STRATEGIES TO IMPROVE SKELETAL MUSCLE PROTEIN TURNOVER DURING DIETARY ENERGY RESTRICTION

PROTEIN AND RESISTANCE EXERCISE STRATEGIES TO IMPROVE SKELETAL MUSCLE PROTEIN TURNOVER DURING DIETARY ENERGY RESTRICTION

By

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LAY ABSTRACT

Dietary energy restriction is commonly used to promote weight/fat loss; however, a potential negative consequence of dietary energy restriction is the loss of skeletal muscle mass. This thesis examines the impact of dietary energy restriction on the two processes that regulate skeletal muscle mass: muscle protein synthesis and muscle protein breakdown. Additionally, this thesis investigates the role of protein intake and resistance exercise as strategies to prevent diet-induced changes in muscle protein synthesis and breakdown. The studies within this thesis demonstrate that during energy restriction rates of muscle protein synthesis are reduced whilst muscle protein breakdown is unchanged. Importantly, consuming high quality protein such as whey protein and performing resistance exercise prevent the diet-induced decline in rates of muscle protein synthesis. These findings provide new and insightful information for the design of weight loss programs that aim to preserve skeletal muscle whilst also promoting the loss of body fat.

ABSTRACT

Weight loss through dietary energy restriction (ER) is an effective method to promote fat mass loss. However, a negative consequence of ER is the loss of lean body mass (LBM), particularly skeletal muscle, which is induced by an imbalance between rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Increased protein intake and resistance exercise (RE) during ER promote the retention of LBM. Currently, the relative contribution of MPS and MPB to diet-induced LBM loss, and the effect of protein intake and RE on these variables is not well characterized. In Study 1 we compared the acute (hour-to-hour) MPS response to the ingestion of whey and soy protein, before and after 14 days of ER (-750kcal/d). The results of Study 1 indicated that whey protein was superior to soy protein in stimulating MPS before and after ER. In Studies 2 and 3 we examined the effect of 10 days of a marked 40% energy restriction on acute postabsorptive MPS and MPB and integrated (day-to-day) MPS. Using unilateral RE, we examined the effects of protein (1.2g protein/kg/g or 2.4g protein/kg/d) at rest and in combination with resistance exercise. The results of Study 2 showed that there were no changes in acute MPB or markers of proteolysis with ER. The results of Study 3 indicated that acute and integrated MPS were reduced following ER at both protein levels (1.2g protein/kg/g or 2.4g protein/kg/d), but RE was able to prevent this decline. Taken together, these studies demonstrate that reductions in MPS are the likely reason for LBM loss during short-term dietary energy restriction, and strategies such as RE and high quality protein

intake can help to prevent the decline in MPS. These findings provide information for the design of weight loss programs that wish to preserve skeletal muscle.

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LIST OF ABBREVIATIONS

ABR	Absolute breakdown rate
Akt	Protein kinase B
ALS	Autophagic-lysosomal system
AMPK	Adenosine monophosphate-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ASR	Absolute synthesis rate
Atrogin-1	Muscle Atrophy F-box
AUC_{neg}	Area under the curve below baseline
AUC_{pos}	Area under the curve above baseline
BMI	Body mass index
C_{max}	Maximum concentration
CHO	Carbohydrate
DIAAS	Digestible indispensable amino acid score
DXA	Dual-energy x-ray absorptiometry
D_2O	Deuterium oxide
EAA	Essential amino acids
EB	Energy balance
eEF2K	Eukaryotic elongation factor-2 kinase
eIF4E	Eukaryotic initiation factor 4E
eIF4G	Eukaryotic initiation factor 4G
ER	Energy restriction
EX	Energy deficit, exercised leg
REST	Energy deficit, rested leg
FBR	Fractional breakdown rate
FSR	Fractional synthetic rate
Foxo3a	Forkhead box O3
Gabarapl1	GABA Type A Receptor Associated Protein Like 1
GAP	GTPase activating protein
GTPase	Guanosine triphosphatase
HP	Higher protein group
LC3	Microtubule-associated protein 1A/1B-light chain 3
LBM	Lean body mass
LP	Lower protein group
MAFbx	Muscle Atrophy F-box
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mTORC1	Mechanistic target of rapamycin protein complex 1
MuRF1	Muscle RING Finger 1
Myo-PS	Myofibrillar protein synthesis
PDCAAS	Protein digestibility corrected amino acid score

PI3K	Phosphatidylinositide 3-kinases
P70S6K1	Protein of 70 kDa S6 kinase 1
Ra	Rate of appearance
RDA	Recommended dietary allowance
REDD	Regulated in development and DNA damage responses
RE	Resistance exercise
REE	Resting energy expenditure
Rheb	Ras homolog enriched in brain
Rps6	Ribosomal protein S6
TAA	Total amino acids
T_{max}	Time of maximum concentration
TRAF6	E3 ubiquitin ligase tumor necrosis factor receptor (TNFR)-associated factor 6
TSC2	Tuberous sclerosis 2
T2D	Type II Diabetes Mellitus
Ulk1	Unc-51-like kinase 1
UPS	Ubiquitin-proteasome system
4E-BP1	4E-binding protein-1

PREFACE
DECLARATION OF ACADEMIC ACHIEVEMENT

FORMAT AND ORGANIZATION OF THESIS

This thesis is prepared in the "sandwich" format as outlined in the School of Graduate Studies Guide for the Preparation of Theses. It includes a general introduction, three original research papers prepared in journal article format, and a general discussion. The candidate is the first author on all of the manuscripts. At the time of the thesis preparation, Chapter 2 was published in a peer-reviewed journal and Chapters 3 and 4 were in review.

CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIPS

Chapter 2 (Study 1)

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Contribution:

A.J. Hector, G.R. Marcotte, T.A. Churchward-Venne, C.H. Murphy, L. Breen, M. von Allmen, S.K. Baker, and S.M. Phillips conducted the research. A.J. Hector and S.M. Phillips analyzed the data. All authors assisted in editing the manuscript. A.J. Hector and S.M. Phillips had primary responsibility for the final content. All authors read and approved the final manuscript.

Chapter 3 (Study 2)

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Contribution:

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. All authors edited and approved the final version.

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Chapter 1:
INTRODUCTION

1.1 Introduction

Obesity, characterized by the accumulation of excess adipose tissue, is a growing world-wide public health epidemic. Canadian data show that in 2014, 21.8% of adult men and 18.7% of adult women were classified as obese (body mass index (BMI) 30 kg/m^2 or higher), while 40% of adult men and 27.5% of adult women were classified as overweight (BMI 25 to <30 kg/m^2) (1). Overweight and obese individuals are at significantly greater risk of developing comorbidities versus normal weight individuals, including type II *diabetes mellitus* (T2D), coronary heart disease, cancers, and sleep disorders, all of which pose a significant health risk to the individual and lead to an incumbent socioeconomic burden (2). Therefore, effective strategies to promote the reduction of excess adiposity and reduce the prevalence of overweight and obese adults are desperately needed.

Weight loss to reduce excess body adiposity is achieved through an energy deficit, which can be accomplished by energy restriction, increased energy expenditure, or a combination of both energy restriction and expenditure. An energy deficit can result in a reduction in fat mass through an increased release of free fatty acids (lipolysis) from adipose tissue, which can be used as a source of fuel by other organs (3). Compared to weight loss surgeries (4, 5) or pharmacotherapy (6), lifestyle modification (i.e. dietary energy restriction and exercise) remains the most cost effective, scalable, and widely available method to most individuals attempting to lose weight (7, 8). Additionally, lifestyle modifications have a low side effect profile and offer other health benefits, such

as improved cardiovascular function and muscular strength with exercise (9, 10). A wealth of weight loss information comes from the National Weight Control Registry, which was established in 1993 to examine the characteristics of individuals maintaining at least a 13.6-kg weight loss for longer than one year. Analyses from these data demonstrate that 89% of participants changed diet and physical activity habits to lose weight (7). Therefore, developing effective dietary modification and exercise programs to promote successful weight loss will provide valuable information to continue to address the obesity epidemic.

1.1.1 Importance of skeletal muscle: Implications for weight loss

Although weight loss through dietary energy restriction is effective at reducing fat mass and improving metabolic and physical health (7), a potential negative consequence of dietary energy restriction is the loss of lean body mass (LBM), particularly skeletal muscle mass (11). Skeletal muscle accounts for approximately 40% of body mass and is the largest organ in the body, providing a store of amino acids for other tissues in the body (i.e. heart, liver, brain) (12, 13). Skeletal muscle is important for maintaining glycemic control (14, 15), as a site of fat oxidation (16), and is obviously critical for mobility (17). Skeletal muscle is also a significant contributor to resting energy expenditure, a portion of which comes from the energy consuming processes of muscle protein synthesis (MPS) and breakdown (MPB) (18). Thus, maintaining skeletal muscle mass can promote favourable health adaptations to weight loss.

Loss of skeletal muscle mass comprises approximately 25% of total weight lost during energy restriction (19). Therefore, the focus of weight loss should not only be on the total weight lost, but also on body composition changes with a goal of a high fat-to-lean mass loss ratio. A study by Heymsfield et al. (20) used two large randomized controlled weight loss trials (CALERIE (21) and Kiel (20)) to characterize different phases of weight loss, with phase I lasting 4-6 weeks and is associated with rapid weight loss, a higher proportion of weight loss as LBM and a decline in resting energy expenditure (20). Thus, strategies that target the early phase of weight loss when LBM loss is most prominent is critical in order to enhance the physical and metabolic adaptations to weight loss.

1.2 Muscle Protein Turnover

Skeletal muscle protein turnover is an energy consuming process (22, 23) that consists of a constant flux between the incorporation of free amino acids into muscle protein (muscle protein synthesis, MPS) and the release of protein-bound amino acids into the intracellular pool (muscle protein breakdown, MPB) (24). In the postabsorptive state, rates of MPB exceed those of MPS resulting in a net negative protein balance (i.e., MPS – MPB is negative). In the postprandial state, MPS is temporarily stimulated by a hyperaminoacidemia (predominantly due to the essential aminoacidemia), while MPB is suppressed mainly due to a protein-induced hyperinsulinemia (25) and the result is a net positive protein balance. In healthy younger individuals, the periods of positive and negative protein balance

are roughly equal, resulting in a maintenance of muscle mass over time (26, 27). Loaded muscle contraction (i.e. resistance exercise, RE) also results in a stimulation of muscle protein turnover. Indeed, in the postabsorptive state, RE stimulates both MPS and MPB, but MPS to a much greater degree resulting in a slightly less negative protein turnover compared to rates at rest (28, 29) that can last up to 24hr post-exercise (30). Importantly, RE sensitizes skeletal muscle to the anabolic effect of dietary protein. For example, the consumption of dietary protein in close temporal proximity to RE results in a synergistic increase in rates of MPS, leading to a protracted state of net positive protein turnover (26, 29, 31). It is for these reasons that repeated bouts of RE coupled with adequate protein consumption lead to muscle hypertrophy (32, 33).

1.2.1 Mechanisms of MPS in human skeletal muscle: Influence of amino acid ingestion and resistance exercise

The process of MPS is regulated mainly through the mechanistic target of rapamycin protein complex 1 (mTORC1) signalling pathway (34), a pathway involved in ribosomal biogenesis as well as the control of translation initiation and elongation (35), which is illustrated in **Figure 1**. The mTOR protein is a 289kDa protein with many regulatory proteins that control its activation (35, 36). One important regulatory protein is the guanosine triphosphatase (GTPase) Ras homolog enriched in brain (Rheb). When in a guanosine triphosphate (GTP)-bound state, Rheb promotes mTORC1 activation; however, when in the guanosine

diphosphate (GDP)-bound state, mTORC1 is not activated by Rheb. Activation of Rheb is controlled by the upstream GTPase activating protein (GAP) tuberous sclerosis 2 (TSC2) (37). When TSC2 is activated, such as during time of low cellular energy by adenosine monophosphate-activated protein kinase (AMPK) phosphorylation on residues Ser^{1387} and Thr^{1271} (38), TSC2 activates Rheb (i.e. activates its GTPase activity), thereby promoting the GDP-bound state of Rheb and inhibiting the activity of mTORC1 (39). In contrast, in response to growth factors, Akt (protein kinase B [PKB]) activation on Thr^{308} and Ser^{473} phosphorylates TSC2 on residues Ser^{939} and Thr^{1462} which inhibits the ability of TSC2 to activate Rheb (40), thus Rheb remains in the GTP-bound state and can activate mTORC1.

Once active, mTORC1 interacts with many downstream substrates including the ribosomal protein of 70 kDa S6 kinase 1 (p70S6K1) and 4E-binding protein-1 (4E-BP1) (41-43). Both p70S6K1 and 4E-BP1 are important for the stimulation of MPS (44, 45). Active mTORC1 phosphorylates p70S6K1 on Thr^{389} which then acts on ribosomal protein S6 (rps6) to upregulate translation initiation of ribosomal proteins (46, 47). The 4E-BP1 protein is targeted by mTORC1 via phosphorylation on $Thr^{37/46}$. Phosphorylation of 4E-BP1 reduces its affinity for eukaryotic initiation factor 4E (eIF4E), enabling eIF4E to interact with eukaryotic initiation factor 4G (eIF4G) to form the 43S preinitiation complex. These steps are crucial to begin protein synthesis (41). Activation of mTORC1 also results in the phosphorylation and inactivation of eukaryotic elongation factor-2 kinase

(eEF2K), resulting in dephosphorylation (activation) of eEF2 which promotes translation elongation (48).

While there are 20 amino acids, it is the provision of only the essential amino acids that stimulates rates of MPS (49, 50) through mTORC1 (43, 51, 52). However, although non-essential amino acids are not required for the stimulation of MPS (53-55), they may have an important role in sustaining rates of MPS beyond the acute maximal stimulation (50). In young men, consumption of 10g of essential amino acids resulted in a 60% increase in MPS, that is accompanied by increased phosphorylation of mTOR (Ser^{2448}), ribosomal S6 kinase 1 (Thr^{389}), and eukaryotic initiation factor 4E binding protein 1 ($Thr^{37/46}$) (43). The importance of mTORC1 activation for the stimulation of MPS was demonstrated by Dickenson et al., who administered rapamycin (an mTORC1 inhibitor) to male and female participants and found that following ingestion of 10g of essential amino acids, there was a complete block of the usual stimulation of MPS, and the activation of mTORC1 signaling proteins was attenuated (43). It is important to note that in this experiment rapamycin did not affect basal rates of MPS, which may be regulated through other mechanisms or only require low/minimal activation of mTORC1 (43).

RE results in a stimulation of MPS (28), but also the stimulation of MPB (28). The consumption of dietary protein in close proximity to RE is required to promote a synergistic increase in MPS, resulting in net positive protein balance (56). The increased rates of MPS in response to RE also result in elevations in the

phosphorylation status of mTORC1 Ser^{2448} and the downstream target of mTORC1, p70S6K1 Thr^{389} (44, 56-59). Thus, mTORC1 is well established as a central nexus for MPS in response to RE (60-62). In a repeat of their experiment with amino acids (43), the same researchers also examined whether rapamycin prevented RE-induced increases in mTORC1-mediated rates of MPS (63). Indeed, Drummond et al. (63) reported that the administration of rapamycin 2 h prior to RE significantly reduced MPS during the initial 1-2 h recovery period. The reduction in MPS was accompanied by blunted mTOR Ser^{2448} and p70S6K1 Thr^{389} phosphorylation 1h post exercise. Such findings (64) when taken together with previous work (43), provide compelling evidence that amino acid ingestion and RE stimulate rates of MPS in an mTORC1 dependent manner.

1.2.2 Mechanisms of skeletal muscle protein breakdown in human skeletal muscle

The degradation of skeletal muscle proteins is necessary to replace damaged proteins and to provide a source of glucose and amino acids for other organs during times of stress, such as fasting (65, 66). Two main systems coordinate the removal of amino acids from muscle: the ubiquitin-proteasomal system (UPS); and the autophagic-lysosomal system (ALS), both of which will be discussed in greater detail in the following sections.

An important regulator of both the UPS and ALS is the transcription factor Forkhead box O3 protein (FoxO3a protein) that undergoes specific

phosphorylation in response to cellular signals including amino acids and insulin, or low energy status (67-70). For example, in response to growth factors, phosphorylation of FoxO3a by Akt on residues Thr^{32} and Ser^{253} leads to the cytosolic retention of FoxO3a, inhibiting the transcription of genes involved in the UPS and ALS (69). However, in response to low energy in the cell, 5' adenosine monophosphate-activated protein kinase (AMPK) phosphorylates FoxO3a on $Ser^{413/588}$, leading to its translocation to the nucleus where the transcription of genes involved in the UPS and ALS are promoted. Indeed, skeletal muscle knockout of AMPK in fasting mice results in impaired skeletal muscle autophagy and hypoglycemia (66). Additionally, the deletion of skeletal muscle FoxO3a in mice prevents autophagy, ubiquitination of proteins, and muscle loss due to fasting (71). Another potential regulator of both the UPS and ALS is the E3 ubiquitin ligase tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (72, 73). Knockout of TRAF6 in mouse muscle showed reduced atrophy (74) and expression of the UPS and ALS pathways (72), while the increased expression of TRAF6 in skeletal muscle has been demonstrated during starvation in mice (73).

The UPS has been considered the main pathway leading to both soluble and myofibrillar protein breakdown (75). When genes involved in the UPS are silenced or knocked out in mice, there is a significant reduction in muscle atrophy (76). An increase in components of the UPS (genes, proteins) has been consistently demonstrated during increased metabolic demand such as acutely

following RE (77, 78), fasting (79) and conditions of inflammation and muscle wasting (e.g., sepsis, renal failure, cancer cachexia) (80-82). The mechanism of action of the UPS involves three key enzymatic reactions with ubiquitin first being bound to the E1-ubiquitin activating enzyme in an ATP-dependent process. Ubiquitin is then transferred to the E2-ubiquitin conjugating enzyme and E3-ubiquitin ligases conjugate the ubiquitin monomer to the specific protein (usually on a lysine residue) targeted for degradation. A minimum of four Ub monomers are required to be attached to the target protein for the 26S proteasome to recognize the signal and degrade the ubiquitinated protein, also in an ATP-dependent manner (83). The FoxO3a transcription factor controls the transcription of two major muscle specific E3 ubiquitin ligases: atrogin-1/MAFbx (Muscle Atrophy F-box) and MuRF1 (Muscle RING Finger 1) (67). The Atrogin1/MAFbx ligase conjugates ubiquitin to the translation initiation factor eIF3f (84), and MyoD (85) while MuRF-1 conjugates ubiquitin to cleaved myofibrillar proteins (86).

The intact myofibrillar proteins are somewhat protected from ubiquitin-proteasomal degradation (87). Caspase-3, an aspartic acid-specific protease has been shown to be crucial for muscle proteolysis (88). Procaspase-3 is a stable dimer with low enzymatic activity (89) that requires cleavage of an intersubunit linker by initiator caspases (such as Caspase-9) to become the activated caspase-3 (90). Inhibition of caspase-3 in rats with acute diabetes suppressed the accelerated muscle proteolysis (88).

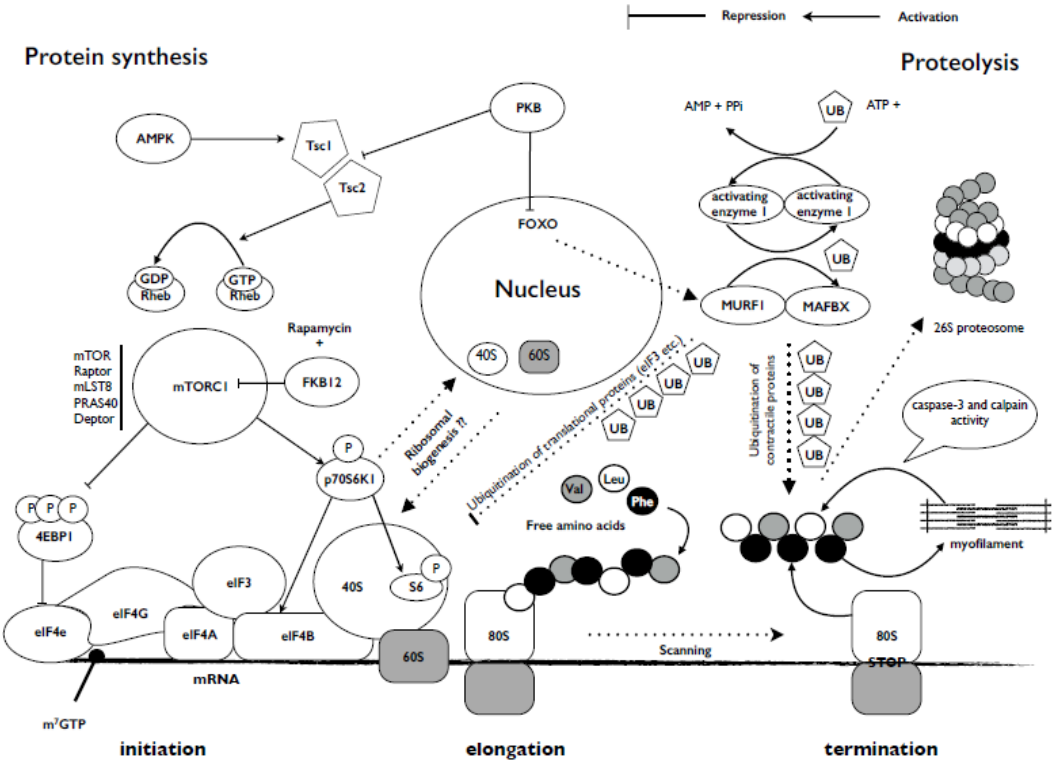

Figure 1. Integration of the skeletal muscle protein synthesis and the ubiquitin proteasome pathways. PKB: protein kinase B, AMPK: 5' AMP-activated protein kinase, TSC: tuberous sclerosis, Rheb: Ras homolog enriched in brain, GTP: guanosine triphosphate, mTORC1: mechanistic target of rapamycin complex 1, 4EBP1: EIF4E binding protein 1, p70S6K: ribosomal protein S6 kinase, eIF: eukaryotic translation initiation factor, MuRF1: muscle ring finger protein 1, MAFBx: muscle atrophy F box, Ub: ubiquitin, FOXO: forkhead box. Figure used, with permission, from Hector et al. (91).

The autophagic-lysosomal system (ALS) is constitutively active within skeletal muscle and is responsible for the degradation of damaged proteins and organelles to maintain cellular homeostasis (92) (93), and respond to cellular stress, such as cytokines and nutrient deprivation (70). During starvation-induced muscle atrophy in mice, FoxO3a regulates the transcription of several autophagy related genes, including Microtubule-associated protein 1A/1B-light chain

3 (LC3B), GABA Type A Receptor Associated Protein Like 1 (Gabarapl1), and Beclin1 (69, 71) as well as the accumulation of autophagosomes (94).

Autophagy is controlled by both AMPK and mTORC1. The phosphorylation of mTORC1 (Ser^{2448}) in response to amino acids suppresses autophagy by phosphorylation of Unc-51-like kinase 1 (ULK1) at Ser^{757}, preventing ULK1 activation and autophagy initiation (95). FoxO3a activation by AMPK leads to an increase in autophagy related gene expression (69). AMPK phosphorylates ULK1 on Ser^{555} which triggers the initiation of autophagosome formation (95).

During autophagy, intracellular components such as proteins and organelles are first sequestered into double membrane intracellular vacuoles called autophagosomes, which then fuse with lysosomes for the digestion of cell components by lysosomal protein hydrolases. Cellular components are then recycled to yield energy to maintain cellular metabolism (96). Autophagosome formation, elongation and lysosomal fusion are coordinated by groups of autophagy related proteins. For example, activated ULK1 Ser^{555} directly phosphorylates Beclin1 Ser^{14} that is involved in the induction of autophagy (97). Another protein involved in autophagy induction is BNIP3 (69). The formation (lipidation) of the autophagosomal membrane proceeds with LC3 (98) and closure of the autophagosomal membrane is associated with Gabarapl1 (98). There are many other proteins involved in each stage of autophagy, however, the proteins listed here represent the more well-studied markers in skeletal muscle (69).

1.2.3 Measurement of skeletal muscle protein turnover using stable isotopic tracers

Stable isotope tracers are non-radioactive elements that contain extra neutrons, rendering them greater in mass from the most naturally occurring form of an element (i.e. ^{13}C and ^{12}C, ^{2}H and ^{1}H, ^{15}N and ^{14}N). This difference in mass allows the stable isotope tracer to be distinguished from the more abundant form of the element while still behaving identically within the body (99). One method of administering stable isotope tracers is through a primed constant intravenous infusion over a period of several hours to achieve a steady tracer:tracee ratio in the plasma (5-10%) and intracellular precursor pool (3-5%) (99). During the steady-state intravenous infusion, timed blood samples and muscle biopsies are taken (for example, during fasted and fed states) and the incorporation of the tracer into specific skeletal muscle fractions (i.e. myofibrillar, mixed muscle) is measured by isotope-ratio mass spectrometry over a period of hours. MPS can then be calculated using the precursor-product equation (28):

$$FSR(\% \cdot hr - 1) = \frac{E(t2) - E(t1)}{Ep \cdot (t2 - t1)}\ x\ 100$$

Where, E(t1) is the protein-bound enrichment of the first biopsy and E(t2) is the protein-bound enrichment of the second biopsy. Ep is the average enrichment of the intracellular fraction (precursor pool) between the two biopsies, and t2 and t1 are the times the biopsies were taken. The equation is multiplied by 100 to convert the value to a percentage. An important assumption of the precursor-product

model is that none of the tracer incorporated into the muscle protein pools is released via proteolysis during the experiment, which is reasonable given the short duration of the measurement (hours) (100). Ideally, the precursor pool enrichment for incorporation into the protein-bound fraction would be aminoacyl-tRNA, however, this aminoacyl-tRNA pool is small and a large muscle sample would be needed to measure this pool. Thus, the intracellular free amino acid pool, the immediate precursor to the aminoacyl-tRNA pool, is often used as a surrogate instead (99). The primed constant intravenous infusion method provides an acute assessment (hours) of MPS in response to certain stimuli (for example, in fasted and fed states), in controlled laboratory conditions. However, this confines participants to a laboratory setting and does not represent the day-to-day activities of participants.

An alternative to the acute intravenous infusion measure of MPS is the use of orally ingested deuterated water (D_2O). This method eliminates the need for an intravenous infusion, allowing participants to be free-living, and can capture an integrated MPS response over a period of days to weeks, which would include all periods of fasting, feeding, activity or inactivity. Following consumption of D_2O, which rapidly accumulates into the total body water, all amino acids become labelled with ^2H via exchange reactions and transamination (101). However, the non-essential amino acid alanine undergoes the most rapid turnover with, on average, 3.7 out of the 4 potential H atoms being exchanged with the ^2H (D) label, permitting better detection. The ^2H labelled alanine can then charge the

aminoacyl-tRNA and be incorporated into muscle proteins. Saliva samples can be used to monitor the change in total body water enrichment over days following D_2O consumption (102), and this can be used as the surrogate precursor for labelled alanine and measurement of MPS by gas chromatography-pyrolysis-isotope ratio mass spectrometry and a modified version of the precursor-product equation described above (103).

Using the rate of integrated MPS (%/d) obtained from the D_2O tracer, a calculation of absolute synthesis rate (ASR) and absolute breakdown rate (ABR) (in grams per day) can be obtained when paired with measures of muscle mass. The equations (104) are:

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

Where FSR is the integrated myofibrillar rate in %/d, LFFM (leg FFM) is the amount of FFM in the leg obtained by dual-energy x-ray absorptiometry (DXA) corrected for the amount of water in the muscle (wet weight-dry weight), and ASP is the average alkali soluble protein concentration present in a muscle (calculated from a biopsy). Similarly, absolute breakdown rate (ABR) can be calculated as:

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

Where FBR = FSR-FGR and FGR (fractional growth rate) is the % change in leg lean mass per day.

The tracee-release method is commonly used to measure mixed MPB (28, 105, 106), and this method has been shown to correspond with values obtained by the arterio-venous balance method, which is the other technique employed to estimate muscle protein balance (105). The tracee-release method measures the dilution in enrichment of the amino acid tracer in the intracellular free amino acid tracer pool by the unlabelled amino acid (tracee) from the muscle to calculate a rate of protein breakdown. Once steady state is achieved, the primed constant infusion of stable isotope tracer is discontinued, and two muscle biopsies are obtained at 40min and 60min following discontinuation of the tracer. This allows for the measurement of the difference in intracellular enrichment between the two biopsies which can be used to model a rate of decay as protein breakdown (105). The equation for MPB is as follows (105):

$$FBR\ (\% \cdot hr) = \frac{EM(t2) - EM(t1)}{P\int_{t1}^{t2} EA(t)dt - (1+P)\int_{t1}^{t2} EM(t)dt}\ x\ (\frac{QM}{T})$$

Where EM(t2)-EM(t1) is the change in enrichment in the muscle intracellular fraction from t1 to t2 when the isotope infusion is discontinued, $\int_{t1}^{t2} EA(t)dt$ is the area under the arterialized blood enrichment decay curve, $\int_{t1}^{t2} EM(t)dt$ is the area under the intracellular muscle enrichment decay curve, QM/T is the ratio of intracellular free tracee concentration and protein-bound tracee concentration , $P = \frac{EM}{(EA-EM)}$ at isotope plateau (accounts for the two precursor pools (arterial blood and protein bound pools).

The assumption in this model is that the arterial blood is the only source of tracer entering the intracellular pool, that is there is no tracer recycling from proteolysis, which is assumed to be negligible given the very short time period (20-60mins) over which the measurements are made (100).

1.2.4 Influence of energy restriction on skeletal muscle protein turnover

In order to develop effective weight loss strategies to promote LBM retention, it is important to understand the effects of energy restriction on muscle protein turnover. There are, however, relatively few studies investigating the effects of energy restriction on muscle protein turnover. Shorter term studies (lasting 5 days to a few weeks) have consistently shown a decrease in postabsorptive (14-27% (107-109)), and postprandial (14-20%) (109, 110)) MPS following moderate energy deficits (300-1000kcal/d). In contrast, longer term studies (lasting a month or longer) have not found impairments in MPS. For example, Villareal et al. examined the effect of weight loss on MPS following 3 months of weight loss in obese older adults and found that postabsorptive MPS was not impaired (111). The effect of longer term weight loss on postprandial MPS is less clear, and could be due to differences in the timing of the measurement (i.e. during weight loss or after returning to energy balance), but one study reported no change in postprandial MPS from pre weight loss values after 3 months of weight loss (112) and Villareal et al. reported an increase compared to pre weight loss values (111). Taken together, data from shorter and longer term studies suggest that

postabsorptive and postprandial MPS is significantly reduced at the early phase during energy restriction, which is further supported by more rapid LBM changes during early caloric restriction (20). The mechanisms underlying the decrease in MPS during weight loss have been shown to be mechanistically linked to the mTORC1 pathway. For example, Pasiakos et al., (107) demonstrated a 19% reduction in MPS as well as a reduction in the phosphorylation of 4EBP-1 $Thr^{37/46}$ in the post-absorptive state during a 10-day 20% energy deficit in healthy individuals. In another study, Pasiakos et al., (110) demonstrated a reduction in postprandial phosphorylation of p70S6K1 (Ser424/Thr421), along with increased expression of mTORC1 inhibitors regulated in development and DNA damage responses 1 and 2 (REDD1 and REDD2). Thus, strategies that promote the activation of the mTORC1 pathway, such as amino acid ingestion and RE, will help to prevent the diet-induced decline in MPS.

Whereas much is known about the effects of energy restriction on rates of MPS, relatively little is known about the effect of energy restriction on rates of MPB. In humans, MPB is often described through changes in static markers of gene expression and proteins that are part of the UPS and ALS. This approach has yielded inconclusive results as to whether MPB changes following short term energy restriction (106, 113, 114). For example, Carbone et al., reported a significant increase in the gene expression of Atrogin-1 and MuRF-1 following a 21d energy deficit (-40% versus requirements), but no change in 26S proteasome activity or caspase-3 activity (113). Smiles et al., (114) did not detect changes in

gene or protein expression in the autophagy pathway following a 5 day weight loss diet. Carbone et al. reported that following a 10-day, 20% energy deficit in normal-weight adults, there was a significant increase (11%) in caspase-3 activity, but no change in genes related to the ubiquitin-proteasome pathway or 26S proteasome activity. Interestingly, despite the small or absent changes in proteolytic pathway markers, there was a 60% increase in rates of postabsorptive MPB (106). To date, no other study has attempted to characterize MPB during energy restriction in humans. **Figure 2** summarizes the current understanding of muscle protein turnover in energy balance and energy restriction.

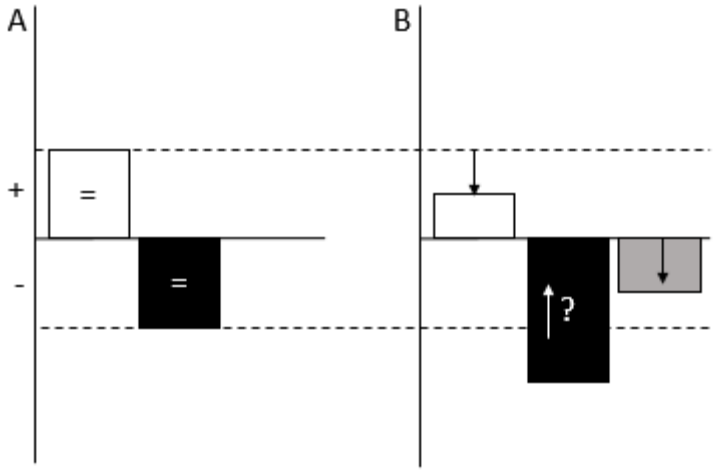

Figure 2. Theoretical representation of muscle protein synthesis (white bars), muscle protein breakdown (black bars) and net protein balance (grey bars). (A) During energy balance, rates of MPS are equal to rates of MPB and net balance is zero. (B) During energy restriction, MPS is reduced, which would lead to negative net protein balance. An increase in MPB would contribute to a much greater reduction in net protein balance. Thus, understanding how MPB changes with weight loss, and studying interventions to improve net protein balance will promote lean mass retention during energy restriction.

Table 1. Summary of randomized controlled trials measuring muscle protein synthesis during energy restriction

	Reference	Participants	Intervention	Outcomes (versus pre weight loss)
Longer-term studies	Campbell et al., 2009	Postmenopausal, overweight women	11 weeks 500kcal/d ER 1g/kg/d protein RE 3x/week	↑ postabsorptive mixed muscle FSR ↔ postprandial mixed muscle FSR No influence of RE
	Villareal et al., 2012	Obese older adults	3 months 500-750kcal/d ER 1g/kg/d protein	↔ postabsorptive mixed muscle FSR ↑ postprandial mixed muscle FSR
	Pasiakos et al., 2013	Young, physically active men and women	21 days 40% ER 1x, 2x, 3x RDA	↔ postabsorptive mixed muscle FSR ↓ postprandial mixed muscle FSR in RDA group only
Shorter-term studies	Pasiakos et al., 2010	Young physically active men and women	10 days 20% ER 1.5g/kg/d protein	↓ 19% postabsorptive mixed muscle FSR ↓ Akt and 4EBP-1 phosphorylation
	Areta et al., 2014	Young resistance trained men and women	5 days ER 30kcal/kg FFM per day 1.4-1.6g/kg/d protein	↓ postabsorptive MPS by 27% In ER, RE stimulated FSR equal to EB Protein ingestion + RE ↑ FSR above resting EB
	Murphy et al., 2015	Overweight/obese older men	4 weeks 300kcal/d ER 1.3g/kg/d protein 2 weeks of RE 3x/wk	↓ postprandial myofibrillar FSR With RE, fed-state myofibrillar FSR ↑ above energy restriction

Table 2. Summary of randomized controlled trials studying muscle protein breakdown during energy restriction

	Reference	Participants	Intervention	Outcomes (versus pre weight loss)
Longer-term study	Carbone et al., 2013	Young physically active men and women	21 days 40% ER 1x, 2x, 3x RDA	↑ MuRF-1 (1.2 fold) and atrogin-1 (1.3 fold) ↔ 26S proteolytic activity or caspase-3 activity
Shorter-term studies	Carbone et al., 2014	Young physically active men and women	10 days 20% ER 1.5g/kg/d protein	60% ↑ FBR 11% ↑ Caspase-3 ↔ Atrogin-1 expression or 26S proteasome activity
	Smiles et al., 2015	Young resistance trained men and women	5 days ER 30kcal/kgFFM per day 1.5g/kg/d protein	↔ gene expression of autophagy markers ↔ protein content or phosphorylation of autophagy markers

1.3 Strategies to maintain skeletal muscle protein balance during energy restriction

The recommended dietary allowance (RDA) for protein intake for adult men and women is 0.8g protein/kg/d (115); however, consumption of more dietary protein than the RDA appears to be necessary to attenuate muscle loss during energy restriction (11). Protein containing a high proportion of essential amino acids (EAA) can stimulate MPS to a greater extent than other protein sources (116), and RE combined with protein intake can have a synergistic effect on rates of MPS (117). Therefore, protein quantity, quality and RE are three variables that could be manipulated to promote LBM retention during energy restriction.

1.3.1 *Influence of protein quality and RE on body composition during energy restriction*

Dietary protein quality can be measured by the protein digestibility corrected amino acid score (PDCAAS) and the digestible indispensable amino acid score (DIAAS). In general, the method to calculate these scores involves measurement of the amino acid composition, digestibility and availability of the proteins (118). The PDCAAS is measured as fecal digestibility and is truncated at a score of 1 even if there are values beyond 1 for high quality proteins (e.g., whey and soy are both set at 1 despite having PDCAAS scores of 1.21 and 1.04, respectively). In contrast, DIAAS values use ileal digestibility and are not truncated, giving different values for high quality proteins (e.g. whey 1.1 and soy 0.97) (118).

These different scores are important when we consider that whey protein is superior to soy protein at rest and following exercise in stimulating MPS (119). One important difference between whey and soy protein is that whey protein has a higher composition of essential amino acids and importantly leucine, a key amino acid known to stimulate MPS (49, 120). Additionally, leucine has the potential to inhibit adipocyte lipogenesis and stimulate lipolysis (121, 122) and thus may play a synergistic role in promoting both adipose tissue loss and LBM retention. Another important difference between whey and soy proteins is the contribution of amino acids to peripheral tissues, which is lower in soy than whey due to a greater splanchnic extraction following soy protein ingestion, meaning fewer amino acids are available to the peripheral tissues (such as skeletal muscle) to support MPS (123).

In the context of weight loss, there are a handful of studies examining the impact of protein quality on body composition. Josse et al., demonstrated that during energy restriction while subjects performed both resistance and aerobic exercise, the consumption of a higher protein, dairy rich diet (representing 30% of energy intake) promoted greater fat mass loss and lean mass retention than a lower dairy-containing diet (124). In addition, two meta-analyses showed that higher dairy consumption resulted in greater lean mass retention and fat mass loss during short term energy restriction (125, 126). What remains to be answered from these studies is what constituent components of the dairy foods are responsible for these effects. Currently, there is a need to further elucidate the

mechanisms and role of whey protein compared to other protein sources on muscle and fat metabolism during energy restriction.

1.3.2 *Influence of protein quantity and RE on skeletal muscle during energy restriction*

Increased protein intake is another strategy for the stimulation of MPS. In a meta-regression, Krieger et al. demonstrated a protective effect of protein intakes greater than 1.05g/kg/d on lean mass during energy restriction in studies lasting more than 4 weeks (19). Similarly, in a meta-analysis conducted by Wycherley et al., it was shown that protein consumption above the RDA (0.8g/kg/d) resulted in a protective effect on lean mass and resting energy expenditure while promoting fat mass loss in studies lasting ~12 weeks (11). Longland et al. conducted a 4 week weight loss study in young overweight men, placing participants in a 40% dietary energy deficit, while also having participants perform high-intensity exercise 6 times weekly. The results demonstrated a sparing of lean mass in young men consuming 1.2g/kg/d protein, and a gain in lean mass for those individuals consuming 2.4g/kg/d protein (117). Currently, whether there are changes in MPS and/or MPB underlying this effect are not well characterized, which is important information in order to develop effective weight loss programs.

There are few studies that have investigated the impact of higher protein intakes on MPS during weight loss. In a study by Pasiakos et al., it was

demonstrated that consuming the RDA of protein during a 21-day, 40% energy deficit resulted in a blunting of postprandial MPS in response to 20g milk protein. However, consuming twice (2x) or three times (3x) the RDA of protein preserved the MPS responses to the 20g serving of milk protein. The authors discussed that the higher amino acid levels are adequate for basal energy and whole body protein intakes, resulting in more amino acids being used for MPS. Participants in the RDA group lost significantly more LBM (2.3±0.3kg) compared to the 2x (0.8±0.2kg) and 3x (1.2±0.3kg) RDA groups. This data demonstrates that protein consumption has an effect on the maintenance of postprandial MPS and LBM (110). Shorter term studies have also attempted to characterize the effect of energy restriction on MPS. After only 5 days of energy restriction (reduction from 45kcal/kg FFM to 30kcal/kg FFM) and consuming 1.4-1.6g/kg/d protein, Areta et al., reported a 27% reduction in postbsorptive MPS rates. Performing RE stimulated rates of MPS to levels similar to energy balance, and ingestion of 15 and 30 g of protein after the exercise bout increased MPS ∼16 and ∼34% above resting energy balance (108). Although these data provide an understanding of the acute effects of protein intake and RE on MPS during energy restriction, there is still a need to understand the chronic effects of protein quantity alone and in combination with RE on muscle protein turnover during energy restriction.

1.4 Objectives

The primary objective of the studies conducted as part of this thesis was to examine the effect of dietary energy restriction on muscle protein turnover (synthesis and breakdown) and the effect of protein quality, quantity, and RE on attenuating weight loss-induced changes in muscle protein turnover. It is well characterized that acute MPS is reduced following short-term dietary energy restriction (107-109). Additionally, we know that protein and RE are anabolic stimuli to muscle (26), but their effect on MPS and MPB during energy restriction are not fully elucidated. Furthermore, very few studies have characterized MPB during energy restriction (106). Thus, there is a need to further understand the mechanisms underlying muscle loss during dietary energy restriction, and the effect of protein and RE on muscle protein turnover. A better understanding of these mechanisms will provide valuable information to develop effective lifestyle-oriented strategies to preserve muscle during energy restriction.

1.4.1 Studies and hypotheses

In Study 1, we examined how protein quality affected rates of myofibrillar protein synthesis and lipolysis during 2 weeks of dietary energy restriction. Before energy restriction, we measured postabsorptive and postprandial (following consumption of a whey, soy or carbohydrate (CHO) supplement) MPS using L-*ring*-[$^{13}C_6$]phenylalanine and lipolysis using $^{2}H_5$-glycerol. Participants then completed a 2 week controlled diet providing a -750kcal/d deficit based on estimated energy

requirements, that included two supplements per day containing either whey or soy protein (1.2g/kg/d protein total) or CHO (0.74g/kg/d protein total). Following the 2 weeks of energy restriction, we measured postabsorptive and postprandial (following consumption of a whey, soy or CHO supplement) MPS and lipolysis. We hypothesized that whey protein consumption would stimulate MPS to a greater extent than soy or CHO before and after weight loss, and lipolysis would be reduced after feeding, but to a greater extent following ingestion of CHO.

In Study 2 we examined the effect of dietary energy restriction with differing protein quantities (1.2g/kg/d and 2.4g/kg/d) at rest and following RE training on mixed MPB and markers of the ubiquitin-proteasome and autophagic-lysosomal pathways. We measured postabsorptive MPB with ^{15}N-Phenylalanine using the tracee-release method with muscle biopsies to measure gene and protein expression at rest following a controlled 5 day energy balance diet. Participants then began a controlled 10 day 40% reduction in energy intake based on rested energy expenditure measurements with a sedentary activity factor, and performed 5 unilateral RE sessions (the last bout completed on day 9 of the diet). Following 10 days of energy restriction, we measured postabsorptive MPB and gene/protein expression in both the rested and RE legs. We hypothesized that MPB would not be elevated, and may be adaptively reduced following weight loss, which would correspond to the gene and protein expression.

In Study 3 we examined the effect of dietary energy restriction with differing protein quantities (1.2g/kg/d protein and 2.4g/kg/d protein) and RE on

acute mixed MPS and integrated MPS. We measured postabsorptive mixed MPS in both protein groups following a 5 day energy balance diet and following the 10 day energy restricted diet in both the rested and exercised legs using *ring*-[$^{13}C_6$]phenylalanine. We used the MPB rates from Study 2 to calculate postabsorptive net protein balance (%/hour). We measured integrated MPS with D_2O during a 5 day energy balance phase at rest, and during a 10 day dietary energy deficit phase in the rested leg and contralateral RE leg (5 unilateral sessions). We measured body composition before and after weight loss and used the integrated MPS data to calculate absolute (g/d) rates of MPS and MPB. We calculated net protein balance (g/d) by subtracting absolute MPS and MPB. We hypothesized that MPS and net protein balance would be reduced following ER in the rested leg, but exercise and higher protein intake would help to preserve rates of MPS and net protein balance.

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CHAPTER 2:

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Whey Protein Supplementation Preserves Postprandial Myofibrillar Protein Synthesis during Short-Term Energy Restriction in Overweight and Obese Adults[1–3]

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Abstract

Background: Higher dietary energy as protein during weight loss results in a greater loss of fat mass and retention of muscle mass; however, the impact of protein quality on the rates of myofibrillar protein synthesis (MPS) and lipolysis, processes that are important in the maintenance of muscle and loss of fat, respectively, are unknown.

Objective: We aimed to determine how the consumption of different sources of proteins (soy or whey) during a controlled short-term (14-d) hypoenergetic diet affected MPS and lipolysis.

Methods: Men (n = 19) and women (n = 21) (age 35–65 y; body mass index 28–50 kg/m^2) completed a 14-d controlled hypoenergetic diet (−750 kcal/d). Participants were randomly assigned, double blind, to receive twice-daily supplements of isolated whey (27 g/supplement) or soy (26g/supplement), providing a total protein intake of 1.3 ± 0.1 g/(kg · d), or isoenergetic carbohydrate (25 g maltodextrin/supplement) resulting in a total protein intake of 0.7 ± 0.1 g/(kg · d). Before and after the dietary intervention, primed continuous infusions of L-[ring-$^{13}C_6$] phenylalanine and [$^2H_5$]glycerol were used to measure postabsorptive and postprandial rates of MPS and lipolysis.

Results: Preintervention, MPS was stimulated more ($P < 0.05$) with ingestion of whey than with soy or carbohydrate. Postintervention, postabsorptive MPS decreased similarly in all groups (all $P < 0.05$). Postprandial MPS was reduced by 9 ± 1% in the whey group, which was less ($P < 0.05$) than the reduction in soy and carbohydrate groups (28 ± 5% and 31 ± 5%, respectively; both $P < 0.05$) after the intervention. Lipolysis was suppressed during the postprandial period ($P < 0.05$), but more so with ingestion of carbohydrate ($P < 0.05$) than soy or whey.

Conclusion: We conclude that whey protein supplementation attenuated the decline in postprandial rates of MPS after weight loss, which may be of importance in the preservation of lean mass during longer-term weight loss interventions. This trial was registered at clinicaltrials.gov as NCT01530646. *J Nutr* 2015;145:246–52.

Keywords: leucine, lean body mass, protein quality, hypocaloric, myofibrillar protein synthesis

Introduction

High quality weight loss is a term used to describe the loss of weight during hypocaloric feeding with the lowest possible ratio of lean body mass (LBM)[7] to fat mass. It is well known that LBM is a major contributor to resting energy expenditure, mobility, and glucose disposal (1), whereas excess fat mass, particularly visceral adipose tissue, contributes to inflammation in obesity, which is correlated with increased risk of developing cardiovascular disease and type 2 diabetes (2, 3). We proposed that weight loss plans should aim to maximize retention of LBM and reduction of fat mass in order to achieve the greatest metabolic health benefits.

The maintenance of muscle mass, a large component of LBM, is determined by the balance between myofibrillar protein

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[3]Supplemental Figure 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[7]Abbreviations used: FSR, fractional synthetic rate; LBM, lean body mass; MPB, myofibrillar protein breakdown; MPS, myofibrillar protein synthesis; Ra, rate of appearance.

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synthesis (MPS) and myofibrillar protein breakdown (MPB) (4). During an energy deficit, rates of MPS are blunted in the postabsorptive (5–7) and postprandial (6) states, which would clearly lead to loss of lean mass. One strategy to promote retention of muscle mass includes the consumption of dietary protein at intakes greater than the RDA of 0.8 g/(kg · d). Multiple studies and meta-analyses showed that consumption of protein at amounts greater than the RDA improves lean mass retention (8–10) and increases fat mass loss during energy restriction (8, 9). Interestingly, Pasiakos et al. (6) reported that postprandial rates of MPS were reduced during energy restriction in participants consuming the RDA for protein, but not when protein intakes were 2 or 3 times the RDA.

The source of protein (animal vs. plant) may also be an important factor affecting body composition changes during weight loss. For example, during energy restriction with exercise, the consumption of higher protein meals (30% of total energy intake) rich in dairy-source proteins promoted greater fat mass loss and lean mass retention (11). Admittedly, the contribution of other constituents of dairy foods responsible for these effects and the underlying physiologic mechanisms are unclear (12). Whey protein is one potential protein component that was speculated to contribute to the bioactivity of dairy (13). The branched-chain amino acid leucine is found in high proportions in whey and was shown to be a potent stimulator of muscle protein synthesis in humans (14, 15). In addition, data exist (at least in adipocytes and muscle cells) to suggest that leucine alone may have a synergistic role in muscle and adipocyte cells because it inhibits adipocyte lipogenesis and stimulates lipolysis (16, 17). Therefore, whey protein (containing leucine) may be a dairy constituent that is important for greater fat mass loss and lean mass retention. However, with regards to lipolysis, little data exist to expand this knowledge in humans. A meta-analysis conducted by Miller et al. (18) found a modest effect from whey protein supplementation on LBM retention and fat mass loss compared with carbohydrate during energy deficit; however, it was noted that currently there are not enough studies to compare whey protein with other protein sources.

The effect of protein source during weight loss requires further study; thus, the aim of this study was to examine the efficacy of supplementation with whey vs. soy protein compared with an isoenergetic control (carbohydrate) in affecting protein turnover. We hypothesized that there would be greater stimulation of MPS before and after weight loss with consumption of whey than with consumption of soy and that both would be more effective than carbohydrate. We also investigated the impact of protein source vs. carbohydrate on lipolysis and hypothesized that the rate of lipolysis would be suppressed with feeding, but less so with the ingestion of whey and soy than with carbohydrate because of the greater insulin response with carbohydrate ingestion.

Methods

Participants. Baseline anthropometric characteristics of the participants are given in **Table 1**. A total of 50 participants were recruited through posters and newspaper advertisements. Each participant gave their written, informed consent after being screened for eligibility. Nine subjects declined participation before the trial and 1 terminated participation during the trial for personal reasons. Inclusion criteria were the following: BMI 28–50 kg/m^2, 35–65 y old, nonsmoker, nondiabetic, and otherwise healthy on the basis of participant responses to a standard medical screening questionnaire. No participants were undertaking a weight loss or exercise program at the time of enrollment. Participants were asked to maintain their regular physical activity level until after study completion. Participants were informed of the experimental procedures to be used, the purpose of the study, and all potential risks before providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and was in accordance with standards set by the Canadian Tri-Council Policy (19) on the use of human participants in research.

TABLE 1 Baseline participant characteristics of the whey, soy and carbohydrate groups[1]

	Whey	Soy	Carbohydrate	*P*
Sex, M/F	7/7	7/7	5/7	
Age, y	52 ± 2	52 ± 2	48 ± 3	0.257
BMI, kg/m^2	34.7 ± 1.1	34.8 ± 1.5	36.9 ± 1.2	0.423
Body fat, %	36.0 ± 1.9	35.9 ± 1.7	37.9 ± 2.2	0.699
Lean mass, kg	60.6 ± 3.3	61.6 ± 3.9	60.4 ± 4.0	0.794
Trunk fat, kg	8.9 ± 0.8	9.8 ± 1.3	9.5 ± 1.1	0.805

[1] Values are means ± SEMs unless otherwise indicated.

Before the study commenced, participants' height and body mass were measured (Rice Lake Weighing Systems) and participants were asked to complete a 3-d food journal (2 weekdays and 1 weekend day) to provide an estimate of their habitual dietary intake. These records were analyzed with the use of a commercially available software program (The Food Processor, ESHA). Participants were instructed not to consume any vitamin or mineral supplements, particularly calcium or vitamin D, for the duration of the study. Participants also were instructed not to consume alcohol for the duration of the study.

Study design. The timeline of the overall study is shown in **Supplemental Figure 1**A. In a double-blind investigation, 40 men and women were randomly assigned to a hypoenergetic diet with twice daily supplementation with isolated whey protein, isolated soy protein, or an isoenergetic amount of carbohydrate. Groups were matched and stratified by age, sex, and BMI. Participants' energy requirements were calculated with the use of the Mifflin-St Jeor equation (20), with an appropriate activity factor (calculated for each participant based on an activity log) by a registered dietician. Three days before the start of the experimental infusion trial, participants were provided with a 3-d weight maintenance diet designed to provide 100% of their estimated energy requirements and at a protein intake of 1 g/(kg · d). Participants were supplied with all the food required for the entire duration of the study. To enhance participant compliance, this mainly was in the form of prepackaged meals (Copper County Foods).

After the weight maintenance diet, participants underwent their first infusion trial. Briefly, after an overnight fast, participants consumed a standardized breakfast at 0530 consisting of Ensure Plus (15% protein, 29% fat, 56% carbohydrate; Abbott) providing 6 kcal/kg body mass at home before arriving to the laboratory. Participants arrived at the laboratory at McMaster University at 0730. A 20-gauge catheter was inserted into the antecubital vein of one arm of each participant, and a baseline blood sample was drawn. A second catheter was inserted in the contralateral arm and primed continuous infusions of ring-[$^{13}C_6$]-phenylalanine [0.05 μmol/(kg · min); 2.0 μmol/kg prime] and [$^2H_5$]-glycerol [0.1 μmol/(kg · min); 1.5 μmol/kg prime] (Cambridge Isotope Laboratories) were initiated. After 3 h of tracer infusion, a muscle biopsy was obtained from the vastus lateralis, after which participants consumed their assigned study beverage composed of isonitrogenous quantities of whey (27 g protein; Agropur Isochill 8000 Whey Protein Concentrate), soy protein isolate (26 g protein; SoyPro950M, International Trade Company), or an isoenergetic amount of carbohydrate (<1 g protein, 25 g maltodextrin; Globe Plus). Protein beverages were enriched to 4% with ring-[$^{13}C_6$]-phenylalanine according to their phenylalanine content. After a 3-h postprandial period, a second muscle biopsy (fed state) was obtained from the vastus lateralis (Supplemental Figure 1B).

The day after the infusion protocol, participants consumed prepackaged meals marking the onset of the 14-d weight loss program providing a 750 kcal/d deficit from subjects' estimated energy requirements based on the Mifflin St. Jeor equation. In addition to the meals,

participants also consumed study supplements (whey, soy, or carbohydrate), which were included within the energy allowance. Whey and soy supplements were isonitrogenous and both were isoenergetic with the carbohydrate control supplement. The supplements (27 g whey, 26 g soy, or 25 g carbohydrate) were consumed twice a day: midmorning (between breakfast and lunch) and midafternoon (between lunch and dinner). After the 14-d weight loss period, a second infusion was performed. This was identical to the first infusion with the exception of one extra biopsy before the infusion began to account for the new baseline isotope enrichment level.

Blood analyses. Plasma amino acid concentrations were determined through use of the Phenomenex EZ:faast amino acid analysis kit with gas chromatography-mass spectrometry (GC Model 6890 Network, Agilent Technologies; MSD model 5973 Network, Agilent Technologies). Insulin concentrations were determined in plasma through use of solid-phase, 2-site chemiluminescence immunometric assays (Immulite; Intermedico) and glucose was measured through use of the glucose oxidase method.

Glycerol concentration and enrichment was measured through use of gas chromatography-mass spectrometry (GC Model 6890 Network, Agilent Technologies; MSD model 5973 Network, Agilent Technologies) to measure whole body lipolysis. Briefly, 50μL plasma was deproteinized on ice in 500 μL 0.3N $Ba(OH)_2$ and 500 μL 0.3N $ZnSO_4$ for 20 min, and then centrifuged at 500 × *g* for 20 min at 4°C. The supernatant was collected and flowed through an ion exchange column consisting of 1 mL Dowex cationic resin (50WX8–200 resin; Sigma-Aldrich) and 1 mL Dowex anionic resin (1 × 8 chloride form; Sigma-Aldrich). The resin was washed 4 times with 1 mL of distilled deionized water and all flow-through was collected. Samples were then dried. The glycerol rate of appearance (Ra) was calculated through use of different equations in the fasted state (steady state) and after supplement ingestion (nonsteady state) as defined by the Steele equation (21). To calculate fasted glycerol Ra, the glycerol enrichment measured in the first blood sample of the infusion day (0 h) and after 2 h of infusion was used. To calculate fed glycerol Ra between 1 and 2 h after supplement ingestion, both the glycerol concentration and the enrichment 1 and 2 h after consuming the study supplement were used in the equation.

Muscle analyses. Approximately 50 mg wet muscle was homogenized on ice in buffer [10 μL/mg muscle of 25 mmol/L Tris 0.5% v:v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, Roche; PhosSTOP, Roche Applied Science)] and centrifuged at 15,000 × *g* for 10 min at 4°C to separate the supernatant (sarcoplasmic) and pellet (myofibrillar) fractions. The myofibrillar fraction was stored at −80°C for future processing.

To determine myofibrillar protein-bound enrichments, the myofibrillar fraction (pellet) was washed with distilled deionized water and then purified from collagen in sodium hydroxide (NaOH). The myofibrillar fraction was then hydrolyzed for 72 h in 0.1 M HCl and Dowex (50WX8–200 resin; Sigma-Aldrich) at 110°C and mixed on a vortex every 24 h. The free amino acids were purified with the use of Dowex ion exchange chromatography, and the N-acetyl-n-propyl derivative was prepared and run on an isotope ratio MS to measure bound enrichment of ring-[$^{13}C_6$]-phenylalanine as described previously (16). The fractional synthetic rate (FSR) of myofibrillar protein was calculated with the use of the standard precursor-product equation and methods as described previously (22, 23). The postintervention infusion trial included a baseline muscle biopsy before the infusion began to account for changes in protein-bound enrichment from the preintervention infusion trial.

Body composition. Body composition was determined at the same time of day under the same nutritional conditions before and after the protocol. A DXA scan (QDR-4500A; Hologic software version 12.31) was performed with the use of a standardized protocol with participants positioned similarly on the scan table (24). Trunk fat was measured by sectioning lumbar 1 to lumbar 4 of the spine from the DXA scan. This was reported be an accurate surrogate measure of visceral adipose tissue (25).

Statistical analyses. Statistical analyses were performed with the use of SPSS version 18.0. A univariate (treatment) ANOVA was performed to compare all baseline anthropometric and dietary variables (all dietary variables in **Table 2**) between groups. A univariate ANCOVA was performed with energy deficit (difference from requirement) as the covariate on changes in body mass and fat mass. A repeated measures ANOVA (treatment × time) was performed for the analyses of plasma amino acid–related variables, glucose and insulin concentration time course and AUC, western blot, and glycerol Ra. A repeated measures ANOVA (time) was performed on the myofibrillar FSR within groups and a repeated measures ANOVA (treatment × time) was performed on the change in myofibrillar FSR from postabsorptive to postprandial states pre- and postdiet. Significant differences in ANOVA were isolated with Tukey's post hoc test. Significance was set at $P < 0.05$. Data are presented as means ± SEMs.

Results

Participant characteristics. Baseline participant characteristics are shown in Table 1. There were no differences between treatment groups (all $P > 0.05$) for any of the variables.

Dietary manipulation. The 14-d energy restriction diet is shown in Table 2. The whey and soy groups consumed significantly more protein ($P < 0.01$) than the carbohydrate group, and there were no differences between whey and soy groups. Despite instruction and provision of prepackaged diets, the calculated energy deficit (difference between foods consumed and estimated requirement) was significantly higher ($P = 0.007$) in the carbohydrate group than in the whey group.

Body composition. Body composition changes are shown in **Table 3**. All groups lost LBM, fat mass, total body mass, and trunk fat mass, with a main effect ($P < 0.05$) for time; however, there were no significant between-group differences. Because of the significant difference in the energy deficit between the whey and carbohydrate groups, additional statistical analyses were performed (ANCOVA) with the use of energy deficit (difference from estimated requirement) as a covariate. There were no significant differences between groups in fat mass ($P = 0.83$) or total body mass change ($P = 0.76$) indicating that the small but statistically significant differences in the energy deficit (Table 2) did not affect changes in fat mass or total body mass loss between the whey and carbohydrate groups.

Plasma glucose and plasma insulin. Changes in plasma glucose followed expected patterns after ingestion of the respective supplements (**Figure 1**A and B). AUC for the 3-h sampling period was significantly higher (main effect for treatment, $P < 0.001$)

TABLE 2 Composition of 14-d weight loss diet including supplements consumed by the overweight or obese participants in the whey, soy, and carbohydrate groups[1]

	Whey	Soy	Carbohydrate
Protein intake, g/(kg · d)	1.3 ± 0.1[a]	1.3 ± 0.1[a]	0.7 ± 0.1[b]
Fat intake, g/d	48 ± 4[a]	47 ± 5[a]	48 ± 4[a]
Carbohydrate intake, g/d	206 ± 18[a]	214 ± 20[a]	226 ± 14[b]
Energy intake, kcal/d	1750 ± 123[a]	1760 ± 142[a]	1640 ± 97[a]
Estimated energy deficit, kcal/d	−680 ± 37[a]	−750 ± 38[ab]	−860 ± 39[b]
Protein, en%	29 ± 0.8[a]	30 ± 1.4[a]	19 ± 0.6[b]
Carbohydrate, en%	47 ± 0.8[a]	48 ± 0.8[a]	56 ± 0.3[b]
Fat, en%	25 ± 0.4[a]	24 ± 0.7[a]	27 ± 0.5[b]

[1] Values are means ± SEMs. Whey and soy groups, $n = 14$; carbohydrate group, $n = 12$. Means in a row without a common letter differ between groups, $P < 0.05$. en%, percentage of energy.

TABLE 3 DXA measurements of body composition pre- and postintervention and the change in body composition between pre- and postintervention after the 14-d diet in overweight or obese participants consuming a whey, soy, or carbohydrate supplement[1]

	Whey			Soy			Carbohydrate		
	Pre	Post	Δ	Pre	Post	Δ	Pre	Post	Δ
Total body mass, kg	98.8 ± 4.4	96.7 ± 4.5*	−2.1 ± 0.3	104 ± 6.6	101 ± 6.7*	−2.7 ± 0.3	105 ± 4.9	103 ± 4.8*	−2.1 ± 0.3
Lean body mass, kg	60.6 ± 3.3	59.9 ± 3.5*	−0.6 ± 0.5	63.5 ± 3.9	62.2 ± 4.1*	−1.3 ± 0.4	62.6 ± 4.0	62.2 ± 3.8*	−0.4 ± 0.3
Fat mass, kg	35.5 ± 2.4	34.1 ± 2.3*	−1.4 ± 0.4	37.6 ± 3.3	36.1 ± 3.3*	−1.5 ± 0.2	39.6 ± 3.0	38.0 ± 3.1*	−1.6 ± 0.2
Trunk fat mass, kg	9.1 ± 0.8	8.6 ± 0.8*	−0.5 ± 0.1	9.6 ± 1.3	9.1 ± 1.2*	−0.5 ± 0.1	9.8 ± 1.1	9.3 ± 1.1*	−0.5 ± 0.1

[1] Values are means ± SEMs. Whey and soy groups, $n = 14$; carbohydrate group, $n = 12$. *Post means within a row significantly different from Pre means ($P < 0.05$). Post, postintervention; Pre, preintervention.

in the carbohydrate group than in the whey and soy groups (Figure 1E). Plasma insulin concentrations had trends that were similar to those for glucose, with the concentrations of insulin in the carbohydrate group significantly greater ($P < 0.001$) than those in the whey and soy groups pre- and postintervention (Figure 1C and D). Insulin AUC for the 3-h sampling period was significantly higher in the carbohydrate group than in the whey and soy groups ($P < 0.001$) (Figure 1F).

Whole body lipolysis. Glycerol Ra is shown in **Figure 2** as a measure of whole body lipolysis. There was a significant effect for time ($P < 0.001$), with glycerol Ra decreasing in all groups with feeding. The Ra in the carbohydrate group was significantly lower than in the whey and soy groups after supplement ingestion ($P < 0.001$). There was no significant difference between pre- and postintervention values in any group.

Plasma amino acid concentrations. AUC, maximum concentration, time of maximum concentration, and AUC below baseline for total amino acids, plasma leucine, and the sum of essential amino acids are shown in **Table 4**. The total amino acid, leucine, and sum of essential amino acids AUC and maximum concentration were significantly different between the whey, soy, and carbohydrate groups, with higher values in the whey group than in the soy and carbohydrate groups ($P < 0.001$), and higher values in the soy group than in the carbohydrate group ($P < 0.01$).

Myofibrillar protein synthesis. Rates of MPS in the fasted (postabsorptive) and fed (postprandial) states are shown in **Figure 3**A. Baseline postabsorptive rates of MPS were similar across groups. In response to supplement ingestion, MPS increased significantly in the whey (pre- and postintervention, $P < 0.001$) and soy (preintervention, $P = 0.002$ and postintervention, $P = 0.001$) groups before and after the diet. There was no significant effect from ingestion of the carbohydrate supplement on postprandial MPS before ($P = 0.67$) or after ($P = 0.55$) the diet. After the weight loss diet, there was a significant decrease in postabsorptive (all groups, $P < 0.001$) and postprandial (whey, $P < 0.001$; soy, $P = 0.001$; carbohydrate, $P = 0.022$) MPS in all groups. The decrease in postabsorptive MPS did not differ between the whey (−15 ± 4%), soy (−25 ± 4%), and carbohydrate (−20 ± 4%) groups ($P > 0.05$); however, postprandial rates of MPS were reduced by 9% in the whey group, which was significantly less than the reduction in the soy (−28%; $P = 0.021$) and carbohydrate (−31%; $P = 0.013$) groups after the 14-d weight loss intervention. Figure 3B shows the change in MPS from the postabsorptive to the postprandial state before and after the diet. In response to the supplement, FSR increased significantly more after whey ingestion than soy or carbohydrate ingestion ($P < 0.001$) both pre- and post diet.

Discussion

The novel finding from this study was that twice-daily consumption of whey protein resulted in an attenuation of the

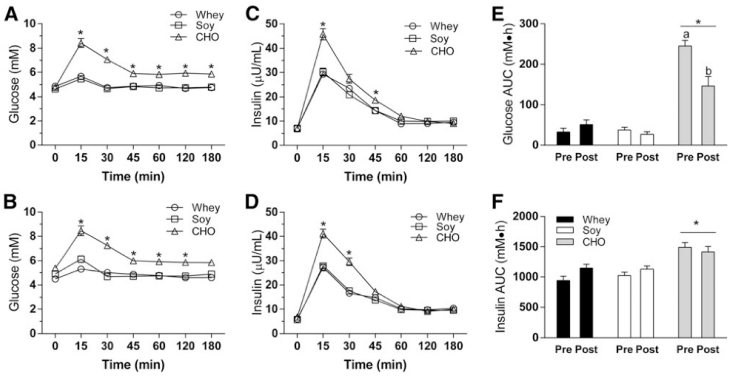

FIGURE 1 Blood glucose (A and B) and insulin (C and D) concentrations during pre– (A and C) and post– (B and D) weight loss. *Differences between groups ($P < 0.001$); carbohydrate group significantly different from whey and soy groups. Within-group comparisons omitted for clarity. AUC of glucose (E) and insulin (F) after supplement ingestion. *Significantly different from whey and soy groups ($P < 0.001$); means with different letters within the same time point are different from each other, $P < 0.05$. Values are means ± SEMs, $n = 14$ for whey and soy groups, and $n = 12$ for carbohydrate group. Time 0 = time of supplement ingestion. CHO, carbohydrate; Post, post–weight loss; Pre, pre–weight loss.

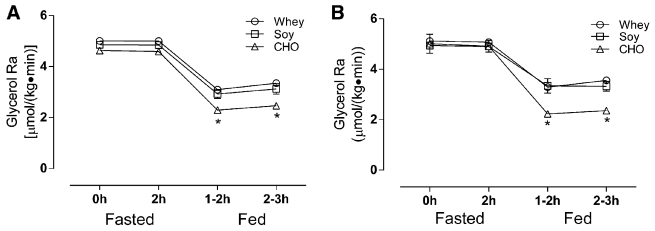

FIGURE 2 Glycerol Ra in the postabsorptive (Fasted) and postprandial (Fed) periods early (1–2h) and later (2–3h) after supplement ingestion, before (A) and after (B) the dietary intervention. Within-group comparisons omitted for clarity. *Carbohydrate significantly different from whey and soy groups ($P < 0.001$). Values are means ± SEMs, $n = 14$ for whey and soy groups, and $n = 12$ for carbohydrate group. CHO, carbohydrate; Ra, rate of appearance.

postprandial decline in MPS during a short-term dietary hypoenergetic diet vs. twice-daily supplementation with soy protein or carbohydrate. This is an important discovery because it indicates that proteins such as whey may be more effective at preserving MPS and potentially LBM in longer-term weight loss interventions. In addition, rates of lipolysis, although suppressed in all conditions, were suppressed to a greater extent after carbohydrate ingestion than with the ingestion of whey or soy protein. Although no group effects were observed for measures of body composition during this trial, this was almost assuredly because of the short-term nature of the intervention and a lack of sensitivity toward detecting changes with the use of DXA.

After the 14-d hypoenergetic diet, postabsorptive rates of MPS decreased in all groups. This finding is consistent with previous studies demonstrating a reduction in postabsorptive rates of MPS after short-term weight loss (5, 7). Interestingly, we observed that supplementation with whey protein resulted in the greatest retention of the postprandial MPS response over soy and carbohydrate after the intervention. Similar findings were demonstrated by Pasiakos et al. (6), who showed that consumption of 2 and 3 times the RDA for protein preserved postprandial MPS rates after an energy deficit, whereas consuming the RDA for protein resulted in an impaired rate of postprandial MPS after the diet. Pasiakos et al. (6) suggested that the amino acids in the RDA group may have been sequestered as a source of energy instead of being used for MPS; consequently, more protein would be required to optimally stimulate MPS. The difference between whey and soy protein supplementation could be the result of the greater leucine content in whey, which results in greater postprandial hyperleucinemia and stimulus for MPS (26). Indeed, our data showed a greater peak and net (AUC) exposure to leucine and essential amino acids with whey than with soy and carbohydrate. There is evidence that whey protein, as opposed to soy protein, results in amino acids being directed more toward peripheral (i.e., muscular) rather than splanchnic tissues (27). Our findings are congruent with this concept and support our earlier work demonstrating a greater MPS response after whey ingestion than with soy protein ingestion (26).

Despite the increased MPS response with whey ingestion compared with soy, no difference in whole body lipolysis was observed between the whey and soy groups. This finding was surprising especially during a hypoenergetic diet, because the energy consuming process of MPS (28) is likely to require energy from endogenous sources (such as fat). In support of this theory, high protein intakes were shown to stimulate greater fat oxidation during energy restriction than do high carbohydrate intakes (29), and lead to greater fat mass loss after 10 wk (30). In addition, we demonstrated that whey protein consumption

TABLE 4 Aminoacidemia-related variables for leucine and the sum of essential amino acids and total amino acids after whey, soy, or carbohydrate ingestion in obese or overweight subjects before and after the 14-d diet[1]

	Whey		Soy		Carbohydrate	
	Pre	Post	Pre	Post	Pre	Post
Leucine						
AUC_{pos}, mM · h	28,600 ± 1890^{a}	26,200 ± 1100^{a}	11,000 ± 1240^{b}	12,700 ± 1150^{b}	46 ± 18^{c}	573 ± 296^{c}
C_{max}, mM	361 ± 17^{a}	352 ± 16^{a}	248 ± 14^{b}	242 ± 9^{b}	110 ± 5^{c}	97 ± 6^{c}
T_{max}, min	54 ± 8^{a}	55 ± 3^{a}	47 ± 2^{a}	51 ± 3^{a}	11 ± 2^{b}	22 ± 4^{b}
AUC_{neg}, mM · h	−4 ± 4^{a}	−4 ± 4^{a}	−8 ± 8^{a}	−5 ± 5^{a}	−3270 ± 412^{b}	−1420 ± 371^{b*}
ΣEAA						
AUC_{pos}, mM · h	45,000 ± 3720^{a}	42,200 ± 2050^{a}	21,300 ± 3000^{b}	25,900 ± 3510^{b}	104 ± 42^{c}	2580 ± 1120^{c}
C_{max}, mM	785 ± 32^{a}	735 ± 19^{a}	620 ± 24^{b}	619 ± 15^{b}	373 ± 15^{c}	372 ± 13^{c}
T_{max}, min	49 ± 2^{a}	54 ± 2^{a}	48 ± 12^{a}	53 ± 2^{a}	23 ± 3^{b}	33 ± 4^{b}
AUC_{neg}, mM · h	−33 ± 23^{a}	−31 ± 31^{a}	−28 ± 20^{a}	−40 ± 40^{a}	−8420 ± 1350^{b}	−3040 ± 1100^{b*}
ΣTAA						
AUC_{pos}, mM · h	123,000 ± 9540^{a}	114,000 ± 5740^{a}	50,500 ± 7870^{b}	6880 ± 7650^{b}	181 ± 74^{c}	5650 ± 2440^{c}
C_{max}, mM	2100 ± 79^{a}	1980 ± 51^{a}	1620 ± 69^{b}	1600 ± 46^{b}	920 ± 40^{c}	938 ± 41^{c}
T_{max}, min	47 ± 3^{a}	55 ± 3^{a}	45 ± 3^{a}	50 ± 3^{a}	25 ± 4^{b}	32 ± 3^{b}
AUC_{neg}, mM · h	−91 ± 64^{a}	−74 ± 74^{a}	−481 ± 457^{a}	−106 ± 97^{a}	−21,400 ± 3380^{b}	−7960 ± 3060^{b*}

[1]Values are means ± SEMs. Means within a row without a common letter differ between groups, $P < 0.05$. *Mean significantly different from preintervention mean, within that group for that variable, $P < 0.05$. AUC_{neg}, AUC below baseline; AUC_{pos}, AUC above baseline; C_{max}, maximum concentration; EAA, essential amino acid; Post, postintervention; Pre, preintervention; TAA, total amino acid; T_{max}, time to maximum concentration.

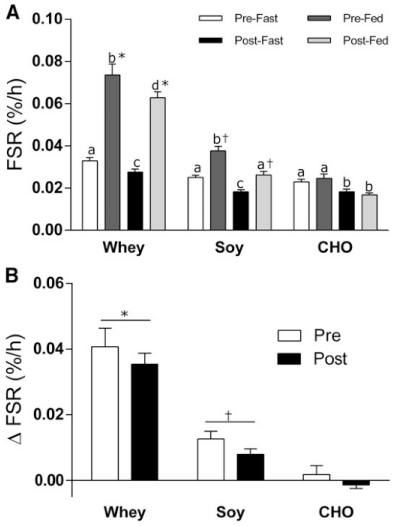

FIGURE 3 FSR in the fasted (postabsorptive) and fed (postprandial) states before and after the 14-d diet (A). Means without a common letter differ, $P < 0.05$, within groups. *Significantly different ($P < 0.01$) from soy and carbohydrate groups in the same condition (i.e., fed-state). †Significantly different ($P < 0.01$) from carbohydrate group in the same condition. Feeding-induced change in FSR from the postabsorptive to postprandial states before and after the 14-d dietary intervention (B). *Both means significantly different ($P < 0.01$) from soy and carbohydrate groups; †Both means significantly different ($P < 0.01$) from carbohydrate group. Values are means ± SEMs, $n = 14$ for whey and soy groups, and $n = 12$ for carbohydrate group. CHO, carbohydrate; FSR, fractional synthetic rate; Post, postintervention; Pre, preintervention.

results in greater plasma hyperleucinemia, which makes it available to peripheral tissues such as muscle and fat. This is significant because leucine administration to a coculture of adipocyte and muscle cells resulted in decreased fatty acid synthase gene expression in adipocytes and increased FA oxidation in muscle cells, resulting in a reduction in energy storage in adipocyte cells and increased energy use (presumably for protein synthesis) in skeletal muscle cells. These results suggest that leucine affects energy partitioning between adipose tissue and lean mass (16). Therefore, mechanistically, whey protein has the potential to be a dairy component known to promote fat loss. Previous work also demonstrated that high protein from dairy promotes greater fat mass loss (11). Future work would be needed to determine the effect of whey protein supplementation on fat and lean mass over a longer-term energy restriction intervention. Interestingly, a recent publication documented that, when combined with exercise, whey protein supplementation was highly effective at reducing visceral adipose tissue mass and increasing LBM (31).

Recently, it was reported that during a short-term hypoenergetic period there was a 60% increase in rates of mixed muscle protein breakdown (32), despite the fact that participants consumed 1.5 g/(kg · d) protein, which is an intake known to preserve lean mass (8). This is a surprising finding, given that both postabsorptive and postprandial rates of MPS are also reduced during a hypocaloric period (5–7) and that in nonpathologic situations, rates of MPS and MPB do not change divergently (33, 34). In fact, if a hypocaloric diet-induced increase in MPB is real, then, to our knowledge, such an increase at the same time that there is a decline in MPS would be the first report of a divergent change in these 2 processes, at least in a nonpathologic state. We estimate that if MPB were elevated by the degree suggested (32), combined with the reductions in MPS that we and others (5–7) observed in both the postabsorptive and postprandial states, then losses of LBM would have been more than 2 times greater than those we observed (Table 3). Thus, we find it difficult to reconcile the apparent large increase in MPB reported (32) at the same time that we and others observed a reduction in MPS. We acknowledge that much of the data on muscle protein turnover are from short-term trials and that, in a longer-term trial, reductions in postabsorptive and postprandial MPS were not observed (35). Clearly, there is a need for further trials in hypoenergetic states to ascertain the relative roles that MPS and MPB play in the determination of muscle mass.

Although all participants lost fat and lean mass, there were no body composition advantages (i.e., lean mass sparing or increased fat mass loss) observed between groups. We propose that the lack of a treatment effect on body composition may be a result of the short duration of the study. Wycherley et al. (8) demonstrated in a meta-analysis and Krieger et al. (9) in a meta-regression that higher protein hypoenergetic diets preserve lean mass in studies lasting 4 wk or longer; thus, we would have had to extend our study to observe differences had they existed.

The use of DXA to measure body composition is one important limitation in this study, and a more accurate model, such as the 4-compartment model, may have been more effective (36). It is likely that 2 wk of this energy-restricted diet was not long enough to elicit body composition changes that were outside the error of measurement of the DXA. In a study of overweight women, the measurement error by the same instrument we used was reported as 1.2% for fat and 1.1% for LBM (37). In our study, this would equate to an average loss of ~0.7 kg lean mass, which was not exceeded by the whey (−0.6kg) or carbohydrate (−0.4kg) groups. Therefore, the variations observed in this study were still within the error of the repeat measurement with use of DXA. Importantly, because the strength of our study was to examine the acute changes with energy restriction, we were underpowered to detect changes in body composition.

In conclusion, our data demonstrated that whey protein supplementation during energy restriction provided greater stimulation of MPS and maintenance of postprandial MPS rates. To show changes in body composition, we would need to have extended our study. These results demonstrate the impact of protein quality on MPS during energy restriction, and may be of importance in the development of nutritional strategies to promote higher-quality weight loss, which involves the loss of a high ratio of fat to LBM.

Acknowledgments

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The Journal of Nutrition
Errata

Erratum for Vadiveloo et al. Greater healthful food variety as measured by the US Healthy Food Diversity Index is associated with lower odds of metabolic syndrome and its components in US adults. J Nutr 2015;145:564–71.

In the above mentioned article, the second author's surname is spelled incorrectly. In the author list on page 564, "Niyati Parkeh" should be "Niyati Parekh." Footnote 2 should read as follows: "Author disclosures: M Vadiveloo, N Parekh, and J Mattei, no conflicts of interest."

doi:10.3945/jn.115.214031

Erratum for Hector et al. Whey protein supplementation preserves postprandial myofibrillar protein synthesis during short-term energy restriction in overweight and obese adults. J Nutr 2015;145:246–52.

In the above mentioned article, the lean mass values in Table 1 should be 63.5 ± 3.9 kg (soy) and 62.6 ± 4.0 kg (carbohydrate). These changes do not alter the overall conclusions of the article.

doi:10.3945/jn.115.214361

CHAPTER 3:

Impact of resistance exercise and dietary protein intake on skeletal muscle protein breakdown during pronounced short-term energy restriction in overweight young men. *Data submitted to FASEB J*

ABSTRACT

The preservation of lean body mass requires a balance between rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Currently, the contribution of MPB to lean body mass (LBM) loss during energy restriction in overweight young men is unknown. We aimed to determine the impacts of dietary protein intake and resistance exercise on MPB during a controlled short-term energy restricted (ER) diet. Adult men (BMI=28.6±0.6kg/m^2, age=22±1y) underwent 10 days of a 40% reduction in energy intake, while performing unilateral resistance exercise and consuming lower protein (LP) (1.2g/kg/d, n=12) or higher protein (HP) (2.4g/kg/d, n=12). Pre- and post-intervention testing included primed constant infusion of ^{15}N-phenylalanine to measure acute (hourly) MPB in the fasted state, and skeletal muscle biopsies to measure gene and protein expression of the ubiquitin-proteasome and autophagic-lysosomal pathways. There was no change in MPB (energy balance=0.080±0.01%/hr, ER rested legs=0.078±0.008%/hr, ER exercised legs= 0.079±0.006%/hr). Similarly, neither gene nor protein expression of the ubiquitin-proteasome and autophagic-lysosomal pathways were altered following ER. We conclude that changes in MPB are not a significant contributor to LBM loss early in ER in young overweight men.

INTRODUCTION

The maintenance of muscle mass is dependent on a balance between fasted and fed state changes in rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). In energy balance, postprandial periods of positive protein balance are equal to postabsorptive periods of negative protein balance, resulting in a net neutral protein balance, and maintenance of muscle mass (1). During energy restriction (ER), rates of MPS are reduced in the fasted and fed states (2-5) resulting in an overall decline in net protein balance that could partly underpin a reduction in LBM. Whereas relatively more is known about MPS during ER, little is known about the effect of ER on rates of MPB. It is important, in order to gain a full understanding of the mechanisms of LBM loss during ER, that MPB be studied more thoroughly.

Molecular markers of MPB during ER in humans, such as changes in gene and protein expression of components and targets involved in the ubiquitin-proteasome and autophagic-lysosomal pathways, have yielded discordant results (6-8). For example, following 21 days of a 40% energy deficit, increases of 20% and 30% in the genes encoding MuRF-1 and Atrogin-1 were reported but there was no increase in the activities of subunits of the 26S proteasome, or caspase-3, and dietary protein intake (studied across a 3-fold range of protein intake: 0.8-2.4 g/kg/d) did not affect the expression of these atrogenes (6). Few direct measurements of MPB during ER have been made, however, one report showed that following a 10 day energy deficit in healthy adults (consuming 1.5g/kg/d

protein) there was a 60% increase in rates of postabsorptive MPB (7). Such a large increase in MPB (7) is difficult to reconcile when considered with data showing ER-induced decline in MPS (2, 5). If MPS were to decline and MPB increase then the loss of LBM during an energy deficit would be much greater than what has been reported. The primary aim of this study was to examine the effect of short term (10 day) ER (40% reduction in energy from that required for weight maintenance) on acute (hourly) rates of MPB. We used unilateral (single leg) resistance exercise to examine the impact of loading exercise on MPB. We also wished to examine the impact of differing dietary protein intakes on responses of MPB, thus, subjects were randomly assigned to consume a higher (2.4g protein/kg/d) versus lower (1.2g protein/kg/d) protein intake. To provide mechanistic insight, a secondary aim was to examine the effects of these interventions on the expression of genes and proteins involved in the ubiquitin-proteasome and autophagic-lysosomal proteolytic pathways. We hypothesized, given the known rates of loss of skeletal muscle during ER, that following ER MPB would not be significantly elevated or would be adaptively reduced in accordance with the known decline in MPS (2-4, 9).

MATERIALS AND METHODS

Participants: A total of 25 non-smoking, non-diabetic men (BMI=28.6±0.6kg/m^2, age=22±1y) were recruited through posters and gave their written, informed consent after being screened for eligibility. Participants were not undertaking ER

or any form regular 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, all experimental procedures and risks before providing written consent. The study was approved by the Hamilton Health Sciences Research Ethics Board and was in accordance with standards set by the Canadian Tri-council Policy on the use of human participants in research (10). This trial was registered at clinicaltrials.gov as NCT02406040. Before the 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. A CONSORT diagram of the participant flow-through this parallel group trial is shown in **Figure 1.**

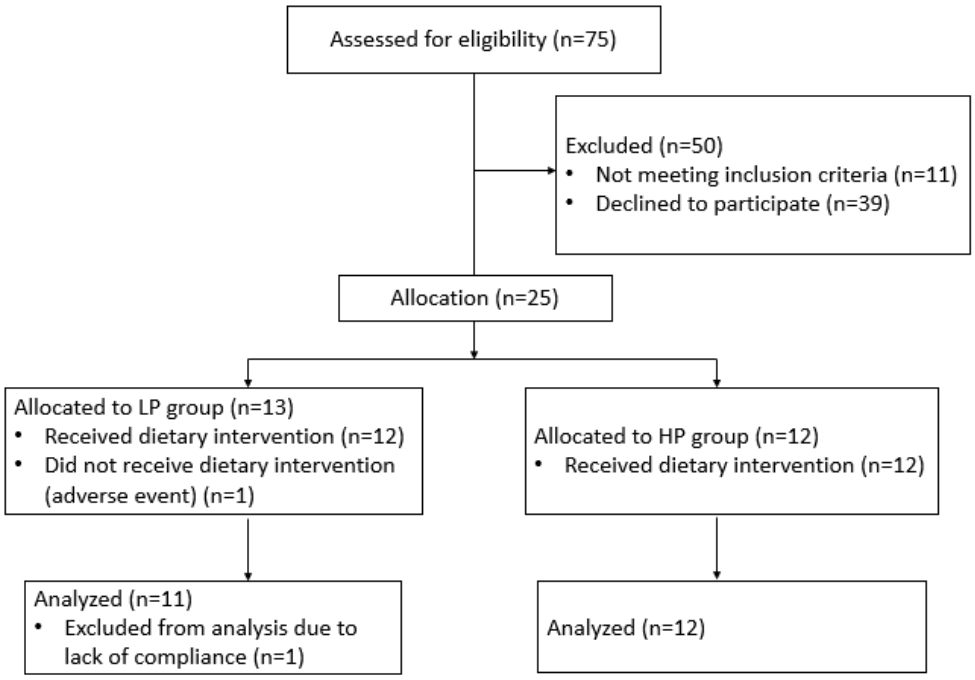

Figure 1. CONSORT diagram of participant recruitment and testing through the parallel group trial.

Study Design: In a single-blind investigation, 24 men completed a 10 day energy restricted diet containing either 1.2g/kg/d protein or 2.4g/kg/d protein. Groups were matched based on age, BMI, body fat, LBM, 10-repetition maximum (10RM) unilateral leg press (Maxam Fitness, Hamilton, ON, CAN) and 10RM unilateral leg extension (Atlantis C-105, Laval, QC, CAN; Table 1), with matched pairs for BMI, 10RM Leg Press and 10RM leg extension. Participants' energy requirements were determined using indirect calorimetry to establish resting metabolic rate (Moxus metabolic system, AEI Technologies, Pittsburgh, PA, USA) with an appropriate activity factor assigned using each individual participants' activity logs (11). The legs to be exercised were randomly selected

and counter-balanced for dominance based on strength so that equal numbers of dominant and non-dominant limbs were tested in each group.

Participants began the study by consuming an energy balanced diet designed to provide 100% of their energy requirements and a protein intake of 1.2g/kg/d. Participants were provided with all the food for the study, consisting of frozen meals (Heart to Home Meals, Brampton, ON, CAN) and prepackaged snacks. Following the energy balance diet, participants underwent a stable isotope infusion trial, shown in **Figure 2** to measure acute mixed MPB. Briefly, following an overnight fast, participants arrived at the laboratory and a 20 gauge catheter was inserted into a vein in the hand/forearm for blood sampling and infusion. A primed continuous filtered (0.2μm) infusion of ^{15}N-Phenylalanine (2μmol/kg, 0.05μmol/kg/min; Cambridge Isotope Laboratories, Tewksbury, MA, USA) was initiated. Following 150mins of infusion, the ^{15}N-Phenylalanine tracer was discontinued for calculation of muscle protein breakdown using the tracee-release method (12). At 40mins and 60mins following cessation of the ^{15}N-Phenylalanine tracer, muscle biopsies from the *vastus lateralis* were obtained. Muscle biopsy samples were cleared of visible blood and connective tissue and rapidly frozen in liquid nitrogen.

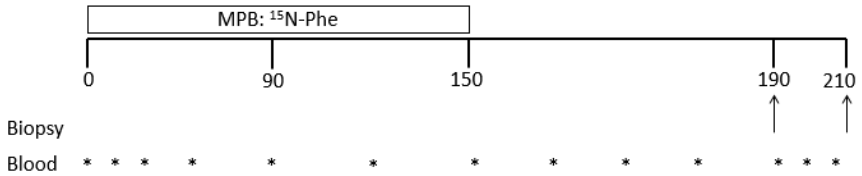

Figure 2. Infusion protocol. Following an overnight fast, participants arrived to the laboratory and a 20-gauge catheter was inserted into the vein of one arm and a baseline blood sample was taken before a 0.9% saline drip was started to keep the catheter patent for repeated blood sampling. A second catheter was placed in the contralateral arm for a primed continuous infusion of ^{15}N-Phenylalanine (2μmol/kg, 0.05μmol/kg/min). Following 150mins of infusion, the ^{15}N-Phenylalanine tracer was discontinued for calculation of MPB. During the first infusion (energy balance) only one leg (rested) was analyzed. However, following energy restriction, both the rested and exercised legs were assessed.

The day following the first infusion protocol, participants began the 10 day energy restricted diet, placing each participant in a 40% energy deficit from their individual calculated energy requirements. In addition to the meals, participants also consumed two chocolate flavoured protein supplements per day, which were included in their caloric requirements. The higher protein group consumed macronutrients in a ratio of 35:50:15 as energy from protein:carbohydrate:fat (PRO:CHO:FAT) that included, in a blinded manner, two high protein supplements per day containing 35g whey protein isolate in 250mL skimmed (<1% fat) milk. The lower protein group consumed macronutrients in a ratio of 15:50:35% (PRO:CHO:FAT) which included one high protein supplement (35g whey protein isolate, in 250mL 2% milk) and one chocolate flavoured placebo (250mL 2% milk).

During the energy restricted diet, participants arrived to the laboratory on five separate occasions for the performance of unilateral resistance exercise sessions spread evenly over the 10 day ER diet, with no more than two resistance exercise days in a row. Exercise sessions 1-4 were performed at any time of day but not before eating breakfast. The fifth exercise set was performed 48h prior to the last infusion trial, and hydration/water consumption was ad libitum for every exercise session. The unilateral exercise sessions consisted of 3 sets of 10 repetitions at 85% of predetermined 10 repetition maximum (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 8-12 repetition range on the last set. Immediately following the exercise sessions, participants consumed one high protein supplement.

Following 10 days of the energy restricted diet, participants arrived to the laboratory following an overnight fast for a second infusion protocol. This protocol was identical to the infusion outlined that took place prior to ER to measure acute mixed MPB, and gene/protein expression with the exception that biopsies were obtained from both the 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 DXA scan (GE Lunar iDXA;

Mississauga, ON, CAN) was performed with a standardized protocol with participants lying similarly on the bed.

Plasma enrichments. Plasma (50 μL) was deproteinized in 500 μL of acetonitrile. The HFB derivative was prepared and the enrichment of ^{15}N-Phenylalanine was determined by GC-MS (Hewlett Packard 6890; MSD model 5973 Network, Agilent Technologies, Santa Clara, CA, USA).

Intracellular enrichments. Approximately 25 mg of frozen muscle was first washed in 1X phosphate buffered saline to remove blood. The muscle was homogenized with a Teflon pestle in 500 uL of perchloric acid (PCA) to precipitate the muscle proteins. The sample was then centrifuged at 10000 *g* at 4°C for 5 minutes. The supernatant was collected and the procedure was repeated twice more for a total volume of 1.5mL of supernatant. The PCA supernatant was purified with the use of Dowex ion exchange chromatography and the heptafluorobutyl (HFB) derivative was prepared. The enrichment of ^{15}N-Phenylalanine was determined by GC-MS (Hewlett Packard 6890; MSD model 5973 Network, Agilent Technologies Santa Clara, CA, USA).

To determine intracellular phenylalanine concentration, one sample from each participant had 0.225uL/mg wet muscle of 0.001uM D8-Phenylalanine (Cambridge Isotope Laboratories, Tewksbury, MA, USA) added as an internal standard. The concentration of Phenylalanine was calculated as previously

described (13). Mixed MPB was calculated using the tracee-release method (14), where the dilution in the enrichment of ^{15}N-phenylalanine in the intracellular free amino acid pool is a function of unlabelled phenylalanine arising from release from the protein-bound fraction of the muscle (muscle protein breakdown), the decay of ^{15}N-phenylalanine in the blood following cessation of the intravenous infusion, and the concentration of unlabelled intracellular free and protein-bound phenylalanine. Thus, the equation to calculate MPB is:

$$FBR\ (\% \cdot hr) = \frac{EM(t2) - EM(t1)}{P\int_{t1}^{t2} EA(t)dt - (1+P)\int_{t1}^{t2} EM(t)dt}\ x\ (\frac{QM}{T})$$

Where EM(t2)-EM(t1) is the change in enrichment in the muscle intracellular fraction from t1 (40mins) to t2 (60mins) when the isotope infusion is discontinued, $\int_{t1}^{t2} EA(t)dt$ is the area under the arterialized blood enrichment decay curve, $\int_{t1}^{t2} EM(t)dt$ is the area under the intracellular muscle enrichment decay curve, QM/T is the ratio of intracellular free tracee concentration and protein-bound tracee concentration , $P = \frac{EM}{(EA-EM)}$ at isotope plateau.

Western Blotting. The expression of intracellular signaling proteins was assessed using *sodium dodecyl sulfate*-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting. Approximately 50 mg of wet muscle was homogenized on ice in buffer (500µL of 25 mmol/L Tris 0.5% v:v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, and PhosSTOP; Roche Diagnostics Laval, QC, CAN)) and centrifuged at

1500 *g* for 10 min at 4°C to separate the supernatant (sarcoplasmic) and pellet (myofibrillar) fractions. Total protein concentrations of the sarcoplasmic fraction were determined using the bicinchoninic acid assay (BCA Assay) (Thermo Scientific, Rockford, IL, USA). 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 polyvinylidene fluoride (PVDF) membranes, and exposed overnight at 4°C to primary antibodies following block for 2h in 5% BSA. Membranes were washed in Tris-Buffered saline and Tween 20 (TBST), and incubated in Anti-rabbit IgG conjugates with horseradish peroxidase secondary antibody (GE Healthcare Life Sciences, Mississauga, ON, CAN) for 1h at room temperature. Signals were detected using chemiluminescence SuperSignalWest Dura Extended Duration Substrate (Thermo Fisher Scientific, Burlington, ON, CAN) on a FluorChem SP Imaging system (Alpha Innotech, Santa Clara, CA, USA), bands were quantified on ImageJ scanning densitometry (National Institute of Health, USA), and data normalized relative to α-tubulin loading control. Antibodies used were purchased from Cell Signaling Technology (Danvers, MA, USA) as follows: LC3 (1:500, #2775), Caspase-3 (1:500, #9662), Ubiquitin (1:1000, #3933), p-Foxo3a (Ser253) (1:1000, #9466), TRAF6 (1:1000, #8028), p-TSC2 (Thr1462) (1:1000, #3617).

RNA extraction, reverse transcription and polymerase chain reaction:

Approximately 20 mg of skeletal muscle was used to isolate RNA using the trizol

phenol-chloroform procedure, as previously described (15). Reverse transcription was performed using Applied Biosystems High Capacity cDNA reverse transcription kit (Foster City, CA, USA). GAPDH was used as the housekeeping gene. The relative amounts of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method (16). Primer sequences for atrogin-1, MuRF-1, Foxo3a, LC3, Gabarapl1, Ulk2, Beclin1, BNIP3 and TRAF6 have been previously published (17, 18).

Statistical analyses: Statistical analyses were performed using SPSS version 19 (IBM, Armonk, NY, USA). A mixed model analysis of variance (ANOVA) was conducted on body composition, mixed protein breakdown, plasma enrichment, protein expression and gene expression (group x time). Linear regression was performed on plasma and intracellular free phenylalanine enrichment. Diet analysis, baseline characteristics, and unilateral resistance exercise were analysed by independent sample 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 the participants are given in Table 1. There were no significant differences between groups for any of the variables at study entry (all $p>0.05$).

Table 1. Participants' baseline characteristics

	Lower Protein Group	**Higher Protein Group**	**p value**
Age. Y	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
Lean Body Mass, kg	62±7	60±8	0.64
Leg Press Strength (10 Repetition Maximum), kg	111±44	110±19	0.93
Leg Extension Strength (10 Repetition Maximum), kg	41±14	38±9	0.62

Dietary Manipulation. The composition of the 5-day energy balance diets is provided in Table 2. There were no significant differences between groups for any of the variables. The composition of the 10-day ER diet is shown in Table 2. The high 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 7/12 participants in the higher protein group and 7/11 participants in the lower protein group guessed their weight loss diet (higher or lower protein group) correctly.

Table 2. Diet composition

	Lower Protein Group	**Higher Protein Group**	**p**
5d Energy Balance 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
% energy, PRO:CHO:FAT	13:51:36	13:51:36	0.48:0.81:0.29
10d Energy Restriction 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

*Significantly different from lower protein group

Unilateral Resistance Exercise. Total volume (kg) for unilateral leg press was not different in the lower (18675±6121) and higher (19188±5155) protein groups, p=0.83. Total volume (kg) for unilateral leg extension was not different between the lower (6051±1999) and higher (5744±1250) protein groups, p=0.66.

Body Composition. Body composition changes are shown in Table 3. There was a main effect of energy restriction on body mass (p<0.001), fat mass (p<0.001) and total lean body mass (p<0.001) with no interaction (p>0.05) for each

measurement. The change in appendicular lean mass showed a main effect of exercise (p=0.003), but no interaction (p=0.29).

Table 3. Body mass and composition

	Lower Protein Group			**Higher Protein Group**		
	Pre	**Post**	**Change**	**Pre**	**Post**	**Change**
TBM, 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
FM, 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
% FM	27.9± 6.0	27.7± 6.1	-0.3±0.5	29.7± 6.0	29.2± 6.2*	-0.6±1.0
LM EX, 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]
LM REST, 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]

*Significantly different from pre-weight loss mean within that group. Values without a common letter differ within that group. TBM: total body mass, LBM: lean body mass, LM: lean mass, FM: fat mass, EX: exercised leg, REST: rested leg

Mixed muscle protein breakdown. Postabsorptive mixed muscle protein breakdown (%/hr) rates are shown in **Figure 3**. Mixed MPB was not different between EB and ER time points in either group (time: p=0.17, interaction: p=0.57). Plasma and intracellular enrichments of ^{15}N-Phenylalanine are shown in **Figure 4**. The slope of the decay line of ^{15}N-Phenylalanine was the same between all infusion trials.

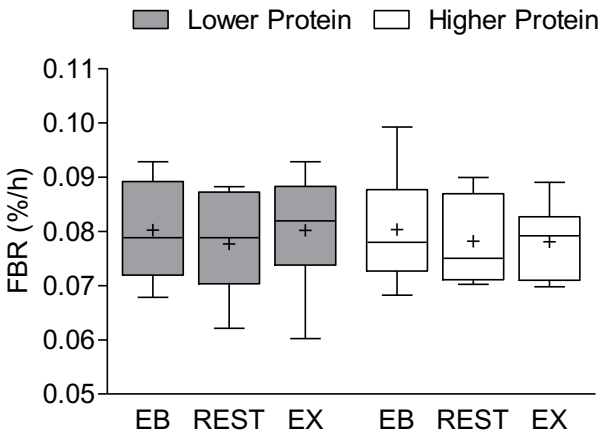

Figure 3. Mixed muscle protein breakdown, (FBR, %/hr). EB: energy balance, REST: energy restriction rested leg, EX: energy restriction exercised leg.

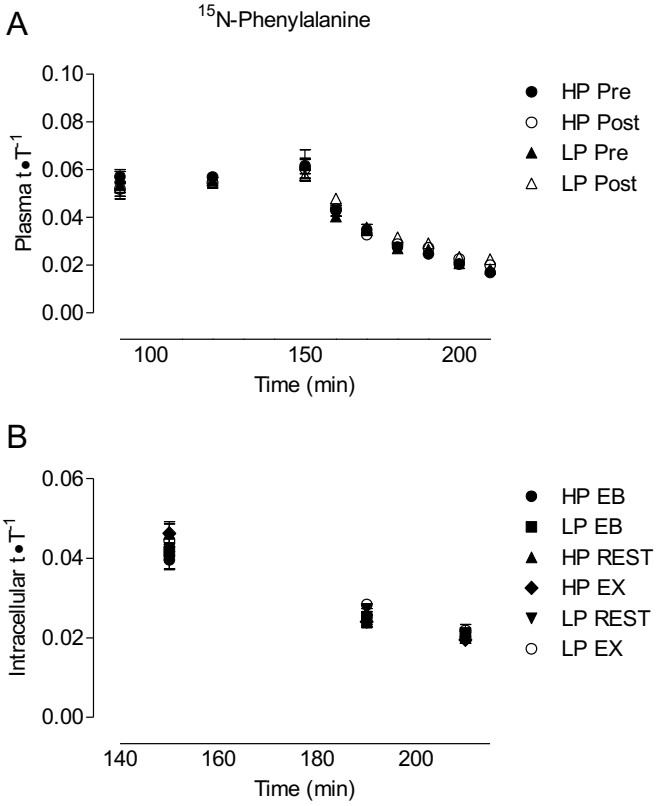

Figure 4. (A) Plasma enrichments of ^{15}N-Phenylalanine. (B) Intracellular enrichments of ^{15}N-Phenylalanine. HP: higher protein group, LP: lower protein group, Pre: pre energy restriction, Post: post energy restriction, REST: energy restriction exercised leg, EX: energy restriction exercised leg.

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

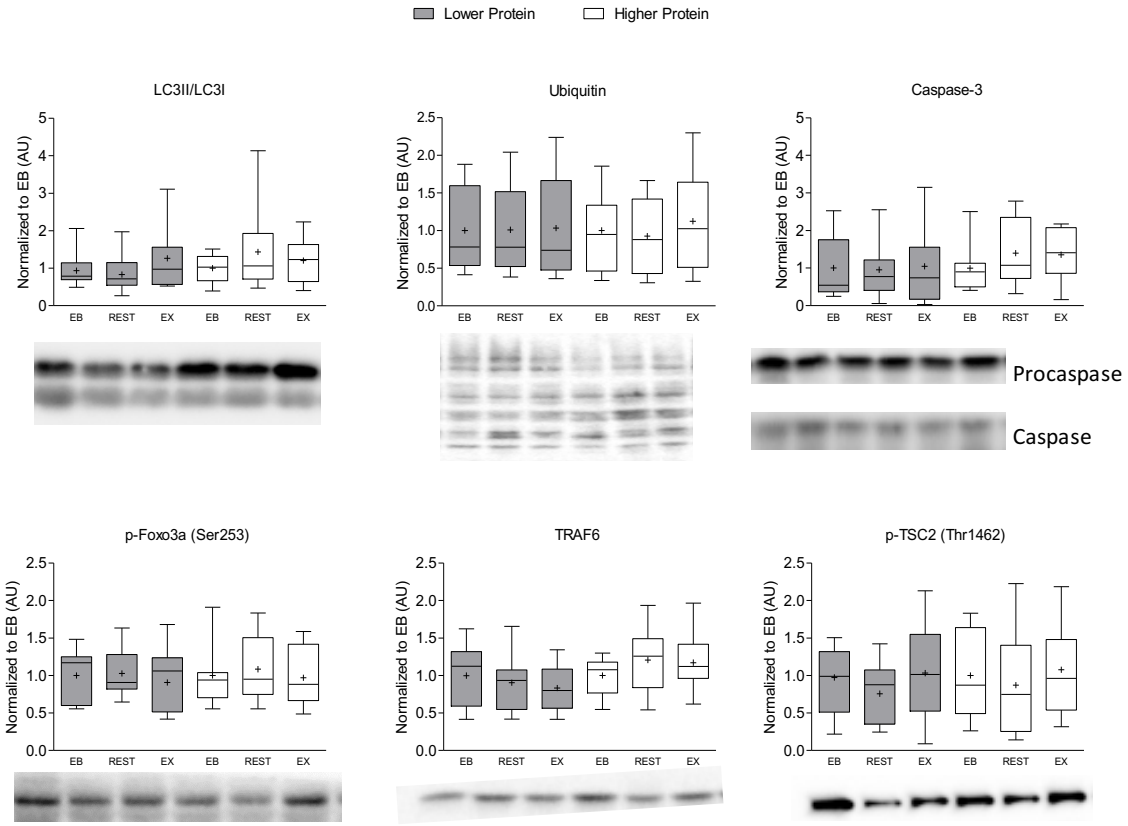

Figure 5. Western blot quantification with representative images. Data are normalized to the average weight maintenance value. EB: energy balance, REST: ER rested leg, EX: ER exercised leg, LC3: Microtubule-associated protein 1A/1B-light chain 3, Foxo3a: Forkhead box O3, TRAF6: TNF receptor-associated factor 6, TSC2: Tuberous Sclerosis Complex 2.

Gene Expression: Expression of all genes (**Figure 6**) except TRAF6 was not different between groups or across time. Atrogin-1 (time p=0.91, group × time p=0.97), MuRF1 (time p=0.09, group × time p=0.64), FoxO3a (time p=0.44, group × time p=0.80), LC3 (time p=0.26 group × time p=0.67), Gabarapl1 (time p=0.07, group × time p=0.26), Ulk2 (time p=0.84, group × time p=0.80), Beclin1 (time p=0.49, group × time p=0.36), BNIP3 (time p=0.06, time × group p=0.25), TRAF6 (time p=0.01, group × time p=0.94), in the rested leg of the lower protein group, expression of TRAF6 was significantly greater than the exercised leg (p=0.04).

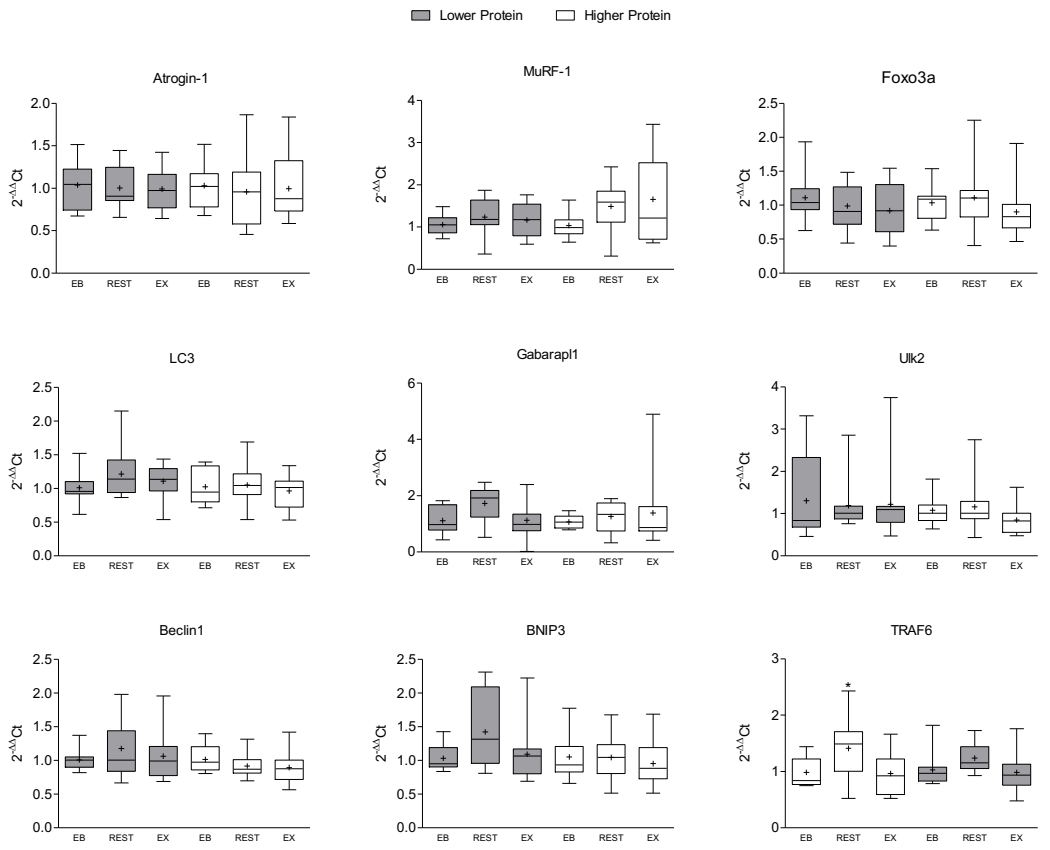

Figure 6. Gene expression of targets in the ubiquitin proteasome and autophagic-lysosomal pathways. * Significantly different than EX within that group. EB:

energy balance, REST: energy restriction rested leg, EX: energy restriction exercised leg, MuRF-1: Muscle RING-finger protein-1, Foxo3a: Forkhead box O3, LC3: Microtubule-associated protein 1A/1B-light chain 3, Gabarapl1: GABA Type A Receptor Associated Protein Like 1, Ulk2: Unc-51 Like Autophagy Activating Kinase 2, BNIP3: BCL2 Interacting Protein 3, TRAF6: TNF receptor-associated factor 6.

DISCUSSION

We found that muscle proteolysis, measured as acute hourly rates of MPB and gene and protein expression of markers in the ubiquitin-proteasome and autophagic-lysosomal pathways were unchanged throughout the ER and exercise intervention. These results are in contrast to data showing that postabsorptive MPB was increased by 60% following 10 d of a 20% energy deficit (with participants consuming 1.5g protein/kg/d) (7), despite our subjects being in a 40% energy deficit. The disparate findings between these studies could be due to differences in participant characteristics who were, according to BMI, of normal weight (7) compared to overweight in the current study. For example, Heymsfield et al. (19), plotted the fraction of weight lost as LBM during semi-starvation in young men against baseline adiposity and reported that the men in the lowest quintile of baseline % fat lost more LBM than the men in the higher quintiles of % fat (19). Therefore, individuals who are leaner would be more susceptible to LBM loss. However, whether the LBM loss in lean individuals who are in ER is due to such a large increase in MPB as observed by Carbone et al. (7) requires further investigation, especially when we consider previously reported declines in

MPS in the same energy restricted participants (3) which would contribute to losses in LBM.

Congruent with the lack of change in acute rates of MPB in the current study, we did not observe any changes in the gene or protein expression of targets in the ubiquitin-proteasome and autophagic-lysosomal pathways, findings that are consistent with some short-term studies in humans (4, 7, 8). Other ER studies have observed only small increases in these proteolytic genes and proteins. For example, Carbone et al. (7) observed no change in 26S proteasome proteolytic activity, and an 11% increase in caspase-3 protein expression despite reporting a 60% increase in MPB. Thus, it appears equivocal that these proteolytic pathways are upregulated with ER in young men. In the current study, we observed a slightly elevated (1.4 fold) level of TRAF6 mRNA [a regulator of both the ubiquitin-proteasome and autophagic-lysosomal pathways, (20)] in the rested leg of the lower protein group; however, this was not significantly elevated above expression seen in EB and the protein content of TRAF6 was unchanged.

Despite the novelty of our data, we acknowledge some limitations of the current study. Firstly, the short-term nature of the study resulted in small changes in body composition; however, we were still able to detect a significantly smaller reduction in LBM in the exercised leg compared to the rested leg in both groups and hypothesize that an effect of dietary protein would take more time and potentially a larger sample size to detect when the outcomes were being measured using DXA to estimate LBM (21). 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 the groups as closely as possible, we acknowledge the variability of the data due to inherent inter-subject differences.

In summary, we demonstrate that MPB and markers of the ubiquitin proteasome and autophagic-lysosomal pathways do not change despite a marked ER in overweight young men. We propose that our findings indicate that at least in the short-term, ER-mediated losses of LBM are not mediated through increased proteolysis.

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mass gain and fat mass loss: a randomized trial. *Am J Clin Nutr* **103**, 738-746

CHAPTER 4:

Impact of resistance exercise and dietary protein intake on skeletal muscle protein synthesis and turnover during pronounced short-term energy restriction in overweight young men. *Data submitted to FASEB J.*

ABSTRACT

The preservation of lean body mass (LBM) may be important during dietary energy restriction (ER) and requires balanced and equal rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Currently, no study has measured MPS and MPB simultaneously to obtain a measure of muscle protein turnover during ER. We aimed to determine the impacts of dietary protein intake and resistance exercise on muscle protein turnover during a controlled short-term energy deficit. Adult men (BMI=28.6±0.6kg/m^2, age=22±1y) underwent 10 days of a 40% reduction in energy intake, while performing unilateral resistance exercise and consuming lower protein (LP) (1.2g/kg/d, n=12) or higher protein (HP) (2.4g/kg/d, n=12). Pre- and post-intervention testing included primed constant infusion of *ring*-[$^{13}C_6$]-phenylalanine to measure acute postabsorptive MPS, and deuterated water to measure integrated (day-to-day) MPS. Using the DXA results from Chapter 3 we calculated absolute (g/d) breakdown, synthesis and turnover. Similarly, using the rates of MPB from Chapter 3, we calculated net protein balance in the postabsorptive state. There was a decrease in acute MPS following ER (HP=0.059±0.01%/hr to 0.051±0.01%/hr, LP=0.061±0.01%/hr to 0.045±0.01%/hr, $p<0.05$) that was attenuated with resistance exercise (HP= 0.067±0.01%/hr and LP= 0.061±0.016%/hr), and integrated MPS followed a similar pattern. There was no change in the absolute breakdown rate, which is congruent with the findings of MPB from Chapter 3. We conclude that reductions

in MPS are the main mechanism underpinning reductions in muscle protein turnover that lead to LBM loss early in ER in adult men.

INTRODUCTION

Dietary energy restriction (ER) is commonly employed to reduce total body mass, however, a potentially unfavourable consequence of ER is a loss of lean body mass (LBM) (1). Loss of LBM can comprise up to 25% of body mass lost (2) and can have deleterious consequences. Given that that largest component in LBM is skeletal muscle, and that skeletal muscle is a highly metabolically active tissue, LBM loss during ER could have important impacts on mobility and aspects of metabolic health (2). Thus, strategies that promote the retention of LBM during ER are of clinical importance especially, for example, in the elderly.

The maintenance of skeletal muscle mass is dependent on the balance between fasted and fed state changes in the rates of skeletal muscle protein synthesis (MPS) and skeletal muscle protein breakdown (MPB). In energy balance, periods of positive and negative protein balance are equal, resulting in a net neutral protein balance and stable muscle mass (3). During ER, rates of MPS are reduced in both the postabsorptive and postprandial states (4-7), which would lead to an overall decline in net protein balance that could partly underpin a reduction in LBM. However, higher protein intakes and resistance exercise attenuate LBM losses and can even result in gains in LBM during ER (8). Indeed, we showed that even during a marked (40% below energy requirements) energy

deficit, young men consuming 2.4g/kg/d protein and performing resistance exercise demonstrated significant increases in LBM (8). Resistance exercise and protein ingestion are known to attenuate the energy deficit-induced declines in resting MPS (6), which may explain why LBM does not decline. In addition, consuming 2-3 times the recommended dietary allowance (RDA) (1.6 and 2.4g/kg/d, respectively) for protein during ER preserved the MPS responses to a 20g serving of milk protein compared to 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 unavoidable, such as military training or operations (10).

The primary aim of this study was to examine the impact of short term (10d) ER (40% reduction in energy intake from that required for weight maintenance) on acute (hourly) rates of mixed MPS and integrated myofibrillar protein synthesis (myo-PS; daily). We used unilateral resistance exercise to examine the impact of loading exercise on the same variables. Subjects were randomly assigned to consume a higher (2.4g protein/kg/d) or a lower (1.2g protein/kg/d) protein intake. Thus, we also examined how differing levels of dietary protein intake affected the MPS responses, and used the MPB and body composition data from Chapter 3 to calculate postabsorptive and absolute (g/d) muscle protein turnover. We hypothesized, given the known rates of loss of skeletal muscle during ER, that mixed MPS and integrated myo-PS would be

reduced following ER but to a lesser extent in the resistance exercised leg. We also hypothesized, given the body composition differences reported by Longland et al. (8) that the higher (2.4g protein/kg/d) protein intake would act synergistically with exercise to stimulate mixed and myofibrillar MPS greater than that seen in subjects with the lower (1.2 g protein/kg/d) protein intake.

MATERIALS AND METHODS

Experimental Design. Details regarding the participants, controlled diet and physical activity interventions have been described in Chapter 3. Briefly, In a single-blind investigation, 24 non-diabetic, non-smoking men, body mass index (BMI) 28.6±0.6kg/m^2, age 22±1y, underwent 10 days of a 40% reduction in energy intake, while performing unilateral resistance exercise and consuming lower protein (LP) (1.2g/kg/d, n=12) or higher protein (HP) (2.4g/kg/d, n=12). A schematic of the overall study is shown in **Figure 1a**. Prior to beginning any dietary control (no longer than one week), a muscle biopsy was obtained to assess baseline deuterium enrichment levels. Five days prior to the first infusion protocol, all participants consumed 100mL of 70% deuterated water (D_2O) (Cambridge Isotope Laboratories, Tewksbury, MA, USA), began collecting daily saliva samples in the morning before any food/water or brushing teeth, and began an energy balance diet designed to provide 100% of their energy requirements and a protein intake of 1.2g/kg/d. Participants were provided with all the food for the study, consisting of frozen meals (Heart to Home Meals, Brampton, ON, CAN)

and prepackaged snacks. Following the energy balance diet, participants underwent a stable isotope infusion trial, shown in **Figure 1b** to measure acute mixed MPS. Briefly, following 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. A primed continuous filtered (0.2µm) infusion of *ring*-[$^{13}C_6$]-phenylalanine (2.0µmol/kg; 0.05 µmol/kg/min; Cambridge Isotope Laboratories, Tewksbury, MA, USA) was initiated. Approximately 50mg of muscle was collected at the 190min biopsy for measurement of acute mixed MPS, and integrated myoPS using the ingested D_2O. Muscle biopsy samples were cleared of visible blood and connective tissue and rapidly frozen in liquid nitrogen.

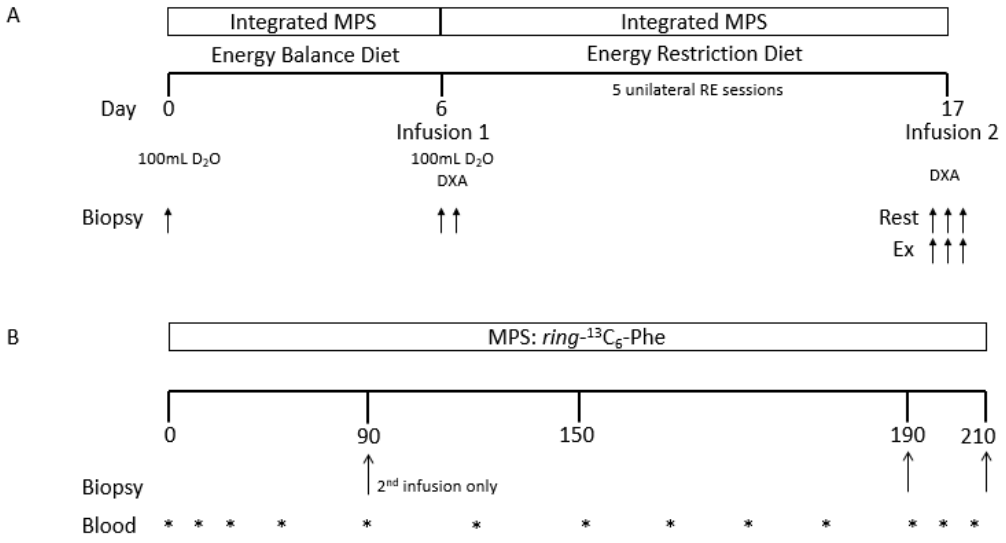

Figure 1. (A) Timeline of study. Prior to any dietary control a baseline muscle biopsy was obtained. Five days prior to the first infusion protocol, participants ingested a bolus of deuterated water to measure basal integrated myofibrillar protein synthesis (myo-PS), and consumed an energy balance diet. Participants then completed an acute infusion protocol to measure postabsorptive rates of

mixed muscle protein synthesis (MPS) as well as a dual-energy x-ray absorptiometry (DXA) scan to measure body composition. Participants then consumed a second bolus of deuterated water and completed the energy restriction diet (40% energy deficit, 2.4 or 1.2 g/kg/d protein) with five unilateral resistance exercise sessions. Following 10 days of this intervention, a second infusion was conducted to assess changes in acute MPS and body composition was measured by DXA. (B) Infusion protocol. Following 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 taken 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*-[$^{13}C_6$]phenylalanine (2.0μmol/kg; 0.05 μmol/kg/min). During the first infusion (energy balance) only one leg (rested) was analyzed. However, following weight loss, both the rested and exercised legs were assessed.

The day following this first infusion protocol, participants consumed another 100mL bolus of D_2O with daily saliva samples (obtained in the morning before any food/water or brushing teeth) and began the 10 day energy restricted diet with unilateral resistance exercise sessions described in Chapter 3. Exercise sessions 1-4 were performed at any time of day but not before eating breakfast. The fifth exercise set was performed 48h prior to the last infusion trial, and hydration/water consumption was ad libitum for every exercise session. Following 10 days of the energy restricted diet, participants arrived to the laboratory following an overnight fast for a second infusion protocol. This protocol was identical to the pre-ER infusion protocol to measure acute mixed MPS and myo-PS with the exception that a biopsy at 90 mins was also obtained to account for new baseline isotope enrichment levels of *ring*-[$^{13}C_6$]-phenylalanine from the previous infusion protocol, and biopsies were obtained from both the rested and exercised legs.

Integrated Myofibrillar Protein Synthesis. Approximately 50 mg of wet muscle was homogenized on ice in buffer (500μL of 25 mmol/L Tris 0.5% v:v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease Inhibitor Mini-Tabs, and PhosSTOP; Roche Diagnostics Laval, QC, CAN)) and centrifuged at 1500 *g* for 10 min at 4°C to separate the supernatant (sarcoplasmic) and pellet (myofibrillar) fractions. To determine myofibrillar protein-bound enrichments, the myofibrillar fraction (pellet) was washed with distilled deionized water and then purified from collagen in sodium hydroxide (NaOH) (11). The myofibrillar fraction was then hydrolyzed for 72 h in 1M HCl and Dowex (50WX8–200 resin; Sigma-Aldrich, Oakville, ON, CAN) at 110°C and mixed on a vortex every 24 hours. The free amino acids were purified with the use of Dowex ion exchange chromatography. The *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 %/day was calculated with the use of the standard precursor-product equation as described previously (12). Saliva samples were analyzed for ^{2}H 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 (13)

Leg absolute synthetic (ASR) and breakdown (ABR) rates were calculated as previously described (14). Briefly, approximately 15mg of frozen muscle tissue was freeze dried and weighed, then homogenized in 0.2M PCA and spun to form

a pellet. The supernatant was discarded and this process was repeated twice more. 800uL of 0.3M NaOH was then added to the pellet which was dissolved for 30min at 37°C. The supernatant was then used to quantify the alkali soluble protein at A280 on a Nanodrop. Using the rates of integrated myofibrillar protein synthesis, leg lean tissue mass derived from DXA and average alkali soluble protein over the 10 day period, ASR and ABR were calculated using the following equations:

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

Where FSR is the integrated myofibrillar protein synthesis rate in %/d, LFFM (leg FFM) 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 is the average alkali soluble protein concentration.

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

Where FBR=FSR-FGR and FGR is the % change in leg lean mass per day.

Plasma enrichments. Plasma (50 μL) was deproteinized in 500 μL of acetonitrile. The HFB derivative was prepared and the enrichment of *ring*-[$^{13}C_6$]phenylalanine was determined by GC-MS (Hewlett Packard 6890; MSD model 5973 Network, Agilent Technologies, Santa Clara, CA, USA).

Intracellular enrichments. Approximately 25 mg of frozen muscle was prepared as described in Chapter 3. Briefly, the muscle was rinsed in phosphate buffered saline and then homogenized with a Teflon pestle in 500 uL of perchloric acid (PCA) to precipitate the muscle proteins. The sample was then centrifuged at 10,000 *g* at 4°C for 5 minutes, the supernatant was collected and the procedure was repeated twice more. The PCA supernatant was purified with the use of Dowex ion exchange chromatography and the heptafluorobutyl (HFB) derivative was prepared. The enrichment of *ring*-[$^{13}C_6$]phenylalanine was determined by GC-MS (Hewlett Packard 6890; MSD model 5973 Network, Agilent Technologies Santa Clara, CA, USA).

Mixed muscle protein synthesis: The precipitated mixed protein pellet obtained from homogenization in PCA (from intracellular enrichments) was washed in distilled deionized water, washed three times in absolute ethanol, then placed at 50°C to dry. The dried pellet was weighed and then hydrolyzed for 72 hours in 0.1 M HCl and Dowex (50WX8–200 resin; Sigma-Aldrich, Oakville, ON, CAN) at 110°C and mixed on a vortex every 24 hours. The free amino acids were purified with the use of 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-[$^{13}C_6$]-phenylalanine as described previously (16). Mixed muscle protein synthesis in %/hour was calculated using the standard precursor-product equation as previously described (15).

Statistical analyses: Statistical analyses were performed using SPSS version 19 (IBM, Armonk, NY, USA). A mixed model analysis of variance (ANOVA) was conducted on integrated myo-PS, mixed protein turnover, absolute protein turnover, plasma enrichment (group x time). Linear regression was performed on plasma and intracellular free phenylalanine enrichment. Diet analysis, baseline characteristics, and unilateral resistance exercise were analysed by independent sample 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.

RESULTS

Integrated Myofibrillar Protein Synthesis. Integrated myo-PS (%/d) values are shown in **Figure 2**. Integrated myo-PS is in **Figure 2A** and showed a time-by-group interaction ($p=0.03$). Integrated myo-PS in the exercised leg was preserved compared to energy balance (EB) in the HP group ($p=0.21$) but only partially preserved in the LP group following ER ($p=0.03$ *vs* EB and $p<0.001$ *vs* REST). The change in integrated myo-PS (**Figure 2B**) was significantly greater in the rested leg than the exercised leg in both the higher protein ($p<0.001$) and lower protein ($p<0.001$) groups with no difference between protein groups ($p=0.44$). Body water enrichment (**Figure 3**) showed a linear decline following dosing (a

linear regression equation explained a significant proportion of the variance; r^2=0.99).

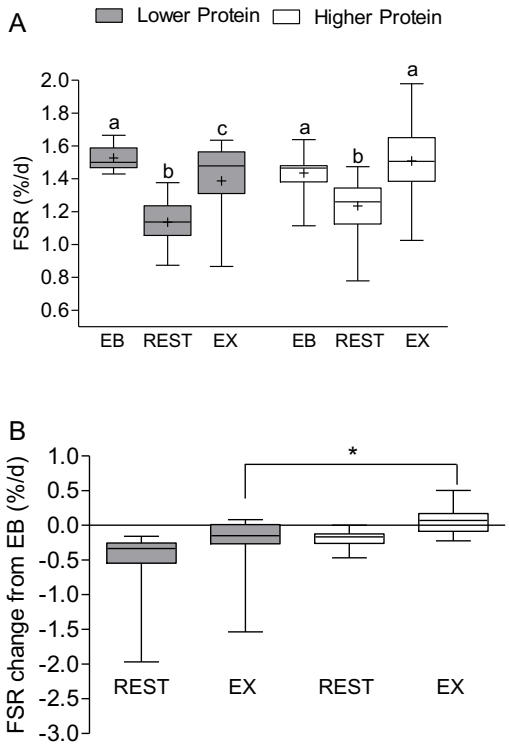

Figure 2. (A) Integrated myofibrillar protein synthesis (myo-PS). Letters represent within-group statistics, and means without a common letter differ. (B) Change in myo-PS from energy balance. * Significantly different from REST leg. EB: energy balance, REST: ER rested leg, EX: ER exercised leg

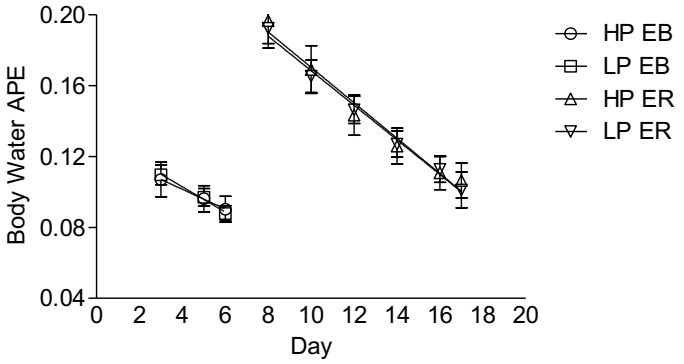

Figure 3. Body water (saliva) atom percent excess (APE). HP EB: higher protein, energy balance, LP EB: lower protein, energy balance, HP ER: higher protein energy restriction, LP ER: lower protein energy restriction.

Absolute Synthesis and Breakdown. Calculation of absolute synthesis and breakdown (g/d) is shown in **Figure 4**. The fractional growth rate (FGR) of leg lean tissue (%/d) showed a main effect of time ($p<0.01$) but no interaction ($p=0.34$; **Figure 4A**). The FGR was higher in the higher protein exercised leg compared to the corresponding rested leg ($p=0.01$; **Figure 4A**). The absolute synthesis rate (g/d, ASR) showed a main effect of time ($p<0.001$; **Figure 4B**), but no interaction ($p=0.87$). The ASR was significantly lower in the rested legs compared to the exercised legs of both groups ($p=0.001$; **Figure 4B**). The absolute breakdown rate (ABR; **Figure 4C**) was not different between groups or legs (legs $p=0.39$, group × legs $p=0.53$). Net protein Balance (g/d, ASR-ABR; **Figure 4D**) had 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 to the corresponding rested leg ($p=0.01$).

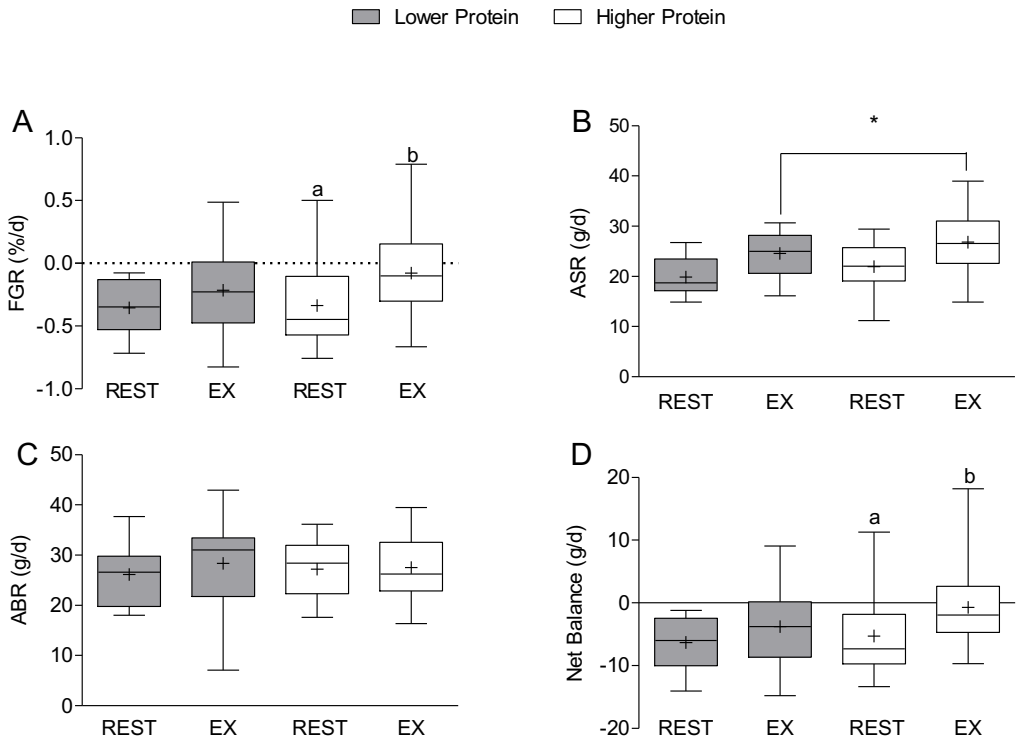

Figure 4. (A) Fractional growth rate (FGR), (B) absolute synthesis rate (ASR), (C) absolute breakdown rate (ABR) and (D) absolute net protein balance. Letters represent within-group statistics and means without a common letter differ significantly (P<0.05). *significantly different from REST. REST: ER rested leg, EX: ER exercised leg (P<0.05).

Mixed muscle protein turnover. Postabsorptive mixed muscle protein synthesis (%/hr), and net protein balance change from EB (%/hr) are shown in **Figure 5** (panels A and B, respectively). There was a main effect of time (p<0.001) but no interaction (p=0.07; **Figure 5A**). Mixed MPS was significantly reduced from EB in the rested leg in both groups following ER (HP p=0.01, LP p<0.001; **Figure 5A**). 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 following the last exercise bout (p=0.01). The exercised leg had a significantly smaller change in net protein balance from EB than the rested leg in both groups (p<0.001; **Figure 5B**), and there was a strong trend for a between-group difference in the exercised legs (p=0.054). Plasma and intracellular enrichments are shown in **Figure 6**. There were no differences between treatments or across time for the *ring*-[$^{13}C_6$]phenylalanine plasma enrichment. The slope of plasma free *ring*-[$^{13}C_6$]phenylalanine enrichment by time was not different from zero for either group.

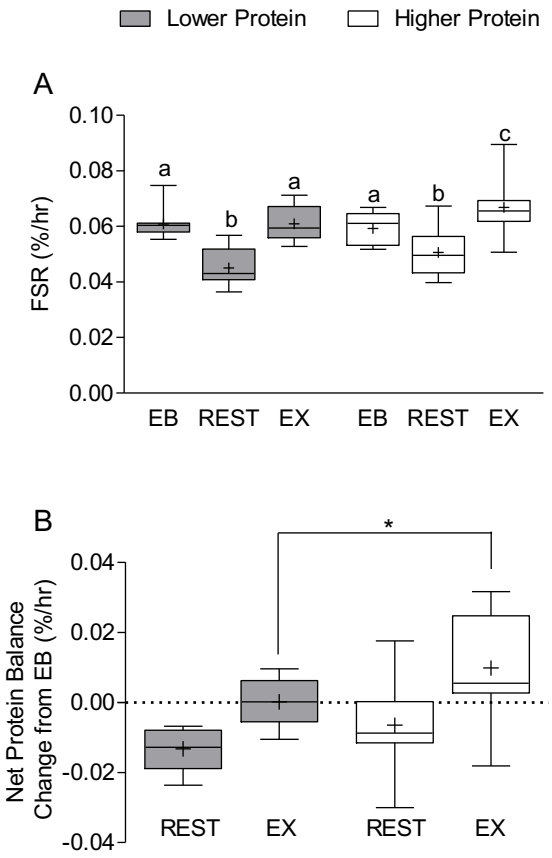

Figure 5. (A) Mixed muscle protein synthesis, (FSR, %/hr), (B) net protein balance change from energy balance, (EB, %/hr). Letters represent within-group

statistics and means without a common letter differ within each group. EB: energy balance, REST: energy restriction rested leg, EX: energy restriction exercised leg

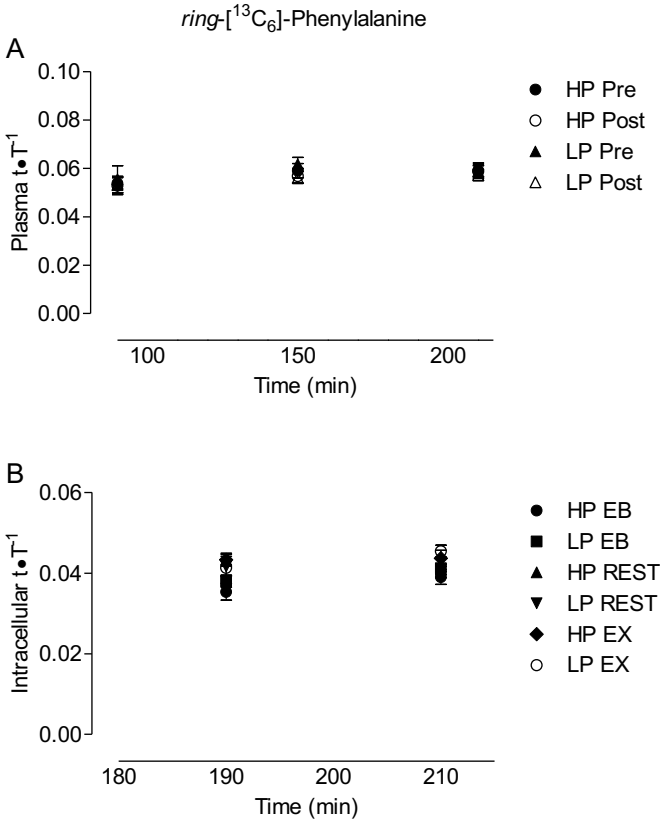

Figure 6. (A) Plasma enrichments of *ring*-[$^{13}C_6$]phenylalanine. (B) Intracellular enrichments of *ring*-[$^{13}C_6$]phenylalanine. HP: higher protein group, LP: lower protein group, Pre: pre energy restriction, Post: post energy restriction, REST: energy restriction exercised leg, EX: energy restriction exercised leg.

DISCUSSION

We observed that ER resulted in a significant reduction in integrated (daily) myo-PS, which was alleviated by resistance exercise. Additionally, ER resulted in a significant reduction in acute (hourly) postabsorptive mixed MPS, which was also mitigated by resistance exercise that was performed 48h prior. Consistent with the

changes in MPS, in Chapter 3 we reported a significantly greater reduction in LBM in the rested compared to the exercised legs, regardless of protein intake, following ER in both groups. Importantly, ABR was unchanged throughout the diet and exercise intervention, confirming the results of MPB obtained in Chapter 3. These results demonstrate that the main early-stage driver of LBM loss during a marked energy deficit in young overweight men was a significant decline in MPS with little-to-no change in MPB.

The 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 rates of acute (hourly) 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 while participants consumed 1.5g/kg/d protein. Murphy et al. (16), reported a significant reduction in integrated myo-PS in overweight older men following 2 weeks of ER, which was increased following a second two week 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 is in general agreement with data in older men (16). Additionally, we demonstrate that even in ER, resistance exercise has the capacity to stimulate postabsorptive mixed MPS even up to 48h following an exercise bout, consistent with work from situations of energy balance (17) and further demonstrating the potency of resistance exercise to stimulate MPS (6) and provide a fundamentally anabolic signal to skeletal muscle. An important consideration is

that individuals spend most of their waking hours in the postprandial state, assuming they do not skip meals. The use of ingested D_2O allowed an integrated measure encompassing postprandial, postabsorptive, and fasted (sleeping) periods. In the current study, the average decline of integrated myo-PS in the rested legs was -0.36 ±0.08%/d, which was close to the average change in leg LBM per day of -0.34±0.06%/d. Thus, even if we assume only 10 hours per day 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 could in any way be a significant mechanism underpinning loss of lean mass during ER in overweight young men.

By simultaneously assessing rates of MPS and MPB, we were able to calculate 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 (Figure 5A). The reduction in the net protein balance, versus energy balance, calculated using the rates of acute mixed MPB from Chapter 3 and the rates of acute mixed muscle MPS in the current study, was significantly greater in the rested leg than the exercised leg in both groups, and it is interesting to note the strong trend for a differential effect between the exercised legs, tending to be elevated from EB in the higher protein group ($p=0.054$). To further support our acute protein turnover data, we calculated absolute (g/d) myofibrillar protein synthesis, 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 to the exercised leg. Absolute net protein balance was elevated in the exercised leg compared to the rested leg of the higher protein group, which is again in agreement with the fundamentally anabolic nature of resistance exercise. In order to parallel the study design from Longland et al., we opted to keep carbohydrate intake constant between the higher and lower protein groups due to the known effects carbohydrate has on exercise performance and to ensure similar insulinemia (18). Thus, we cannot attribute the effects on muscle protein turnover we report solely to protein intake because there were also differences in fat intakes between these two groups. However, there is not, to our knowledge, any data to suggest that differences in daily dietary fat intakes that are not exceeding the acceptable macronutrient distribution range would affect changes in muscle protein turnover. Hammond et al. (19), reported that high fat feeding (3.5g/kg) post-exercise suppressed p70S6K1 activity; however, the protein supplement provided to all participants post-exercise in the current study contained a maximum of 5g of fat (from 2% milk), thus we would not expect the same effect to occur (19).

A limitation of the current study is due to the timing of biopsies used to examine gene and protein expression, which were taken only 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 associated markers would be in the fasted state (see Chapter 3) (20). Nonetheless, the one target related to MPS that we did analyze [p-TSC2 (Thr1462)] did not change pre-to post-ER. Due to the

numerous muscle biopsies already being performed on the 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 signalling related to MPS if an effect were present. However, the measurement of integrated MPS would include 14-16hrs per day in the postprandial state, and in this study we did detect a reduction in integrated MPS. Thus, it is likely that signalling pathways related to MPS would also be reduced. We acknowledge that there is data showing a reduction in phosphorylation of Akt and 4EBP-1 following 10 days of ER (5), as well as a reduction in the MPS response to protein ingestion during ER (4, 7). Data from rodents suggests that ER results in a downregulation of the phosphorylation of protein synthesis related proteins (Akt, mTOR, rps6 and p70S6K) (21) and the reduction in active ribosomes (22). Thus, strategies that target the mTORC1 pathway such as resistance exercise and increased protein intake (3) are important for the preservation of MPS and LBM during ER.

In summary, we show that in young overweight men, ER results in a decrease in MPS, but resistance exercise with protein intakes three times higher than the RDA can mitigate this decline. Furthermore, we demonstrate that MPB does not change despite a marked ER in overweight young men. Finally, we provide a comprehensive assessment of the processes governing changes in skeletal muscle mass during ER in overweight young men. Our findings may be of importance for the design of weight loss programs that wish to preserve skeletal muscle and promote loss of fat mass.

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CHAPTER 5:

GENERAL DISCUSSION

5.1 Introduction

In overweight or obese persons, weight loss is associated with improved risk for a number of chronic conditions. Nonetheless, weight loss, ignoring the contribution of body water losses, consists of varying proportions of fat mass and lean body mass (LBM) loss, with a meta-analysis putting the ratio of lean to fat mass loss at ~3:1 (1). High quality weight loss, defined here, as weight loss with the highest possible ratio of fat to LBM loss is, for a variety of reasons, a goal for many individuals. Consumption of higher protein-containing diets rich in dairy protein sources during dietary energy restriction, particularly when combined with resistance exercise, are effective in promoting high quality weight loss (2-4). To date, the mechanisms underlying the beneficial effects of protein intake and resistance exercise on LBM retention and fat mass loss during dietary energy restriction were not well understood.

The aim of the present thesis was to further elucidate the impact of dietary protein intake and resistance exercise during dietary energy restriction on skeletal muscle protein synthesis (MPS) and skeletal muscle protein breakdown (MPB) as mechanisms underpinning muscle mass. We also examined, in one study, lipolysis as a mechanism of fat mass control. To the best of our knowledge, this is the first time that both MPS and MPB have been simultaneously reported as a measure of muscle protein turnover during dietary energy restriction. Additionally, the use of deuterated water as an oral isotope tracer for protein metabolism provides an integrated assessment of MPS throughout all activities of daily living. We used

this method in experiments reported in this thesis to calculate the loss of LBM during energy restriction in grams per day, providing a clinically relevant quantification of LBM changes and mechanistic insight.

In Study 1 (Chapter 2) we demonstrated that whey protein ingestion stimulated myofibrillar protein synthesis to a greater extent than soy protein ingestion both before, and after 14 days of dietary energy restriction. Importantly, the decline in postprandial myofibrillar protein synthesis following energy restriction was less pronounced with whey protein supplementation than soy protein. Additionally, we demonstrated a reduction in lipolysis with feeding, that was significantly greater following ingestion of a carbohydrate supplement compared to protein, but there were no significant differences between the whey and soy protein groups or pre-and post-weight loss. In Study 2 (Chapter 3) we demonstrated that MPB was not changed following a marked (40%) dietary energy deficit, which gained support given the absence of changes in gene and protein expression of the ubiquitin proteasome and autophagic-lysosomal pathways. In Study 3 (Chapter 4) we demonstrated that higher protein intakes combined with resistance exercise preserved postabsorptive mixed MPS, integrated myofibrillar protein synthesis, and muscle protein turnover during energy restriction. Taken together, these findings demonstrate that higher protein intake when combined with resistance exercise were important strategies for the preservation of MPS during diet-induced weight loss. This chapter discusses the

findings of this thesis in more detail, specifically in relation to previous work completed in this field, and highlights some areas of future directions.

5.2 Influence of protein quality on acute measures of myofibrillar protein synthesis and lipolysis

Consumption of an energy-restricted diet higher in dairy protein has been shown to promote the retention of muscle and loss of fat mass (3, 5, 6). One constituent of dairy known to be a potent stimulator of MPS is whey protein. Whey has a higher content of the amino acid leucine than almost all commercially available isolated sources of protein (7). Additionally, leucine has the potential to inhibit adipocyte lipogenesis and stimulate lipolysis (8, 9) and thus may play a synergistic role in promoting both fat mass loss and LBM retention. However, whether whey protein is superior to other protein sources such as soy protein, which has the same PDCAAS protein quality score as whey, for LBM retention and fat mass loss during energy restriction has not been examined. In Study 1, we compared the effect of whey and soy protein supplementation and a carbohydrate control on MPS and lipolysis as two potential mechanisms to preserve skeletal muscle and promote fat mass loss, respectively. We observed that whey protein supplementation was superior to soy in stimulating postprandial MPS, and whey protein also alleviated the diet-induced decline in postprandial MPS that was observed with soy and carbohydrate supplementation. Additionally, we observed a decline in lipolysis with consumption of each supplement, with the greatest

decline following consumption of carbohydrate, but there were no differences pre- and post-weight loss, or between the whey and soy protein groups.

The superior stimulation of MPS following consumption of whey compared to soy protein is consistent with studies in energy balance (7) and the leucine content of whey compared to soy protein (10). Indeed, we observed that consumption of whey protein resulted in a greater leucinemia both pre-and post-weight loss compared to soy protein. Additionally, the blood aminoacidemia for the same amount of total protein ingestion was higher in whey compared to soy, providing more amino acids as building blocks for supporting MPS (11). The amino acid profiles observed in Study 1 are consistent with previous research investigating the delivery of amino acids to peripheral tissues by differing protein sources. Such studies have demonstrated that the essential amino acids (higher in whey than soy) are less readily taken up by the splanchnic region than the non-essential amino acids (12). Finally, the preservation of postprandial MPS following energy restriction with whey protein ingestion compared to soy protein may be of importance in the preservation of LBM (13).

Greater preservation of LBM during energy restriction is commonly associated with greater loss of fat mass (2, 14, 15). For example, Josse et al. reported a significantly greater LBM retention and fat mass loss in a higher protein (as dairy) group (28% protein, 41% carbohydrate and 31% fat) compared to an adequate protein group (18% protein, 58% carbohydrate and 24% fat) between 8-16 weeks of a weight loss (-500kcal/d) and exercise diet (3). Another

study reported that when whey protein is provided as a supplement during a weight loss diet, there was a greater loss of fat mass and retention of lean mass in the whey protein supplemented group (consuming 0.8g protein/kg/d total) compared to the control group (consuming 0.6g protein/kg/d total) (16). Although we observed better stimulation of MPS with whey protein in Study 1, which could explain the superior retention of LBM observed in the aforementioned studies, we did not find an effect of whey protein supplementation compared to soy protein on whole-body lipolysis, despite the potential for superior decreases in fat mass (3, 16). Since insulin is known to inhibit lipolysis (17) the lack of a difference in lipolysis between whey and soy protein is in line with the similar insulin responses that were observed between whey and soy protein ingestion. However, it is possible that we were not able to detect potential differences between whey and soy protein supplementation with the whole-body measurement of lipolysis. Differences between whey and soy protein supplementation may have been more evident in gene and/or protein expression relating to adipogenesis and/or lipolysis in the adipose tissue (9); this could be an area of future research. It should be noted that LBM accounts for a significant proportion of daily energy expenditure (18), thus it is also possible that the maintenance of LBM could promote greater fat mass loss or resist fat mass gain over time (19). However, whether the acute changes in MPS in Study 1 would translate into better preservation of LBM (and fat mass loss) over time with whey protein compared to soy protein

supplementation during energy restriction requires further research with longer-term weight loss interventions (13).

5.3 Influence of energy restriction on muscle protein breakdown

In comparison to MPS, MPB is relatively understudied in humans. One explanation for the limited data on MPB is that fluctuations in MPS in response to feeding and exercise are 3-4 times larger than fluctuations in MPB in healthy humans. Thus, changes in MPB are arguably less important than changes in MPS for the control of muscle mass in healthy humans (20). Nonetheless, acute rates of MPB have been measured during a 10 day 20% energy restricted diet in healthy normal weight (BMI 23.9±0.7) young men, with reported increases in MPB of 60% (21). These authors reported a surprising increase of 60% in the rates of MPB during dietary energy restriction (21), which is similar to a 65% increase in leg protein breakdown observed following the acute infusion of catabolic hormones (epinephrine, cortisol and glucagon) (22). Thus, given this is a single study (21) and the increase in protein breakdown is larger than one would expect, there is a need to further examine MPB during dietary energy restriction. In Study 2, we investigated the effect of 10 days of an energy deficit of 40% on rates of MPB and markers of proteolysis in overweight young men. We also utilized unilateral resistance exercise in two parallel groups consuming 1.2g protein/kg/d or 2.4g protein/kg/d to simultaneously investigate the impact of exercise and protein dose. The main finding from this study was that there were no changes in

MPB or markers of proteolysis with energy restriction in either group in the rested limb or the limb that had undertaken resistance exercise. An important question is why the findings in Study 2 are so different than the 60% increase in MPB reported by Carbone et al. (21). One important difference in study design relates to the participant characteristics. Specifically, in Study 2 we recruited young overweight men with an average BMI of 29±1 kg/m^2, fat mass of 26±3 kg, and % fat of 29±1 % and Carbone et al. recruited young healthy highly physically active men with a BMI of 24±1 kg/m^2, fat mass of 14±2 kg, and approximately 20% fat. These body composition differences may be important when we consider findings from Heymsfield et al. (23), who reported that the men in the lowest quintile of baseline % fat lost more LBM than the men in the other quintiles of % fat (23). However, whether the LBM loss in leaner individuals is due to such a large increase in MPB as observed by Carbone et al. (21) requires further investigation. Such a conclusion is emphasized when one considers previously reported declines in MPS in the same participants (24) which would contribute to substantial losses of LBM.

Previous studies attempting to characterize muscle proteolysis during diet-induced weight loss have relied only on single point-in-time measures of gene and protein expression of proteolytic markers, and this has resulted in ambiguous results regarding the potential changes in proteolysis following weight loss (21, 25, 26). Interestingly, despite a 60% increase in rates of MPB, there was only a modest 11% increase in caspase-3 activation measured by western blot, and no

change in genes related to the ubiquitin-proteasome pathway or changes in 26S proteasome activity (21). We also observed no change protein and gene markers for the ubiquitin proteasome pathway or the autophagic-lysosome pathway, findings that are consistent with findings from Smiles et al. (26). In known states of overt catabolic muscle loss, such as sepsis or cachexia, protein degradation pathways have been shown to be upregulated (27). When plasma from patients with sepsis was added to skeletal muscle myotubes, there was a significant increase in MuRF-1, atrogin-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activity and ubiquitinated myosin in the myotubes. Importantly, the plasma from the septic shock patients had increased levels, 10-50 fold, of the cytokine IL-6 versus controls (28). Thus, circulating catabolic trigger(s) were present that would trigger the upregulation of proteolytic pathways. In addition to IL-6, other known triggers of muscle atrophy include cortisol and cytokines such as TNFα (29) and importantly, cytokine levels often decrease after weight loss (30).

In starvation-induced muscle atrophy in mice, a potential intramuscular trigger for proteolysis is the tumor necrosis factor receptor-associated 6 (TRAF6), an E3 ubiquitin ligase that functions as a central regulator of multiple pathways including PI3K/Akt, NFκB, and the Endoplasmic Reticulum stress response (31). For example, targeted ablation of TRAF6 during 48hrs of starvation in mice suppressed the expression of many atrophy related genes and proteins including MuRF-1, atrogin-1, Beclin-1, LC3B, and improved the phosphorylation of Akt

and Foxo3a (31). In the studies undertaken in this thesis we did not detect changes in the expression of TRAF6 during a marked energy restriction, which casts some doubt as to whether this pathway is important for muscle atrophy during energy restriction in humans. Importantly, energy restriction is arguably a less extreme scenario than fasting, since calories and nutrients to support metabolic functions are still being provided during energy restriction. For example, although the participants in Study 2 were placed in a marked 40% energy restricted diet (from measured resting metabolic rate and activity factor), the reduced dietary energy intake was still roughly equivalent to the subjects' measured resting energy expenditure (REE) requirements by indirect calorimetry: average REE was 2136±47kcal/d and average caloric intake during 40% energy restriction was 2179±58kcal/d. Therefore, the participants were still consuming enough calories to maintain basic body functions. Thus, although a 40% reduction in energy intake is still a severe reduction in energy, it would be less of a stress than a starvation situation, resulting in no observed change in protein breakdown pathways (such as changes in TRAF6 (31)). Finally, in contrast to starvation in which no food is provided, caloric restriction would still result in moderate feeding-induced rises in insulin, which is known to inhibit proteolysis (32). In this regard, the fed and fasted-state fluctuations in insulin signalling during energy restriction could arguably have been similar to those seen in energy balance, contributing to the lack of observed differences in the mechanisms for proteolysis.

5.4 Influence of protein quantity and resistance exercise on integrated myofibrillar protein synthesis and skeletal muscle protein turnover

The recommended dietary allowance (RDA) for protein intake is currently set at 0.8g/kg/d for adult men and women (33). However, there is evidence that during energy restriction, individuals require protein intakes higher than the RDA to reduce LBM loss (14, 15). Additionally, a higher protein intake (1.2g/kg/d) in combination with resistance exercise during a marked 40% energy deficit has been shown to result in a preservation of LBM while consumption of 2.4g protein/kg/d resulted in a gain in LBM (2). Currently, the mechanisms driving the effects of protein and resistance exercise on LBM preservation during energy restriction are unknown.

In Study 3 we examined the effect of protein quantity (1.2g/kg/d and 2.4g/kg/d) alone and in combination with resistance exercise on integrated myofibrillar protein synthesis (myo-PS) and acute rates of postabsorptive mixed MPS. We observed that following energy restriction, myo-PS and postabsorptive mixed MPS were reduced in both groups regardless of protein intake (1.2 and 2.4g protein/kg/d), but resistance exercise mitigated these declines in MPS at both protein levels. Interestingly, the average percentage decline in integrated myo-PS (-0.36 ±0.08%/d) aligns with the percentage decline in leg LBM (-0.34±0.06%/d), supporting the results from Study 2 in which no change in MPB was detected. Although we did not observe a significant difference between groups (i.e. effect of protein quantity on MPS), we did observe a difference in the within-group

trends, suggestive of an effect of protein quantity. For example, the rate of integrated myo-PS in the resistance exercised leg of the higher protein group (2.4g protein/kg/d) was not different from energy balance (EB), but the rate of integrated myo-PS in the resistance exercised leg of the lower protein group (1.2g protein/kg/d) was below EB. Moreover, the rate of mixed MPS in the resistance-exercised leg of the higher protein group was elevated above EB levels but the rate of mixed MPS in lower protein group was the same as EB. Taken together, these results demonstrate the anabolic potency of resistance exercise during energy restriction.

Given the previously reported differences in body composition changes between consumption of 1.2g protein/kg/d and 2.4g protein/kg/d during 40% energy restriction in young men (2), it is important to address the lack of a statistically significant difference between the groups in Study 3. Firstly, the measurements of acute mixed MPS were taken in the basal fasted state. Gorissen et al. (34) investigated the impact of 2 weeks of habituation to low protein intake (0.7g/kg/d) and higher protein intake (1.5g/kg/d) on basal MPS, and found no differences between groups following the habituation period. Similarly, after 12 weeks of habituation to a very low protein intake (0.4g/kg/d) or high protein intake (2.4g/kg/d) in young men and women, there was no difference between the basal postabsorptive MPS rates (35). Thus, our data confirm these findings that habitual dietary protein intake does not affect basal postabsorptive MPS at rest. Likewise, we did not observe a group interaction from our measurements of

integrated myo-PS, which would have included the basal rates of MPS with all postprandial periods throughout the day. Murphy et al. (36) examined the effect of feeding patterns (balanced protein distribution throughout the day compared to skewed) on integrated myo-PS and did not find a between-group difference, despite observing between-group differences in MPS during an acute stable isotope infusion (37). They hypothesized that the overnight fasted period may have diluted the postprandial effects on integrated myo-PS (37). Thus, considering we did not find changes in postabsorptive acute mixed MPS, it could be hypothesized that these postabsorptive periods diluted any protein quantity effects on integrated myo-PS.

Due to ethical limitations on the number of muscle biopsies being performed in the participants of studies 2 and 3 (9 per participant), we did not assess postprandial MPS. However, Pasiakos et al. (38), compared the MPS responses to a protein drink following 21 days of energy restriction with participants consuming either protein at the RDA, twice (2x) the RDA or three-times (3x) the RDA of protein during energy restriction, and reported that compared to energy balance only the 2x and 3x RDA groups preserved the anabolic response to the protein meal. The authors also reported that reductions in LBM were lower and loss of fat mass was higher in the 2x RDA and 3x RDA groups compared to the RDA group (38). This study by Pasiakos et al. provides mechanistic support for the effect of protein quantity on LBM observed by Longland et al.(2), whereby a greater preservation of postprandial MPS with higher protein intakes (38) (or

higher quality protein as we observed in Chapter 1) supports LBM retention (2). Therefore, the between-group differences in Study 3 may have been more apparent had we measured postprandial MPS. An additional consideration is that the changes observed in Study 3 for integrated myo-PS and basal mixed MPS were small such that a between-group difference would be difficult to detect with the parallel group design and the relatively small participant numbers in each group.

To the best of our knowledge, the calculation of net muscle protein balance has not yet been undertaken during energy restriction. In Study 3, muscle protein turnover was calculated two ways: from changes in integrated MPS and leg lean mass (grams/d); and, in the postabsorptive state (%/d). Both methods for calculating muscle net protein balance yielded similar findings and were mainly driven by changes in MPS. Specifically, there was a significant decrease in muscle protein turnover in the rested leg of both protein groups following energy restriction and there was a preservation of muscle protein turnover (net balance close to zero) with resistance exercise. In the postabsorptive state, there was a trend ($p=0.054$) for a between-group effect whereby the change in net balance from energy balance tended to be higher in the exercised leg of the higher protein group than the exercised leg of the lower protein group. In energy balance, Phillips et al. measured net balance in the postabsorptive state 48hrs following the last exercise bout and reported elevated levels of net balance (compared to the rested state). Thus, our data suggest a potential role of the higher protein diet in

preserving responses to resistance exercise seen in energy balance (39), and this could partly explain the gains in LBM observed by Longland et al. in a group consuming 2.4g protein/kg/d during a 40% energy restricted diet with resistance exercise training (2). We also calculated integrated rates of net balance in absolute (g/d) terms using the estimated changes in leg lean mass from DXA and the changes in integrated myo-PS. This data is in line with the postabsorptive muscle protein turnover trends, and provides more clinically-relevant information to quantify the amount of muscle mass change per day.

A common concern is related to the safety of elevated protein intakes. The acceptable macronutrient distribution range (AMDR) for protein is 10-35% (33), however in Study 3 the higher protein group consumed 42±2% of calories of protein, outside the AMDR. Nonetheless, due to the extensive energy deficit the participants were in, the percentage of total calories becomes much greater for the same amount of protein. Additionally, Antonio et al. (40) reported that after 1 year on a diet containing 2.5-3.3 g/kg/d protein, young resistance trained men displayed no adverse effects on blood lipids, or liver and kidney function.

5.5 Conclusions and Future Directions

Obesity is a prevalent disease and the comorbidities associated with the disease place a large burden on our healthcare system. Thus, the development of lifestyle interventions that promote fat mass loss and lean mass retention are important in order to promote the best physical and metabolic health outcomes.

This thesis examined the impact of protein quality, quantity and resistance exercise during dietary energy restriction on the mechanisms that control skeletal muscle mass: muscle protein synthesis and muscle protein breakdown. The studies within this thesis highlight the importance of performing resistance exercise to preserve muscle protein synthesis and suggest an advantage to the consumption of higher protein-containing diets including higher quality dietary protein such as whey in the preservation of MPS. We demonstrate that reductions in MPS are the main driver of muscle mass loss during energy restriction. Figure 1 schematically summarizes the potential molecular mechanisms controlling skeletal muscle mass during energy restriction that have been highlighted in this thesis. Additionally, we attempted to examine the effect of protein quality on whole-body lipolysis as a mechanism of fat mass loss. Although we detected a significantly greater reduction of lipolysis following consumption of carbohydrate compared to protein, we did not observe a difference between whey and soy protein or pre and post weight loss. Therefore, more work is required to further elucidate the effect of protein quality on fat mass loss during an energy deficit.

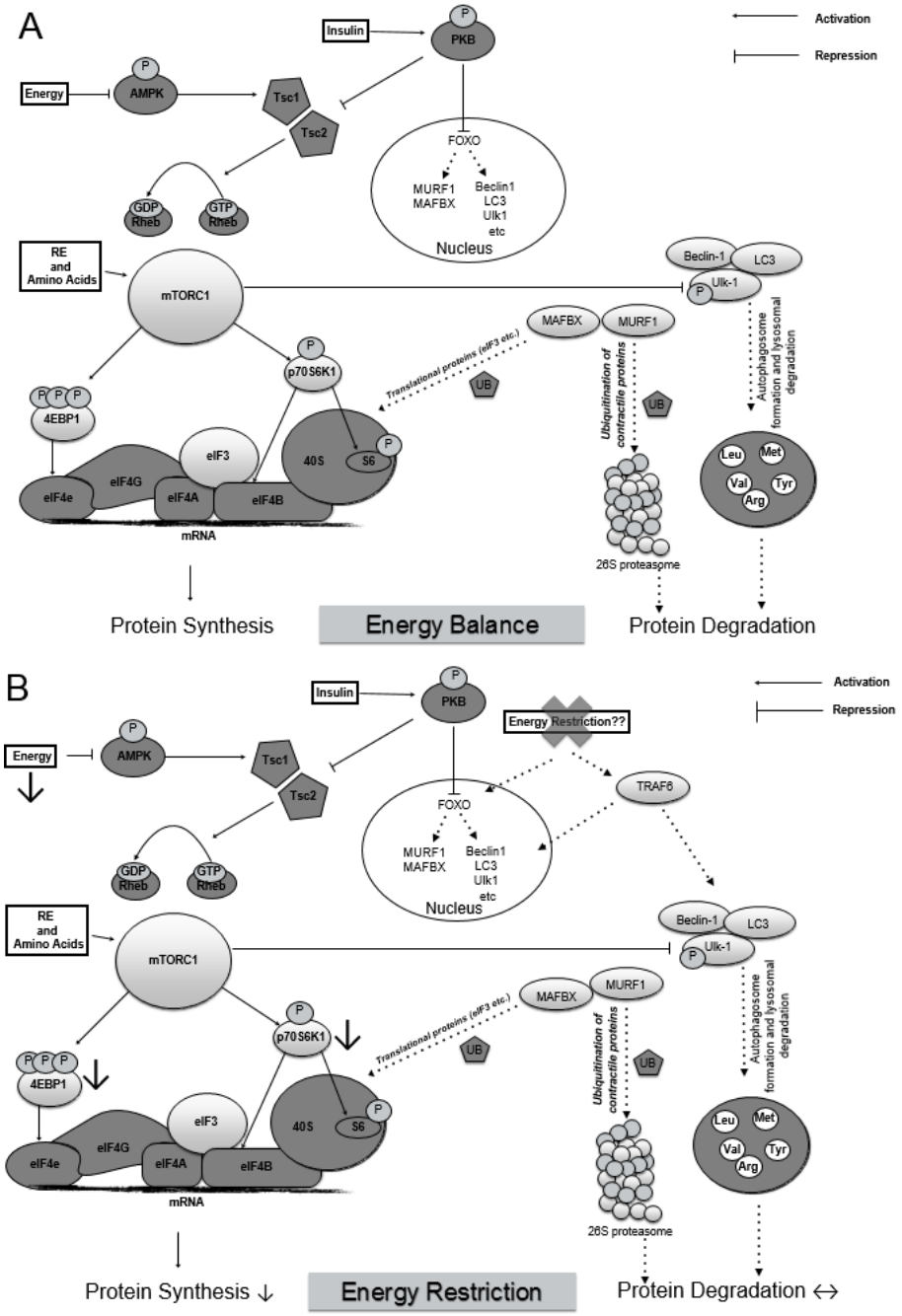

Figure 1. (A) Pathways involved in skeletal muscle protein synthesis and breakdown in energy balance. In energy balance, rates of muscle protein synthesis and breakdown are roughly equal, resulting in a maintenance of skeletal muscle mass. Growth signals such as amino acids, resistance exercise and energy from food promote muscle protein synthesis while insulin reduces protein breakdown (32), resulting in positive net protein balance. For example, the inhibition of

AMPK during high levels of ATP relative to AMP results in reduced activation of TSC2, therefore the GTPase activating activity of TSC2 on Rheb is reduced, promoting the GTP-bound active state of Rheb (41). GTP-bound Rheb promotes the activation of mTORC1, which phosphorylates multiple substrates related to protein synthesis. mTORC1 phosphorylates p70S6K1 on Thr^{389} which then acts on ribosomal protein S6 (rps6) to upregulate translation initiation of ribosomal proteins. Phosphorylation of 4E-BP1 on $Thr^{37/46}$ by mTORC1 reduces its affinity for eukaryotic initiation factor 4E (eIF4E), enabling eIF4E to interact with eukaryotic initiation factor 4G (eIF4G) to form the 43S preinitiation complex (42). In the absence of growth signals (such as in the fasted state at rest), the stimulation of protein synthesis pathways is reduced. Additionally, the inhibition on the protein breakdown pathways is reduced and this results in negative protein balance. For example, reduction in insulin levels in the fasted state results in a reduced activation of Akt (PKB), thus translocation of Foxo3a to the nucleus is permitted, where there is an increased expression of genes involved in the ubiquitin-proteasome and autophagic-lysosome pathways to promote protein breakdown (32, 43, 44).
(B) Pathways of skeletal muscle protein synthesis and breakdown that are affected during energy restriction. During energy restriction, the reductions in energy intake result in a decrease in the activation of the mTORC1 pathway (24, 38). This results in reduced rates of protein synthesis, which are demonstrated in this thesis, and is consistent with previous work in both the fasted and fed states (24, 38). In this thesis, we showed that resistance exercise is a potent stimulator of MPS that can preserve MPS during energy restriction. We also show that protein quality, likely due to higher leucine content of whey protein, is important for the preservation of postprandial MPS during energy restriction. Thus, two interventions that stimulate the mTORC1 pathway (45, 46) are effective at mitigating the diet-induced decline in MPS. Importantly, this thesis highlights the lack of a change in rates of protein degradation during energy restriction. For example, there was no increase in the expression TRAF6, an E3 ubiquitin ligase that has been implicated in the control of the autophagic lysosome and ubiquitin proteasome pathways during starvation-induced atrophy (31). Additionally, there was no change in the phosphorylation of $Foxo3a^{Ser253}$. In summary, this thesis demonstrates that protein breakdown pathways are under similar control as they are in energy balance. Thus, the decrease in protein synthesis due to reduced activation of the mTORC1 pathway during energy restriction causes a negative protein balance that can explain the loss of skeletal muscle. Figures adapted from (47).

In the current thesis we used a variety of stable isotope tracers to assess acute MPS, MPB, lipolysis, and integrated MPS. We also used western blotting and

polymerase chain reaction (PCR) to measure protein and gene expression of targets related to MPB, respectively. A limitation of all studies in this thesis was the ability to detect changes in body composition, which was difficult due to the short-term nature of the studies and the relative insensitivity of DXA in detecting small changes in body composition with a repeated measures design (48). Nonetheless, we were able to use DXA to confirm that all participants did lose LBM and fat mass and we were able to detect an effect of exercise on LBM during weight loss.

In the future, it will be necessary to conduct longer-term interventions to establish the role of whey protein compared to other protein sources on LBM retention and fat mass loss during energy restriction alone or in combination with resistance exercise. Additionally, there is more work required to examine the effect of energy restriction on body composition in different populations attempting to lose weight, such as obese individuals or other persons with clinical conditions.

5.6 References

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