continued whole-body PS. Decreases in whole-body PS during energy deficit can be viewed as an energy-sparing mechanism, because ∼35% of resting energy expenditure is accounted for by basal whole-body protein turnover (42). The system-wide effects of this attenuation can, however, be detrimental over extended periods of time; these detrimental effects include, but are not limited to, decreased enzyme synthesis affecting all body systems, diminished plasma protein concentrations disrupting fluid and electrolyte balance, and reduced immune factor synthesis. Increasing dietary protein content should increase amino acid availability and reduce the need for whole-body PB to provide endogenous amino acids to sustain whole-body PS.

Whole-body protein flux, PS, and PB, measured in healthy young men and women were not different between sexes but all decreased during a 2-d period of energy balance when participants consumed a controlled diet coupled to a significant amount of exercise-induced energy expenditure (∼2300 kcal/d) (13). During a subsequent 2-d, severe energy deficit (∼3680 kcal/d) without change in exercise-induced energy expenditure, whole-body PB in these participants was significantly increased without any alteration in whole-body protein flux or whole-body PS; as a result, net protein balance was more negative during energy deficit relative to energy balance. Similarly, whole-body PB was higher, and whole-body PS and net protein balance were lower, in US Marines participating in strenuous training for 7 d during severe underfeeding (∼4200 kcal/d) (18). Postabsorptive whole-body PS, whole-body PB, and net protein balance all increased after a subsequent 27-d refeeding period, although net protein balance did not return to baseline levels. Supplementing participants' ad libitum diets with low (7 g/d), moderate (84 g/d), or high (133 g/d) amounts of additional protein did not influence the postabsorptive whole-body protein response to the 27-d refeeding period. Kim et al. (43) reported a relatively linear relation between higher-protein intake, within the context of a single mixed-meal, and suppression of whole-body PB and the resulting enhancement of net protein balance. It is important, therefore, to consider the context of reported whole-body protein measures, because postabsorptive measures provide only one side of the protein metabolic response to dietary protein and energy deficit, overlooking the likely anabolic response to nutrient intake.

Differences in whole-body protein turnover related to the window of observation are clearly illustrated in 2 separate but similar studies (14, 15). Subsequent to a 4-d, 54-km cross-country ski march with an accompanying 50% energy deficit (3390 kcal/d), healthy Norwegian soldiers exhibited increased whole-body protein flux, whole-body PS, and whole-body PB when measured during an overnight fast (14). When protein turnover was measured over 24 h in a similar group of Norwegian soldiers after completing a 4-d, 51-km ski march that produced a 50–60% energy deficit (range: 3000–3600 kcal/d), whole-body protein flux, whole-body PS, and whole-body PB were all lower relative to baseline (15). In both studies, whole-body PB exceeded PS after the ski march, but net balance was ∼5-fold more negative when measured during an overnight fast (14) compared with a full 24 h (15). The disparate results surely reflect the different examination windows, one solely postabsorptive and the other including both postabsorptive and postprandial episodes. These observations illustrate the need to interpret whole-body protein turnover in light of the conditions in which PS and PB are measured and to carefully consider the window of observation when designing future studies.

In healthy young men and women, a 40% energy deficit (∼1000 kcal/d) attenuated both postabsorptive whole-body PS and PB relative to energy balance (11). Interestingly, consuming higher amounts of dietary protein (double and triple the RDA) did not mitigate these observed decreases. In fact, those consuming triple the RDA for protein exhibited the highest rate of protein oxidation and associated negative net protein balance. It is logical that consuming more protein results in more being oxidized to meet daily energy demands, as protein becomes a predominant energy source. In these same volunteers, consuming a protein-containing mixed-meal during energy balance stimulated whole-body protein flux and PS and attenuated PB; this effect was not observed during the energy deficit, as the amino acids derived from the meal were likely targeted for energy-generating pathways when underfed.

Similarly, consuming higher amounts of dietary protein (2.0 compared with 1.0 $g \cdot kg^{-1} \cdot d^{-1}$) during a 21-d severe ∼70% energy deficit (∼1840 kcal/d) and accompanying high-altitude (4300 m) acclimatization resulted in significantly higher rates of postabsorptive protein oxidation (24). Net whole-body protein balance, however, was more negative for those consuming the higher-protein diet, again illustrating a potential inverse relation between higher-protein intakes during energy deficit and net protein balance. Postabsorptive whole-body PS and whole-body PB were lower, but net protein balance was unchanged, after 21 d of combined high-altitude acclimatization and energy deficit relative to eucaloric measures performed at sea level. Due to the study design, it is impossible to determine whether high-altitude acclimatization independently influenced the overall suppression of whole-body protein turnover that was expected with the energy deficit. It can be concluded, however, that high-altitude exposure did not counter the inhibition of whole-body protein turnover anticipated, and observed, in response to a sustained energy deficit.

Conclusions

Sustained periods of negative energy balance place a metabolically challenging stress on muscle. Consuming higher-protein diets during periods of moderate negative energy balance can protect muscle mass by attenuating the decline in muscle PS that occurs with underfeeding and by restoring muscle PS to rates observed during energy balance while also suppressing PB at the whole-body and muscle intracellular level. Combining resistance exercise with recovery protein intake potentiates the muscle-sparing advantages achieved by adhering to a higher-protein diet during energy deficit. However, the muscle-sparing efficacy of higher-protein diets is apparently lost as the magnitude of energy deficit increases (e.g., >40%), because dietary protein then becomes predominantly a readily oxidizable energy source for meeting the energy demand.

Identifying the precise energy-deficit threshold at which higher-protein intakes no longer carry net benefit will be important in determining how best to create nutritional countermeasures for real-world scenarios that disrupt muscle and whole-body protein homeostasis. We encourage the use of human volunteers for future applied research, because rodent models can provide incongruous results. Volunteer pools should also be structured and powered such that meaningful comparisons between men and women may be made, because we are unaware of any studies focusing on potential sex-based differences in protein turnover in response to energy deficit. It may also prove beneficial to expand the investigation of muscle protein turnover beyond the predominantly fast-twitch *vastus lateralis* to more aerobic-dependent tissues in order to examine the effects of energy deficit during extended periods of high metabolic demand, such as ultra-endurance athletic training and strenuous military operations. Future studies should also consider the amino acid content of nutritional interventions, because the relative amount and balance of specific amino acids may influence the effect of protein feeding and allow for more efficient use of specific amino acid supplements to offset muscle loss in times of energy deficit and environmental stress. We also suggest that careful thought be given to the examination window for measures of muscle and whole-body PS and PB, as well as molecular regulators, such that any observed effects can be evaluated in the proper context and extrapolated thoughtfully to potential long-term changes in muscle mass.

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