STRATEGIES TO MAXIMIZE SKELETAL MUSCLE PROTEIN SYNTHESIS

IN OLDER ADULTS

NUTRITION AND EXERCISE STRATEGIES TO MAXIMIZE SKELETAL

MUSCLE PROTEIN SYNTHESIS

IN OLDER ADULTS

By

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A Thesis

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ABSTRACT

There is a saturable, dose-response relationship between the amount of protein ingested at a meal, the ensuing hyperaminoacidemia, and the subsequent skeletal muscle protein synthesis (MPS) response. Imposition of an external load, usually practiced as resistance exercise, on skeletal muscle is also a potent stimulus for increasing MPS and adds synergistically to the hyperaminoacidemia-induced rise in MPS. The current thesis examined the potential for meal-focused protein/leucine intake strategies, alone and in combination with resistance exercise, to augment MPS in older men. MPS was measured acutely (hours) using the continuous infusion of L-[ring- ${}^{13}C_6$] phenylalanine (Study 1) or over longerterm, integrated periods via ingestion of deuterated water (Study 2: 2-wk and Study 3: 3-d) while participants were free-living. In Studies 1 and 2 we examined whether a balanced versus a skewed pattern of protein intake across daily meals would enhance MPS during energy restriction (ER) in overweight/obese older men. Study 1 showed that a balanced consumption of protein during ER stimulated acute (%/h) myofibrillar protein synthesis (MyoPS) more effectively than a traditional, skewed distribution. Combining resistance training (RT) with a balanced protein intake pattern restored the lower acute rates of MyoPS during ER to the higher levels observed in energy balance. Study 2 showed no effect of daily protein intake pattern during ER on longer-term integrated MyoPS (%/d). However, the inclusion of RT during ER enhanced integrated MyoPS and the synthesis of numerous individual contractile, sarcoplasmic and mitochondrial

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skeletal muscle proteins with both protein intake patterns. Study 3 showed that leucine co-ingestion with daily meals enhanced integrated (%/d) MyoPS in healthy older men who were in energy balance and was equally effective among those consuming higher (1.2 g/kg/d) and lower (0.8 g/kg/d) protein intakes. Furthermore, the stimulatory effect of leucine co-ingestion on integrated MyoPS was further potentiated with the performance of resistance exercise. Collectively, these studies support the potential for per-meal recommendations, optimizing the protein dose consumed on a per-meal basis and leucine co-ingestion with meals, to augment MyoPS in older men, especially when combined with RT. These data have implications for recommendations to optimize MyoPS and possibly muscle mass in aging persons.

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LIST OF ABBREVIATIONS

ADL	Activities of daily living
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
AUC _{neg}	Area under the curve below baseline
AUC _{pos}	Area under the curve above baseline
BAL	Balanced distribution of daily protein intake
BM	Body mass
СНО	Carbohydrate
C _{max}	Concentration maximum
СТ	Computerized tomography
D_2O	Deuterium oxide
DEXA	Dual-energy x-ray absorptiometry
EAA	Essential amino acids
EB	Energy balance
eEF2	Eukaryotic elongation factor 2
eEF2k	Eukaryotic elongation factor 2 kinase
ER	Energy restriction
FFM	Fat-free mass
FSR	Fractional synthetic rate
GTP	Guanosine triphosphate
GβL	G protein β-subunit-like protein
HbA1 _c	Glycated hemoglobin
HDL	High density lipoprotein
HP	Higher protein
hVps34	Human vacuolar protein sorting-34
Ile	Isoleucine
LDL	Low density lipoprotein
LEU	Leucine supplementation
LP	Lower protein
MAP4K3	Mitogen activated protein kinase kinase kinase
mLST8	Mammalian lethal with sec-13
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mTORC1	Mammalian target of rapamycin complex 1
MyoPS	Myofibrillar protein synthesis
NEAA	Nonessential amino acids
NHANES	National Health and Nutrition Examination Survey
NPB	Net protein balance
PAM	Protein associated with Myc
PLAC	Placebo supplementation
PRAS40	Proline-rich Akt substrate-40

Rags	Ras-related GTPase proteins
Raptor	Regulatory associated protein of mTOR
RDA	Recommended daily allowance
REDD1/2	Regulated in development and DNA damage responses
REE	Resting energy expenditure
REST	Resting conditions
REX	Resistance exercise
Rheb	Ras-homologue enriched in brain
rpS6	Ribosomal protein S6
RT	Resistance exercise training
S6K1	p70 ribosomal S6 kinase 1
SKEW	Skewed distribution of daily protein intake
TAA	Total amino acids
TAUC	Total area under the curve
TCTP	Translationally controlled tumor protein
Tg	Triglycerides
T _{max}	Time to maximum concentration
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
Val	Valine
4EBP-1	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 an overall 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 preparation for submission.

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CHAPTER 1:

INTRODUCTION

1.1 Introduction

Skeletal muscle represents the largest organ in the body accounting for ~40% of body mass. While it is increasingly recognized that skeletal muscle has a number of important metabolic functions (i.e. glycemic control, fat oxidation, endocrine function), skeletal muscle is best recognized for its integral role in locomotion (1-3). Skeletal muscle is fundamental for every interaction with our environment and in performing activities of daily living (ADL). This is likely no truer than in the elderly for whom the performance of ADL are critical for independent living. Activities such as rising from a chair, dressing and walking, performing housework and eating are essential for maintaining independence. As such, when skeletal muscle function is compromised by weakness it is often a precursor to institutionalization. Advancing age is a major contributing factor to the loss of muscle mass, strength and function (4). Indeed, approximately 41% of adults aged 65 years or older report limitations performing at least one of their daily tasks (5). Alarmingly, once individuals show difficulties completing their ADL, without intervention, they are more likely to require hospitalization, experience disability, require long-term care, to experience poor health-related quality of life and are at a greater risk of death (6-8).

Population aging is a global phenomenon (9). By 2050, it is projected that the number of people worldwide older than 60 years will more than double from 841 million to over 2 billion (9). Furthermore, the average life expectancy is estimated to reach 83 years in developed regions (9). While the continued increase

in longevity may be viewed as one of society's greatest achievements, it highlights a critical and urgent need to identify strategies to preserve health and independence in older adults during the years of life expectancy gained.

1.1.1 Sarcopenia

Aging is accompanied by a progressive loss of skeletal muscle mass and function, termed sarcopenia (4). Sarcopenia begins in or around the fifth decade of life and proceeds, based on population estimates, at a rate of $\sim 0.8\%$ per year (10) whereas declines in skeletal muscle strength occur more rapidly at a rate of $\sim 2-3\%$ per year (11). Although there is still no global consensus on the definition and diagnostic criteria for sarcopenia, it is increasingly being recognized as encompassing both quantitative (muscle mass) and qualitative (strength and function) declines in skeletal muscle (4, 12). Sarcopenia constitutes a large component of physical frailty (13) and is an independent risk factor for physical disability (14), falls (14), hospitalization (15), post-operative complications (16), reduced quality of life (17) and death (18). The prevalence of sarcopenia reported in the literature varies widely, likely due to the variations between the populations studied and the various approaches used to diagnose/classify sarcopenia (19). Nevertheless, a common finding has been a high prevalence among older adults that increases with advancing age (14, 20-22). Using the definition proposed by the European Working Group on Sarcopenia, a recent systematic review determined that, among adults \geq 50 years, the prevalence of sarcopenia is up to

33% in community-dwelling populations, with a higher prevalence in acute and long-term care settings (19). This prevalence underscores the crucial importance of developing preventative and therapeutic strategies to counteract the negative impact of sarcopenia (19).

The mechanisms that contribute to sarcopenia are complex, overlapping, and interdependent and may include (but are not limited to) neuromuscular deterioration (23), changing endocrine function (24, 25), low grade systemic inflammation (26), alterations in muscle protein turnover (27, 28), reduced physical activity (29-31) and nutritional deficiencies (32, 33). The contributions of physical activity and nutrition to sarcopenia are of exceptional interest as they represent imminently modifiable risk factors of sarcopenia and, as such, are the focus of this thesis.

1.1.2 Sarcopenic obesity

In addition to the progressive loss of muscle mass, aging is accompanied by a concomitant increase in adiposity, particularly in the visceral depot (29, 34). In recent years the prevalence of obesity has increased markedly among older adults (to ~35-60% in developed countries) (35-37) and has further complicated the condition of sarcopenia. In older persons, obesity is associated with reduced quality of life (38, 39) and an increased risk for numerous comorbidities (40-42). Obesity is also associated with elevated risk for age-related functional impairment (43), disability (39) and institutionalization (44). Moreover, when obesity is

superimposed on sarcopenia, termed by some sarcopenic obesity, there is an even greater risk of physical disability and falls than is seen with either condition in isolation (45). For example, in a cross-sectional population-based study, the odds ratio for two or more self-reported physical disabilities was 8.72 for sarcopenic obesity compared with 3.78 for sarcopenia alone and 1.34 for obesity alone in older men after controlling for potential confounders (46). Similarly, in a longitudinal design, Baumgartner et al. (45) observed that non-disabled elderly with sarcopenic obesity were ~2-3 times more likely to develop disability over 7-years of follow-up than either lean sarcopenic or non-sarcopenic obese individuals, regardless of age, sex, level of habitual physical activity or other morbidities.

Studies have shown that weight loss in older persons improves quality of life (47), physical function (48) and the medical complications associated with obesity (49). However, the management of obesity, and in particular sarcopenic obesity, remains controversial in older adults due to concerns that energy restriction-induced weight loss to reduce excess adiposity may simultaneously accelerate the loss of muscle mass and strength and expedite the decline in physical function (50). Indeed, it is well established that dietary energy restriction, while undoubtedly an effective short-term weight loss strategy, results in the loss of both fat and lean mass (including muscle), with the latter typically accounting for ~25% of total weight lost (51). Consequently, identifying strategies that support weight loss with a high fat-to-lean ratio in older adults is paramount.

1.2 Muscle protein turnover

The process of skeletal muscle protein turnover is constant and ongoing. Protein turnover describes the dynamic processes of synthesis (i.e. incorporation of free amino acids into proteins) and breakdown (release of peptide-bound amino acids into the metabolic free pool) of proteins.

1.2.1 Net protein balance

In the postabsorptive state, rates of muscle protein breakdown (MPB) exceed those of muscle protein synthesis (MPS) resulting in negative net protein balance and muscle protein loss (52). Protein ingestion represents a potent anabolic stimulus that transiently increases MPS and, predominantly via the effects of insulin, suppresses MPB resulting in a positive net muscle protein balance and protein accretion (52). The response of MPS is the principal driver of this 'anabolic' shift towards a positive net protein balance with a comparatively small contribution from the reduction in MPB (53-55). In healthy younger persons the consumption of protein-containing meals over the day results in fluctuations between periods of negative (postabsorptive) and positive (postprandial) net muscle protein balance that are approximately equivalent, thereby serving to maintain a stable muscle mass (52).

In addition to protein intake, the muscle contractile force associated with added external loading (i.e. resistance exercise) is a powerful stimulus for MPS (54, 56, 57). However, resistance exercise also results in a concomitant increase

in MPB such that, in the absence of a hyperaminoacidemia, net muscle protein balance remains negative, albeit to a lesser extent than in the resting state (57). When there is a hyperaminoacidemia after resistance exercise (by ingestion or infusion), rates of MPS surpass those of MPB, resulting in a net positive protein balance (53, 58). Importantly, prior resistance exercise interacts in a synergistic manner to enhance the inherent feeding response both immediately after exercise (59, 60) and at 24 h post-exercise (61), and possibly for longer (62) such that a greater proportion of the day is spent in positive net balance (i.e. MPS>MPB). This forms the basis for increased skeletal muscle protein accretion following prolonged periods of resistance exercise training with adequate energy and protein intake. It follows that chronic imbalances between the processes of MPS and MPB that favor negative net balance (i.e. MPS<MPB) are associated with muscle mass loss.

1.2.2 Measurement of muscle protein synthesis using stable isotopes

It is generally accepted that, in non-diseased states, alterations in MPS play a quantitatively more important role than MPB in mediating long-term changes in muscle mass in humans (63). Furthermore, whereas MPB is technically difficult to measure *in vivo*, MPS can be directly and reliably measured using a variety of techniques. The studies conducted as part of this thesis have utilized stable isotope tracer methodologies to obtain dynamic measures of MPS (64). Stable isotopes (i.e., ¹³C, ²H) are non-radioactive atoms of a particular element that are chemically

identical to the most common form of that element (i.e., ${}^{12}C$, ${}^{1}H$) but differ in atomic mass. The fractional synthetic rate (FSR) of mixed muscle proteins and/or specific muscle protein fractions (i.e. myofibrillar, sarcoplasmic etc.) is usually measured using the primed, continuous intravenous infusion of an isotopically labeled amino acid (i.e. L-[ring- ${}^{13}C_{6}$]phenylalanine) coupled with skeletal muscle biopsy sampling (65). The principle is that a known amount of labeled (tracer) amino acid is infused into the body and is incorporated into muscle protein over time. The rate of MPS can be determined by measuring the change in the muscle protein-bound enrichment over a given period of time (using serial skeletal muscle biopsy samples) and the enrichment in the precursor pool (i.e. plasma free amino acids, muscle intracellular free amino acids, or ideally aminoacyl-tRNA) (66).

The continuous labeled amino acid infusion technique provides sensitive measurements of MPS, and under controlled laboratory conditions, is ideal for assessing the acute response (2-24 hours) to specific stimuli such as feeding or exercise (65). Nevertheless, this technique restricts the acquisition of long-term, potentially more relevant, measures of MPS in free-living settings. An approach that allows the subject greater freedom that can be employed to determine MPS involves the oral administration of deuterium oxide ($^{2}H_{2}O$ or D₂O), which can be used to measure an integrated MPS response. This method takes into account postabsorptive, postprandial, and active/inactive periods, over several hours to days to several weeks in a cumulative manner (67-71). The deuterated water

ingestion technique minimizes participant burden by avoiding the need for intravenous infusions and yields comparable MPS rates to those obtained using traditional L-[ring-¹³C₆]-phenylalanine tracers (67). Furthermore, in addition to providing FSR measurements for mixed muscle proteins and specific muscle protein sub-fractions (70), recent methodological advances have permitted the assessment of the FSR of individual skeletal muscle proteins using ²H-labeling combined with tandem mass spectrometry (72). This exciting development could yield unprecedented insights into the molecular mechanisms underpinning muscle-related conditions as well as the responses to preventive and therapeutic interventions (73).

Like the continuous infusion of labeled amino acids, measurement of MPS using ${}^{2}\text{H}_{2}\text{O}$ employs the precursor-product labeling approach (74). In this regard, ${}^{2}\text{H}_{2}\text{O}$ is orally consumed that rapidly equilibrates within the body water pool. In contrast to labeled amino acids that must be pre-labeled, infused and gain entry into the cell via transporters, ${}^{2}\text{H}$ -labeling of carbon-bound hydrogens of amino acids (i.e., alanine) occurs intracellularly during *de novo* amino acids synthesis and intermediary metabolism (75, 76). Subsequently, ${}^{2}\text{H}$ -labeled amino acids can then charge its respective aminoacyl-tRNA and become incorporated into proteins enabling the measurement of protein-bound enrichment (74). Although with deuterated water ingestion all amino acids become ${}^{2}\text{H}$ -labeled (77, 78), alanine in particular undergoes rapid turnover (79) and has 4 potential sites (α - and β -hydrogens) for ${}^{2}\text{H}$ -labeling to occur via transamination (80), allowing for easier

detection. Another advantage given its rapid turnover, is that alanine closely tracks with the enrichment of body water (²H-labeling of alanine is ~3.7 times that of body water) without perturbation (75, 76). Therefore, easily obtained body water samples (i.e. saliva, urine) can be used to determine precursor enrichment for calculation of FSR (70).

Initial studies using the 2 H₂O-labelling approach to measure MPS in humans provided a large, priming dose followed by daily 'top-up' doses to rapidly obtain and then maintain isotopic steady-state similar to the primed, continuous infusion of a labeled amino acid (69, 71). However, more recently, MPS has been accurately measured over periods of 1-13 days after a single (100-150 mL) oral dose (70, 81). The method is made possible by the predictable kinetics and long elimination half-time of body water combined with the high measurement sensitivity of gas chromatography-pyrolysis-isotope ratio mass spectrometry (GCMS-P-IRMS) (70, 77, 81).

1.2.3 Regulation of muscle protein synthesis with feeding

The hyperaminoacidemia that occurs following the ingestion of dietary protein is a potent stimulator of MPS (82). A saturable dose-response relationship exists between the quantity of protein ingested in a bolus/meal and the response of MPS (27, 59, 83). This relationship is altered by a number of factors such as aging (84), physical activity (31, 59) and dietary energy availability (85, 86). The influence of protein feeding on MPS is primarily attributable to the essential amino acids (EAA) (58, 87), whereas nonessential amino acids (NEAA) appear to be ineffective in this regard (87, 88). Since EAA are the primary drivers of MPS, it follows that the quality of a protein source ingested (encompassing the composition of EAA and ileal digestibility) is another determinant of the postprandial MPS response (89, 90). The feeding-mediated changes in MPS are, however, quite transient and peak ~1.5 hours after the consumption of a large bolus of EAA (15 g) or isolated protein (48 g), and return to postabsorptive rates after ~3 h (91-93).

Stimulation of MPS by EAA is mediated, in part, through activation of the mechanistic target of rapamycin complex 1 (mTORC1) (94). The mTORC1 signaling pathway plays a major role in the stimulation MPS; integrating signals from nutrients (i.e. amino acids), hormones (i.e. insulin, IGF-1) and loading (contraction) to regulate the function of several proteins involved in the control of translation initiation, as well as translation elongation factor 2 (95). Upon activation, mTORC1 phosphorylates 4E Binding Protein-1 (4EBP-1) and p70 ribosomal S6 kinase 1 (S6K1) leading to the activation of two parallel signaling pathways that promote the binding of mRNA to the 43S preinitiation complex, a critical step in translation initiation and the subsequent synthesis of new proteins (96). The importance of mTORC1 activation as a regulator of the feeding-induced rise in MPS has been highlighted by work demonstrating that the administration of rapamycin (a potent mTORC1 inhibitor) attenuates the increase in MPS following EAA ingestion in humans (94) (Figure 1).



Figure 1. A simplified schematic representation of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway driven by muscle contraction, insulin, essential amino acids (leucine), and/or energy. AMPK, AMP-activated protein kinase; Akt, protein kinase B; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2; REDD1/2, regulated in development and DNA damage responses; Rheb, Ras-homologue enriched in brain; TCTP, translationally controlled tumor protein; PAM, protein associated with Myc; Raptor, regulatory associated protein of mTOR; GβL, G protein β-subunit-like protein; MAP4K3, mitogen activated protein kinase kinase kinase kinase-3; hVps34, human vacuolar protein sorting-34; S6K1, p70 ribosomal S6 kinase 1; 4E-BP1, 4E binding protein 1; eEF2k, eukaryotic elongation factor 2 kinase; eEF2, eukaryotic elongation factor 2; rpS6, ribosomal protein S6; PRAS40, proline-rich Akt substrate-40. Used with permission from Drummond et al. (2009) *J Appl Physiol*. 106(4): 1374-84.

In skeletal muscle, it has been clearly demonstrated that of all of the EAA leucine is a particularly potent stimulator of mTORC1 and protein synthesis *in*

vitro and *in vivo* (97-99). For example, a systematic analysis of the effect of each EAA on mTORC1 signaling in C2C12 myotubes demonstrated that leucine was the most potent in its ability to increase the phosphorylation of protein targets of the mTORC1 pathway (97). In rodent models, the independent provision of leucine, but neither isoleucine nor valine, stimulated MPS (98, 100) and this effect is mediated, at least partially, via the activation of the mTORC1 (98). As such, it appears that, in addition to its more obvious role as an amino acid substrate, leucine acts as a signaling molecule to induce the MPS response (96). Indeed, recent work has clearly established that relatively small doses of leucine (3.42 g) ingested independently of other amino acids robustly stimulates MPS in young men (99) corroborating results from over 20 years ago showing that administration of a flooding intravenous dose of leucine stimulated MPS in humans (88).

The exact mechanism(s) by which leucine stimulates mTORC1 signaling remains to be clearly established. Activation of mTORC1 requires interaction between its catalytic subunit, mechanistic target of rapamycin (mTOR) and several regulatory proteins including the Ras homolog enriched in brain (Rheb), regulatory associated protein of mTOR (Raptor), mammalian lethal with sec-13 (mLST8), and ras-related GTPase proteins (Rags) (96). Cell culture studies have demonstrated that leucine promotes guanosine triphosphate (GTP) charging of Rag GTPases and serves to translocate mTOR to the lysosomal membrane where it can interact with its co-activator Rheb (101, 102). Recent work has identified

Sestrin2 as a candidate leucine 'sensor' as leucine disrupts the Sestrin2-GATOR2 interaction by binding to Sestrin2 and liberating GATOR2, a complex necessary for the activation of mTORC1 (102). In addition, it is postulated that vesicular trafficking protein (hVps) 34 (a kinase that promotes the movement of membrane vesicles like the lysosome) (103-105) and/or mitogen activated protein kinase kinase kinase (MAP4K) 3 (106) are involved in the regulation of mTOR activation in response to leucine. However, little work has been conducted in human models of feeding and is therefore an area of future research.

The growing body of research showing that leucine has a unique role as a key activator of the translation initiation process (97) and is capable of stimulating MPS even in the absence of other EAA (99) has led to the development of the 'leucine threshold' hypothesis. This hypothesis posits that a minimum intracellular leucine concentration threshold must be surpassed to stimulate MPS in response to feeding. This hypothesis has some support in that a number of studies show that the consumption of intact proteins that are higher in leucine content (i.e., whey) induce a more robust rise in MPS compared to proteins that are lower in leucine content (i.e., soy) or that have a slower appearance rate of leucine (i.e., micellar casein) (89, 90, 107). For example, Pennings et al. (90) reported that the ingestion of 25 g of whey protein resulted in a higher peak plasma leucine concentration and stimulated postprandial MPS more effectively than an isonitrogenous dose of casein or casein hydrolysate in older men.

plasma leucine concentration and the postprandial MPS response supporting the notion that leucine represents a key factor regulating postprandial MPS (90). On this basis, the 'dose response' of MPS to EAA/protein may not be driven by EAA content *per se* but instead by leucine content (provided sufficient EAA are available to serve as precursors for the synthesis of new muscle) (108).

1.2.4 Influence of aging on muscle protein turnover

Sarcopenic muscle loss is in part due to disturbances in skeletal muscle protein turnover, whereby rates of MPB chronically exceed rates of MPS (52). As such, the possible age-related derangements in protein turnover leading to muscle loss are declines in MPS, increases in MPB, or a combination of these. The available data in humans suggest that normal 'healthy aging' is not accompanied by accelerated rates of MPB (109), although there is some evidence that the suppressive effect of insulin on MPB may be diminished at moderate insulin concentrations (~15 μ IU/mL) equivalent to those following a small, low-glycemic index meal (110). In terms of MPS, the majority of studies report no difference in postabsorptive rates between young and older adults (27, 111, 112). However, elderly individuals show an attenuated response of MPS to normally robust anabolic stimuli such as protein/amino acid intake (27, 84, 112) and resistance exercise (28), a phenomenon that has been termed 'anabolic resistance'. The blunted MPS response to protein feeding in older adults specifically occurs with the ingestion of low-to-moderate doses (i.e., 20g or less) that are similar to the

protein quantities typically consumed within a meal. In contrast, there is no blunting of feeding-induced anabolism when larger protein doses are ingested (~30-40 g) (84, 113). In a recent retrospective analysis of the data from six previously published studies Wall and colleagues (114) demonstrated that, while postabsorptive rates were similar between older and younger men, older men had a 3-fold lower rise in MPS in response to the ingestion of a meal-like (20 g) dose of casein compared to their younger counterparts (112). These data further add to a preponderance of evidence suggesting that 'anabolic resistance' to protein feeding is one of the key factors responsible for age-related muscle loss (115).

The mechanisms underlying the reduced capacity of older adults to stimulate a rise in MPS following the ingestion of meal-like quantities of protein are not fully understood and a multitude of factors likely contribute. As exercise is known to sensitize the muscle protein synthetic machinery to protein feeding (60), while muscle disuse has the opposite effect (31, 116, 117), it has been argued that anabolic resistance may be largely due to lower physical activity levels in older adults (118). In addition, impairments in: protein digestion and amino acid absorption (119); insulin-mediated muscle tissue perfusion (120); muscle amino acid uptake (121); and a reduced amount or degree of activation of key signaling proteins involved in the translational protein machinery (27) have all been reported in older adults and could result in reduced utilization of ingested protein for MPS stimulation.

1.2.5 Influence of energy restriction on muscle protein turnover

The tremendous increase in global obesity rates over the past three decades has affected individuals of all ages and has posed an extraordinary challenge to healthcare systems (122). It is well established that weight loss is associated with a plethora of health benefits in younger and older obese populations including a reduction in co-morbidities, mortality and improved quality of life (123, 124). The fundamental variable determining weight loss is a sustained energy deficit achieved by dietary energy restriction, increased energy expenditure, or their combination. While it is undisputed that energy restriction is an effective weight loss strategy, energy restriction alone results in weight loss comprised of both fat and fat-free mass (FFM), with FFM (composed of ~45% skeletal muscle) generally accounting for $\sim 25\%$ of weight lost (51). Moreover, this loss of FFM is often accompanied by a decline in muscle strength (125-127). Given the critical importance of skeletal muscle in numerous bodily functions (i.e. contribution to basal metabolic rate, physical function, glucose disposal) this weight loss-induced reduction in muscle mass may offset or negate some of the benefits of weight loss. Of particular concern is that, among older adults, the energy restriction-induced reduction in muscle mass and strength (125) may 'accelerate' sarcopenia and further exacerbate disability risk (128). This highlights the need for weight loss interventions in older persons that are tailored towards achieving 'high quality' weight loss, which can be defined as the loss of body weight with the greatest

ratio of fat to lean mass (129).

Understanding the perturbations in muscle protein turnover associated with energy restriction is important to facilitate the development of high quality weight loss strategies. Surprisingly, however, relatively few studies have investigated the effect of energy restriction on muscle protein turnover in humans (85, 86, 130-132). Short-term studies (5 - 21 d) using a moderate energy deficit (~500-1000 kcal/d) have reported a decline in postabsorptive MPS (15 - 27%) (85, 130, 131) and postprandial MPS (~9 - 28%) (85, 86) in young and middle-aged adults. Recently, it was reported that during a short-term energy restricted period (10 d) there was a large (~60%) increase in postabsorptive MPB in young physically active adults (133). However, this finding is controversial given that, if MPB was elevated to the degree suggested (133), combined with the reductions in postabsortive and postprandial MPS consistently reported in the literature (85, 86, 130, 131), then the loss of lean mass should be considerably higher than that typically observed (85, 86). Furthermore, it is well established that the rise in insulin concentration following a meal suppresses MPB in weight stable individuals (134). The impact of energy restriction on postprandial MPB has never been examined; nevertheless, the consumption of a mixed meal was reported to attenuate markers of ubiquitin-mediated proteolysis to a similar extent during weight maintenance and following 21 d of energy restriction (135), which supports the thesis that MPB remains robustly responsive to the postprandial rise in insulin. Given that humans typically spend the majority of their waking hours
in the postprandial state, as well as the fact that MPB is notoriously challenging to accurately measure *in vivo*, further investigation is warranted before any firm conclusions can be drawn regarding the contribution of changes in MPB to the energy restriction-induced loss of skeletal muscle.

Two studies have reported on the influence of longer-term energy restriction on MPS (132, 136) and have yielded equivocal findings. Campbell and colleagues (136) reported that postabsorptive MPS increased and postprandial MPS was unchanged following 11 weeks of moderate energy restriction (energy deficit of \sim 500 kcal/d, subjects' protein intake \sim 1 g protein/kg/d) with or without resistance training in overweight and obese older women. In contrast, following a similar dietary intervention protocol, Villareal et al (132) observed that postabsorptive MPS was unchanged and postprandial MPS was increased in sedentary, obese older adults. While the findings of these two longer-term trials appear to conflict with each other as well as with the short-term studies, there are a number of possible explanations that may account for this. An important methodological consideration in the study by Campbell and colleagues is that MPS was assessed three days after reestablishing energy balance (136). Thus, while these data indicate that returning to energy balance following a period of energy deficit may result in an up-regulation of postabsorptive MPS, the reported MPS responses may not necessarily reflect the MPS rates during a period of energy deficit per se. In the study by Villareal et al. (132) MPS was assessed while participants were still under conditions of energy restriction. The

observation that postabsorptive MPS was unchanged in the latter study is inconsistent with reports that MPS is suppressed following shorter-term (5-14 d) energy restriction in young and middle-aged adults (85, 130, 131). However, following a slightly longer period of energy restriction (21 d) Pasiakos et al. (86) reported postabsorptive rates similar to energy balance in young, physically active adults. Taken together, these data suggest that postabsorptive MPS may be reduced at the onset of energy restriction but adjusts to pre-weight loss levels as the body adapts to prolonged energy insufficiency. The elevation in postprandial MPS following longer-term energy restriction observed by Villareal and colleagues is more difficult to explain, although it cannot be ruled out that this may also reflect an adaptive shift following the early phase of energy restriction and/or that older adults respond to energy restriction differently than younger or middle-aged adults. In contrast to short-term studies in which postprandial MPS was assessed in response to the bolus ingestion of moderate protein doses ($\sim 20 -$ 27 g) (85, 86), Villareal et al. (132) provided a series of small protein doses (10 -15 g) in small aliquots over 2.5 h. As such, it is very surprising that the minimal increase in leucinemia (~1.15-fold) generated by this feeding protocol was actually sufficient to robustly stimulate MPS in an older (less anabolically sensitive) population. Indeed, numerous studies in weight stable older adults have demonstrated that protein doses less than ~20g are insufficient to stimulate MPS above postabsorptive levels (113, 137, 138). Given that the feeding protocol used by Villareal et al. (132) may not reflect the manner in which protein is typically

consumed (i.e. within discrete meals), as well as the limited sample size (n = 6), further work will be necessary to confirm the influence of longer-term energy restriction on postabsorptive and postprandial MPS.

Thus, while the influence of longer-term energy restriction on MPS remains unclear, the data from short-term studies are consistent with a downregulation of postabsorptive and postprandial MPS, at least in young and middleaged populations. The mechanisms mediating the down-regulation of MPS during energy restriction are unknown; however, as MPS is an energetically expensive process, it has been proposed that this may reflect an adaptive response to conserve energy and protein reserves (86). As such, the preservation of the MPS response during energy restriction likely represents an appropriate target to support the maintenance of muscle mass while allowing for fat loss. As in energy balance, protein intake and exercise (especially resistance exercise) appear to be important for modulating rates of MPS during energy restriction (86, 130) and represent key factors for the maintenance of muscle mass during weight loss (136, 139, 140). Recently, it was reported that while the postprandial MPS response to a protein-rich meal was abolished in young adults that consumed the Recommended Daily Allowance (RDA) for protein during 21 d of energy restriction, it was preserved among those that consumed 2 or 3 times the RDA (86). Furthermore, consumption of higher quality protein sources (i.e., whey versus soy) has also been reported to attenuate the energy restriction-induced decline in postprandial MPS (85). These data are consistent with studies showing that higher protein

intakes, and particularly greater consumption of dairy proteins, are associated with greater lean mass maintenance during energy restriction (129, 139, 141). However, the current literature indicates that preservation of lean mass is not possible with a dietary intervention alone (125, 139) and studies that have included exercise (particularly resistance exercise training) as a co-intervention generally show a greater effect of higher protein intake on lean mass and strength preservation (129, 142). This finding highlights the importance of examining combined nutrition and exercise strategies during weight loss.

1.3 The case for per-meal protein intake recommendations in older adults

Dietary protein recommendations are traditionally expressed on a daily basis. For example, the RDA for protein intake for men and women (\geq 19 y of age) is 0.8 g protein/kg/d (143). More recently, however, a number of expert groups have advocated for higher daily protein intakes of 1.0 - 1.2 g/kg/d for older adults to support the preservation of muscle mass and function (144, 145). In addition, there is also a growing appreciation for the need to consider protein intake on a per-meal basis rather than simply focusing on the total daily protein intake (144, 145).

1.3.1 Rationale for considering protein intake on a per-meal basisThe rationale for a per-meal recommendation for protein is based on thedemonstrated existence of a saturable dose-response relationship between the

quantity of protein ingested per meal and the response of MPS as a primary driver of maintenance of muscle mass. As mentioned previously, several studies have demonstrated that as the amount of protein consumed increases there is a graded rise in the rate of MPS up to a saturating or 'optimally effective' protein dose (27, 59, 83, 84). Beyond this optimal protein dose, however, the muscle becomes refractory to increasing protein intake/aminoacidemia and there is no further stimulation of MPS associated with consuming larger protein servings (83, 93).

In young adults, feeding-induced MPS plateaus with the consumption of ~20g (83) or 0.24 g protein/kg (90% confidence interval: 0.18-0.30 g protein/kg) of high quality protein (84). The anabolic resistance to protein feeding in older adults however is characterized by a rightward shift of the same protein dose-MPS response curve (84). Thus, the MPS response to a protein dose equal to or less than that which maximally stimulates MPS in younger men (~ 20 g or 0.24 g/kg body mass [BM]) is attenuated in older adults. Instead, the maximally effective dose of protein for MPS in older men is 0.40 g protein/kg BM (90% confidence interval: 0.21-0.59) (84). Importantly, however, similar maximal postprandial rates of MPS were seen in young and older men at these maximally stimulatory protein doses: 0.067 ± 0.01 %/h vs. 0.057 ± 0.01 %/h in young vs. old, respectively (Figure 2). Thus, these data (84) suggest that, once a sufficient quantity of protein is provided, healthy elderly muscle retains the capacity for enhanced rates of MPS. As such, consideration of per-meal protein intakes may be of particular importance, especially among older populations.

Despite the recent publication of breakpoint data (84), the dose of protein required to maximally stimulate MPS in older adults has yet to be clearly established and, critically, tested in longer-term experiments. Furthermore, while maximally stimulatory for MPS, the efficacy of such a protein dose regime in affecting muscle mass and function is still unclear at this point. An optimally effective protein dose for stimulating MPS is likely affected by a range of factors such as sub-clinical health issues, body composition, habitual physical activity, energy availability, and dietary aspects of the protein feeding (i.e. protein quality, food matrix) (84). Nevertheless, previously published work indicates that 0.4g/kg BM (0.61g/kg of FFM; 90% confidence interval: 0.32-0.89) of high quality protein per feeding is required, under resting conditions, to maximally stimulate MPS (84). This finding has led to a hypothesis termed the 'meal threshold' (146), which states that a balanced distribution of total protein intake with the consumption of at least 30 - 40 g (or 0.4 g/kg BM) of high quality protein permeal would more effectively stimulate rates of MPS throughout the day and, if practiced over months/years, may slow the progression of sarcopenia (147).

Contrary to using solely the MPS response as a target for per-meal protein recommendations there is a hypothesis that in addition to maximally stimulating MPS the suppression of MPB is also an important consideration (148, 149). While the authors of this viewpoint (148) made use of whole-body proteolysis to make the point that higher protein intakes should be recommended, they argued that reductions in whole-body proteolysis reflected, to some degree, a reduction in

muscle proteolysis. Thus, these authors (148) made an argument for even higher per-meal protein intakes than we have recommended here, based on maximal stimulation of net protein balance (NPB) through maximal stimulation of MPS and suppression of MPB. Such a thesis remains to be experimentally tested in longer-term trials, but clearly the first demonstration that such a thesis is viable would be to demonstrate that the reported meal-induced suppression of *wholebody* proteolysis (148, 149) includes a measurable suppression of *muscle* proteolysis and an improved muscle NPB.

When aiming to optimize the daytime MPS response via the per 'meal threshold' approach, the spacing and frequency of protein-containing meals over the day are likely to be important considerations. At rest, approximately 45-90 min after the consumption of a large bolus of EAA (15 g) or isolated protein (48 g), MPS rates rise and remain elevated for ~90 min before returning to fasted levels, even in the presence of hyperaminoacidemia (92, 93). This phenomenon has been termed the 'muscle full' effect (i.e. the upper limit of amino acids at which muscle cells can no longer use them as a substrate for MPS) (93). It follows that, after the ingestion of an EAA/protein bolus, it would likely take ~3-4 h before the muscle protein synthetic machinery is once again able to respond to the hyperaminoacidemia following consumption of another protein-containing meal. Recent evidence suggests that the 'muscle full' effect may be influenced by the feeding-induced aminoacidemia in older adults (92). Mitchell et al. (92) reported that, compared with the rapid and pronounced aminoacidemia (and

leucinemia) characteristic of bolus consumption of EAA, a lower, more gradual aminoacidemia (achieved via the ingestion of small, repeated EAA doses) delays the onset of the muscle full effect such that MPS rates remain elevated 4 hours after the commencement of feeding in older adults. Given that mixed-meal feeding is associated with a lower-amplitude and protracted aminoacidemia compared to an isolated protein or EAA bolus, it may be speculated that, in older adults, protein-containing meals should be separated by ~4-5 h in order to maintain the sensitivity of MPS to repeated stimulation. Practically, this would translate into ~3 protein feedings throughout the day in keeping with the traditional meal pattern of breakfast, lunch and dinner. Recently, nighttime (during sleep) provision of dietary protein has been shown to enhance MPS in older men overnight, which would represent the longest relative postabsorptive period during which MPS is quite low (150). As such, a pre-sleep proteincontaining snack may offer an additional opportunity to consume a protein-rich meal.



Figure 2. Biphase linear regression analyses of relative protein intake per kg body mass and rested myofibrillar fractional synthetic rate (FSR) in healthy older (A) and younger (B) men. Analyses were performed using data from 6 previously published studies (n = 108). Adapted with permission from Moore et al. (2015) *J Gerontol A Biol Sci Med Sci.* 70(1):57–62.

1.3.2 Typical protein intake patterns among older adults

The pattern in which older adults habitually consume protein across the daily meals has been examined (151-154). Studies have consistently reported a 'skewed' pattern of protein intake in which a disproportionately higher amount of a person's daily total protein intake is consumed at only one of the daily meals (usually lunch or dinner). National food survey data indicate that older adults living in the US (154) and UK (155) consume the majority of daily protein in the evening dinner meal (~40-50% of total protein intake), with smaller amounts typically consumed at breakfast (~15%), the midday/lunch meal (~28%), and as snacks (~10%). This translates into lower protein intakes at breakfast (~10 - 15 g) and lunch (~16 - 22 g) that would likely be, based on the arguments put forward in considering a dose-response, suboptimal for the stimulation of MPS (154, 155).

For example, Tieland and colleagues (152) reported that, among both communitydwelling and institutionalized older adults, the lowest proportion of protein was consumed at breakfast (8-12 g), with lunch meals providing slightly more (18-27 g). Protein consumed during dinner was the highest in community-dwelling older adults (~30 g), but tended to be lower (~18 g) among those living in institutional care (152). Importantly, in these studies (151, 153-155) while per-meal intake was measured there was no account of protein quality. Thus, it may be even more important to consider that at the breakfast meal a good deal of protein comes from lower quality proteins from cereal grains (152), which are not high quality protein and could compound even further the issue of a lower protein intake.

While meal patterns in some countries favor a larger midday lunch meal with a lighter evening dinner, a skewed protein distribution is still evident (151, 153). Valenzuela et al. (153) observed that older Mexican men and women consumed the majority of protein at lunch, achieving a per-meal protein intake of ~0.4 g/kg BM, whereas low protein intakes were ingested at breakfast and dinner (~0.21 - 0.26 g/kg BM/meal). Thus, considering that upwards of 30 - 40 g (or 0.4 g/kg BM) of protein per-meal is necessary to elicit a maximal MPS response in older adults, it would appear that free living older adults may reach this threshold only once per day, and institutionalized older adults may not adequately stimulate MPS at all (152, 153) (Figure 3). Thus, a more balanced/even protein intake pattern, with the ingestion of an optimal quantity of protein at each meal, warrants consideration as an applied strategy to more frequently stimulate MPS throughout the day in older adults.



Figure 3. Graphic representation of the amount of protein habitually consumed per-meal by institutionalized older adults (152) relative to the proposed threshold for stimulation of maximal muscle protein synthesis (MPS) with mixed meals in older adults. Note that, the speculated threshold ranges from 0.4 - 0.8 g/kg BM/meal to account for the varied protein quality typically consumed in mixed meals and the range of factors that may influence the level of anabolic resistance among institutionalized older adults (i.e. physical inactivity, illness, malnutrition). This graph demonstrates that the per-meal protein intakes habitually consumed by institutionalized older adults are, in all cases, below the threshold thought to be needed to optimally stimulate MPS. In this example total protein intake is equivalent to the RDA (0.8 g/kg/d) and the largest protein meal is consumed midday in concordance with reported protein intake patterns among institutionalized older adults (152). The bars shown between meals represent protein consumed as between-meal snacks (152).

1.3.3 Evidence for the influence of per-meal protein distribution on muscle

protein synthesis

Several studies have examined the influence of the daily distribution of dietary protein on MPS measured over 12-24 h periods. In agreement with the proposed benefit of an even/balanced protein distribution, Mamerow et al. (156) showed

that when younger adults consumed ~ 30 g (0.4 g/kg BM) of protein at breakfast, lunch and dinner, 24 h mixed MPS was ~25% higher than when they consumed the same total protein intake in a skewed manner (~10 g (0.13 g/kg BM) at breakfast, ~15 g (0.20 g/kg BM) at lunch, ~65 g (0.85 g/kg BM) at dinner). Similarly, in younger men the ingestion of total amount of 80 g of isolated whey protein as four evenly spaced 20 g (0.25 g/kg BM) doses stimulated 12 h myofibrillar protein synthesis and whole body net protein balance to a greater extent than two 40 g (0.48 g/kg BM) doses following a session of resistance exercise (157, 158). Furthermore, a balanced (4 x 20 g doses) pattern of ingestion was reported to be more effective in stimulating MPS than a 'pulsed' distribution (8 x 10 g doses each spaced by 1.5 h) designed to simulate a grazing or a 'little and often' approach to protein consumption (157). Consistent with these findings, compared to the ingestion of 3 meals, a 'grazing' approach to protein feeding has previously been shown to result in higher amino acid oxidation, an outcome which is likely undesirable when the aim is to direct ingested amino acids towards MPS (159). Thus, in younger persons, it appears that a balanced distribution of daily protein, providing a dose of protein known to be optimally effective for stimulating MPS at each meal, is the most anabolic pattern of protein intake both at rest and after exercise.

In older adults, one study has been conducted to examine the influence of daily protein distribution on MPS (160). Kim and colleagues (160) reported that 24 h mixed MPS was similar with a balanced (33/33/33% total protein at

breakfast/lunch/dinner) versus a skewed (15/20/65% total protein at breakfast/lunch/dinner) pattern of protein intake in older adults consuming energy-balanced diets containing either 0.8 g protein/kg/d (1xRDA) or 1.5 g protein/kg/d (\sim 2xRDA) (160). It is difficult to explain the discrepancy between these results and the findings in younger persons (156). However, one possible explanation may be that the per-meal protein dose provided by Kim et al. (160) was still less than maximally stimulatory for older adults. As discussed, and based on a retrospective analysis (84), the dose of protein that maximally stimulates MPS in older adults is ~0.4 g/kg BM (90% confidence interval: 0.21-0.59). In the study by Kim et al. (160) the per-meal protein dose provided to the balanced groups ranged from ~0.2-0.3 g/kg BM/meal in participants randomized to the lower protein level (1xRDA: 0.8 g/kg/d) to 0.5 g/kg BM/meal in those allocated to the higher protein diet (2xRDA: 1.5 g/kg/d). As such, based on the higher per-meal protein ingested (and well within the protein dose we recommend) it is surprising that there was no clear enhancement of MPS in the 2xRDA-balanced group; however, the protein in this study was provided in the context of mixed macronutrient meals (160). Mixed nutrient meals typically contain proteins of varying quality, contain fat and carbohydrate and as such are associated with alterations in amino acid absorption kinetics and reduced plasma amino acid availability (161). As a result, the quantity of protein required to maximally stimulate MPS in a *mixed meal*, as opposed to recommendations based on consumption of *isolated* proteins, may be higher than the optimal level

determined by Moore et al. (84). Mamerow et al. (156) also provided the protein as part of mixed food-based meals in their study in young adults. However, the 30 g/meal protein dose provided to their balanced group equated to ~0.40 g/kg BM/meal which is ~60% higher than the 0.24 g/kg BM/meal dose that we have reported maximally stimulates MPS in young adults (84) and was therefore likely sufficient in spite of protein being provided in mixed meals (156). Alternatively, the cross-over design employed by Mamerow et al. (156) would result in an increased power to detect differences between the two patterns of protein intake compared to the Kim study (160) in which participants were randomized to one of four groups with a relatively small sample size (n=4-6/group).

It is possible the pattern of daily protein distribution may have an increasingly important role under conditions in which muscle mass loss is accelerated in older adults, for example during periods of energy restriction or physical inactivity. Moreover, given the synergistic influence of muscle loading and protein intake on MPS (59), the impact of per-meal protein intake pattern could be enhanced by the performance of resistance exercise. Nevertheless, these hypotheses remain unexplored and further research in this area is required.

1.3.4 Evidence for the influence of per-meal protein distribution on muscle mass and function

Studies in which MPS is measured in response to variations in protein intake pattern provide important 'proof of principal' information and valuable insight into the potentially beneficial effects of longer-term feeding strategies. Nevertheless, it is essential to establish the relationship between daily protein intake pattern and clinically relevant endpoints. Regrettably, there are few studies that have examined the influence of dietary protein distribution on muscle mass, strength and/or functional capacity in older adults. In a cross-sectional design, Bollwein et al. (151) observed that although frail, pre-frail, and non-frail community dwelling older adults (n = 194; age: ≥ 75 y) reported comparable total daily protein intakes, non-frail participants demonstrated a more even per meal protein distribution pattern across their daily meals than frail and pre-frail participants. Specifically, the proportion of daily protein consumed at breakfast was higher in the non-frail group, whereas protein was more skewed toward the lunch/noon meal in the pre-frail and frail groups (151). In addition, analysis of data from the National Health and Nutrition Examination Survey (NHANES) supports a positive association between the frequency of consumption of optimal per-meal protein intake and muscle mass and leg strength in older Americans (Dr. J.P. Loenneke, personal communication).

Two randomized controlled trials have investigated the influence of protein distribution patterns on body composition and/or functional outcomes in older adults. Arnal et al. (162) conducted a 2-wk controlled-feeding intervention in older women (mean: age 68 y, BMI 25 kg/m²) during which total protein intake was distributed across daily meals (provided at 0800 h, 1200 h, 1600 h and 2000 h) in the proportions of 7%, 79%, 0%, and 14% in the skewed group and 22%,

31%, 19%, and 28% in the balanced group. Interestingly, the authors reported that while whole body lean mass was maintained in the skewed group, there was a small, but statistically significant decrease (mean decrease ~0.3 kg) in the balanced per meal protein consuming group. Although the results from the study by Arnal et al. (162) appear to challenge our hypothesized benefit of a balanced protein distribution a crucial consideration is that daily protein intakes during this study were only 30% higher than the RDA at 1.05 g/kg/d. Consequently, the quantities of protein consumed at each meal in the balanced protein group (i.e., 12 – 20 g/meal; 0.23 - 0.33 g/kg BM/meal) were still likely insufficient to maximally stimulate MPS and might not have even raised MPS much above fasting levels (59, 84, 113) (Figure 4). Indeed, the potential advantage of a balanced distribution of protein intake in older adults is likely contingent on the consumption of a higher protein diet (≥ 1.2 g/kg) that allows for the provision of an amount of protein shown to maximally stimulate MPS at each meal (~0.4 g/kg BM).

Bouillanne et al. (163) provided hospitalized, malnourished or at-risk older adults (mean: age 85 y, BMI 21 kg/m²) with 6-wk diets that delivered protein in either a balanced (0800 h: 12 g; 1200 h: 21 g; 1600 h: 14 g; 1900 h: 21 g) or skewed (0800 h: 5 g; 1200: 48 g; 1600 h: 2 g; 1900 h: 11 g) pattern. Consistent with the findings of Arnal et al. (162), the authors reported a significant improvement in lean mass in the skewed group (mean change from baseline +0.9 kg) compared with the balanced group (-0.4 kg), although no changes in handgrip strength or ADLs were detected in either group (163). Notably, an advantage of

the skewed protein distribution compared to the balanced pattern was observed despite the provision of a higher protein intake of 1.3 g/kg/d (163). Nevertheless, as these investigators (163) provided four daily meals, the quantity of protein consumed at each meal in the balanced group (i.e., 0.22 - 0.38 g/kg BM/meal) was again likely insufficient to optimally stimulate MPS at any of the meals. The per meal thesis may be particularly true amongst hospitalized older adults in whom low physical activity (164), energy deficiency (165), and illness/inflammation may be present and all of which have previously been shown to reduce the responsiveness of MPS to hyperaminoacidemia (31, 85, 166). In fact, position stands have advocated for protein intakes as high as 1.5-2.0 g/kg/d in malnourished hospitalized patients (145). Thus, the quantity of protein required to maximally stimulate MPS in compromised older populations may be even higher than the 0.4 g/kg BM meal dose previously reported for healthy elders (Figure 4). In the skewed group, the protein dose of ~48 g (~0.94 g/kg BM) provided at the noon/lunch meal was probably more than sufficient to maximally stimulate MPS and therefore may account for the observed improvement in lean mass (163) (Figure 4).

In summary, available studies indicate the protein distribution pattern has the potential to affect muscle mass. Nonetheless, in our view no study to date has compared a balanced/even pattern of protein intake with sufficient quantities of per-meal protein to a conventional skewed intake, representing an important knowledge gap.



Figure 4. Graphic representation of the amount of protein ingested per-meal in the balanced (A and C) and the skewed (B and D) groups in the studies by Arnal et al. (162) (A and B) and Bouillanne et al. (163) (C and D) relative to the proposed thresholds for stimulation of maximal muscle protein synthesis (MPS) with mixed meals in healthy (A and B) and hospitalized (C and D) older adults. Note that, for the healthy older adults in the study by Arnal et al. (162), the speculated threshold ranges from 0.4 - 0.6 g/kg BM/meal to account for the varied protein quality typically consumed in mixed meals. This intake level would be shifted to greater levels (0.6 g/kg BM or more) in compromised (more 'anabolically resistant' – see text for explanation) older adults in the study by Bouillanne et al. (163). These graphs demonstrate that the per-meal protein intakes provided to the balanced groups were, in all cases, below these thresholds and provide a possible explanation for the lack of observed benefit associated with the balanced protein distribution pattern in these studies.

1.3.5 Difficulties achieving optimal per-meal protein intakes in older adults

The concept of a balanced distribution of optimal per-meal protein necessitates the consumption of daily protein intakes ≥ 1.2 g/kg/d. These intakes are roughly in line with the recent recommendations from two groups of at least 1.0 - 1.2 g/kg/d protein for healthy older adults, with even higher intakes recommended for older adults suffering from acute or chronic diseases (i.e. 1.2 - 1.5 g/kg/d) (144, 145). Nonetheless, population-based studies have consistently reported average daily protein intakes below this level (i.e. ~0.8 - 1.1 g/kg/d) in older persons (32, 152, 167). Furthermore, up to 10% of European community-dwelling older adults and 35% of those in institutional care had protein intakes less than 0.7 g/kg/d (152). Other estimates show that in the US, protein intakes equal to or less than the RDA (0.8 g/kg/d) are seen in up to 25% of older women and 10% of older men (168). Thus, for many older adults the application of the balanced, meal threshold protein distribution concept clearly necessitates not only a redistribution of protein intake, but also an increase in total daily protein intake.

Protein intake tends to be particularly low at breakfast in older adults (8-15 g/kg/d) (152, 154, 167), largely because many traditional breakfast foods (i.e., grain-based cereals, breads, fruit preserves, juice) are higher in carbohydrate and lower in protein content (and would contain mostly lower quality grain-based proteins). Similarly, older adults also frequently consume a grain-based (bread) meal at lunchtime which tends to result in a suboptimal protein intake at that meal (152). As such, breakfast and lunch represent logical meals at which to aim efforts

to improve both total protein intake as well as protein distribution. While simple in theory, the practical implementation of this strategy may be challenging given that food selections tend to be habit-driven among older adults. Furthermore, particularly among more compromised populations of older adults, achieving the relatively large per-meal protein intakes necessary to optimally stimulate MPS repeatedly throughout the day may prove challenging for a number of reasons. Age-related issues such as poor appetite, poor dentition, dysphagia, functional disabilities and food insecurity are known to negatively impact protein intake (144, 145). Consequently, there is considerable interest in investigating potential strategies to augment the MPS response to lower protein doses in the elderly.

1.3.6 Leucine fortification of a suboptimal protein dose

Recent studies have described leucine as the most potent amino acid responsible for triggering the initiation of muscle protein translation (97) and suggest that the leucine content of a protein source is an independent predictor of its capacity to stimulate postprandial MPS (90, 107). Highlighting the potency of this amino acid, the addition of extra leucine (4.25 g) to a small (~6 g) quantity of whey protein, a protein dose which had previously been shown to be less effective in stimulating MPS (83), elicited the same MPS response as an optimal dose (25 g) of whey in young men, both at rest and post-exercise (169). As such, in older adults, increasing the leucine content of a meal-like (suboptimal from the perspective of the stimulation of MPS) dose of dietary protein may effectively compensate for a blunted muscle protein synthetic response. In support of this thesis, Wall and colleagues (114) demonstrated that addition of 2.5 g of crystalline leucine to a meal-like (20g) bolus of casein enhanced postprandial MPS in older men. Similarly, Katsanos et al (170) showed that increasing the leucine content of a low dose (7.5 g) EAA mixture (from 26 to 41%; or from 1.7 to 2.8 g leucine) reversed the attenuated MPS response in older adults. In contrast, Dickinson et al. (171) observed that the ingestion of a leucine-enriched (3.5 g leucine) 10 g EAA dose following resistance exercise did not further enhance the immediate post-exercise MPS response compared to the consumption of a lowerleucine (1.7 g leucine) EAA mixture in older men. Interestingly however, 24 h after the exercise, fasting MPS rates remained elevated only in the group that had consumed the leucine-enriched EAA dose post-exercise. This suggests that the consumption of higher quantities of leucine in the post-exercise period may prolong the anabolic effects of resistance exercise in older adults in the postabsorptive state (171). Whether this strategy would also prolong the sensitivity of skeletal muscle to amino acids in older adults remains to be determined. Nevertheless, in support of this possibility, Casperson et al. (172) showed that 2 weeks of leucine supplementation with meals (4 g/meal and 3 meals/d) augmented the MPS response to a (non-supplemented) low protein (7 g) meal in non-exercising older adults.

Taken together, these data suggest that leucine supplementation of a suboptimal protein dose can augment the acute postprandial MPS response in

older adults (114, 170, 173), with some evidence that higher leucine consumption may have prolonged effects that enhance the sensitivity of skeletal muscle to anabolic stimuli (171, 172). Importantly, however, despite promising results from the acute MPS studies, the three longer-term randomized controlled trials (RCTs) that have examined the influence of leucine supplementation in older adults to date (Table 1) have been unable to show clear benefits on muscle mass and strength (174-176). Of the two studies that did not include exercise, one study involved 3 months of ingesting 2.5 g of crystalline leucine with daily meals (3 meals/d) in healthy elderly men (mean age 71 y) (176), and the other study lasted 6 months with the same dosing regimen in older men with type 2 diabetes (mean age 71 y) (175). Neither study reported a benefit of leucine in promoting lean mass or strength gains (175, 176). In a third study assessing muscle strength/function but not mass, leucine supplementation (5 g crystalline leucine consumed 1 h after lunch and dinner) was combined with resistance exercise training (3 d/week) for 3 months in elderly nursing home residents and adult day care attendees (mean age 84 y) (174). Although only 11 of the 30 original participants completed the 3 month intervention, using imputation techniques the authors determined that leucine supplementation was associated with a trend for greater improvements in maximal voluntary contraction and certain markers of functional performance (timed up and go, chair stands) (174). Nevertheless, although these observations may be of clinical relevance, given the considerable attrition associated with this study, these findings remain tenuous until further

confirmation.

There are a number of reasons why leucine supplementation may not have resulted in any change in muscle mass in these longer-term intervention trials. First, as has been proposed by several authors (172, 175, 176), habitual protein intake is a key determinant of the efficacy of leucine supplementation regimens such that people with lower protein intakes are more likely to benefit from leucine supplementation. The participants in the longer-term studies were already consuming protein intakes higher than the RDA and more in line with current recommendations for an optimal protein intake in older adults (mean intakes were \sim 1.0 g/kg/d) (175, 176) and, consequently, additional leucine may not have had a potent effect in augmenting postprandial MPS (175, 176). Of note, however, Trabal and colleagues (174) observed a trend for leucine supplementation to enhance resistance training-induced gains in muscle strength and function despite habitual protein intakes of 1.2 - 1.4 g/kg/d. It may also be that supplementation for 3-6 months may simply be too short a period of time to detect an effect of leucine supplementation on changes in lean mass. At best, leucine may be able to offset the gradual decline in muscle mass loss that occurs at a population rate of ~ 0.8 % in older adults (10). As such, for a ~ 85 kg man with ~ 35 - 40 kg of muscle mass would normally expect to lose ~140 - 160 g of muscle in a 6 month period, which if it were completely offset or even augmented, would be a change that would be very difficult to detect using the available methods (i.e., dual-energy xray absorptiometry [DEXA], computerized tomography [CT], and/or measures of

muscle fiber area) (177). Third, all of the previous studies investigating the influence of leucine co-ingestion on the acute MPS response in older adults have been conducted following an overnight fast and in the context of either an isolated protein (114) or EAA bolus (170, 171), or as part of a mixed macronutrient meal provided in small aliquots over an extended period (173). In reality protein is most often eaten in a food matrix and is co-ingested with carbohydrate and fat during meals consumed within a discrete period of time. The addition of fats and carbohydrates to protein-containing meals would alter the kinetics of gut amino acid absorption (161) and further work is necessary to delineate whether addition of leucine to a normal, mixed macronutrient meal has the capacity to enhance the MPS response with consumption of food in a regular meal pattern. Finally, previous studies examining the influence of leucine fortification on the acute postprandial MPS response have all used intravenous infusions of stable isotope tracers that are limited in that participants are confined to a laboratory bed and measurements are typically only made over 3-6 h (114, 170, 171, 173). Consequently, the acute measurements of MPS do not account for all aspects of

feeding, resting, sleep and daily activities that accompany free-living conditions. In sum, a number of factors may contribute to the incongruent results seen in the acute (114, 170, 173) versus the long-term outcomes (175, 176).

Reference	Participants	Protein intake	Exercise	Leucine dose	Duration	Methods	Outcomes
Verhoeven et al. (2009)	30 healthy older men	~1.0 g/kg/d	_	3 meals x 2.5 g/meal versus placebo	3 months	СТ	\leftrightarrow upper leg CSA
	(29 completers)					DEXA	\leftrightarrow Lean mass
	$71 \pm 4 y$					1-RM leg	\leftrightarrow Strength
	$26.1 \pm 0.5 \text{ kg/m}^2$					extension/press	
Leenders et al. (2011)	60 older type 2	~1.0 g/kg/d	_	3 meals x 2.5 g/meal versus placebo	6 months	DEXA	\leftrightarrow Lean mass
	(57 completers)					1-RM leg extension/press	↑ Strength (equally in leucine and placebo groups)
	$71 \pm 1 \ y$					I I I I I I I I I I I I I I I I I I I	
	$27.3\pm0.4~kg/m^2$					Skeletal muscle biopsy	↔ Muscle fiber CSA
Trabal et al. (2015)	30 older men/women residing in a nursing home or	$\sim 1.2 - 1.4$ en g/kg/d n a ome or adult leters)	Whole body resistance training (3 d/week; 65% 1-RM x 15 repetitions x 2 sets), balance exercises (1 d/week)	2 meals (lunch and dinner) x 5 g/meal versus placebo	3 months	Dynamometer	↑ Maximal isometric leg strength (trend for greater ↑ in leucine group)
	attending adult					Physical	↑ Select markers of functional
	day care (11 completers)					Performance Battery (PPB)	status (trend for greater † in leucine group)
	$84 \pm 6 \text{ y}$						
	$26.3\pm4.8~kg/m^2$,				

Table 1. Randomized controlled trials examining effect of crystalline leucine supplementation on muscle mass, strength and/or function in older adults. CT, computerized tomography; DEXA, dual-energy x-ray absorptiometry; CSA, cross-sectional area; 1-RM, 1-repetition maximum.

1.3.7 Resistance exercise to enhance the anabolic response to per-meal protein intake strategies

A wealth of literature supports the efficacy of resistance exercise training in augmenting muscle mass, strength and physical performance in older adults (178, 179). In addition, compared to energy restriction alone, the inclusion of resistance training during a hypoenergetic period is associated with an attenuated loss of muscle mass and greater improvements in physical function in obese older adults (180, 181). As such, resistance exercise represents a potent strategy in the prevention and management of both sarcopenia and sarcopenic obesity (182). Resistance exercise sensitizes the muscle protein synthetic machinery to protein feeding resulting in more of the ingested amino acids being incorporated into newly synthesized skeletal muscle protein (60). The enhanced sensitivity of MPS to protein ingestion following intense resistance exercise persists for at least 24 h following the exercise bout (61) indicating that prior exercise can augment the MPS response, not only to the post-exercise meal, but to every meal consumed over a ~1-2 day period. This suggests that the capacity for 'an optimized per-meal approach to protein feeding' to improve daily MPS would be further enhanced with the performance of resistance exercise.

1.4 Objectives

The primary objective of the studies comprising this thesis was to examine the potential for practical, meal-based protein intake strategies to increase MPS in older adults. A number of acute studies have demonstrated that optimizing the protein dose at each meal and/or increasing the leucine content of a suboptimal protein dose can augment

the muscle protein synthetic response over several hours (~4-6 h) in older adults (27, 84, 114, 170). This has led to widespread speculation that implementation of these strategies at every meal consumed within a day would enhance the cumulative MPS response over time and, consequently, may offset sarcopenic muscle loss (145, 147, 183). Critically, however, the integrated MPS responses to these strategies over days/weeks remains to be examined, particularly in free-living settings. Furthermore, whether the MPS responses to these meal-based approaches could be further enhanced with the performance of resistance exercise remains largely unexplored.

1.4.1 Studies and hypotheses

In Study 1 we examined the impact of the distribution pattern of dietary protein over the day on the synthesis of specific muscle protein fractions in overweight/obese older men. We compared the influence of consuming a total of 75 g of whey protein in an even/balanced pattern (25 g protein/'meal' x 3) or a traditional, skewed pattern (10 g at 'breakfast', 15 g at 'lunch', 50 g at 'dinner') on rates of myofibrillar and sarcoplasmic protein synthesis measured over an 11 h period during energy balance, following 2 wk of energy restriction alone and following 2 wk of energy restriction combined with resistance exercise training. This was a 'proof of principal' experiment and rates of MPS were measured acutely (%/h) via the primed, continuous infusion of L-[ring-

 $^{13}C_6$]phenylalanine. Based on previous findings that the feeding-induced rise in mixed MPS was maximally stimulated with a ~30 g dose of protein in older adults (184), we hypothesized that the balanced distribution of protein intake throughout the day would

stimulate myofibrillar protein synthesis (MyoPS) to a greater extent than the skewed distribution. Furthermore, we hypothesized that this effect would be enhanced after undertaking resistance training.

In Study 2 we used the same participants from Study 1 to examine the influence of daily protein distribution (balanced versus skewed) on the longer-term integrated synthetic response (%/d) of myofibrillar protein and individual skeletal muscle proteins over 2-wk of energy restriction alone and 2-wk of energy restriction combined with resistance training. We used oral consumption of 2 H₂O to obtain the integrative MyoPS (%/d) rates in response to the two divergent protein intake patterns in the free-living setting. We hypothesized that, in accordance with our acute findings from Study 1, that a balanced distribution of dietary protein intake throughout the day would stimulate longer-term MyoPS, as well as the synthesis of individual contractile proteins, to a greater extent than a skewed distribution and that this effect would be enhanced while undertaking resistance training.

In Study 3 we used oral ${}^{2}H_{2}O$ to examine the impact of 3 days of leucine coingestion with mixed macronutrient meals on the integrated myofibrillar protein synthetic response (%/d) in free-living older men consuming daily protein intakes at (1.2 g/kg/d; 50% greater than the RDA for protein) or below (0.8 g/kg/d; the RDA for protein) the current recommendations for optimal protein intake in healthy older adults (1.0 -1.2 g/kg/d) (144, 145), both at rest and when combined with resistance exercise. Specifically, we provided participants an energy-adequate diet that was either higher (1.2 g/kg/d) or lower (0.8 g/kg/d) in protein and measured integrative MyoPS in a rested and resistance

exercised leg over a 3-d period in which the participants co-ingested a NEAA placebo with daily meals and over a 3-d period in which they co-ingested a leucine supplement with each meal. Previous studies have demonstrated that the addition of leucine to a suboptimal 'meal-like' protein dose augments the acute (~4-6 h) postprandial muscle protein synthetic response in older adults (114, 170). Therefore, we hypothesized that leucine co-ingestion with the main daily meals would enhance integrative MyoPS in both groups 1) under resting conditions and 2) when combined with the performance resistance exercise.

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CHAPTER 2:

Hypoenergetic diet-induced reductions in myofibrillar protein synthesis are restored with resistance training and balanced daily protein ingestion in older men. Published

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Hypoenergetic diet-induced reductions in myofibrillar protein synthesis are restored with resistance training and balanced daily protein ingestion in older men

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Murphy CH, Churchward-Venne TA, Mitchell CJ, Kolar NM, Kassis A, Karagounis LG, Burke LM, Hawley JA, Phillips SM. Hypoenergetic diet-induced reductions in myofibrillar protein synthesis are restored with resistance training and balanced daily protein ingestion in older men. Am J Physiol Endocrinol Metab 308: E734–E743, 2015. First published March 3, 2015; doi:10.1152/ajpendo.00550.2014.-Strategies to enhance weight loss with a high fat-to-lean ratio in overweight/obese older adults are important since lean loss could exacerbate sarcopenia. We examined how dietary protein distribution affected muscle protein synthesis during energy balance (EB), energy restriction (ER), and energy restriction plus resistance training (ER + RT). A 4-wk ER diet was provided to overweight/obese older men $(66 \pm 4 \text{ yr}, 31 \pm 5 \text{ kg/m}^2)$ who were randomized to either a balanced (BAL: 25% daily protein/meal \times 4) or skewed (SKEW: 7:17:72:4% daily protein/meal; n = 10/group) pattern. Myofibrillar and sarcoplasmic protein fractional synthetic rates (FSR) were measured during a 13-h primed continuous infusion of L-[*ring*- $^{13}C_6$]phenylalanine with BAL and SKEW pattern of protein intake in EB, after 2 wk ER, and after 2 wk ER + RT. Fed-state myofibrillar FSR was lower in ER than EB in both groups (P < 0.001), but was greater in BAL than SKEW (P = 0.014). In ER + RT, fed-state myofibrillar FSR increased above ER in both groups and in BAL was not different from EB (P = 0.903). In SKEW myofibrillar FSR remained lower than EB (P = 0.002) and lower than BAL (P = 0.006). Fed-state sarcoplasmic protein FSR was reduced similarly in ER and ER + RT compared with EB (P < 0.01) in both groups. During ER in overweight/obese older men a BAL consumption of protein stimulated the synthesis of muscle contractile proteins more effectively than traditional, SKEW distribution. Combining RT with a BAL protein distribution "rescued" the lower rates of myofibrillar protein synthesis during moderate ER.

aging; energy restriction; stable isotope

SARCOPENIA, THE PROGRESSIVE loss of skeletal muscle mass with age, is associated with declines in strength and functional capacity, and increased risk for disability and mortality (21, 26). Progression of sarcopenic muscle loss occurs when the rate of muscle protein breakdown chronically exceeds muscle protein synthesis (MPS) (40). As potent sarcopenic "countermeasures," protein ingestion and resistance exercise independently drive the synthesis of new muscle proteins and interact synergistically to stimulate protein synthetic rates (40). However, aging is associated with a reduced MPS response following both protein consumption (34) and resistance exercise (25). This phenomenon, termed "anabolic resistance," has been implicated as an important factor in sarcopenic muscle loss (11, 24).

The dose of ingested protein required to maximally stimulate MPS in older adults remains to be clearly established. Nevertheless, MPS increases in a protein dose-response fashion, and doses in the range of 30-40 g of high-quality protein maximally stimulate MPS under resting conditions (34, 39, 41, 53). Older adults tend to consume protein in a "skewed" fashion, consuming almost 50% of their daily protein at the evening meal (45). Given that MPS is protein dose-responsive (34, 39, 41, 53), it has been speculated that a "balanced" per meal distribution of protein intake, with consumption of at least 30 g of protein/meal, would provide an optimal stimulation of MPS and potentially alleviate muscle loss (36, 47). The recommendation of an intake of 30 g protein/meal would necessitate a protein intake exceeding the current U.S.-Canadian recommended dietary allowance (RDA) of 0.8 g·kg⁻¹·day⁻¹. Nonetheless, numerous scientific committees have recommended higher protein intakes to support the maintenance/ regain of muscle mass and function in older adults (3, 15, 47).

The prevalence of obesity is increasing among older adults and is often concomitant with a low skeletal muscle mass, a scenario termed "sarcopenic obesity" (4). A sustained energy deficit, achieved through a reduction in energy intake and/or increased energy expenditure, is a prerequisite for the loss of body mass; however, energy restriction (ER) alone results in weight loss comprised of both fat and lean body mass, with lean tissue generally accounting for ~25% (50). The addition of exercise, particularly resistance training (RT), to a hypocaloric diet has been shown to be effective in retention of greater amounts of lean tissue (17, 27). Because ER-induced weight loss results in loss of fat and lean tissue this could lead to an "acceleration" of sarcopenic muscle loss. Thus, there is a need

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to identify strategies that facilitate simultaneous fat mass loss and muscle mass retention.

We examined the impact of dietary protein distribution on the synthesis of specific muscle protein fractions during periods of ER, with and without RT, compared with energy balance (EB) in 20 overweight/obese older men. We hypothesized that a balanced (BAL) distribution of dietary protein intake throughout the day would stimulate myofibrillar protein synthesis to a greater magnitude compared with a skewed (SKEW) distribution. Furthermore, we hypothesized that this effect would be enhanced after undertaking RT.

METHODS

Ethical approval. This study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy on the ethical use of human subjects in research (http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web.pdf). Each participant was informed of the purpose of the study, experimental procedures, and potential risks before written consent being provided.

Participants. Twenty overweight and obese older adult men [age 66 \pm 4 yr, body mass index (BMI) 31 \pm 5 kg/m²] were recruited to participate in the study through posters and via local newspaper advertisements. Inclusion criteria were men, 60-75 yr of age, BMI between 27 and 40 kg/m², nonsmokers, and generally healthy according to responses to a standard health screening questionnaire. Exclusion criteria included self-reported diabetes mellitus, cardiovascular disease, renal disease, gastrointestinal disease, musculoskeletal injuries, significant body mass loss in the 3-mo period before the study, vegetarianism, and use of medications known to interfere with muscle metabolism, including statins, β-blockers, hormone replacement therapy, antiarrhythmic drugs, oral hypoglycemic agents, and insulin. Inclusionary medications were low-dose aspirin (81 mg/day), type I and II 5 α -reductase inhibitors (avodart and propecia), xanthine oxidase inhibitors (allopurinol), calcium channel blockers, and selective serotonin reuptake inhibitors. Before commencing the study, participants completed a 5-day weighed food record (3 weekdays and 2 weekend days), and these were analyzed using a commercially available software program (Nutribase version 11.5; Cybersoft, Phoenix, AZ) to assess habitual dietary intake.

Study overview. An overview of the study design is shown in Fig. 1. Participants were provided with a 3-day lead-in diet (days - 3 to 0) designed to provide energy to maintain EB immediately before commencing a 4-wk hypocaloric feeding intervention with RT. Before entry, participants were randomly allocated to one of two groups (n = 10/group) matched for age and BMI (BAL or SKEW). In BAL, participants were provided with diets that evenly distributed dietary protein across their daily meals, and in SKEW the majority of protein was provided as part of the evening meal. The 4-wk intervention

consisted of two \times 2-wk phases. In *weeks 1* and 2 all participants were in ER (Phase 1:ER) and continued their habitual physical activity. In *weeks 3* and 4, while still energy restricted, all participants commenced a supervised RT program (Phase 2:ER + RT) in which they undertook whole body, progressive RT on 3 days/wk. The rates of MPS in response to a BAL or SKEW pattern of protein intake were measured at the conclusion of the energy balanced lead-in diet (*trial 1*, *day 0*), at the end of Phase 1:ER (*trial 2, day 14*), and at the end of Phase 2:ER + RT (*trial 3, day 28*). Participants were required to wear a pedometer and accelerometer (SenseWear v7.0; BodyMedia, Pittsburgh, PA) for 3 days immediately before each experimental infusion trial to monitor the number of daily steps and habitual physical activity.

Diets. At baseline, each participant's energy requirement to maintain EB was calculated using the Mifflin St. Jeor equation (16, 30) with the appropriate activity factor, which was determined for each participant based on their response to a standard habitual physical activity questionnaire before intervention. During the 3-day lead-in phase, participants consumed a diet providing 100% of estimated energy requirements, including 1 g protein·kg⁻¹. During this lead-in period, protein was distributed in a traditional skewed pattern (i.e., small amount of protein at breakfast and lunch and the majority of protein at the evening dinner meal). The macronutrient breakdown of the lead-in diet was 55% energy from carbohydrate, 15% from protein, and 30% from fat. The purpose of the lead-in diet was to ensure participants commenced the study in EB and to minimize the influence of interparticipant differences in habitual protein intake.

During the 4-wk energy-restricted period, participants in both groups consumed a diet providing 300 kcal/day less than their estimated energy requirements to maintain EB. The diets provided 1.3 g protein·kg⁻ day⁻¹, and dietary carbohydrate and fat were both manipulated within the ranges 50-55 and 20-25% of total energy intake, respectively, to achieve the target energy intake for each participant. Although the diets in both groups contained the same total amount of daily protein, they differed in the distribution pattern. In BAL, protein was evenly distributed across the four daily meals ($\sim 25\%$ of total protein intake at breakfast, lunch, dinner, and prebed snack), with each meal providing ≥ 30 g (≥ 0.33 g/kg) protein. In SKEW the majority of daily protein intake was provided with the evening dinner meal (~7% at breakfast, ~17% at lunch, ~72% at dinner, and ~4% prebed). In SKEW, the protein content of breakfast, lunch, and the prebed snack was <20 g (<0.22 g/kg), and dinner provided 70-110 g (~0.94 g/kg), depending on each participant's daily protein requirement. In both groups, meals contained a variety of plant- and animalbased protein sources. In BAL, a ready-to-drink whey protein micelle (WPM) beverage (240 g: 25 g protein, 3 g carbohydrate, 0.6 g fat; Nestle, Lausanne, Switzerland) was consumed as part of breakfast and the prebed snack as a practical means of achieving target protein intakes at these meals, which are typically low in protein (43, 45). SKEW did not consume a WPM supplement throughout the intervention and therefore received their total daily protein intake from food sources only. To



Fig. 1. Schematic overview of study design. DEXA, dual-energy X-ray absorptiometry; EB, energy balance; ER, energy restriction; ER + RT, energy restriction and resistance training; PA, physical activity assessed using accelerometry; SKEW, skewed; BAL, balanced.

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minimize any potential psychological influence of consuming a protein supplement in BAL, participants in SKEW consumed a protein-free, low-energy placebo drink (240 g: 0.2 g protein, 3 g carbohydrate, 0.6 g fat; Nestle) similar in appearance, smell, and taste to the WPM beverage, with breakfast and the prebed meal. Participants were not told their group allocation and were blinded to the composition of their assigned study beverage. Although complete blinding to a dietary intervention is difficult, we took several measures to minimize obvious differences between the diets. Provision of the study beverages with breakfast and the prebed snack allowed us to provide similar foods to both groups for these meals (i.e., breakfast cereals, milk, fruit, granola bars, juice, and nuts). Similar foods were also provided for lunch and dinner in both groups (i.e., skimmed milk and prepackaged frozen meals) although the serving sizes of meat within the meals differed between groups.

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All study diets were designed by a research dietitian who met with each participant individually to customize meal plans in-line with their personal food preferences. To enhance compliance, participants were supplied with all of the food required for the duration of the study, which consisted of meals that required minimal preparation. The meal plans specified the time of day meals should be consumed (breakfast: 0700, lunch: 1200, dinner: 1700, prebed snack: 2200), and participants were instructed to mark food items that were consumed in a log. Eating food not provided by the study was discouraged, but, if this occurred, the participant logged the extra food that was consumed. The daily logs were returned by participants and checked by the research dietitian on a weekly basis.

Exercise training. During weeks 3 and 4 participants underwent a progressive, low-load, high-volume RT program (31) consisting of three training sessions per week (6 sessions total) with at least 1 day between each session. Each session consisted of two upper body exercises (chest press, seated row; Hur) and three lower body exercises (leg press, leg extension, leg curl; Hur). Training sessions in week 3 consisted of two sets and in week 4 consisted of three sets (4 sets in the final training session) of each exercise performed to the point of volitional fatigue. A strength test was conducted at least 1 day before the start of the lead-in phase of the intervention to determine the maximum load that each participant could lift for 20-30 repetitions of each exercise. This load was used for the first set of each exercise in the first training session. Once a participant was able to complete more than 30 repetitions with a given load, the weight was increased to maintain each participant within the target repetition range of 20-30. Trained study personnel individually supervised each training session, verbally encouraged participants, and completed training logs detailing the load and repetitions for every session. All training sessions were performed in the morning before breakfast, and the final training session was performed 48 h before the third experimental infusion trial.

Anthropometrics and body composition. Height was measured to the nearest 0.1 cm using a stadiometer, and body mass was assessed to the nearest 0.1 kg using a calibrated scale. Whole body dual-energy X-ray absorptiometry scans (QDR-4500A, software version 12.31; Hologic, Bedford, MA) were carried out after an overnight fast by the same trained technician to determine fat mass and (fat and bone free) lean mass. Appendicular skeletal muscle mass (ASMM) was measured as the sum of arm and leg lean mass.

Experimental infusion protocol. Participants reported to the laboratory via automobile or public transportation at $\sim 0\hat{000}$ following a 10- to 12-h overnight fast. Upon arrival a catheter was inserted in an antecubital vein to obtain a baseline blood sample before initiating a 0.9% saline drip to keep the catheter patent to allow for repeated arterialized blood sampling. Arterialized blood samples (13) were obtained repeatedly during the infusion trial by wrapping a heating blanket around the forearm. A second catheter was inserted into the antecubital vein of the opposite arm, and a primed, continuous infusion (0.05 µmol·kg⁻¹·min⁻¹, 2.0 µmol/kg prime) of L-[ring-13C6]phenylalanine (Cambridge Isotope Laboratories, Woburn, MA) was initiated and maintained for the next 13 h while the participants rested comfortably on a bed. Participants consumed a WPM supplement (Nestle) 2, 5, and 8 h following the onset of the primed constant infusion with the purpose of simulating a BAL or SKEW meal pattern. Both groups consumed a total of 75 g of WPM during each trial. In BAL, 25 g of WPM was consumed at 2 h (breakfast), 5 h (lunch), and 8 h (dinner). In SKEW, the WPM dose was 10 g at 2 h, 15 g at 5 h, and 50 g at 8 h. All protein "meals" were from original ready-to-drink beverages and were enriched to 4% with L-[ring- ${}^{3}C_{6}$]phenylalanine to minimize disturbances in isotopic equilibrium following amino acid ingestion (6). The spacing of 3 h between each protein "meal" was selected based on previous work showing that MPS is stimulated and returns to basal levels within 3 h of protein ingestion (2). The period following the "dinner" protein meal was extended to 5 h to ensure we did not "miss" the influence a potentially more prolonged stimulation of MPS in SKEW with the large dinner protein dose. Plasma samples were obtained before and 15, 30, 45, 60, 120, and 180 min after all meals and additionally 240 and 300 min after dinner during all trials. In trials 2 and 3, skeletal muscle biopsies were obtained directly before the initiation of the primed continuous infusion, at 2 h (immediately before ingestion of the first WPM feeding) and 13 h following infusion initiation. In trial 1 biopsies were obtained only at 2 and 13 h, and the fasted (0-2 h) fractional synthetic rate (FSR) was calculated based on the ¹³C enrichment of mixed plasma proteins obtained from the preinfusion blood sample and the skeletal muscle biopsy following 2 h of tracer incorporation (5). Muscle biopsies were obtained from the vastus lateralis muscle using a 5-mm Bergström needle adapted for manual suction under 2% xylocaine local anesthesia. The tissue samples were freed from visible fat and connective tissue and frozen immediately in liquid nitrogen for further analysis. Details of the infusion protocol are outlined in Fig. 2.

FED MPS



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Fasted MPS

Fig. 2. Schematic of the experimental infusion protocol performed in EB (*trial 1*), after 2 wk of ER (*trial 2*), and after 2 wk of ER + RT (*trial 3*). MPS, muscle protein synthesis (myofibrillar and sarcoplasmic); WPM, whey protein micelle drink.

PROTEIN DISTRIBUTION AND MUSCLE PROTEIN SYNTHESIS

Table 1. Baseline participant characteristics

SKEW 66 ± 4 1.74 ± 0.05 95.8 ± 13.9 21 4 ± 4.6	P 0.35 0.46 0.82
66 ± 4 1.74 ± 0.05 95.8 ± 13.9 21.4 ± 4.6	0.35 0.40 0.82
1.74 ± 0.05 95.8 ± 13.9 21.4 ± 4.6	0.46
95.8 ± 13.9	0.82
21.4 ± 4.6	0.0
51.4 ± 4.0	0.94
28.8 ± 9.0	0.93
66.0 ± 5.1	0.73
27.7 ± 2.7	0.75
5.6 ± 0.5	0.76
2.1 ± 1.1	0.23
5.2 ± 0.2	0.48
$6,880 \pm 3,899$	0.49
	31.4 ± 4.6 28.8 ± 9.0 66.0 ± 5.1 27.7 ± 2.7 5.6 ± 0.5 2.1 ± 1.1 5.2 ± 0.2 6.880 ± 3.899

Values are means \pm SD; n = 10 participants/group. BAL, balanced; SKEW, skewed; ASMM, appendicular skeletal muscle mass, HOMA; homeostatic model assessment of insulin resistance.

Analytical procedures. Blood glucose concentration was measured using a blood glucose meter (OneTouch Ultra 2; Lifescan, Milpitas, CA) within 2 min of blood collection. Plasma insulin concentration was measured using a commercially available immunoassay kit (ALPCO Diagnostics, Salem, NH). Plasma amino acid concentrations were analyzed via gas-chromatography-mass spectrometry using the Phenomenex EZfaast (Torrance, CA) amino acid analysis kit per the manufacturer's instructions. Plasma L-[ring-¹³C₆]phenylalanine enrichment was determined as described previously (19).

Myofibrillar- and sarcoplasmic-enriched protein fractions were isolated as previously described (35). Amino acids were liberated by adding 1 M HCl and DOWEX (50WX8-200 resin Sigma-Aldrich) and heating at 110°C for 72 h, with vortex mixing every 24 h. Free amino acids were purified using DOWEX ion exchange chromatography and converted to their *N*-acetyl-*n*-propyl ester derivatives for analysis by gas chromatography combustion isotope ratio mass spectrometry (Hewlett Packard 6890, IRMS model Delta Plus XP; Thermo Finnagan, Waltham, MA) as described previously (35).

Calculations. Total area under the concentration vs. time curve (TAUC) and concentration maximum (C_{max}) were calculated for insulin and for amino acid data for each protein meal (breakfast: 2–5 h, lunch: 5–8 h, dinner: 8–13 h). The FSR of myofibrillar and sarcoplasmic protein were calculated using the standard precursor-product equation:

$$FSR = \left[\left(E_{2b} - E_{1b} \right) / \left(E_{p} \times t \right) \right] \times 100$$

where E_{sb} is the protein-bound enrichment from *biopsy x* according to the protocol (Fig. 2), E_p is the mean integrated plasma L-[*ring*-¹³C₆]phenylalanine enrichment during the time period for determination of amino acid incorporation, and *t* is the tracer incorporation time in hours. The utilization of "tracer naïve" participants allowed us to use a preinfusion blood sample (i.e., a mixed plasma protein fraction) as the baseline enrichment (E_{1b}) for calculation of basal (i.e., fasted) FSR in *trial 1*, an approach that has been validated (5). *Trials 2* and *3* included a baseline muscle biopsy before the infusion began to account for changes in protein-bound enrichment from *trial 1*.

Statistical analysis. All analyses were performed using SPSS (version 22.0, Chicago, IL). The Shapiro-Wilk test was used to check data for normality. If data were not normally distributed, values were transformed by using the square root or ln of the value. The statistical analysis was performed on transformed data, but nontransformed data are presented in graphic or tabular form for clarity. Mauchly's test of sphericity was used to assess homogeneity of variances, and if this assumption was violated the Greenhouse-Geisser correction of the degrees of freedom was used. Baseline characteristics (body composition, dietary intake parameters, physical activity level) were compared between groups using an unpaired *t*-test. Myofibrillar and sarcoplasmic FSR were analyzed separately using a two-factor

(group × trial) mixed-model ANOVA for each feeding state (fasted and fed). Other variables were analyzed using a three-factor (group × trial × meal) mixed-model ANOVA, as appropriate. Significant main effects were further analyzed using simple planned contrasts. Tukey's post hoc test with a Bonferroni correction for multiple comparisons was performed whenever a significant interaction was found to isolate specific differences. Statistical significance was accepted when $P \leq$ 0.05. Results are presented as means ± SE in text and Figs. 1–4 and as means ± SD in Tables 1–4.

RESULTS

Participants. Participant characteristics are shown in Table 1. All participants who commenced the intervention completed the study and were included in the final analysis. At baseline, there were no significant differences between groups for any of the anthropometric or descriptive variables examined. All participants had HbA1c levels below the Canadian Diabetes Association prediabetes diagnostic criteria of 6.0-6.4% (18). Baseline dietary intake before beginning the study is shown in Table 2. One participant in the SKEW group failed to return his diet record, and therefore baseline dietary intake data are reported for nine participants in that group. Both groups reported consuming protein, as expected, in a skewed pattern over the day in their habitual diets, eating the majority of protein at the evening meal. At baseline, BAL reported a slightly higher number of daily eating occasions during which \geq 30 g of protein were consumed compared with SKEW (1.6 \pm 0.7 vs. 1.0 \pm 0.3 occasions/day, P = 0.034; Table 2). There were no other differences in baseline dietary intake variables between groups.

Physical activity levels and RT variables. There were no differences between groups in daily steps (BAL: 9,080 \pm 790; SKEW: 6,782 \pm 541), time spent engaged in moderate physical activity (BAL 1.9 \pm 0.3; SKEW 1.7 \pm 0.2 h/day), or average metabolic equivalents (BAL: 1.4 \pm 0.1; SKEW: 1.4 \pm 0.0) in the 3-day period before each experimental infusion trial (all P > 0.5). There were no differences between groups for the product of load (kg) \times volume (no. of repetitions) for exercises performed during the training sessions (all P > 0.5; data not shown).

 Table 2. Baseline dietary intake measured by 5-day weighed diet record

	Balanced	Skewed	P Value
Energy, kcal/day	$2,503 \pm 719$	$2,203 \pm 468$	0.30
Fat, g/day	102 ± 36	87 ± 29	0.34
Fat, g·kg body mass ⁻¹ ·day ⁻¹	1.1 ± 0.5	0.9 ± 0.3	0.36
Fat, %total energy intake	38 ± 7	35 ± 6	0.58
CHO, g/day	269 ± 97	246 ± 63	0.56
CHO, g·kg body mass ⁻¹ ·day ⁻¹	2.8 ± 0.9	2.6 ± 0.7	0.65
CHO, %total energy intake	40 ± 6	42 ± 9	0.54
Protein, g/day	108 ± 24	95 ± 16	0.18
Protein, g·kg body mass ⁻¹ ·day ⁻¹	1.1 ± 0.2	1.0 ± 0.2	0.23
Protein, g·kg FFM ⁻¹ ·day ⁻¹	1.6 ± 0.3	1.4 ± 0.3	0.19
Protein, %total energy intake	18 ± 3	18 ± 4	0.99
Alcohol, %total energy intake	4 ± 5	4 ± 4	0.88
Daily eating occasions	4.8 ± 0.9	4.9 ± 1.2	0.82
Eating occasions ≥ 30 g protein	1.6 ± 0.7	1.0 ± 0.3	0.03
Breakfast protein content, g	16 ± 12	13 ± 5	0.32
Lunch protein content, g	30 ± 7	26 ± 12	0.36
Dinner protein content, g	54 ± 10	47 ± 12	0.17
Prebed meal protein content, g	3 ± 4	3 ± 4	0.75

Values are means \pm SD; n = 10 (BAL) and 9 (SKEW). CHO, carbohydrate; FFM, fat-free mass.

Changes in body composition and anthropometry. A post hoc power calculation demonstrated that, with our sample size of n = 10/group, we only had 27% power to detect betweengroup differences in body composition changes. Therefore, the body composition data were pooled for analysis. Total body mass decreased over the 4-wk hypocaloric feeding intervention (P < 0.01), and body mass loss was greater in Phase 1:ER (pooled mean change: -2.5 ± 0.3 kg) than Phase 2:ER + RT (pooled mean change: -1.4 ± 0.2 kg; main effect for phase < 0.01). Body fat decreased over the intervention (P <0.001) with no difference between phases (Phase 1:ER $-1.3 \pm$ 0.2 kg, Phase 2:ER + RT -1.1 ± 0.2 kg; P = 0.75). Whole body lean mass decreased over Phase 1:ER (pooled mean change -1.1 ± 0.1 kg, P = 0.003) of the intervention; however, there were no further changes in Phase 2:ER + RT (pooled mean change -0.2 ± 0.1 kg, P = 0.64). Trunk lean mass decreased in a similar pattern to whole body lean mass with a loss occurring over Phase 1:ER (pooled mean change -0.8 ± 0.0 kg, P = 0.001) but no further change occurring during Phase 2:ER + RT (pooled mean change -0.2 ± 0.1 kg, P = 0.523). ASMM (legs and arms) was unchanged in both phases.

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Plasma insulin. Fasting insulin concentration and homeostatic model assessment of insulin resistance (HOMA-IR) were similar in BAL and SKEW in all trials. There was a trial × group interaction (P < 0.05) such that in BAL fasting insulin concentration and HOMA-IR were reduced in *trial* 2 and *trial* 3 compared with *trial* 1 (P < 0.01), whereas in SKEW fasting insulin and HOMA-IR decreased in *trial* 2 (P < 0.05) but returned to baseline levels in *trial* 3. There was a meal × group interaction for plasma insulin such that TAUC was greater in BAL than SKEW following breakfast and lunch (P < 0.05), but there was no difference between groups following the dinner meal (P = 0.49; Table 3).

Plasma amino acids. The TAUC and C_{max} for plasma \geq total amino acids (TAA), \geq essential amino acids (EAA), and leucine in response to the three protein meals consumed during each experimental trial day are presented in Table 4. There was a meal \times group interaction such that TAUC and C_{max} for \geq TAA, \geq EAA, and leucine were greater in BAL than SKEW following breakfast and greater in SKEW than BAL following dinner (P < 0.05). TAUC and C_{max} after lunch were also greater in BAL than SKEW for \geq EAA and leucine, and heucine, and the flucture of \geq EAA and leucine, and \geq EAA and \geq EAAA

simple planned contrasts revealed that TAUC and C_{max} were greater in *trial 2* (ER) than *trial 1* (EB; P < 0.01) and *trial 3* (ER + RT; P < 0.01). There was a trial × group interaction for \sum TAA such that C_{max} was greater in *trial 2* than the other trials in SKEW (P < 0.01) but not in BAL (Table 4). Plasma concentrations of leucine over time are illustrated in Fig. 3. No statistical analysis was performed on the concentration vs. time data.

Muscle protein synthesis. In the fasted state, there was a main effect for trial (P = 0.008) but not for group (P = 0.352) for myofibrillar FSR. Simple planned contrasts revealed that myofibrillar FSR was $\sim 14\%$ lower in ER (trial 2) and ER + RT (trial 3) vs. EB (trial 1; P < 0.05; Fig. 4A). In the fed state, there were main effects for trial (P = 0.000) and group (P =0.007) and a trial \times group interaction for myofibrillar FSR (P = 0.035). Tukey's post hoc test revealed that myofibrillar FSR was lower in ER than EB in both groups (P = 0.000) but was ~19% higher in BAL than SKEW (P = 0.014). In ER + RT, fed-state myofibrillar FSR increased above ER in both groups (P < 0.05) and in BAL was not different from EB (P = 0.903). In contrast, in SKEW myofibrillar FSR remained ~14% lower than EB (P = 0.002) and ~16% lower than BAL (P = 0.006, Fig. 4C). For sarcoplasmic FSR, in both the fasted and fed state, there was a main effect for trial (P = 0.000) but not for group. Simple planned contrasts revealed that sarcoplasmic FSR was reduced to a similar extent in ER and ER + RT compared with EB in both the fasted ($\sim 22\%$) and the fed state (~19%, P < 0.01, Fig. 4, B and D).

DISCUSSION

This study demonstrates for the first time that, during ER in overweight/obese older men, consumption of a balanced distribution of daily protein intake (i.e., 3×25 g evenly spaced doses of protein) acutely stimulated the synthesis of muscle contractile proteins more effectively than a skewed distribution of the same amount of protein (i.e., 10 g at breakfast, 15 g at lunch, 50 g at dinner). Furthermore, we show that combining RT with a balanced protein distribution restored depressed rates of myofibrillar protein synthesis during ER to those observed during EB.

The recommendation of ER in overweight older adults remains controversial (49), primarily because of concerns that weight loss to improve metabolic health in overweight older

Table 3. Fasting plasma insulin concentrations and TAUC plasma insulin in response to breakfast, lunch, and dinner consisting of BAL or SKEW distribution of protein intake

	Trial 1		Trial 2		Tric	Trial 3		
	BAL ^{\$}	SKEW	BAL ^{\$}	SKEW	BAL ^{\$}	SKEW	Meal $ imes$ Group	Trial \times Group
Fasting Insulin, µU/ml TAUC	15.2 ± 2.8	8.4 ± 2.8	10.8 ± 2.4	$7.1\pm2.4^{\rm S}$	10.0 ± 2.2	8.4 ± 2.2	0.00	0.03
Breakfast*† Lunch* Dinner#	$87 \pm 44 \\ 68 \pm 27 \\ 93 \pm 49$	$35 \pm 21 \\ 35 \pm 20 \\ 93 \pm 54$	$66 \pm 44 \\ 56 \pm 32 \\ 75 \pm 43$	$28 \pm 12 \\ 30 \pm 16 \\ 86 \pm 41$	$62 \pm 35 \\ 44 \pm 24 \\ 63 \pm 33$	$28 \pm 11 \\ 31 \pm 15 \\ 87 \pm 42$		

Values are means \pm SD; n = 10 participants/group. TAUC, total area under the concentration vs. time curve. Data were analyzed using a 3-factor (group × trial × meal) mixed-model ANOVA with simple planned contrasts and Tukey's post hoc test where appropriate. Plasma insulin concentrations were measured immediately before and 30 and 60 min after all meals and additionally 120 and 180 min after breakfast and lunch, and 240 min after dinner during *trial* 1 (energy balance), *trial* 2 (after 2 wk of energy restriction), and *trial* 3 (after 2 wk energy restriction + resistance training). Postprandial plasma insulin concentrations are expressed as TAUC (μ U·ml^{-1.4} h⁻¹ for breakfast and lunch, μ U·ml^{-1.4} h⁻¹ for dinner). *Different from other meals in SKEW only. *Different from other trials.

Table 4. TAUC and C_{max} plasma ΣTAA , ΣEAA , and leucine in response to breakfast, lunch, and dinner consisting of BAL or SKEW distribution of protein intake

	Trial 1		Trial 2 Trial 3		Trial 2				
	BAL	SKEW	BAL	SKEW	BAL	SKEW	Trial	ıl Meal × Group	Trial $ imes$ Group
TAUC									
TAA							0.09	0.00	0.17
Breakfast*	$11,004 \pm 7,093$	$7,503 \pm 1,866$	$9,566 \pm 566$	$9,401 \pm 1,103$	$8,847 \pm 1,215$	$7,306 \pm 547$			
Lunch	$8,948 \pm 2,639$	$8,127 \pm 1,985$	$9,037 \pm 1,448$	$9,248 \pm 774$	$8,858 \pm 1,074$	$7,876 \pm 843$			
Dinner*†	$14,027 \pm 3,429$	$16,466 \pm 3,810$	$14,386 \pm 1,613$	$18,819 \pm 1,799$	$14,130 \pm 1,682$	$15,322 \pm 1,626$			
EAA ^{\$}							0.01	0.00	0.19
Breakfast*#	$3,774 \pm 674$	$2,806 \pm 769$	$4,252 \pm 371$	$3,828 \pm 318$	$3,806 \pm 500$	$2,905 \pm 197$			
Lunch*#	$3,998 \pm 1,029$	$3,297 \pm 875$	$4,160 \pm 787$	$3,983 \pm 302$	$3,995 \pm 407$	$3,347 \pm 392$			
Dinner*†	$6,248 \pm 1,080$	$7,909 \pm 2,124$	$6,631 \pm 714$	$9,220 \pm 732$	$6,395 \pm 646$	$7,593 \pm 949$			
Leucine ^{\$}							0.01	0.00	0.26
Breakfast*†	781 ± 110	489 ± 122	899 ± 113	670 ± 66	784 ± 104	500 ± 34			
Lunch*†	941 ± 157	664 ± 174	975 ± 201	800 ± 80	898 ± 63	624 ± 63			
Dinner*†	$1,442 \pm 191$	$1,918 \pm 534$	$1,523 \pm 198$	$2,189 \pm 159$	$1,433 \pm 182$	$1,821 \pm 205$			
Cmax									
TAA‡							0.00	0.00	0.04
Breakfast*	$3,605 \pm 657$	$3,218 \pm 704$	$4,059 \pm 265$	$4,176 \pm 526$	$3,881 \pm 615$	$3,100 \pm 303$			
Lunch	$3,859 \pm 888$	$3,548 \pm 677$	$3,914 \pm 534$	$4,048 \pm 445$	$3,729 \pm 638$	$3,377 \pm 284$			
Dinner*#	$4,082 \pm 925$	$4,193 \pm 687$	$4,165 \pm 653$	$5,059 \pm 462$	$3,786 \pm 313$	$4,186 \pm 644$			
EAA ^{\$}							0.00	0.00	0.06
Breakfast*#	$1,618 \pm 256$	$1,318 \pm 302$	$1,921 \pm 159$	$1,838 \pm 211$	$1,803 \pm 295$	$1,326 \pm 120$			
Lunch*#	$1,832 \pm 438$	$1,534 \pm 312$	$1,933 \pm 338$	$1,905 \pm 230$	$1,769 \pm 265$	$1,542 \pm 149$			
Dinner*†	$2,008 \pm 349$	$2,155 \pm 434$	$2,098 \pm 325$	$2,658 \pm 150$	$1,905 \pm 154$	$2,182 \pm 389$			
Leucine ⁸							0.00	0.00	0.16
Breakfast*†	367 ± 53	257 ± 50	436 ± 53	363 ± 67	403 ± 64	258 ± 27			
Lunch*†	454 ± 102	336 ± 71	486 ± 83	426 ± 62	423 ± 49	330 ± 26			
Dinner*†	499 ± 69	558 ± 116	542 ± 97	673 ± 48	479 ± 43	554 ± 91			

Values are means \pm SD; n = 10 participants/group. C_{max} , concentration maximum; TAA, total amino acids; EAA, essential amino acids. Data were analyzed using a 3-factor (group \times trial \times meal) mixed-model ANOVA with simple planned contrasts and Tukey's post hoc test where appropriate. Plasma amino acids concentrations were measured immediately before and 15, 30, 45, 60, 120, and 180 min after all meals and additionally 240 and 300 min after diment during trial 1 (energy balance), trial 2 (after 2 wk of energy restriction), and trial 3 (after 2 wk energy restriction + resistance training). Plasma amino acid concentrations are expressed as TAUC (nmol-ml⁻¹.3 h⁻¹ for breakfast and lunch, nmol-ml⁻¹.5 h⁻¹ for dinner) and C_{max} (nmol/ml). *Different between groups. †Different from other meals within the same group. #Different from other meals in SKEW only. *Different from other trials in SKEW only.

adults, while associated with health benefits, may exacerbate muscle loss (4, 49). Several studies have examined the effect of ER on MPS (1, 9, 37, 38, 46) with equivocal results. Such a lack of consistency between the results from studies may be the result of differences in dietary interventions, the methodologies used to assess MPS, the conditions under which MPS measurements were performed, and the characteristics of the population studied. Nonetheless, short-term studies have shown a decline in rested fasted MPS with ER (1, 38) and a reduced capacity to stimulate MPS (37). Similarly, in the present study we observed that short-term ER in older men was accompanied

by a reduction in fasted- and fed-state MPS in both the myofibrillar and sarcoplasmic protein fractions. Because MPS is an energetically expensive process, the ER-induced down-regulation of fasted- and fed-state MPS rates may reflect an adaptive response to conserve energy and protein reserves. Taken together, these data suggests that preservation of the MPS response during ER is a viable goal to support the maintenance of muscle mass while allowing for clinically indicated fat loss in a variety of populations.

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Accumulating evidence indicates that consuming dietary protein at levels beyond the RDA of $0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ atten-



Fig. 3. Plasma concentrations (nmol/ml) of leucine in the BAL and SKEW protein consumption groups in EB (*trial 1, A*), after 2 wk of ER (*trial 2, B*), and after 2 wk of ER + RT (*trial 3, C*). Arrows indicate a protein meal. Values are means (error bars omitted for clarity), n = 10 participants/group.



Fig. 4. Myofibrillar (A and C) and sarcoplasmic (B and D) protein fractional synthetic rate (FSR, %/h) in the fasted (A and B; 0–2 h) and fed (C and D; 2–13 h) state in BAL and SKEW protein consumption groups in EB (*trial* 1), after 2 wk of ER (*trial* 2), and after 2 wk of ER + RT (*trial* 3). Note the difference in scales of the axes in A and B vs. C and D. Values are means \pm SE, n = 20 (10 participants/group). Dissimilar letters demonstrate within-group differences (P < 0.05). *Different from SKEW in the same trial (P < 0.05).

uates lean mass loss during periods of ER in young and older adults (27, 33, 52). The mechanism for this effect is unknown but may be through preservation of the anabolic sensitivity of skeletal muscle to protein-containing meals (37). Our data demonstrate that the quantity of protein consumed over the day is not the sole determinant of the potential to stimulate MPS in conditions of ER and suggest that the distribution of daily protein intake may also be important in attenuating the ERinduced decrement in myofibrillar protein synthesis when measured acutely. Indeed, although myofibrillar protein synthesis decreased with both patterns of protein feeding following 2 wk of ER (Phase 1) in the fed state, this reduction was less pronounced when protein intake was consumed in a balanced/ even pattern across daily meals compared with the traditional dietary pattern of skewed protein intake at the evening meal (Fig. 4*C*).

We observed that a balanced pattern of protein consumption was even more effective when combined with RT, resulting in the restoration of myofibrillar protein synthesis to levels observed during EB. This synergistic effect of a balanced protein distribution and RT may be attributed to the fact that resistance exercise sensitizes the muscle protein synthetic machinery to protein feeding, resulting in an enhanced MPS response (7, 35), an effect that has also recently been demonstrated under conditions of ER in young adults (1). Our data extend these previous findings and show that, during ER, the synergistic effect of RT and protein feeding is still present 48 h after the last exercise bout. In the present study the effect of daily protein distribution alone and in combination with RT was specific to the myofibrillar fraction, and we observed no influence of protein distribution pattern on the rate of sarcoplasmic protein synthesis. These findings are perhaps to be expected, since previous work has demonstrated that sarcoplasmic protein synthesis is less responsive to amino acid availability (14, 32) and resistance exercise than myofibrillar proteins (8, 35). Moreover, maintenance of myofibrillar protein synthetic rates is arguably of greater priority given that it is the loss of contractile proteins with aging that plays a greater role in the decrease in muscle mass and strength underpinning sarcopenia (40).

The superior capacity of a balanced protein distribution to stimulate myofibrillar protein synthesis over the day in ER, both in the presence and absence of prior resistance exercise, was likely the result of greater stimulation following breakfast and lunch in BAL than SKEW during the infusion trials. In support of this hypothesis, we observed that the TAUC and C_{max} for $\sum EAA$ and for leucine were greater in the BAL compared with the SKEW group following breakfast and lunch. An increase in plasma amino acid concentrations is a potent stimulator of MPS, an effect that is graded, saturable, and primarily attributable to the EAA (48). Furthermore, leucine plays a unique role as a key activator of the translation initiation process (23), and accumulating evidence supports the notion that a minimum concentration of leucine (systemic or intracellular) threshold must be surpassed to stimulate MPS in response to feeding (10, 34, 51). As such, the greater fed-state

myofibrillar protein synthetic response in the balanced group compared with the skewed group during ER and ER + RT is likely attributable to the aminoacidemia during the infusion trials, rather than a chronic effect resulting from the consumption of daily protein intake in a balanced pattern throughout the 4-wk hypocaloric feeding period.

It could also be speculated that the greater postprandial insulinemia following the breakfast and lunch protein feedings in the balanced group compared with the skewed group may be, at least in part, responsible for the higher MPS in BAL during the ER and ER + RT infusion trials. Indeed, the feeding-induced rise in plasma insulin concentration represents a key factor driving postprandial perfusion, allowing subsequent amino acid delivery to the muscle and/or activating anabolic signaling (29). Nevertheless, the role of insulin appears to be permissive rather than stimulatory in the presence of hyperaminoacidemia (44). Gorissen et al. recently reported that carbohydrate coingestion with dietary protein did not modulate MPS in older adults despite an increase in plasma insulin concentrations from ${\sim}18~\mu\text{IU/ml}$ (protein alone) to $-65 \mu IU/ml$ (protein + carbohydrate) (20). Therefore, it appears unlikely that the peak insulin concentrations of ~18-27 µIU/ml following the breakfast and lunch protein feedings in the skewed group would have limited the MPS response in the current study.

In contrast to the ER and ER + RT conditions, we observed no influence of protein distribution under conditions of EB. This is in agreement with recently published work by Kim and colleagues showing that 24-h mixed MPS was similar with an even (33:33:33% total protein at breakfast/lunch/dinner) vs. a skewed (15:20:65% total protein at breakfast/lunch/dinner) pattern of protein intake in older adults under conditions of EB (22). The latter findings, in addition to ours, are in contrast to a study by Mamerow et al. showing that the consumption of -30 g of protein at each mixed macronutrient meal stimulated 24-h mixed MPS to a greater extent than skewing protein intake toward the evening meal in younger adults in EB (28). It is difficult to explain the discrepancy between the results of these studies. However, one possible explanation may be the provision of an insufficient dose of protein per meal in the studies examining older adults. Whereas the exact amount of protein required to maximally stimulate MPS in older adults is unclear, previous estimations put the per meal dose at 30-40 g (39, 41, 42). Recently, we suggested a dose of ${\sim}0.4~{\rm g}$ protein·kg⁻¹·meal⁻¹ or 0.6 g protein·kg FFM⁻¹·meal⁻¹ in older persons (34). For the participants in the current study, this recommendation would be equivalent to ~40 g/meal on average, with estimated optimal intakes of up to ~ 48 g/meal in some of the heavier participants. Therefore, the per meal protein dose of 25 g of WPM (the decision on which was made before our previous results becoming available) consumed by the BAL group during our acute feeding protocol was likely insufficient to maximally stimulate MPS. Although the per meal protein intakes in the even/balanced groups in the study by Kim et al. were $\sim 0.3-0.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{meal}^{-1}$, protein was provided in the context of mixed macronutrient meals which, because of alterations in amino acid absorption and uptake kinetics, may further increase the quantity of protein required to maximally stimulate MPS (22, 51). In contrast, the 30 g dose of protein provided per meal in the Mamerow study equates to ~ 0.39 g/kg, which is $\sim 60\%$ higher than the 0.24 $g \cdot kg^{-1} \cdot meal^{-1}$ dose reported to maximally stimulate MPS in young adults (34) and was therefore likely sufficient even though protein was provided in mixed meals.

Intriguingly, that we observed greater myofibrillar protein synthesis over the day with a balanced protein distribution than a skewed distribution under conditions of ER and ER + RT in the present study, even despite a potentially suboptimal per meal protein dose, indicates that the distribution of daily protein likely becomes increasingly important in weight loss situations in older adults. Alternatively, that the influence of protein distribution on myofibrillar protein synthesis was specific to the ER conditions may relate to a potentially greater level of insulin resistance at baseline in the BAL group. Although we did not directly measure insulin resistance in the current study, fasting insulin concentration and HOMA-IR score were higher (although not statistically) in the BAL group compared with the SKEW group on trial 1 (EB). As such, it could be argued that the insulin-mediated increase in endothelial-dependent vasodilation and subsequent amino acid delivery to muscle may have been impaired to a greater extent in the BAL group, thus attenuating the MPS response (44). If this were the case, it is possible that it may account for our inability to detect a favorable effect of the BAL protein distribution on myofibrillar protein synthesis in trial I (EB), whereas during trial 2 (ER) and trial 3 (ER + RT) when fasting insulin concentration and HOMA-IR were more similar between groups (Table 3) a positive influence of BAL protein distribution on myofibrillar protein synthesis was apparent. In opposition to this notion, however, a number of studies to date report no influence of measures of insulin sensitivity and glycemic control on the postprandial MPS response (7, 8).

To date no study has examined the influence of protein distribution on MPS in conditions of ER, and further work is needed to elucidate the mechanisms as to why a balanced protein feeding pattern was beneficial. It will be important in future studies to confirm the influence of protein distribution on MPS during ER in the context of mixed meals comprised of real foods. An assumption of the constant labeled amino acid infusion technique used to measure MPS in the current study is that the tracer labeling in the precursor pool remains in a relative steady state during the infusion protocol. Because mixed meals have unpredictable amino acid absorption kinetics, we opted to feed isolated protein during the infusion trials to minimize disturbances in the precursor pool tracer enrichment. This study was designed to be a tightly controlled, "proof of principle" study to evaluate the MPS response to two different patterns of protein intake. As such, it should be noted that the metabolic responses during the infusion trials (insulinemia, aminoacidemia, etc.) do not fully reflect the BAL and SKEW diets participants were fed between trials.

In conclusion, we demonstrate that, during ER in older men, a balanced distribution of daily protein in amounts previously shown to increase MPS in elderly persons (12) acutely stimulated the synthesis of muscle contractile proteins more effectively than a skewed protein intake with consumption of the majority of protein in the evening meal. Combining RT with a balanced protein distribution rescued rates of myofibrillar protein synthesis during ER to the levels observed during EB. Although further work is required to determine whether our acute observations translate to mixed macronutrient meals and into a long-term functional response, we contend that the

combination of RT and a balanced distribution of daily protein in the context of a higher protein diet may represent an effective strategy to allow for fat mass loss during ER without exacerbating sarcopenic muscle loss. In the face of the rising rates of obesity among the growing aging population, these results have potential implications for clinical practice in healthcare professionals working with community-dwelling and institutionalized older adults who have indications for weight loss.

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DISCLOSURES

Both Kassis and Karagounis are employees of Nestec SA a subsidiary of Nestle who was a linkage partner in this grant.

AUTHOR CONTRIBUTIONS

Author contributions: C.H.M., A.K., L.G.K., L.M.B., J.A.H., and S.M.P. conception and design of research; C.H.M., T.A.C.-V., C.J.M., N.M.K., and S.M.P. performed experiments; C.H.M., T.A.C.-V., C.J.M., N.M.K., J.A.H., and S.M.P. interpreted results of experiments; C.H.M. and S.M.P. prepared figures; C.H.M., L.M.B., J.A.H., and S.M.P. drafted manuscript; C.H.M., T.A.C.-V., C.J.M., N.M.K., L.G.K., L.M.B., J.A.H., and S.M.P. edited and revised manuscript; C.H.M., T.A.C.-V., C.J.M., N.M.K., L.G.K., J.A.H., and S.M.P. and S.M.P. and S.M.P. drafted manuscript; C.H.M., T.A.C.-V., C.J.M., N.M.K., A.K., LG.K., L.M.B., J.A.H., and S.M.P. edited and revised manuscript; C.H.M., T.A.C.-V., C.J.M., N.M.K., A.K., L.G.K., J.A.H., and S.M.P. edited and revised manuscript.

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CHAPTER 3:

Longer-term integrated myofibrillar protein synthetic rates and proteome kinetics are altered by resistance training but not dietary protein distribution in older men

during weight loss. In preparation.

Longer-term integrated myofibrillar protein synthetic rates and proteome kinetics are altered by resistance training but not dietary protein distribution in older men during weight loss

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Running Head: Integrative muscle protein synthesis during weight loss

ABSTRACT

We previously showed that during energy restriction (ER) a balanced consumption of protein stimulated myofibrillar protein synthesis (MyoPS) more effectively than a skewed distribution in older men performing resistance training (RT). We aimed here to identify how dietary protein distribution and RT influenced longer-term (2wk), integrated MyoPS during ER. Longer-term MyoPS and proteome kinetics were measured during 2-wk of ER alone (Phase 1:ER) and 2-wk of ER plus RT (Phase 2:ER+RT), in overweight/obese older men ($66 \pm 4 \text{ y}$, $31 \pm 5 \text{ kg/m}^2$). Participants who were randomized to consume their dietary protein in either a balanced (BAL: 25% daily protein/meal x 4 meals) or skewed (SKEW: 7:17:72:4% daily protein/meal) pattern (n = 10/group). Participants ingested 2 H₂O and skeletal muscle biopsies obtained at the beginning and conclusion of ER and ER+RT were used to quantify integrative MyoPS and the synthesis of individual skeletal muscle proteins. Longer-term MyoPS was not affected by protein distribution pattern during either Phase 1 or 2 (Phase 1 - ER: BAL= $1.24 \pm 0.31\%/d$, SKEW= $1.26 \pm 0.37\%/d$; Phase 2 - ER + RT: BAL=1.64 ± 0.48%/d, SKEW=1.52 ± 0.66%/d). However, MyoPS was ~26% higher during Phase 2:ER+RT than Phase 1:ER (p = 0.023). The synthesis of 175 (of 190 measured) individual contractile, cytosolic and mitochondrial skeletal muscle proteins were significantly higher during ER+RT than ER, with no difference between the BAL and SKEW groups. We report that RT, but not the pattern of daily protein ingestion, increased longer-term MyoPS and the synthesis of a number of individual skeletal muscle proteins. This study demonstrates the potency of the RT stimulus and supports the importance of its inclusion during weight loss in older adults.

INTRODUCTION

Sarcopenia, the progressive loss of skeletal muscle mass with age, is associated with a decline in strength and functional capacity, and increased risk for numerous adverse health and quality of life-based outcomes (1, 2). The condition of sarcopenia is made more complex with the prevalence of obesity in older adults (3-5). In older adults obesity is associated with increased risk for comorbidities (6-8) and exacerbates age-related functional decline and disability risk (9), particularly when superimposed on sarcopenia (10, 11). The recommendation of weight loss in overweight older adults remains somewhat controversial (12), primarily due to concerns that dietary energy restriction (ER) interventions designed to reduce excess adiposity may simultaneously accelerate muscle loss (12, 13). Consequently, there is a critical need to identify lifestyle strategies that support weight loss with a high ratio of fat-to-lean loss in older adults (7).

Both aging (14) and ER (15) have been shown to attenuate the muscle protein synthetic response to protein feeding. Recently, we reported that during ER in obese older men a balanced ingestion of dietary protein more effectively stimulated the acute synthesis of myofibrillar proteins than a skewed distribution (16). Furthermore, we showed that combining resistance training (RT) with a balanced protein distribution restored the lower rates of myofibrillar protein synthesis (MyoPS) during ER to those observed during energy balance (EB). These data support the possibility that combining RT and a balanced distribution of daily protein during ER would represent an effective strategy to slow or abate muscle loss during weight loss in older persons. Nevertheless, the short-term (13 h), laboratory-based nature of the MyoPS measurements restricted our

ability to extrapolate our findings since they do not account for all aspects of daily activity and diet. A more novel approach to determine muscle protein synthesis involves the oral administration of deuterium oxide (D_2O or 2H_2O) which can be used to measure the integrated response over a longer time period among free-living participants. Furthermore, methods were recently described that combine D_2O incorporation with a proteomics approach for the dynamic assessment of the synthesis of individual skeletal muscle proteins (17, 18). This approach can, for the first time, capture the integrated responses of numerous myofibrillar, sarcoplasmic and mitochondrial proteins to an intervention providing unprecedented insight into skeletal muscle adaptations (19).

The purpose of the current study was to examine the impact of daily protein distribution on the longer-term, integrated rate of MyoPS and the synthesis of individual skeletal muscle proteins over 2-wk of ER alone and 2-wk of ER+RT in overweight and obese older men. We hypothesized that, in accordance with our acute findings, a balanced distribution of dietary protein intake throughout the day would stimulate longer-term MyoPS (%/d) and the synthesis of individual myofibrillar skeletal muscle proteins (%/d) to a greater magnitude compared to a skewed distribution and that this effect would be enhanced while undertaking RT.

PARTICIPANTS AND METHODS

Experimental design. Details regarding the participants and controlled diet and physical activity interventions have been reported previously (16). Briefly, after providing informed, written consent, 20 overweight and obese but generally healthy older men (age

 66 ± 4 yr, BMI 31 ± 5 kg/m²) underwent a controlled 4-wk hypocaloric diet (300kcal/d less than estimated energy requirements, 1.3 g protein/kg/d from mixture of plant and animal sources; Figure 1). Participants were randomly allocated to one of two groups (n=10/group) matched for age and BMI: balanced (BAL) or skewed (SKEW). Total protein intake was distributed across four daily meals (breakfast, lunch, dinner, pre-bed snack) in the proportions 25:25:25:25% in participants in the BAL group and 7:17:72:4% in the SKEW group. In BAL, a ready-to-drink whey protein micelle (WPM) beverage (25g protein; Nestle, Lausanne, Switzerland) was consumed as part of breakfast and as a pre-bed snack in order to achieve target protein intakes at these meals. SKEW received their total daily protein intake from food sources only and consumed a protein-free, low energy placebo drink (0.2 g protein; Nestle, Lausanne, Switzerland) matched for appearance, smell and taste to the WPM beverage with breakfast and pre-bed. The 4-wk intervention consisted of two, 2-wk phases. In weeks 1 and 2 all participants were in energy restriction (Phase 1:ER) and continued their habitual physical activity. In weeks 3 and 4, while still energy restricted, all participants commenced a supervised, whole body, resistance training program 3 d/wk (Phase 2:ER+RT).

Isotope protocol. ²H₂O labeling of newly synthesized skeletal muscle proteins was achieved using oral consumption of ²H₂O which commenced on the first day of the ER diet and was continued throughout the entire 4-wk intervention. A target deuterium (²H) enrichment in total body water of 1–2% was achieved during the first 5 d (60 mL 3 times/d = 180 mL/d) and was maintained for the remaining 23 d of the intervention (60 mL/d). All 60 mL doses were separated by at least 3 h. Total body water ²H enrichment

can be used as a surrogate for plasma alanine labeling (18, 20, 21) and was determined from saliva swabs collected at every second day throughout the 4-wk labeling period as described previously (22, 23). Participants were instructed not to eat or drink anything for 30 min before saliva sampling and samples were stored at -80°C until analysis. Muscle biopsies were obtained from the *vastus lateralis* at on days 0, 14 (end of Phase 1:ER) and 28 (end of Phase 2:ER + RT) (Figure 1).



Figure 1. Schematic overview of study design adapted from Murphy et al. (16). DEXA, dual x-ray absorptiometry; EB, energy balance; ER, energy restriction; ER+RT, energy restriction and resistance training.

Body water enrichment. ²H enrichment in saliva was determined using a previously described method (17). Saliva samples were diluted 1:100 and placed into the caps of inverted sealed screw-capped vials for overnight distillation at 80°C. Body water ²H

enrichments are determined by direct measurement of deuterium molar percentage excess (MPE) in water distilled from the saliva against a ${}^{2}\text{H}_{2}\text{O}$ standard curve using a laser water isotope analyzer (Los Gatos Research [LGR], Los Gatos, CA, USA).

Myofibrillar protein synthesis. Myofibrillar-enriched proteins were isolated as previously described (24). Amino acids were liberated by adding 1 M HCL and DOWEX (50WX8-200 resin Sigma-Aldrich Ltd) and heating at 110°C for 72 h, with vortex mixing every 24 h. Free amino acids were purified using DOWEX ion exchange chromatography, and converted to their pentafluorobenzyl-N,N-di(pentafluorobenzyl)-NEAA derivatives (PFB derivatives) as described previously (25). Gas chromatography mass spectrometry (GCMS) was performed in negative chemical ionization (NCI) mode with helium as the carrier gas, and mass-to-charge (m/z) ratios 424-426 corresponding to the M0, M1 and M2 mass isotopomers of derivatized alanine were analyzed by selected ion monitoring (SIM).

Excess fractional M+1 enrichment (EM1) was the normalized change in isotopomer intensity calculated as:

 $EM1 = [(M1)_{sample}/(M0+M1)_{sample}] - [(M1)_{standard}/(M0+M1)_{standard}]$

where sample and standard refer to the sample and an unenriched pentafluorobenzyl triacetyl alanine derivative, respectively. The fraction of myofibrillar protein that was newly synthesized during the labeling period (f) was calculated as the ratio of the measured EM1 to the asymptotic value of EM1 (EM1_{max}), the latter representing EM1 in alanine in fully turned-over proteins at the ${}^{2}H_{2}O$ enrichment measured in the system and calculated as $f = EM1_{sample}/EM1_{max}$, as described previously (25).

To calculate absolute rates of whole body myofibrillar protein synthesis, skeletal muscle mass was estimated according to the model of Kim et al. (26) using dual energy X-ray absorptiometry (DEXA; QDR-4500A, software version 12.31, Hologic, Bedford, MA) scans obtained at baseline, at the end of Phase 1:ER and at the end of Phase 2:ER+RT. Assuming that muscles are 18% protein and myofibrillar protein accounts for 66% of the total, we calculated whole body myofibrillar protein synthesis in g/d as shown by the following equation (27):

Absolute rate of MyoPS $(g/d) = [(muscle mass (kg) \times proportion of myofibrillar protein per kg muscle) \times myofibrillar FSR (%/d)] * 1000$

LCMS/MS analysis for proteome dynamics. Muscle samples were thawed and homogenized for 75 s in PBS containing 1 mM PMSF and 5 mM EDTA using a Mini-BeadBeater 8 (BioSpec, Bartlesville, OK) placed on ice for 1 min. This procedure was repeated twice and the resulting homogenate is diluted to 10% (w/v) in PBS containing 1 mM PMSF. Protein from prepared homogenates was uniformly reduced by incubation in 10 mM DTT and SDS-PAGE sample loading buffer for 5 min at 95 °C. The reduced samples were then alkylated by incubating in 15 mM iodoacetamide for 1 h at room temperature. Proteins were then fractionated by SDS-PAGE (BioRad). Using in-gel molecular weight markers, each sample was divided into 10 molecular weight regions and subjected to overnight trypsin digestion at 37°C (Trypsin Gold, Promega, Madison, WI). The peptides from the resulting samples are extracted from the gel, dried, reconstituted in 5% acetonitrile/5% formic acid for analysis by LCMS.

The isotopic distributions of peptides were measured using an Agilent 6520QToF with Chip Nano source (Agilent, Santa Clara CA). Each sample was injected two times per analysis. Mobile phase for the LC is 3% v/v acetonitrile, 0.1% formic acid, in 18MOhm water (Buffer A) and 95% acetonitrile, 0.1% formic acid in 18M Ω water (Buffer B). During the first injection, data dependent MSMS fragmentation spectra were collected with the instrument set to collect 4 MS scans per second with up to 6 MSMS spectra from each scan. MSMS fragmentation data were analyzed using the Agilent software package Spectrum Mill (B0.3) and protein identifications was based on the Uniprot/Swissprot database (08/2010). The kinetic information in the isotopomer patterns was extracted from the MS scan data using the Mass Hunter software package (B0.4) from Agilent. The peptide list with calculated neutral mass, elemental formula, and retention time was used to filter the observed isotope clusters. A visual basic application was used to calculate peptide elemental composition from lists of peptide sequences and to calculate isotopomer patterns over a range of precursor body ${}^{2}H_{2}O$ enrichments (p), for the number (n) of C-H positions actively incorporating ²H from body water. Fractional synthesis rates of proteins were calculated by deconvoluting the mass isotopomer pattern of newly-synthesized species as compared to unlabeled species, as described previously (17).

Statistical analyses. All analyses were performed using SPSS (version 22.0, Chicago, IL, USA). Skeletal muscle mass data were analyzed using a 2x3 (group x time) mixed-model ANOVA. MyoPS was analyzed using a 2x2 (group x phase) mixed-model ANOVA. Proteome kinetic data were analyzed using a 2x2 (group x phase) mixed-model ANOVA,

differences were considered significant with a false discovery rate of 0.2 after a Benjamini-Hochberg procedure was performed to adjust for the multiple comparisons. Statistical significance was accepted when $p \le .05$. Results are presented as means \pm SD.

RESULTS

Myofibrillar protein synthesis. Relative MyoPS (%/d) was similar between the BAL and SKEW groups (p = 0.75) in both the ER and ER+RT phases, however, there was a main effect for phase (p = 0.023) such that MyoFSR was higher during ER+RT (BAL 1.64 ± 0.48 ; SKEW 1.52 ± 0.66 %/d) than ER (BAL 1.24 ± 0.31 ; SKEW 1.26 ± 0.37 %/d; Figure 2 A). Estimated skeletal muscle mass was similar between groups (p = 0.95) and did not change over the 4-wk intervention (baseline: 31.8 ± 3.4 kg, end of Phase 1: 31.6 ± 3.6 kg, end of Phase 2: 31.6 ± 3.8 kg; p = 0.42). Absolute MyoPS (g/d) was also greater during ER + RT (BAL 62 ± 20 ; SKEW 56 ± 23 g/d) than ER (BAL 47 ± 13 ; SKEW 47 ± 16 g/d; p = 0.031) with no difference between groups (p = 0.68; Figure 2 B).



Figure 2. Relative (%/d; A) and absolute (g/d; B) myofibrillar fractional synthetic rate measured using 2 H₂O labeling in overweight and obese older men who underwent 2 wk of energy restriction (Phase 1:ER) and 2 wk of energy restriction + resistance training (Phase 2:ER + RT) with balanced (BAL) or skewed (SKEW) protein distribution. Boxes represent the 25th to 75th percentiles, horizontal lines and the crosses within the boxes represent median and mean values, respectively. The whiskers represent the minimum and maximum. *n* = 10/group. Data were analyzed using a 2-factor (group x phase) mixed-model ANOVA. *Different from Phase 1:ER; p < 0.05.

Synthesis of individual skeletal muscle proteins. Fractional synthetic rate (FSR) data were obtained for 190 individual skeletal muscle proteins in both ER and ER+RT in at least 2 participants per group (Table 1). Mean FSRs ranged from a minimum of 0.2 %/d to a maximum of 10.8%/d in Phase 1:ER and from 0.6 %/d to 15.5 %/d in Phase 2:ER+RT (Table 1). FSR increased with RT in 175 of the 190 proteins with no difference between groups (significant with Benjamini-Hochberg correction for multiple comparisons; Table 1). Figure 2 shows the FSRs of several of the individual myofibrillar (A), sarcoplasmic (B) and mitochondrial (C) proteins that were responsive to RT.

Table 1. Fractional synthetic rate (FSR) for 190 individual skeletal muscle proteins during
2-wk of energy restriction (ER) and 2-wk of energy plus resistance training (ER + RT)

	BAL PHASE 1:ER	SKEW PHASE 1:ER	BAL PHASE 2:ER + RT	SKEW PHASE 2:ER + RT	Benjamini- Hochberg significance
Sarcoplasmic endoplasmic reticulum calcium ATPase 1	0.98 ± 0.19 (10)	0.85 ± 0.23 (9)	2.34 ± 0.71 (10)	1.87 ± 0.66 (9)	Sig
Actin alpha skeletal muscle	0.17 ± 0.08 (10)	0.15 ± 0.09 (8)	0.70 ± 0.18 (10)	0.63 ±0.27 (9)	Sig
Glycogen phosphorylase muscle form	0.96 ± 0.18 (10)	0.71 ± 0.20 (9)	2.11 ± 0.72 (10)	1.94 ±0.73 (9)	Sig
Myosin-2	0.65 ± 0.25 (10)	0.70 ± 0.29 (9)	1.75 ± 0.44 (10)	1.60 ±0.62 (9)	Sig
Sarcoplasmic endoplasmic reticulum calcium ATPase	0.96 ± 0.24 (10)	0.78 ± 0.15 (9)	$245 \pm 0.72(10)$	1 99 +0 66 (9)	Sig
Myosin-7	$0.63 \pm 0.31 (10)$	0.80 ± 0.44 (9)	1.71 ± 0.41 (10)	1.69 ±0.72 (9)	Sig
Creatine kinase M type	0.52 ± 0.12 (10)	0.42 ± 0.12 (9)	1.23 ± 0.39 (10)	1.13 ±0.37 (9)	Sig
Serum albumin	2.55 ± 0.57 (10)	2.40 ± 0.49 (9)	4.20 ± 1.26 (10)	4.28 ±1.41 (9)	Sig
Fructose bisphosphate aldolase A	0.84 ± 0.14 (10)	0.71 ± 0.17 (9)	1.89 ± 0.48 (10)	1.77 ±0.48 (9)	Sig
Filamin C	1.71 ± 0.32 (10)	1.40 ± 0.26 (9)	3.89 ± 1.37 (10)	3.68 ±1.40 (9)	Sig
Myosin-1	0.58 ± 0.18 (10)	0.69 ± 0.39 (8)	1.60 ± 0.43 (10)	1.46 ±0.51 (9)	Sig
Alpha actinin-2	0.74 ± 0.13 (10)	0.69 ± 0.14 (9)	1.37 ± 0.36 (10)	1.22 ±0.45 (9)	Sig
Myoglobin	0.58 ± 0.10 (10)	0.56 ± 0.11 (9)	0.76 ± 0.30 (10)	0.69 ±0.18 (9)	Sig
Tropomyosin beta chain	0.52 ± 0.16 (10)	0.40 ± 0.10 (9)	1.37 ± 0.37 (10)	1.27 ±0.41 (9)	Sig
Myosin-6	0.56 ± 0.32 (10)	0.64 ± 0.40 (9)	1.69 ± 0.41 (10)	1.65 ±0.72 (9)	Sig
Tropomyosin alpha-1 chain	0.37 ± 0.16 (10)	0.31 ± 0.12 (9)	1.37 ± 0.42 (10)	1.22 ±0.39 (9)	Sig
Myomesin-2	0.42 ± 0.13 (10)	0.44 ± 0.14 (8)	1.40 ± 0.26 (10)	1.43 ±0.75 (9)	Sig
6-phosphofructokinase, muscle type	1.39 ± 0.32 (10)	1.13 ± 0.26 (9)	3.78 ± 1.54 (10)	3.32 ±1.15 (9)	Sig
Glycogen debranching enzyme	0.99 ± 0.11 (10)	0.74 ± 0.26 (9)	2.39 ± 0.67 (10)	2.08 ±0.70 (9)	Sig
ATP synthase subunit beta mitochondrial	0.72 ± 0.23 (10)	0.60 ± 0.18 (9)	2.17 ± 0.80 (10)	1.84 ±0.66 (9)	Sig
Glyceraldehyde 3 phosphate dehydrogenase	0.34 ± 0.10 (10)	0.27 ±0.14 (9)	1.62 ±0. 40 (10)	1.33 ±0.49 (9)	Sig
Beta-enolase	0.41 ±0.11 (10)	0.36 ±0.15 (8)	1.31 ±0.37 (10)	1.23 ±0.43 (9)	Sig
Myosin-4	0.63 ±0.27 (10)	0.63 ±0.35 (8)	1.53 ±0.44 (10)	1.43 ±0.53 (9)	Sig
Carbonic anhydrase 3	0.28 ±0.10 (10)	0.24 ±0.10 (9)	1.00 ±0.39 (10)	0.86 ±0.27 (9)	Sig
Myosin-binding protein C slow-type	2.49 ±0.41 (10)	2.47 ±0.71 (9)	5.09 ±1.23 (10)	5.15 ±1.90 (9)	Sig
Tropomyosin alpha-3 chain	0.69 ±0.14 (10)	0.60 ±0.25 (9)	1.52 ±0.38 (10)	1.48 ±0.51 (9)	Sig
Pyruvate kinase isozymes M1/M2	0.69 ±0.20 (10)	0.60 ±0.20 (9)	2.35 ±0.93 (10)	2.00 ±0.75 (9)	Sig
Myosin light chain 1/3 skeletal muscle isoform	0.27 ±0.12 (10)	0.24 ±0.18 (7)	0.95 ±0.34 (10)	0.85 ±0.35 (9)	Sig
Triosephosphate isomerase	0.33 ±0.14 (10)	0.22 ±0.11 (9)	1.24 ±0.36 (10)	1.17 ±0.43 (9)	Sig
Hemoglobin subunit beta	0.79 ±0.20 (10)	0.55 ±0.15 (9)	1.33 ±0.28 (10)	1.26 ± 0.26 (9)	Sig
Myosin regulatory light chain 2 skeletal muscle isoform	0 33 +0 14 (10)	$0.31 \pm 0.18(8)$	0.98 +0.36 (10)	0 86 +0 31 (9)	Sig
	0.00 ±0.14 (10)	0.01 ±0.10 (0)	0.20 ±0.20 (10)	0.00 ±0.01 (7)	515

Actin alpha cardiac muscle					
1	0.36 ±0.08 (10)	0.25 ±0.14 (9)	0.65 ±0.20 (10)	0.65 ±0.26 (9)	Sig
Aconitate hydratase, mitochondrial	0.86 ±0.26 (10)	0.72 ±0.33 (9)	2.85 ±0.95 (10)	2.67 ±1.07 (9)	Sig
Phosphoglucomutase1	0.55 ±0.12 (10)	0.44 ±0.18 (9)	1.52 ±0.57 (10)	1.38 ±0.46 (9)	Sig
ATP synthase subunit alpha, mitochondrial	1.01 ±0.27 (10)	0.79 ±0.29 (9)	2.56 ±1.02 (10)	2.14 ±0.74 (9)	Sig
Troponin C skeletal muscle	1.76 ±0.21 (10)	1.59 ±0.41 (9)	3.15 ±0.87 (10)	2.84 ±0.68 (9)	Sig
Adenylate kinase isoenzyme 1	0.30 ±0.09 (10)	0.30 ±0.1 (9)	1.44 ±0.99 (10)	0.83 ±0.37 (9)	Sig
Creatine kinase S-type, mitochondrial	0.62 ±0.24 (10)	0.44 ±0.23 (9)	2.05 ±0.71 (10)	1.72 ±0.70 (9)	Sig
Calsequestrin-1	0.69 ±0.24 (10)	0.59 ±0.26 (9)	0.85 ±0.27 (10)	0.74 ±0.37 (9)	Sig
Glycogen phosphorylase brainform	0.98 ±0.22 (10)	0.71 ±0.18 (9)	2.14 ±0.73 (10)	1.90 ±0.68 (9)	Sig
Troponin I fast skeletal muscle	0.95 ±0.21 (10) 0	0.81 ±0.29 (9)	1.97 ±0.51 (10)	1.76 ±0.46 (9)	Sig
Titin	2.29 ±1.12 (7)	1.57 ±0.42 (8)	2.69 ±0.77 (10)	2.63 ±0.83 (9)	Sig
Myosin-8	0.65 ±0.39 (9)	0.65 ±0.35 (8)	1.58 ±0.46 (10)	1.47 ±0.47 (9)	Sig
Myosin-3	0.62 ±0.24 (9)	0.78 ±0.64 (9)	2.02 ±0.96 (10)	1.52 ±0.61 (9)	Sig
Phosphoglycerate kinase 1	0.26 ±0.12 (10)	0.26 ±0.14 (8)	1.21 ±0.58 (10)	1.05 ±0.46 (9)	Sig
Sarcoplasmic/endoplasmic					
ATPase3	0.49 ±0.20 (10)	0.35 ±0.15 (9)	2.23 ±0.64 (10)	1.77 ±0.58 (9)	Sig
Myosin regulatory light					
muscle isoform	1.28 ±0.52 (10)	1.08 ±0.41 (9)	1.65 ±0.66 (10)	1.67 ±0.79 (9)	Sig
Alpha-crystallin B chain	1.40 ±0.32 (10)	1.08 ±0.26 (9)	3.54 ±1.36 (10)	3.23 ±1.05 (9)	Sig
Phosphoglycerate mutase 2	0.46 ±0.17 (10)	0.47 ±0.17 (9)	1.28 ±0.37 (10)	1.16 ±0.41(9)	Sig
Four and a half LIM	1.02 (0.04 (10)	1 10 10 52 (0)		1.55 +0.25 (0)	<u>a</u> .
domains protein 1	1.23 ±0.34 (10)	1.10 ±0.53 (9)	2.07 ±0.67 (10)	1.55 ±0.36 (8)	Sig
ADP/ATP translocase 1	1.06 ±0.65 (9)	0.90 ±0.31 (8)	2.02 ±0.53 (10)	1.76 ±0.62 (9)	Sig
Myosin-13 Troponin T. fast skeletal	0.70 ±0.19 (10)	0.89 ±0.84 (9)	1.50 ±0.44 (10)	1.33 ±0.50 (9)	Sig
muscle	0.75 ±0.25 (10)	0.73 ±0.24 (8)	2.28 ±0.76 (10)	1.86 ±0.57 (9)	Sig
Ryanodine receptor 1	2.68 ±0.9 (9)	2.10 ±0.47(9)	5.34 ±1.82 (10)	3.80 ±1.19 (9)	Sig
Myomesin-1	1.20 ±0.29 (9)	1.16 ±0.39(9)	2.38 ±0.89 (10)	2.10 ±0.86 (9)	Sig
Phosphatidylethanolamine- binding protein 1	0.34 ± 0.08 (8)	0.24 +0.17 (9)	1.21 +0.48 (10)	1.07 +0.47 (9)	Sig
Protein DI-1	$0.41 \pm 0.18(10)$	0 51 +0.10 (9)	1.36 +0.91 (10)	1.11 +0.54 (9)	Sig
Troponin I, slow skeletal muscle	2.17 +0.63 (10)	1.92 +0.53 (9)	3.60 +1.11 (10)	3.37 +1.09 (9)	Sig
Heat shock cognate 71 kDa	1.30 +0.23 (10)	1.10 +0.24 (9)	3.23 +1.29 (10)	2.88 +1.17 (9)	Sig
Isocitrate dehydrogenase (NADP) mitochondrial	0.67 +0.29 (10)	0 44 +0 33 (9)	1 87 +0 63 (10)	1 70 +0 81 (9)	Sig
Heat shock protein beta-6	2 17 ±0.46 (10)	1 84 +0 39 (9)	4 95 +1 73 (10)	4 52 ±1 83 (9)	Sig
PDZ and LIM domain	2.17 20.10 (10)	1.01 ±0.07 (7)	1.95 ±1.75 (10)	1.52 ±1.65 (5)	515
protein 3 Clucosa 6 phosphata	2.49 ±0.36 (9)	2.14 ±0.58 (9)	4.66 ±2.51 (10)	3.44 ±0.99 (9)	Sig
isomerase	0.31 ±0.15 (9)	0.31 ±0.14 (8)	1.71 ±0.70 (10)	1.33 ±0.70 (9)	Sig
Aspartate					
cytoplasmic	0.50 ±0.25 (10)	0.39 ±0.18 (7)	1.57 ±0. 34 (10)	1.51 ±0.44 (9)	Sig
Troponin C slow skeletal	1 92 +0 41 (10)	1 81 +0 52 (9)	3 58 +0 95 (10)	3 37 +1 35 (9)	Sig
and curdiac muscies	1.72 -0.71 (10)	1.01 ±0.04 (7)	$3.30 \pm 0.75 (10)$	5.57 ±1.55 (7)	JIE

2-oxoglutarate					
dehydrogenase,mitochondr	$1.89 \pm 0.45(10)$	1 60 ±0 71 (9)	4 98 +2 34 (10)	4 28 +1 69 (9)	Sig
ATP synthase subunit b	1.07 ±0.45 (10)	1.00 ±0.71 ())	4.90 ±2.94 (10)	4.20 ±1.07 (7)	515
mitochondrial	0.50 ±0.35 (9)	0.45 ±0.26 (9)	1.68 ±0.74 (10)	1.22 ±0.63 (9)	Sig
Alpha-actinin-3	0.65 ±0.17 (10)	0.55 ±0.11 (9)	1.44 ±0.35 (10)	1.20 ±0.55 (9)	Sig
Peroxiredoxin-2	0.65 ±0.20 (10)	0.53 ±0.21 (9)	2.03 ±0.63 (10)	1.57 ±0.49 (9)	Sig
Acetyl-CoA					
acetyltransferase.	$0.84 \pm 0.23(10)$	0 63 +0 29 (9)	1.88 +0.57 (10)	1.85 ±0.64 (9)	Sig
Cofilin 2	0.70 ± 0.22 (10)	0.50 ±0.10 (8)	2.26 ± 1.40 (10)	1 82 ±0.62 (0)	Sig
ATP synthase subunit O.	0.79 ±0.22 (10)	0.39 ±0.19 (8)	2.20 ±1.40 (10)	1.83 ±0.02 (9)	Sig
mitochondrial	0.50 ±0.22 (10)	0.46 ±0.16 (9)	1.36 ±0.61 (10)	1.14 ±0.49 (9)	Sig
L-lactate dehydrogenase A	0.21 +0.10 (10)	0.24 +0.12 (9)	2.02 10.82 (10)	1.50 10.85 (0)	C:-
Cytochrome b-c1 complex	0.31 ±0.19 (10)	0.24 ±0.13 (8)	2.03 ±0.82 (10)	1.59 ±0.85 (9)	Sig
subunit 2, mitochondrial	0.87 ±0.33 (10)	0.84 ±0.49 (9)	1.85 ±0.53 (10)	1.80 ±0.60 (9)	Sig
Heat shock protein beta-1	1.00 ±0.11 (10)	0.79 ±0.20 (9)	3.58 ±1.72 (10)	3.11 ±1.37 (9)	Sig
NADH-ubiquinone					e
oxidoreductase 75 kDa	1 (4 + 0.57 (0)	1 (2 +0.01 (0)	5 (5 12 22 (10)	5 40 11 72 (0)	G.
ATP synthese subunit d	1.64 ±0.57 (9)	1.62 ±0.91 (8)	5.65 ±2.22 (10)	5.40 ±1.73 (9)	Sig
mitochondrial	0.57 ±0.18 (10)	0.53 ±0.24 (9)	1.72 ±0.80 (10)	1.36 ±0.67 (9)	Sig
Transitional endoplasmic					
reticulum ATPase	3.35 ±0.66 (9)	2.66 ±0.49 (9)	8.79 ±4.05 (10)	6.65 ±2.70 (9)	Sig
Mu 2	0.81 ±0.36 (10)	0.57 ±0.25 (9)	1.12 ±0.89 (10)	1.06 ±0.52 (9)	Sig
Heat shock 70 kDa protein					~-8
1A/1B	1.34 ±0.35 (9)	1.18 ±0.25 (9)	3.08 ±0.86 (10)	2.87 ±1.21 (9)	Sig
Fructose-bisphosphate	$0.58 \pm 0.20(10)$	$0.52 \pm 0.14 (9)$	1 91 +0 50 (10)	1 82 +0 68 (9)	Sig
Puromycin-sensitive	0.50 ±0.20 (10)	0.52 ±0.14 ())	1.91 ±0.50 (10)	1.02 ±0.00 ())	Sig
aminopeptidase	0.78 ±0.15 (10)	0.71 ±0.39 (9)	2.36 ±1.19 (10)	1.87 ±0.81 (9)	Sig
Aspartate					
mitochondrial	$0.52 \pm 0.27 (10)$	$0.58 \pm 0.31 (9)$	$1.25 \pm 0.40(10)$	$1.25 \pm 0.45 (9)$	Sig
Homoglohin syhynit delte	0.40 ± 0.25 (10)	0.50 ±0.22 (7)	1 25 ±0 22 (10)	1.27 ±0.48 (0)	S: 2
Cytochrome c oxidase	0.49 ±0.23 (10)	0.30 ±0.22 (7)	1.55 ±0.55 (10)	1.27 ±0.48 (9)	Sig
subunit 4 isoform 1					
mitochondrial	0.69 ±0.49 (8)	0.74 ±0.61 (9)	2.04 ±0.94 (10)	1.84 ±0.65 (9)	Sig
Alpha-actinin-1	0.90 ±0.44 (10)	0.95 ±0.25 (9)	1.39 ±0.42 (10)	1.25 ±0.62 (9)	Sig
Cytochrome b-c1 complex	0.42 +0.22 (0)	0.41 +0.27 (7)	1.09.10.75 (10)	1 74 10 72 (0)	C:-
Malate dehydrogenase.	0.42 ±0.22 (9)	$0.41 \pm 0.27(7)$	1.98 ±0.75 (10)	1.74 ±0.72 (9)	Sig
mitochondrial	0.76 ±0.2 (9)	0.54 ±0.19 (9)	1.77 ±1.04 (10)	1.60 ±0.82 (9)	Sig
Superoxide dismutase	0.75 10.06 (10)	0.60.10.10.00	2 22 10 72 (10)	1 (5 10 5 (0)	<i>c</i> .
(Mn), mitochondrial	$0.75 \pm 0.26 (10)$	0.60 ±0.19 (9)	2.28 ±0.78 (10)	1.65 ±0.56 (9)	Sig
Sarcalumenin	0.25 ±0.1 (7)	0.29 ±0.17 (6)	1.92 ±0.67 (10)	1.56 ±0.61 (9)	Sig
Desmin	1.96 ±0.17 (5)	1.76 ±0.69 (2)	8.68 ±3.76 (10)	7.70 ±2.84 (8)	Sig
Vinculin	1.34 ±0.32 (10)	1.28 ±0.24 (9)	4.05 ±1.70 (10)	3.51 ±1.58 (9)	Sig
Ubiquitin-like modifier-		1 10 10 20 (0)			<i>a</i> :
activating-enzyme I Heat shock protein HSP	1.44 ±0.32 (10)	1.18 ±0.30 (9)	3.//±1.58(10)	3.02 ±1.22 (9)	Sig
90-beta	2.14 ±0.67 (9)	2.40 ±1.68 (8)	15.30 ±15.88(10)	13.36 ±7.80 (9)	Sig
Tripartite motif-containing	, , , , , , , , , , , , , , , , , , ,				
protein 72	0.83 ±0.28 (8)	0.66 ±0.30 (6)	3.05 ±1.35 (10)	2.77 ±1.23 (9)	Sig
Gamma-enolase	0.19 ±0.08 (8)	0.25 ±0.23 (6)	1.43 ±0.53 (10)	1.32 ±0.58 (9)	Sig
Glutathione transferase P	0.62 ±0.23 (10)	0.49 ±0.12 (9)	2.22 ±1.44 (10)	1.76 ±0.98 (9)	Sig

Very long-chain specific					
acyl-CoA dehydrogenase,mitochondr					
ia	0.76 ±0.37 (9)	0.74 ±0.29 (8)	3.81 ±1.34 (10)	2.98 ±1.2 (8)	Sig
Protein 2	1.33 ±0.23 (10)	1.15 ±0.23 (9)	2.99 ±1.06 (10)	2.81 ±1.22 (9)	Sig
Alpha-actinin-4	0.73 ±0.16 (10)	0.72 ±0.20 (9)	1.32 ±0.37 (10)	1.30 ±0.64 (9)	Sig
Mitochondrial inner membrane protein	0.65 +0.2 (7)	0.58 +0.2 (7)	2.02 +0.8 (10)	1.76 +0.76 (9)	Sig
Cytochrome b-c1 complex			()		~-8
subunit Rieske, mitochondrial	$0.87 \pm 0.25 (9)$	$0.95 \pm 0.67 (9)$	3.07 +1.08 (10)	3.04 +1.44 (9)	Sig
Myosin-binding protein C,					~-8
fast-type Calcium-binding	2.12 ±1.08 (9)	1.42 ±0.46 (8)	4.93 ±2.92 (10)	4.24 ±2.10 (9)	Sig
mitochondrial					
Carrier protein Aralar1	0.66 ±0.45 (9)	0.51 ±0.38 (8)	2.58 ±0.94 (10)	2.08 ±0.95 (8)	Sig
Peroxiredoxin-1	0.96 ±0.90 (7)	0.50 ±0.22 (8)	2.41 ±1.55 (10)	1.99 ±1. 30 (9)	Sig
Annexin A6	1.12 ±0.7 (7)	0.66 ±0.25 (4)	4.12 ±3.44 (10)	2.68 ±1.35 (9)	Sig
Peroxiredoxin-6	1.25 ±0.44 (9)	1.09 ±0.48 (8)	2.36 ±1.34 (10)	1.59 ±0.79 (9)	Sig
Flavin reductase (NADPH)	1.12 ±0.40 (10)	0.89 ±0.29 (9)	1.76 ±0.65 (10)	1.69 ±0.54 (9)	Sig
Ig kappa chain C region	0.84 ±0.55 (8)	0.96 ±0.29 (7)	3.32 ±1.15 (10)	3.75 ±1.08 (9)	Sig
Cytochrome c oxidase subunit 2	$1.08 \pm 0.31 (10)$	0.84 ±0.38 (9)	2.13 ±0.80 (10)	1.91 ±0.64 (9)	Sig
Thioredoxin-dependent					
peroxide reductase mitochondrial	$0.49 \pm 0.26(9)$	$0.35 \pm 0.19 (9)$	2.60 +1.20 (10)	1.86 ±0.69 (9)	Sig
Protein-arginine deiminase					~-8
type-2 Fatty acid-binding protein	0.38 ±0.32 (4)	0.56 ±0.39 (2)	3.33 ±2.66 (10)	2.76 ±1.34 (9)	Sig
heart	1.03 ±0.59 (8)	0.88 ±0.60 (8)	1.60 ±0.73 (10)	1.12 ±0.72 (8)	Sig
14-3-3 protein gamma	1.60 ±0.27 (10)	1.48 ±0.41 (9)	3.89 ±1.32 (10)	3.54 ±1.32 (9)	Sig
Dihydrolipoyl					
mitochondrial	1.30 ±0.29 (9)	1.11 ±0.41 (8)	2.60 ±1.02 (10)	2.37 ±0.87 (9)	Sig
Elongation factor 1-alpha 2	2.57 ±1.90 (6)	1.82 ±0.51 (6)	4.93 ±2.12 (10)	3.86 ±1.42 (8)	Sig
Heat shock protein HSP	$4.41 \pm 2.10(2)$	4 45 ±0 87 (2)	$10.02 \pm 5.20(10)$	12.56 ± 0.20 (0)	Sig
Silemin A	$4.41 \pm 2.10(3)$	4.43 ±0.87 (3)	$10.02 \pm 3.20 (10)$	$13.30 \pm 9.20(9)$	Sig
NADH dehydrogenase	1.24 ±0.5 (10)	0.81 ±0.27 (8)	4.51 ±1.80 (10)	4.22 ±1.73 (9)	Sig
(ubiquinone) flavoprotein,	1 22 10 42 (0)	1 27 10 50 (0)	2 22 11 20 (10)	2.02 + 1.64(0)	C:-
Malate dehydrogenase,	1.23 ±0.43 (9)	1.27 ±0.59 (9)	5.38 ±1.39 (10)	5.92 ±1.04 (9)	51g
cytoplasmic	0.39 ±0.39 (10)	0.58 ±0.7 (8)	1.47 ±0.60 (9)	1.58 ±0.92 (9)	Sig
Kelch-like protein 41	2.20 ±0.56 (9)	1.64 ±0.62 (7)	5.71 ±3.22 (10)	5.28 ±1.79 (8)	Sig
Glycogen starch synthase, muscle	$0.94 \pm 0.5 (8)$	1.01 ±0.43 (9)	4.14 ± 1.02 (10)	3.78 ±1.26 (9)	Sig
Dihydrolipoyllysine-					
residue acetyltransferase component of pyruvatede					
dehydrogenase	0.73 ±- (2)	0.61 ±0.38 (4)	1.28 ±0.53 (9)	1.17 ±0.89 (7)	Sig
Elongation factor 2	2.16 ±0.53 (8)	1.56 ±0.2 (6)	5.30 ±2.17 (8)	4.39 ±2.09 (7)	Sig
Succinate dehydrogenase					
subunit, mitochondrial	0.90 ±0.54 (5)	1.55 ±0.84 (3)	3.06 ±0.84 (10)	3.07 ±1.07 (8)	Sig
Fructose-1,6-	$0.66 \pm 0.33(10)$	0.74 ± 0.3 (8)	$2.78 \pm 0.94(10)$	2.97 ± 1.25 (0)	Sig
Cofilin_1	$1.01 \pm 0.21 (0)$	$0.74 \pm 0.3 (0)$	$2.76 \pm 0.94 (10)$ $3.00 \pm 2.54 (10)$	2.77 ± 1.23 (7) 2.32 ± 1.78 (0)	Sia
Comm-1	1.01 ±0.21 (9)	$0.03 \pm 0.19(0)$	5.00 ±2.54 (10)	$2.32 \pm 1.10(7)$	Sig
Peptidyl-prolyl cis-trans					
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isomerase A	1.22 ±0.43 (9)	1.01 ±0.50 (8)	3.09 ±2.18 (10)	2.48 ±1.61 (9)	Sig
Leucine rich repeat-	1.98 ± 0.44 (9)	$1.68 \pm 0.36(0)$	$280 \pm 102(10)$	$265 \pm 0.72(9)$	Sig
LIM domain-binding	1.98 ±0.44 (9)	1.08 ±0.30 (9)	2.89 ±1.02 (10)	2.03 ±0.72 (9)	Sig
protein 3	1.09 ±0.54 (9)	1.07 ±0.51 (9)	2.58 ±0.79 (10)	2.05 ±0.72 (9)	Sig
Glycerol-3 phosphate					
dehydrogenase (NAD(+)),	0.55 +0.11 (5)	0.41.10.24.(7)	1.07.10.06 (10)	1 70 +0 00 (0)	<u>.</u>
cytoplasmic Isochorismatase domain	0.55 ±0.11 (5)	0.41 ±0.24 (7)	1.97 ±0.86 (10)	1.70 ±0.90 (9)	Sig
containing protein 2,					
mitochondrial	1.37 ±0.59 (9)	1.30 ±0.63 (8)	3.38 ±1.80 (9)	2.09 ±0.60 (6)	Sig
Myomesin-3	0.53 ±0.13 (6)	0.45 ±0.16 (6)	1.65 ±0.73 (10)	1.13 ±0.67 (8)	Sig
Tubulin alpha-4A chain	2.05 ±0.47 (5)	1.74 ±0.68 (3)	4.54 ±1.80 (10)	4.27 ±2.17 (8)	Sig
1-4-3-3 protein epsilon	1.40 ±0.4 (10)	1.09 ±0.49 (9)	3.89 ±2.30 (10)	2.81 ±1.01 (9)	Sig
Troponin T, slow skeletal	0.01.10.05.(10)	0.00.00.00	0.50 10.05 (10)		<i>a</i> .
muscle	$0.81 \pm 0.35 (10)$	0.63 ±0.42 (9)	2.73 ±0.96 (10)	2.44 ±1.12 (9)	Sig
chain	$0.32 \pm 0.37 (7)$	$0.24 \pm 0.17(3)$	$4.86 \pm 3.80(7)$	3.92 +4.68 (8)	Sig
Carboxymethylenebutenoli					~-8
dase homolog	0.38 ±0.21 (9)	0.34 ±0.29 (4)	1.29 ±0.70 (10)	1.18 ±0.84 (8)	Sig
Glutathione S-transferase	0.00 +0.42(10)		1.05 \ 0.70 (10)	3.22 ±4.95	<i>a</i> :
Mu I Protein L isoaspartate	$0.90 \pm 0.43(10)$	0.60 ±0.22 (/)	1.95 ±0.78 (10)	9	Sig
(Daspartate) O-				1.48 ± 0.44	
methyltransferase	0.45 ±0.15 (8)	0.21 ±0.15 (6)	2.49 ±0.96 (10)	7	Sig
Citrate synthase,				1.23 ±0.72	
mitochondrial	0.76 ±0.35 (7)	0.57 ±0.38 (6)	1.58 ±0.88 (10)	9	Sig
Glutathione S-transferase	0.05 ± 0.44 (8)	$0.65 \pm 0.20(7)$	$1.05 \pm 0.78 (10)$	3.22 ± 4.95	Sig
Cytochrome c oxidase	0.95 ±0.44 (8)	$0.03 \pm 0.30(7)$	1.95 ±0. 78 (10)	9 1 80 +1 03	Sig
subunit 5A mitochondrial	1.14 ±0.28 (6)	0.46 ±0.23 (8)	2.71 ±1.14 (7)	7	Sig
Heat shock protein beta-7	2.72 ±0.66 (8)	2.96 ±1.17 (7)	8.32 ±4.21 (10)	8.44 ±4.74 (9)	Sig
Filamin-B	2,10,+0,77 (6)	1.76 +0.29 (4)	4.21 +2.06 (10)	4 23 +1.80 (9)	Sig
Myc box-dependent-	2110 20117 (0)	11/0 2012/ (1)		1120 21100 ())	518
interacting protein 1	1.33 ±0.41 (6)	1.14 ±0.51 (5)	4.45 ±1.51 (8)	3.67 ±1.66 (5)	Sig
Alpha-1antitrypsin	4.72 ±1.31 (7)	5.17 ±0.85 (6)	12.95 ±9.90 (9)	14.30 ±4.71 (7)	Sig
AMP deaminase 1	0.77 ±0.43 (4)	0.22 ±0.17 (3)	1.48 ±0.87 (9)	1.58 ±0.94 (5)	Sig
GTP-binding protein SAR	2.76 1 21 (0)	2 10 10 75 (0)	10.10.110.51.(10)	0.00 + 1.50 (0)	<i>a</i> :
1b Glutathione Stransferase	3.76±1.21 (9)	3.19 ±0.75 (8)	13.10±12.51 (10)	8.08 ±4.60 (8)	Sig
Mu 3	0.57 ±0.26 (7)	0.76 ±0.95 (6)	1.82 ±0.66 (8)	1.15 ±0.47 (8)	Sig
Ferritin heavychain	2.04 ±0.86 (8)	1.70 ±0.92 (7)	5.74 ±3.09 (10)	4.74 ±2.79 (9)	Sig
Heat shock protein beta-2	0.80 ±0.14 (9)	0.72 ±0.31 (8)	1.93 ±0.85 (10)	1.33 ±0.56 (8)	Sig
Protein-cysteine N-					
palmitoyltransferase	$0.43 \pm 0.18(7)$	$0.50 \pm 0.35(9)$	$0.90 \pm 0.47(10)$	$1.18 \pm 1.10(7)$	Sig
	0.45 ±0.16 (7)	0.50 ±0.55 ())	0.90 ±0.47 (10)	1.10 ±1.10 (7)	Sig
Lumican Protosomo subunit boto	2.48 ±0.62 (5)	2.22 ±0.54 (5)	15.52 ±11.05 (9)	7.82 ±4.32 (6)	Sig
type-1	2.09 ±0.50 (6)	1.77 ±0.35 (6)	3.99 ±1.93 (10)	3.26 ±1.71 (7)	Sig
Cytochrome c1, heme					0
protein, mitochondrial	0.29 ±0.15 (5)	0.41 ±0.11 (5)	1.36 ±0.34 (9)	1.41 ±0.65 (7)	Sig
Peroxiredoxin-5,	1 10 10 15 (2)	0.00.10.00.00	0.07 11 1 (10)	0.77 11 12 (0)	с.
6-phosphofructokinase	1.10 ±0.45 (3)	0.00 ±0.23 (6)	2.8/ ±1.1 (10)	2.0/±1.12(9)	51g
liver type	0.94 ±0.45 (10)	0.87 ±0.39 (8)	2.29 ±0.88 (9)	2.09 ±0.85 (8)	Sig
Hexokinase-1	1.15 +0.26 (9)	0.98 +0.36 (8)	3.33 +1.32 (9)	2.83 +1.20 (6)	Sig
	1.51 (0.07 (0)		2.02 11.02 (0)	2.54 10.55 (0)	~15
iggamma-i chain C region	$1.51 \pm 0.0/(3)$	2.34 ±0.29 (2)	3.22 ±1.29 (9)	3.34 ±0.66 (8)	Sig

Translationally-controlled					
tumor protein	5.49 ±2.54 (7)	3.98 ±1.25 (8)	12.74 ±9.23 (10)	7.24 ±3.38 (8)	Sig
Dual specificity protein	1.26 ± 0.52 (6)	0.81 ± 0.48 (8)	$4.62 \pm 2.1 (10)$	$3.61 \pm 1.60(7)$	Sig
NADH dehvdrogenase	1.20 ±0.52 (0)	0.81 ±0.48 (8)	4.02 ±2.1 (10)	5.01 ±1.00 (7)	Sig
(ubiquinone) iron sulfur					
protein 7 mitochondrial	2.94 ±0.64 (8)	2.77 ±0.84 (8)	8.47 ±8.36 (10)	5.76 ±2.10 (8)	Sig
Puromycin-sensitive					
aminopeptidase-like	$0.46 \pm 0.21(0)$	0.31 ± 0.20 (8)	$274 \pm 0.85(7)$	$213 \pm 140(6)$	Sig
Short-chain specific acyl-	0.40 ±0.21 (9)	0.31 ±0.20 (8)	2.74 ±0.85 (7)	2.15 ±1.40 (0)	Sig
CoA dehydrogenase,					
mitochondrial	1.47 ±0.36 (5)	1.46 ±0.79 (5)	2.01 ±0.38 (6)	2.79 ±1.44 (8)	Sig
Hydroxyacyl-coenzyme A					
dehydrogenase	0.07 ± 0.11 (C)	0.58 +0.22 (5)	1 1 (+0.59 (7)	0.75 ± 0.22 (C)	C:-
Collagen alpha 1 (VI)	0.97 ±0.11 (6)	0.58 ±0.22 (5)	1.10 ±0.58 (7)	0.75 ±0.55 (6)	Sig
chain	$0.70 \pm 0.36(6)$	$0.84 \pm 0.45(7)$	4 62 +2 93 (9)	4 13 +5 18 (9)	Sig
Phosphorylase b kinase					~-8
regulatory subunit alpha					
skeletal muscle isoform	1.20 ±0.29 (6)	1.00 ±0.33 (4)	3.08 ±1.1 (7)	2.66 ±1.41 (5)	Sig
SH3 domain-binding	1.76 ± 0.26 (8)	1 22 ±0 22 (8)	2 22 ±1 79 (7)	2.00 ± 2.50 (6)	C: a
giutamic acid rich protein Pseudouridine-5' -	1.70 ±0.20 (8)	1.55 ±0.55 (8)	3.32 ±1.78 (7)	3.90 ±2.50 (6)	51g
monophosphatase	$0.52 \pm 0.29 (5)$	0.31 ±0.04 (3)	1.70 ±0.99 (9)	1.54 ±0.43 (5)	Sig
NADH dehydrogenase					
(ubiquinone) 1 alpha					
subcomplex subunit 7	2.27 ±1.15 (6)	1.23 ±0.39 (5)	3.46 ±1.97 (6)	3.42 ±1.67 (4)	Sig
Cytochrome b5 type B	1.15 ±0.46 (5)	1.02 ±0.43 (5)	2.44 ±1.10 (6)	2.94 ±1.51 (6)	Sig
26S proteasome non-					
ATPase regulatory subunit		0.50.10.15.40	0.05 + 1.15 (0)		<i>a</i> .
] A demodente laine en 2	0.83 ±0.17 (4)	0.63 ±0.15 (4)	2.95 ±1.15 (6)	2.79 ±1.70 (5)	Sig
Adenylate Kinase 2 mitochondrial	$0.50 \pm 0.19(4)$	$0.55 \pm 0.16(2)$	$1.63 \pm 0.90(5)$	$1.09 \pm 0.31(3)$	Sig
Maleylacetoacetate	0.50 ±0.17 (4)	0.55 ±0.10 (2)	1.05 ±0.70 (5)	1.09 ±0.51 (5)	515
isomerase	0.42 ±0.30 (4)	0.40 ±0.29 (5)	1.27 ±0.17 (3)	1.36 ±0.52 (6)	Sig
Hemoglobin subunit alpha	1 16 ±0 25 (10)	1 13 +0 26 (9)	1 27 +0 26 (10)	1 22 +0 32 (9)	Not sig
	1.10 ±0.25 (10)	1.15 ±0.20 (5)	1.27 ±0.20 (10)	1.22 ±0.32 (0)	i tot sig
Apolipoprotein A-I	10.43 ±2.66 (9)	10.21 ±4.12 (9)	9.81 ±5.07 (10)	14.34 ±9.93 (9)	Not sig
ligase 1	1 19 +0 55 (4)	1 29 +1 5 (5)	1 83 +0 50 (10)	1 23 +0 72 (8)	Not sig
Superoxide dismutase	1.17 ±0.55 (4)	1.2) ±1.5 (5)	1.05 ±0.50 (10)	1.25 ±0.72 (0)	1101 315
(CuZn)	1.97 (2)	1.26 ±0.67 (3)	1.69 ±0.97 (10)	1.22 ±0.68 (9)	Not sig
Pyruvate dehydrogenase					
E1 component subunit					
alpha, somatic form	$2.04 \pm 1.36(8)$	1 58 ±0 65 (8)	$2.18 \pm 0.75(10)$	$1.81 \pm 0.82 (9)$	Not sig
Intochondria	2.04 ±1.50 (0)	1.56 ±0.05 (6)	2.10 ±0.75 (10)	1266 ± 10.68	Not sig
Polyubiquitin-C	10.78 ±3.09 (7)	8.01 ±2.10 (7)	9.77 ±6.02 (8)	(7)	Not sig
Detionlon 2	1.02 ± 0.14 (6)	1 18 +0 41 (0)	$212 \pm 0.00(10)$	1.24 ± 0.00 (0)	Notaio
Nucleoside diphosphate	1.05 ±0.14 (0)	1.18 ±0.41 (9)	2.13 ±0.90 (10)	1.54 ±0.90 (9)	Not sig
kinase B	1.840.76 (6)	0.76 ±0.12 (4)	2.19 ±1.18 (9)	2.22 ±1.99 (8)	Not sig
NADH dehydrogenase					
(ubiquinone) 1 beta					
subcomplex subunit 9	1.16 ±0.76 (8)	0.58 ±0.37 (5)	1.25 ±0.47 (8)	1.26 ±0.48 (6)	Not sig
Nucleoside diphosphate	1.84 ± 0.76 (6)	0.76 ± 0.12 (4)	$1.60 \pm 0.64.(7)$	2 21 ±2 00 (9)	Notain
KIIIdSC A	1.04 ±0.70 (0)	0.70 ±0.12 (4)	1.00 ±0.04 (7)	2.21 ±2.00 (8)	TNUE SIG
Ras-related protein Rab-7a	1.24 ±0.36 (5)	1.15 ±0.36 (8)	11.39 ±8.63 (8)	6.32 ±4.30 (6)	Not sig
NADH dehydrogenase					
subcomplex subunit 13	$0.38 \pm 0.28(2)$	$0.52 \pm 0.42 (4)$	1.68 +0.87 (8)	$0.62 \pm 0.47(3)$	Not sig
ATP synthase subunit g.	0.20 ±0.20 (2)	(1)	1.00 ±0.07 (0)	0.02 20.77 (3)	1101 515
mitochondrial	0.98 ±0.24 (5)	0.88 ±0.57 (6)	1.50 ±0.59 (3)	0.59 ±0.25 (4)	Not sig

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Cytochrome c oxidase subunit 6B1	0.83 ±0.79 (2)	0.26 ±0.18 (2)	2.08 ±1.49 (3)	1.80 ±0.76 (5)	Not sig
NADH-dehydrogenase					
(ubiquinone) 1 alpha					
subcomplex subunit 5	3.00 ±0.47 (2)	2.03 ±0.66 (4)	3.06 ±1.75 (3)	2.70 ±2.06 (2)	Not sig

Values are means \pm SD (*n*). FSR, fractional synthetic rate (%/d); ER, energy restriction; ER+RT, energy restriction plus resistance training. Sig, significant according to Benjamini-Hochberg procedure to adjust for the multiple comparisons with a false discovery rate of 0.2.







Figure 2. Fractional synthetic rate (FSR) of selected individual myofibrillar (A), sarcoplasmic (B) and mitochondrial (C) proteins in overweight and obese older men who underwent 2 wk of energy restriction (Phase 1:ER) and 2 wk of energy restriction + resistance training (Phase 2:ER + RT) with balanced (BAL) or skewed (SKEW) protein distribution. FSR for all proteins shown were higher during Phase 2:ER + RT compared to Phase 1:ER with no differences between groups. Proteome kinetic data were analyzed using a 2x2 (group x phase) mixed-model ANOVA, differences were considered significant with a false discovery rate of 0.2 after a Benjamini-Hochberg procedure was performed to adjust for the multiple comparisons.

DISCUSSION

We followed up on our previous work (16) and used D₂O to measure the integrated MyoPS response and the synthesis of individual skeletal muscle proteins over 2-wk of ER and 2-wk of ER+RT in overweight/obese older men who consumed their dietary protein in either a balanced or a skewed pattern. Contrary to our previous acute findings and our hypothesis, we found no influence of the distribution of daily protein on integrative MyoPS or proteome kinetics during either phase of the study. However, similar to our previous findings, integrative MyoPS and the synthetic rates of several individual myofibrillar, sarcoplasmic and mitochondrial proteins were higher when RT was performed during ER compared to ER alone.

The observation that there was no influence of the distribution of daily protein on the longer-term integrative MyoPS (%/d) response contrasts with our previously reported findings that a balanced distribution (3 x 25 g evenly spaced doses of protein) versus a skewed distribution of the same amount of protein (10 g at breakfast, 15 g at lunch, 50 g at dinner) more effectively stimulated MyoPS acutely during ER (16). There are a number of reasons that may account for this discrepancy. First, the conditions under which the acute and longer-term measurements were conducted were different. The previous study was a 'proof of principal' experiment in which MyoPS (%/h) was measured under tightly controlled laboratory conditions and over a short (13 h) period while participants consumed precisely timed whey protein feedings (16). Here we report on longer-term MyoPS (%/d) measured over a 2-wk free-living period in which protein was consumed during meals. Although participants were provided with pre-prepared meals for the

duration of the study and advised on the time of day each meal should be consumed we had no control over many variables that could potentially modify MyoPS: exact timing and consumption of prescribed meals, activities of daily living, stress, and sleep duration. Moreover, an important finding from our acute study was that the influence of protein distribution on MyoPS was specific to the fed and not the fasted rates of MyoPS (16). Since the longer-term measure of MyoPS integrates fasted- and fed-state rates it is likely that these fasted periods, particularly the extended overnight fast, may have 'diluted' feeding-specific effects on MyoPS. In addition, in the acute protocol protein was consumed as an isolated, whey protein in liquid form whereas dietary protein was mainly provided in mixed macronutrient meals in the longer-term protocol. Previous work has shown that the achievement of a rapid and pronounced increase in plasma essential amino acid (EAA)/leucine concentrations, which is characteristic of whey protein ingestion, is associated with increased rates of MyoPS compared to a slow rate of appearance of these amino acids (28). Consumption of solid food and co-ingestion of fiber and other nutrients (carbohydrate and fat) modifies amino acid digestion and absorption kinetics and leads to a relative blunting of the aminoacidemia/leucinemia seen with isolated protein sources (29). Therefore, it may be that, when protein is consumed as part of a mixed meal, quantities greater than the \sim 30 g/meal provided to the balanced group in the current study are necessary to achieve the hyperaminoacidemia and increase in intracellular leucine availability required to maximally stimulate MyoPS.

A shortcoming of our study is that we did not measure longer-term MyoPS at baseline while the participants were in energy balance (EB). Therefore it is unclear

whether there were ER-induced changes in longer-term integrated MyoPS. In our previous study we did measure acute MyoPS at baseline in EB and showed that RT combined with a balanced, but not a skewed, distribution of protein ingestion restored the lower fed-state rates of acute MyoPS during ER to the higher EB levels (16). Nevertheless, although not statistically significant, the rates of longer-term, integrated MyoPS during ER+RT were at least qualitatively in line with greater MyoPS in the balanced group $(1.64 \pm 0.15 \%/d)$ compared to the skewed group $(1.52 \pm 0.21 \%/d)$.

In contrast to the lack of demonstrable effect of protein distribution on longerterm MyoPS, we report a clear effect of RT on integrative MyoPS during ER in older men. Illustrating the potency of this stimulus, we observed that just 6 sessions of low-load, high volume RT performed over 2 weeks was sufficient to increase longer-term MyoPS by ~26% compared to ER alone. This finding is in line with our acute data showing higher rates of MyoPS at the end of ER+RT versus ER (16) and supports previous work showing that the incorporation of RT can attenuate muscle mass loss during ER in older adults (30).

We report for the first time that performance of RT during ER increased the synthesis of 175 (of 190 measured) individual skeletal muscle proteins in the myofibrillar, sarcoplasmic and mitochondrial protein fraction, compared to ER alone. To the best of our knowledge, we are the second study to date to measure the synthetic rates of a large number of individual skeletal muscle proteins in humans (18), and the first to report these in response to RT. Our data indicate that, even in the presence of ER, performance of RT elevated the synthesis of a broad array of individual skeletal muscle proteins across the

proteome including not only contractile proteins but also sarcoplasmic and mitochondrial proteins (Table 1; Figure 2). Interestingly, Bell et al (31) recently reported that a bout of resistance exercise resulted in no change in the sub-fractional rate of sarcoplasmic protein synthesis, which in that study would have included both cytosolic and mitochondrial proteins, measured over 24-48 h using ²H₂O labeling in older men. Similarly, our acute findings indicated that sarcoplasmic protein synthesis was comparable after ER and ER+RT in both the BAL and SKEW groups (16). The apparent inconsistency between the results of the latter studies and our individual proteome kinetics data may be explained by the dramatic variability in protein turnover rates within the sarcoplasmic pool, highlighting the potential for sub-fractional analysis to mask dynamic changes in individual proteins within a tissue fraction. Indeed, the ability to discern the FSR for individual skeletal muscle proteins, rather than global tissue or even sub-fractional synthetic rates, provides unique insight into the response to an intervention that would otherwise not be possible. The potential applications of this are far reaching and future work using proteome dynamic techniques could, potentially, lead to the identification of a 'biological blueprint' of the skeletal muscle response to resistance training (and many other) interventions. This could have profound implications for optimizing exercise and other treatment interventions for the prevention and management of sarcopenia as well as other musculoskeletal conditions.

In conclusion, we report no influence of the pattern of protein ingestion over the day on the integrated rate of MyoPS measured over 2-wk of ER alone or 2-wk of ER+RT in overweight/obese older men. We provide novel data showing that short-term RT

increases both longer-term integrated MyoPS and the synthesis of a number of individual skeletal muscle proteins under conditions of ER in older adults. This highlights the potency of RT, particularly in ER, and supports its inclusion during ER to achieve weight loss with a high fat-to-lean ratio in aging persons.

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CHAPTER 4:

Leucine supplementation enhances integrative myofibrillar protein synthesis in free-

living older men consuming lower and higher protein diets. In preparation.

Leucine supplementation enhances integrative myofibrillar protein synthesis in freeliving older men consuming lower and higher protein diets

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Running Head: Leucine supplementation and integrative muscle protein synthesis

ABSTRACT

Background: Leucine co-ingestion with lower protein-containing meals may overcome the blunted muscle protein synthetic response in the elderly, but may only be effective among those consuming lower dietary protein intakes.

Objective: We examined the impact of leucine co-ingestion with mixed macronutrient meals on integrated 3-d rates of myofibrillar protein synthesis (MyoPS) in free-living older men consuming daily protein intakes above (1.2 g/kg/d) or at the RDA (0.8 g/kg/d), in a rested (REST) and resistance exercise (REX) condition.

Design: Twenty healthy older men (age 72 ± 5 y, BMI 28 ± 3 kg/m²) were randomized to receive lower protein (LP = 0.8 g/kg/d) or higher protein (HP = 1.2 g/kg/d) diets while ingesting a placebo (PLAC) and leucine (LEU; 5 g/meal) supplement with their 3 main daily meals. A bout of unilateral REX was performed during the PLAC and LEU treatments. Ingested ²H₂O to label alanine with skeletal muscle biopsies was used to measure the integrated rate of MyoPS over the 3-d of PLAC and LEU in the REST and REX leg.

Results: Leucinemia was higher in LEU than PLAC (main effect for treatment p < 0.001). MyoPS was similar in LP and HP during both treatments (p = 0.39) but was higher in the LEU versus PLAC in the REST (pooled mean: LEU 1.57 ± 0.11; PLAC 1.48 ± 0.08 %/d, main effect for treatment p < 0.001) and REX (pooled mean: LEU 1.87 ± 0.09; PLAC 1.71 ± 0.10 %/d, main effect for treatment, p < 0.001) legs.

Conclusions: Leucine co-ingestion with daily meals enhanced integrated MyoPS in freeliving older men in REST and REX conditions and was equally effective among those consuming daily protein intakes at and above the RDA. The potential for long-term leucine co-ingestion to attenuate sarcopenic muscle loss, particularly in combination with REX, warrants further study. This trial was registered (clinicaltrials.gov) NCT02371278.

INTRODUCTION

The progressive loss of skeletal muscle mass with advancing age, termed sarcopenia, is associated with declines in muscle strength and function and contributes substantially to frailty onset, disability, physical dependence, and mortality among older adults (1-3). Sarcopenic muscle loss is in part due to disturbances in skeletal muscle protein turnover, whereby rates of muscle protein breakdown (MPB) chronically exceed those of muscle protein synthesis (MPS). Increased external loading of muscle and hyperaminoacidemia are powerful stimuli for MPS and represent two major factors that affect muscle mass (4). Aging is associated with an attenuated MPS response compared to younger persons that is particularly apparent with small-to-moderate 'meal-like' quantities of dietary protein (~20 g protein/meal or less), a phenomenon that has been termed 'anabolic resistance' and is implicated as a key factor underpinning sarcopenia (5-7). While the anabolic resistance to protein feeding can be overcome with the ingestion of larger protein servings (~0.4g protein/kg/meal, equivalent to $\sim 30 - 40$ g protein/meal); many older adults may find it challenging to consume these quantities of food-based protein on a per-meal basis (7). Consequently, there is considerable interest in potential strategies to augment the MPS response to lower protein doses in the elderly.

Of the essential amino acids (EAA), leucine has a pivotal role as a key activator of

translation initiation (8) and accumulating evidence supports the thesis that leucinemia, and increases in intracellular leucine concentrations, serve as the 'signal' driving the MPS response to dietary protein ingestion (9-12). Importantly, however, despite promising results from acute studies examining the impact of leucine ingestion on MPS (9-12), longer-term intervention trials have been unable to show clear benefits of leucine supplementation on muscle mass and strength in older adults (13-15). A number of factors may contribute to the disparity between the results from acute leucine supplementation (9-12) and the long-term outcomes (13-15). For example, the participants in the longer-term studies were already consuming protein intakes above the RDA and in line with recent recommendations for optimal protein intake in healthy older adults (1.0 - 1.2 g/kg/d). Thus, the additional leucine may have had a minimal impact in augmenting postprandial MPS and ultimately muscle mass (13, 14). Nevertheless, numerous age-related issues are known to negatively impact protein intake (16) and the significant proportion of elderly persons who are unable to meet optimal protein intake guidelines (17, 18) may benefit to a greater extent from leucine supplementation of smaller doses of protein. It is also possible that acute measurements of MPS do not adequately capture longer-term phenotypic (i.e., muscle mass) outcomes. For example, MPS studies were conducted over short time periods (~3-6 h) under controlled laboratory conditions (i.e. following overnight fast, participants typically confined to a laboratory bed) and leucine was co-ingested with either an isolated protein (19) or EAA bolus (10, 20), or as part of a mixed macronutrient meal provided in small aliquots over several hours (21). Protein is, however, most often consumed as food and is co-ingested with

carbohydrate and fat during meals within a discrete eating occasion. Food form (i.e. solid versus liquid) and the addition of fats and carbohydrates can markedly alter the kinetics of intestinal amino acid absorption and subsequent aminoacidemia (22, 23). Further work is necessary to delineate whether the co-ingestion of leucine with normal, mixed macronutrient meals has the capacity to enhance the cumulative, longer-term MPS response in older adults in a free-living environment.

We aimed to examine the impact of 3 days of leucine co-ingestion with mixed macronutrient meals on the integrated myofibrillar protein synthetic response in freeliving older men consuming daily protein intakes at the current RDA (0.8 g/kg/d) or at current recommendations for optimal protein intake in healthy older adults (1.2 g/kg/d) (16, 24), both at rest and when combined with resistance exercise. We hypothesized that leucine co-ingestion with the main daily meals would enhance integrative myofibrillar protein synthesis (MyoPS) in both groups 1) under resting conditions and 2) when combined with the performance resistance exercise.

METHODS

Ethical Approval. This study was approved by the Hamilton Health Sciences Research Ethics Board and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy on the ethical use of human subjects in research (<u>http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web.pdf</u>). Each participant was informed of the purpose of the study, experimental procedures, and potential risks prior to written consent being provided. This trial was registered at

clinicaltrials.gov as NCT02371278.

Participants. Figure 1 shows a flowchart of the progress from recruitment through completion of the study. Twenty older adult men (age 72 ± 5 y, body mass index (BMI) $28 \pm 3 \text{ kg/m}^2$) were recruited to participate in the study through posters and via local newspaper advertisements. Inclusion criteria were: men, 65-85 y of age, BMI between 20-35 kg/m², non-smokers, and generally healthy according to responses to a standard health screening questionnaire. Exclusion criteria included: diabetes mellitus, cardiovascular disease, renal disease, gastrointestinal disease, neuromuscular disease, infectious disease, cancer, significant body mass changes in the 1 month period prior to the study, vegetarianism, and use of medications known to interfere with muscle metabolism including beta-blockers, high dose non-steroidal anti-inflammatory drugs, corticosteroids, hormone replacement therapy, warfarin, prescription strength acne medications, oral hypoglycaemic agents and insulin.



Figure 1. Participant flow chart.

Study overview. An overview of the study design is shown in Figure 2. Before entry, participants were randomized to one of two groups matched for age and BMI: higher protein (HP: 1.2 g/kg/d) or lower protein (LP: 0.8 g/kg/d) intake (n=10/group). Following baseline testing, participants commenced a 9-d controlled diet designed to meet energy requirements for weight maintenance and to provide a daily protein intake according to each participant's group allocation (i.e. 0.8 or 1.2 g/kg/d). Days -3 to -1 of the diet served

as a pre-study dietary habituation period and no supplements were consumed during this time. During days 0 - 2 participants consumed a placebo supplement (PLAC) with breakfast, lunch and dinner and during days 3 - 5 participants consumed an isonitogenous and energy-matched, to PLAC, supplement containing 5 g of leucine with each of the three daily meals (leucine treatment; LEU). The PLAC and LEU supplement contents are given in Table 1. Participants performed a bout of unilateral leg extension exercise at the start of the PLAC (day 0) and LEU (day 3) treatments. The integrated rate of MyoPS was measured in the exercised and rested leg over the 3 d of PLAC (day 0 - 2) and LEU (day 3 - 5) using orally ingested ²H₂O and muscle biopsies obtained from the *vastus lateralis* on the morning of days 0, 3 and 6. Aminoacidemia, insulinemia and glycaemia were determined in response to the co-ingestion of the supplements with the daily meals during the PLAC (day 2) and LEU (day 5) treatments.



Figure 2. Schematic overview of study design. REE, resting energy expenditure; DEXA, dual-energy x-ray absorptiometry; PA, physical activity.

Baseline testing. Prior to commencing the study participants were asked to wear a pedometer (Piezo SC-StepXTM, StepsCount, Deep River, ON, Canada) and to complete a physical activity and weighed food record (Nutribase version 11.5, Cybersoft Inc., Phoenix, AZ, USA) for 3 days (2 weekdays and 1 weekend day) to assess habitual physical activity levels and dietary intake. Approximately 1 week before commencing the study diet participants reported to the laboratory in the morning in the fasted state. Participants arrived by car and were instructed to minimize physical activity prior to the visit. Height was measured to the nearest 0.1 cm using a stadiometer and body mass was assessed to the nearest 0.1 kg using a calibrated scale. A venous blood sample was

obtained and resting energy expenditure (REE) was measured using a ventilated hood system (Moxus modular oxygen uptake system, AEI Technologies, Pittsburgh, PA, USA). REE testing was performed in a thermoneutral environment with participants lying quietly in a supine position. After a 10-min adaptation to the hood, VO_2 and VCO_2 were measured continuously for 30 min. Following the REE measurement participants underwent unilateral strength testing for the right knee extensors using a seated knee extension device (Atlantis C-605 unilateral leg extension). Participants performed a series of graded-load knee extensions to determine their 10-repetition maximum strength (10-RM) and the maximum load that they could lift for 15 - 24 repetitions.

Diets. Each participant's energy requirement to maintain energy balance was determined using REE with the appropriate activity factor (25). Activity factors were determined for each participant based on their baseline physical activity records and daily step counts. Each morning throughout the study body mass was recorded prior to food or drink consumption and a fasted dual-energy X-ray absorptiometry scan (DEXA; GE-LUNAR iDXA, Aymes Medical, Newmarket, ON) was performed immediately before and after the 9-d diet to confirm that participants were maintained in energy balance throughout the intervention. For participants in the LP group the diet provided a protein intake of 0.8 g/kg/d reflecting the current RDA for protein in adults \geq 19 y (26). In the HP group the diet provided 1.2 g protein/kg/d in line with the recent recommendations from a number of expert committees for an optimal protein intake in healthy older adults (16, 24). The increased protein intake in HP was achieved by reducing the proportion of energy from carbohydrate, while the proportion of energy from fat (30%) was kept constant between

the two groups. In both groups, protein was provided from a variety of plant- and animalbased sources. In order to emulate the skewed protein intake pattern typically consumed by older adults (17, 27) protein was distributed across breakfast, lunch, dinner and an evening snack in the proportions ~15%: 25%: 50%: 10%. Participants were required to abstain from alcohol for the duration of the 9-d diet.

To enhance compliance study diets were designed by a research dietitian who met with each participant individually to customize meal plans according with their personal food preferences. Participants were supplied with all of the food required for the duration of the study which consisted of prepackaged, frozen meals (Copper County frozen foods; Cambridge, ON) and other items that required minimal preparation (i.e. granola bars, fruit cups, juices). Participants were asked to consume breakfast within 30 min of waking and were instructed to space meals ~4-6 hours apart. This meal spacing was selected to reflect a traditional meal pattern and in an attempt to ensure that a 'muscle full' effect persisting from the previous meal did not hamper the MyoPS response to the subsequent meal (28, 29). To assess compliance participants were instructed to record the time of day meals were eaten and the percentage of their food items consumed in a log. Participants were strongly encouraged to consume all of the food provided to them and to avoid eating food not provided by the study, but if this occurred, the participant were asked to record these deviations in their meal plan log.

Supplementation. All participants were provided with identically flavored (lemon), supplemental beverages (Infinit Nutrition, Windsor, ON) and were instructed to consume one beverage midway through each main meal (breakfast, lunch and dinner) from day 0 –

5 of the intervention diet. No supplement was consumed with the evening snack. During day 0 - 2 (PLAC) participants consumed a placebo beverage containing 5 g of nonessential amino acids (NEAA; alanine and glycine) and during day 3 - 5 (LEU) the beverage contained 5 g of crystalline leucine (Table 1). The order of the supplementation periods (i.e. PLAC followed by LEU) was not randomized but was always PLAC prior to LEU to avoid any potential chronic influence of prior leucine supplementation on the MyoPS response (30). Individual servings of the supplement were packaged into a sealed pouch by Infinit Nutrition in powder form and participants were asked mix this with 250 mL of water at home prior to consumption. The beverages were provided in a single-blind manner and were isonitrogeous, energy-matched and indistinguishable in odor, color, and taste (Table 1).

	Supplements	
	LEU	PLAC
Leucine	5.0	0.0
Alanine	0.0	3.5
Glycine	0.0	1.5
Maltodextrin	3.0	3.0
Sucrose	7.0	7.0
Stevia	0.2	0.2
Citric acid	1.2	1.2
Flavoring	1.2	0.3
Totals		
\sum TAA (g)	5.0	5.0
Carbohydrate (g)	10.0	10.0
Energy (kcal)	60.0	60.0

Table 1. Nutritional composition of the supplements per single serving (g)

LEU; leucine supplement, PLAC; placebo supplement, TAA, total amino acids.

Physical activity. Each participant's habitual daily step count was determined based on the average of their pedometer counts measured for 3 d at baseline. To ensure that activity levels remained consistent throughout the intervention participants were asked maintain their daily step count within 10% of their average value. This was monitored with a pedometer and recorded by the participants daily on their meal plan logs. Participants were asked to abstain from any resistance exercise (RE) other than the exercises performed as part of the intervention.

Blood sampling trials. To characterize the 'typical' patter of aminoacidemia, insulinemia and glycaemia in response to the daily meals and supplements in the PLAC and LEU phases of the study, participants underwent a blood sampling trial on the final day of PLAC (day 2) and on the final day of LEU (day 5). Participants reported to the laboratory following an overnight fast at ~0630h. Upon arrival a catheter was inserted into an antecubital vein for blood sampling and participants were provided with their individualized breakfast, lunch and dinner meals in the laboratory. Breakfast was provided at 0700h, lunch 240 min following the completion of breakfast (~1130h) and dinner 300 min after the completion of lunch (~1700h). Blood samples were obtained before breakfast and 20, 40, 60, 90, 120, 180, 240 min following the ingestion of each meal, as well as 300 min following the consumption of lunch (Figure 3). On day 2 (placebo trial) the PLAC supplement was consumed and on day 5 (leucine trial) the LEU supplement was consumed midway through each meal. To attenuate the suppressive effects of inactivity on MyoPS and to simulate normal daily movement during this trial participants were asked to walk around the University campus between meals. Steps were

monitored using a pedometer and participants were required to walk until they reached their habitual daily step count.



Figure 3. Blood sampling trial protocol. Note that each meal was consumed over ~30 min as reflected by the width of the 'meal intake' boxes. The placebo and leucine supplements were consumed midway through each meal during the placebo and leucine trials, respectively. Participants consumed their evening snack upon returning home after the trial.

Isotope protocol. Oral consumption of ${}^{2}H_{2}O$ (70 Atom%, Cambridge Isotope

Laboratories, Tewksbury, MA) was used to label newly synthesized myofibrillar proteins as previously described (31). Participants reported to the laboratory in the fasted state on day 0 and, following collection of a saliva sample and a muscle biopsy from the *vastus lateralis*, participants consumed a single 100 ml oral bolus of ²H₂O at approximately 0900h. Immediately following the ²H₂O bolus participants performed a bout of unilateral leg extension exercise consisting of three sets at the previously determined maximum load they could lift for 15-24 repetitions (~50% of 1-RM) until volitional failure. The exercise was performed using the left leg and with a rest interval of 2 min between sets.

This unilateral study design meant that the rested leg was exposed to the effect of feeding/supplementation alone, whereas the exercised leg was exposed to the combination of feeding/supplementation and resistance exercise (REX). On day 3 participants performed an identical REX session with the exception that the exercise was performed on the right leg and that repetitions were clamped at the same number of repetitions per set achieved on day 0. The exercise was performed on different legs in the PLAC and LEU treatments in view of previous work suggesting that the acute transcriptional (32) and protein synthetic (33) responses to an initial bout of REX may vary substantially from the responses to subsequent bouts in untrained individuals. Therefore, varying the exercise leg allowed us to examine the MyoPS response to REX in an 'exercise-naïve' leg during both the PLAC and LEU treatments. Following both exercise sessions participants consumed their individualized breakfast (with the PLAC beverage on day 0 and with the LEU beverage on day 3) in the laboratory before returning home. Bilateral muscle biopsies were obtained prior to the exercise on day 3 (end of PLAC) and on day 6 (end of LEU). All muscle biopsies were obtained using a 5mm Bergström needle adapted for manual suction under 2% xylocaine local anaesthesia. The tissue samples were freed from visible fat and connective tissue and frozen immediately in liquid nitrogen for further analysis. All muscle biopsies were obtained in the fasted state between 0900h and 1000h.

Total body water ²H enrichment can be used as a surrogate for plasma alanine labeling (33) and was determined from saliva swabs collected by participants at ~0900h prior to, and every morning following, the ingestion of the ²H₂O bolus (33). Participants

were instructed not to eat or drink anything for 30 min before saliva sampling and samples were stored at -20°C prior to analysis.

Analytical methods. Plasma glucose concentrations were measured using the glucose oxidase method (YSI 2300; Yellow Springs, OH). Plasma insulin concentrations were measured using the dual-site chemiluminescent method (Siemens Immulite 2000; Malvern, PA). Plasma amino acid concentrations were analyzed via gas-chromatographymass spectrometry (GC-MS) using the Phenomenex EZfaast[™] (Torrance, CA) amino acid analysis kit as per the manufacturer's instructions.

Myofibrillar-enriched proteins were isolated from the muscle biopsies as previously described (34). The incorporation of deuterium (²H) into protein bound alanine was determined with a Thermo Finnigan Delta V isotope ratio mass spectrometry coupled to a Thermo Trace GC Ultra with a gas chromatography combustion interface III and Conflow IV (Metabolic Solutions, Nashua, NH) as previously described (35). Saliva samples were analyzed for ²H enrichment by cavity ring-down spectroscopy using a Liquid Water Isotope Analyzer with automated injection system (Los Gatos Research, Mountain View, CA) as previously described (35).

Calculations. The fractional synthetic rate (FSR) of myofibrillar protein synthesis was determined from the incorporation of deuterium labeled alanine into protein, using the enrichment of body water (corrected for the mean number of deuterium moieties incorporated per alanine, 3.7) as the surrogate precursor labeling between subsequent biopsies. In brief, the standard equation:

FSR $(\%/d) = [(APE_{Ala})]/[(APE_P) \times t] \times 100$

Where, $APE_{Ala} =$ deuterium enrichment of protein bound alanine, $APE_P =$ mean precursor enrichment over the time period and *t* is the time between biopsies, was used. **Statistical analyses.** All analyses were performed using SPSS (version 22.0, Chicago, IL, USA). Exercise and dietary intervention variables were analyzed using a two-factor mixed-model (group x treatment) ANOVA. Area under the curve (AUC) and concentration maximum (C_{max}) for plasma concentrations (glucose, insulin, amino acids) were examined using a three-factor (group x treatment x meal) mixed-model ANOVA. MyoPS was analyzed using a three-factor (group x treatment x condition [REST vs REX]) mixed-model ANOVA. Tukey's posthoc test with a Bonferroni correction for multiple comparisons was performed whenever a significant interaction was found to isolate specific differences. Statistical significance was accepted when $p \le 0.05$. Results are presented as means \pm SEM in graphs and as means \pm SD in text and tables.

RESULTS

Participants. Participant characteristics are shown in Table 2. Participants' habitual dietary intake assessed at baseline is shown in Table 3.

	LP	HP
Age (y)	72 ± 4	73 ± 6
Height (m)	1.7 ± 0.1	1.7 ± 0.1
Body mass (kg)	85.2 ± 9.9	81.4 ± 0.1
Body mass index (kg/m ²)	28.2 ± 2.5	26.7 ± 3.1
Fat mass (kg)	26.3 ± 6.3	24.1 ± 7.1
Lean body mass (kg)	55.7 ± 4.2	54.0 ± 5.3
Fasting blood glucose (mM)	5.5 ± 0.4	5.6 ± 0.3
Fasting insulin (µU/mL)	7.7 ± 2.3	7.1 ± 2.1
$HbA1_{c}(\%)$	5.4 ± 0.4	5.3 ± 0.2
LDL (mM)	3.0 ± 0.7	2.5 ± 0.5
HDL (mM)	1.2 ± 0.2	1.2 ± 0.3
Tg (mM)	0.97 ± 0.29	1.04 ± 0.52
Average steps per day	9065 ± 3598	11860 ± 6329

Table 2. Baseline participant characteristics

Values are means \pm SD (n = 10/group). LP, lower protein group; HP, higher protein group; HbA1_c; glycated hemoglobin; LDL; low density lipoprotein, HDL; high density lipoprotein, Tg; triglycerides.

Table 3. Baseline dietary intake measured by 3-day weighed diet record

	LP	HP
Energy (kcal/d)	2143 ± 359	2133 ± 417
Fat (g/d)	87 ± 23	75 ± 22
Fat (% total energy intake)	37 ± 6	33 ± 7
CHO (g/d)	237 ± 48	257 ± 61
CHO (% total energy intake)	41 ± 6	44 ± 6
Protein (g/d)	97 ± 20	89 ± 23
Protein (g/kg BM/d)	1.2 ± 0.3	1.1 ± 0.4
Protein (% total energy intake)	19 ± 2	17 ± 2
Alcohol (% total energy intake)	3 ± 5	6 ± 5

Values are means \pm SD (n = 10/group). LP, lower protein group; HP, higher protein; CHO, carbohydrate; BM, body mass.

Physical activity and exercise variables. Participants maintained their habitual daily

step counts throughout the intervention (p = 0.28) and step counts were not different

between the groups (p = 0.23, PLAC: LP = 9159 \pm 3601, HP = 12116 \pm 6131; LEU: LP =

 9870 ± 3168 , HP = 12194 ± 5378). The volume (load [kg] x number of repetitions) of leg extension exercise performed was similar during the PLAC and LEU phases (p = 0.61). **Body composition, diet and supplementation.** There was no change in body mass (Day $-3 LP = 85.2 \pm 10.0$, Day 6 LP = 85.3 ± 10.2 ; and Day $-3 HP = 81.4 \pm 9.1$, Day 6 HP = 81.3 ± 9.1 , p = 0.76) or in any of the body composition variables over the course of the study (all p > 0.05, data not shown) indicating that participants in both groups were maintained in a state of energy balance throughout the intervention. The intervention diet provided energy from protein, carbohydrate and fat in the proportions of ~10%: 60%: 30% in LP and 15%, 55%, 30% in HP, respectively. There was a high level of reported compliance with the prescribed intervention diet with no difference between groups (PLAC: $LP = 98 \pm 3\%$, $HP = 98 \pm 2\%$; LEU: $LP = 98 \pm 3\%$, $HP = 95 \pm 8\%$, p = 0.32). Reported adherence with supplementation was also high with no group differences (PLAC: LP = $100 \pm 0\%$, HP = $99 \pm 3\%$; LEU: LP = $100 \pm 2\%$, HP = $99 \pm 3\%$, p = 0.46). Combined intakes from diet and supplements throughout the intervention are summarized in Table 4. Total leucine intake from diet plus supplements during LEU (LP: $\sim 0.24 \pm$ 0.02 g/kg/d, HP: ~0.28 ± 0.03 g/kg/d in HP) remained substantially lower that the upper tolerable and safe limit in humans of 0.53 g/kg/d (36).

Plasma glucose, insulin and amino acid concentrations. Fasting glucose and insulin concentrations (Figure 4), as well as HOMA-IR (PLAC: $LP = 2.0 \pm 0.2$, $HP = 2.0 \pm 0.2$, LEU: $LP = 2.2 \pm 0.2$, $HP = 2.0 \pm 0.2$), were similar in LP and HP in both treatments (p > 0.05). Glucose AUC and C_{max} following breakfast, lunch and dinner did not differ between groups or treatments (p > 0.05). Insulin AUC following the meals did not differ

between groups (p = 0.965) or treatments (p = 0.707). However, there was a meal x treatment interaction for insulin C_{max} such that it was higher following dinner in LEU (40.2 ± 9.7 µIU/mL) compared to PLAC (32.2 ± 9.6 µIU/mL; p = 0.003), with no difference between groups (p = 0.928).

Plasma concentrations over time for the sum of the EAAs, leucine, isoleucine (Ile) and valine (Val) are shown in Figure 5. There were no differences in fasting concentrations of plasma leucine and \sum EAAs between groups or treatments. There was a treatment x group interaction for fasting plasma Ile concentration (p < 0.001) and a trend for a treatment x group interaction for fasting plasma Val concentration (p = 0.05). Tukey's posthoc tests revealed that fasting plasma Ile concentration was lower in LP during LEU compared to PLAC (PLAC 65 ± 9, LEU 46 ± 12 nmol/mL, p = 0.002) whereas fasting Ile concentration was higher during LEU compared to PLAC in HP (PLAC 46 ± 9, LEU 60 ± 3 nmol/mL, p = 0.023). Fasting plasma Val was similar in PLAC and LEU in LP (PLAC 141 ± 20, LEU 143 ± 33 nmol/mL, p = 0.786) but were higher in LEU in the HP group (PLAC 123 ± 20, LEU 153 ± 36 nmol/mL, p = 0.005).

The AUC_{pos}, C_{max}, T_{max}, and AUC_{neg} were analyzed for plasma $\sum EAAs$, leucine, Ile and Val for each meal and are shown for $\sum EAAs$ and leucine in Table 5. For plasma leucine AUC_{pos} and C_{max} were higher, and AUC_{neg} was lower, in LEU compared to PLAC (p = 0.000) with no difference between groups (p > 0.05). The T_{max} for leucine occurred later in PLAC than LEU (p = 0.000) with no difference between groups (p = 0.069). AUC_{pos} and C_{max} for $\sum EAAs$ were higher in HP compared to LP (< 0.01) and were higher in both groups during LEU compared to PLAC (p < 0.05). The T_{max} for $\sum EAAs$ occurred later in HP compared to LP (p = 0.004) with no difference between treatments (p = 0.977). AUC_{pos} for Ile and Val did not differ between groups or treatments (p > 0.05). There was a group x treatment interaction for Ile AUC_{neg} (p = 0.048) such that it was greater in LEU than PLAC in HP (p = 0.022) but not in LP. Val AUC_{neg} did not differ between groups or treatments (p > 0.05). There was a group x treatment interaction for Ile and Val C_{max} such that it was similar in PLAC and LEU in LP but was higher in LEU than PLAC in HP (p < 0.05).
Treatment	Group	Meal	Energy		Protein			Fat	Cl	HO	Fibre	Leucine
			kcal/kg	g	g/kg BM	g/kg FFM	g	g/kg BM	g	g/kg BM	g	g
		В	8 ± 1	13 ± 3	0.15 ± 0.03	0.23 ± 0.04	16 ± 6	0.2 ± 0.1	126 ± 19	1.5 ± 0.1	7 ± 2	1.0 ± 0.2
		L	9 ± 1	17 ± 3	0.20 ± 0.03	0.31 ± 0.05	31 ± 5	0.4 ± 0.1	110 ± 22	1.3 ± 0.2	8 ± 2	1.4 ± 0.3
	LP	D	11 ± 1	31 ± 4	0.36 ± 0.04	0.55 ± 0.05	35 ± 9	0.4 ± 0.1	123 ± 18	1.5 ± 0.2	10 ± 2	2.4 ± 0.3
		S	5 ± 1	6 ± 2	0.07 ± 0.01	0.11 ± 0.02	12 ± 3	0.1 ± 0.1	68 ± 19	0.8 ± 0.2	4 ± 1	0.5 ± 0.1
		Total	33 ± 3	67 ± 9	0.79 ± 0.03	1.20 ± 0.09	94 ± 16	1.1 ± 0.1	428 ± 60	5.0 ± 0.5	29 ± 5	5.4 ± 0.7
PLAC												
		В	7 ± 1	18 ± 3	0.22 ± 0.03	$0.34\ \pm 0.07$	19 ± 3	0.2 ± 0.0	85 ± 20	1.1 ± 0.3	6 ± 1	1.5 ± 0.3
		L	10 ± 2	27 ± 3	0.34 ± 0.03	0.51 ± 0.07	28 ± 5	0.4 ± 0.1	111 ± 13	1.4 ± 0.3	9 ± 1	2.2 ± 0.3
	HP	D	13 ± 2	49 ± 6	0.61 ± 0.04	0.92 ± 0.11	32 ± 4	0.4 ± 0.1	126 ± 12	1.6 ± 0.3	10 ± 2	3.9 ± 0.5
		S	5 ± 2	7 ± 2	0.09 ± 0.03	0.13 ± 0.04	14 ± 4	0.2 ± 0.5	74 ± 22	0.9 ± 0.3	6 ± 4	0.5 ± 0.1
		Total	35 ± 6	$102 \pm$	1.26 ± 0.06	1.90 ± 0.21	92 ± 10	1.2 ± 0.2	395 ± 51	4.9 ± 1.0	30 ± 5	8.2 ± 0.9
		В	8 ± 1	13 ± 3	0.15 ± 0.03	0.23 ± 0.04	16 ± 6	0.2 ± 0.1	126 ± 19	1.5 ± 0.1	7 ± 2	6.0 ± 0.2
		L	9 ± 1	17 ± 4	0.20 ± 0.03	0.31 ± 0.05	31 ± 6	0.4 ± 0.1	110 ± 23	1.3 ± 0.2	8 ± 2	6.4 ± 0.3
	LP	D	11 ± 1	31 ± 4	0.36 ± 0.04	0.55 ± 0.05	34 ± 8	0.4 ± 0.1	123 ± 15	1.5 ± 0.2	10 ± 2	7.4 ± 0.3
		S	5 ± 1	6 ± 2	0.07 ± 0.01	0.11 ± 0.03	12 ± 3	0.1 ± 0.0	71 ± 18	0.8 ± 0.2	4 ± 1	0.6 ± 0.2
		Total	33 ± 3	67 ± 9	0.79 ± 0.03	1.21 ± 0.09	93 ± 16	1.1 ± 0.1	429 ± 61	5.0 ± 0.5	30 ± 5	20.4 ± 0.7
LEU												
		В	7 ± 1	18± 3	0.22 ± 0.03	0.34 ± 0.07	19 ± 3	0.2 ± 0.3	85 ± 20	1.0 ± 0.3	6 ± 1	6.5 ± 0.3
		L	10 ± 2	27 ± 4	0.33 ± 0.05	0.50 ± 0.07	28 ± 6	0.4 ± 0.1	111 ± 17	1.4 ± 0.3	9 ± 2	7.1 ± 0.3
	HP	D	12 ± 3	46 ± 8	0.57 ± 0.08	0.85 ± 0.09	30 ± 6	0.4 ± 0.1	120 ± 24	1.5 ± 0.4	9 ± 1	8.7 ± 0.6
		S	5 ± 1	7 ± 2	0.09 ± 0.03	0.13 ± 0.04	14 ± 4	0.2 ± 0.1	70 ± 18	0.9 ± 0.3	6 ± 4	0.6 ± 0.2
		Total	35 ± 7	98 ± 13	1.21 ± 0.12	1.82 ± 0.16	90 ± 14	1.1 ± 0.2	386 ± 64	4.8 ± 1.1	30 ± 6	22.9 ± 1.0

Table 4. Dietary intake during the placebo (PLAC; day 0 to 2) and leucine (LEU; day 3 to 5) treatment periods

Values are means \pm SD. CHO, carbohydrate; BM, body mass; FFM, fat free (bone free) mass; LP, lower protein group; HP, higher protein group; B, breakfast; L, lunch; D, dinner; S, evening snack. Intakes were recorded by participants on the meal plan food logs

and include food and supplements. Dietary leucine intake was estimated assuming that on average leucine accounts for ~8% of the amino acid content of protein (37).



Figure 4. Plasma concentrations of glucose (mmol/L; A) and insulin (μ IU/mL; B) in response to the co-ingestion of the placebo and leucine supplements with breakfast, lunch and dinner in the lower protein (LP) and higher protein (HP) groups. Arrows indicate a meal co-ingested with a supplement (placebo or leucine). Values are means ± SEM, n =10/group.



Figure 5. Plasma concentrations of essential amino acids (EAA, nmol/mL; A), leucine (nmol/mL; B), isoleucine (nmol/mL; C) and valine (nmol/mL; D) in response to the co-ingestion of the placebo and leucine supplements with breakfast, lunch and dinner in the low protein (LP) and high protein (HP) groups. Arrows indicate a meal co-ingested with a supplement (placebo or leucine). Values are means \pm SEM, n =10/group.

Meal	Meal	PL	AC	LEU		
		LP	HP	LP	HP	
Leucine						
AUCpos†	В	1078 ± 1010	3050 ± 2554	36052 ± 12458	40927 ± 13230	
	L	4346 ± 2586	6662 ± 3115	36280 ± 14380	34638 ± 11187	
	D	7544 ± 2683	8920 ± 5166	41137 ± 15386	38098 ± 13154	
Cmax (µM)†	В	118 ± 22	141 ± 18	452 ± 154	449 ± 107	
	L	152 ± 25	168 ± 25	442 ± 134	479 ± 104	
	D	190 ± 23	182 ± 21	462 ± 119	456 ± 84	
Tmax (min)†	В	50 ± 11	50 ± 25	28 ± 14	53 ± 51	
	L	73 ± 24	91 ± 57	26 ± 10	28 ± 14	
	D	56 ± 8	60 ± 23	20 ± 0	24 ± 8	
AUCneg†	В	1372 ± 849	682 ± 854	0 ± 0	0 ± 0	
	L	564 ± 546	431 ± 450	90 ± 285	0 ± 0	
	D	247 ± 420	343 ± 737	0 ± 0	0 ± 0	
ΣΕΑΑ						
∠ AUCpos*†	В	34435 ± 14710	44915 ± 15532	36921 ± 17837	48832 ± 19424	
I I	L	47590 ± 13594	92867 ± 25774	50874 ± 17391	100530 ± 33046	
	D	72688 ± 21907	102968 ± 28867	75952 ± 21065	108287 ± 27460	
Cmax (µM)*†	В	871 ± 91	911 ± 59	861 ± 95	975 ± 83	
	L	949 ± 75	1114 ± 187	961 ± 90	1207 ± 235	
	D	1098 ± 81	1271 ± 199	$1104\ \pm 83$	$1287\ \pm 164$	
Tmax (min)*	В	38 ± 18	64 ± 32	47 ± 19	64 ± 32	

Table 5. Plasma leucine and \sum EAA responses to breakfast, lunch and dinner with placebo (PLAC) and leucine (LEU) supplementation

	L D	$69 \pm 84 \\ 59 \pm 14$	$93 \pm 41 \\ 87 \pm 22$	$\begin{array}{c} 55\pm49\\ 59\pm14\end{array}$	$\begin{array}{c} 102\pm40\\ 84\pm24 \end{array}$
AUCneg	В	974 ± 2494	392 ± 687	36 ± 113	513 ± 1082
	L	709 ± 1912	213 ± 674	144 ± 455	48 ± 152
	D	0 ± 0	117 ± 370	36 ± 113	0 ± 0

Values are means \pm SD (n = 10/group). LP, lower protein group; HP, higher protein group; B, breakfast; L, lunch; D, dinner; AUC_{pos}, AUC above baseline; C_{max}, maximum concentration; T_{max}, time of maximum concentration; AUC_{neg}, AUC below baseline; EAA, essential amino acids. Data were analyzed using a 3-factor (group x treatment x meal) mixed-model ANOVA. *Main effect for group, †main effect for treatment. Plasma amino acids concentrations were measured immediately before and 20, 40, 60, 90, 120, 180 and 240 min after all meals and additionally 300 min after lunch.

Body water enrichment. Figure 6A shows the mean body water enrichment over the 6 d MyoPS measurement period. A single bolus of 100 mL of 70% 2 H₂O led to a body water enrichment of 0.131 ± 0.013% 24 h post ingestion (range: 0.114 – 0.171%). Body water enrichment followed an exponential decay pattern (r² = 0.99), decaying slowly over the 6 d period reaching 0.084 ± 0.008 % (range: 0.073 - 0.105%) at the end of the study.

Myofibrillar protein synthesis. MyoPS was similar between the LP and HP groups (p = 0.39), however there were main effects for treatment (p < 0.001) and condition (p < 0.001), as well as a treatment x condition interaction (p = 0.016; Figure 6B). Tukey's post hoc test revealed that myofibrillar FSR was higher in LEU than PLAC in the rested condition (pooled mean: LEU 1.57 \pm 0.11; PLAC 1.48 \pm 0.08 %/d, p < 0.001) as well as in the REX condition (pooled mean: LEU 1.87 \pm 0.09; PLAC 1.71 \pm 0.10 %/d, p < 0.001). MyoPS was higher in the resistance-exercised leg compared to the rested leg in both PLAC and LEU (p < 0.001).



Figure 6. A) Exponential time course of body water enrichment over 6 days following oral bolus of 100 mL ${}^{2}\text{H}_{2}\text{O}$ (70 atoms %). APE, atom percent excess. Values are mean ± 95% CI. B) The integrated myofibrillar fractional synthetic rate (%/d) in the rested (REST) and the exercised (REX) leg in older men consuming lower protein (LP: 0.8 g/kg/d) or higher protein (HP: 1.2 g/kg/d) intakes who underwent 3 d of placebo supplementation and 3 d of leucine supplementation with meals. Boxes represent the 25th to 75th percentiles, horizontal lines and the crosses within the boxes represent median and mean values, respectively. The whiskers represent the minimum and maximum. n = 10/group. Data were analyzed using a 3-factor (group x treatment x condition) mixed-

model ANOVA with Tukey's post hoc test following a treatment x condition interaction (p = 0.016). *Different from placebo treatment in same condition; p < 0.001, † different from rested condition in same treatment; p < 0.001.

DISCUSSION

We discovered that the co-ingestion of 5 g of leucine with normal daily meals enhanced integrative MyoPS in free-living older men and was equally effective in those consuming daily protein intakes at the RDA for protein (0.8 g/kg/d) and at a more 'optimal' level of protein (1.2 g/kg/d) (16, 24). Furthermore, we report that, in addition to enhancing integrative MyoPS under rested (non-exercised) conditions, leucine co-ingestion with meals further potentiated the stimulatory effect of resistance exercise on integrative MyoPS. These are the first data to show under free-living conditions, across days, that supplementation with leucine has a marked stimulatory effect on MyoPS regardless of protein intake and external muscle loading.

It is established that aging is associated with a blunted muscle protein synthetic response to the ingestion of low to moderate quantities of protein (i.e. < ~ 20 g/meal) and this is considered to be a major factor contributing to the progression of sarcopenia (7, 38). Although it has been reported that the attenuated MyoPS response to protein feeding can be overcome with the consumption of greater quantities of high quality protein equivalent to ~ 0.4 g/kg/meal ($\sim 30 - 40$ g/meal) (7), practically, this translates into relatively large per-meal servings of protein-rich foods (i.e., 30 - 40 g protein is 6 large eggs, 1 L milk, ~ 125 g chicken breast, ~ 170 g regular ground beef); and many older adults are likely unable to achieve these optimal per-meal protein intakes at each meal (17). A number of issues are known to negatively affect food and protein intake in the elderly such as poor

appetite, poor dentition, dysphagia, functional disabilities, and food insecurity (16, 24). Furthermore, in some cases, dietary restrictions imposed to slow the progression of certain chronic diseases (i.e., chronic renal disease) may prevent older adults from achieving the protein intakes required to optimize MPS and attenuate muscle loss (39). Here we demonstrate that a practical dietary strategy of leucine co-ingestion (as a liquid supplement) with mixed meals consumed as part of a normal diet can augment the integrated rates of MyoPS in older adults without increasing the total amount of protein consumed. Rather than using capsules of crystalline leucine, we provided the amino acid in a low fluid volume (250 mL), which was well tolerated and did not affect the ability of participants to finish meals. Given that leucine is also readily available and relatively inexpensive, our results indicate that leucine co-ingestion could represent a viable strategy to enhance MyoPS in older adults without compromising dietary intake and broader nutrition goals.

The current data are consistent with a number of studies showing that increasing the leucine content of a low dose of EAA or protein can enhance the MPS response when measured acutely in older adults at rest (10, 19, 21). Importantly, however, the MyoPS rates we present here reflect integrated rates measured over several days in free-living older adults consuming normal diets and performing their usual daily activities. As such, expanding on previous studies in which MPS was measured acutely (over several hours) under controlled laboratory conditions, our data provide MyoPS rates that are more representative of 'real-world' MyoPS and support a robust effect of leucine supplementation in an applied setting. We propose our findings highlight the potential

clinical relevance of leucine co-ingestion with meals as a practical nutritional strategy for the prevention and management of sarcopenia. Our findings also provide broader support for increased leucine requirements in older persons to maintain muscle mass a concept congruent with higher protein requirements in older persons (40, 41).

Few longer-term (3 - 6 months) randomized controlled trials have examined the influence of chronic leucine supplementation on indices of muscle mass and/or function in older adults (13-15). Of the two studies that did not include exercise, one study involved 3 months of leucine co-ingestion with daily meals (3 meals/d x 2.5 g leucine/meal as 5 x 500mg capsules of crystalline leucine) in healthy elderly men (13), and the other study lasted 6 months with the same dosing regimen in older men with type 2 diabetes (14). Neither study reported a benefit of leucine supplementation in promoting lean mass or strength gains (13, 14). One speculated reason for why leucine supplementation did not enhance muscle mass/strength in the longer-term studies is that the participants in these studies were already consuming protein intakes higher than the RDA (13, 14). The participants in these studies reported habitually consuming protein intakes of ~ 1.0 g/kg/d which are in line with current recommendations for more optimal protein intake in older adults (1.0 - 1.2 g/kg/d) (16, 24). As such, additional leucine may have had a minimal effect in augmenting postprandial MPS (13, 14). Our data argue against this hypothesis and show that leucine co-ingestion with meals was equally as effective in augmenting integrated MPS in those consuming lower (0.8 g protein/kg/d) or higher (1.2 g protein/kg/d) protein-containing diets. An alternative explanation of the inability of leucine to impact muscle mass is be that 3-6 months may simply be too short

a period of time to detect an effect of leucine supplementation on changes in lean mass. At best, in the absence of resistance exercise training, leucine supplementation may be able to offset loss of muscle mass during a supplemental period, which occurs at a rate of ~0.8 %/yr in older adults (42). As such, for a ~85 kg man with ~30-35 kg of muscle mass this translates into a loss of ~120-140 g of muscle in a 6 month period, a change (even if augmented) that would be very difficult to detect using the available methods (i.e., DEXA, CT, and/or measures of muscle fiber area) (43).

It is also possible that the per-meal leucine dose provided in the studies by Verhoeven (13) and Leenders (14) (2.5 g/meal) were sub-optimal to enhance MPS when provided alongside *mixed meals*. Although acute studies in older adults have demonstrated an increase in MPS with the addition of small amounts of leucine (1.1 - 2.5)g) to 'meal-like' doses of EAA and isolated protein (10, 19), the higher dose provided in the current study (5 g leucine/meal) may be necessary to augment MPS when the protein/leucine is ingested with fat and carbohydrate in a solid food matrix. The presence of fat and carbohydrate, as well as food form (i.e. solid versus liquid meals), have previously been shown to influence amino acid digestion and gut absorption kinetics (22, 23). As evidence of this point, we observed that the ingestion of 5 g of crystalline leucine in a liquid form with mixed meals resulted in peak plasma leucine concentrations of ~ 450 nmol/mL which are below the concentrations observed with the addition of just 2.5 g of leucine to a 20 g liquid bolus of isolated casein (~650 nmol/mL) (19) or the ingestion of a leucine-enriched (2.8 g leucine) EAA beverage (~700 nmol/mL) (10). Furthermore, using a higher dose of ~4.5 g leucine/meal English et al. (44) recently reported that

leucine co-ingestion with meals attenuated the decline in muscle strength and endurance in older adults subjected to 2 weeks of bed rest. Overall, the current study provides a strong case for the potential clinical relevance of leucine co-ingestion with meals and emphasizes the need for further chronic leucine supplementation studies using longer intervention periods or larger sample sizes, possibly with higher leucine doses provided per meal.

A possible concern with feeding high concentrations of leucine is that branchedchain amino acid antagonism may result in a decline in plasma and intramuscular concentrations of the other branched-chain amino acids (BCAA), valine and isoleucine, and may limit MPS. BCAA share the same intestinal (45) and transsarcolemmal (46) transporters and the same catabolic enzymes. As such, high leucine intakes could reduce BCAA concentrations by reducing their intestinal absorption and/or uptake into muscle, and by stimulating the activity of enzymes responsible for BCAA catabolism (47). Indeed, previous longer-term leucine supplementation studies have documented declines, albeit within the normal physiological range, in postabsorptive plasma concentrations of valine and isoleucine within the first 2-4 weeks of the intervention with concentrations plateauing thereafter (13, 14). In the present study we observed no clear evidence of declines in postabsorptive or postprandial concentrations of the BCAA and in fact, observed slight increases in postabsorptive concentrations of isoleucine and valine in the HP group. Although postabsorptive isoleucine concentrations declined following 3 d of leucine supplementation in LP the physiological significance of this is unclear given that levels remained within the normal range and were similar to baseline postabsorptive

concentrations in the HP group. Furthermore, integrated MyoPS increased similarly during the leucine treatment in LP and HP suggesting that the lower postabsorptive isoleucine concentration in LP did not hamper the MyoPS response. Nonetheless, we cannot rule out that declines in postabsorptive BCAA concentration with longer-term supplementation may become more physiologically relevant, particularly among those consuming lower protein (and BCAA) diets (i.e. ≤ 0.8 g/kg/d).

It is well established that resistance exercise is a potent stimulus for MPS and resistance training is still considered the most effective and safe treatment for improving muscle mass, strength and function in older adults (48, 49). That leucine co-ingestion with meals further enhanced the stimulatory effect of resistance exercise on MyoPS in the current study indicates that the combination of leucine supplementation and resistance exercise may be a particularly effective strategy for the preservation of muscle mass in older adults. Providing support for this thesis, Trabal et al. (15) recently reported a trend for greater improvements in maximal voluntary contraction and certain markers of functional performance following a 12-wk resistance training program in older nursing home residents and adult daycare attendees that consumed 5 g of leucine (versus a NEAA placebo) 1 h after lunch and dinner. Nevertheless, participant attrition was high in the latter study and further studies should be conducted to explore the potential for leucine supplementation to potentiate resistance training-induced improvements in strength and function in older adults.

Given that the MyoPS data in the current study reflect an integrated rate of synthesis over 3 days it is not possible to determine whether leucine supplementation

increased MyoPS during the postprandial periods, the postabsorptive periods or both. Nevertheless, in view of a large body of previous work showing that increasing the leucine content of a suboptimal dose of protein can augment the postprandial MPS response (10, 19, 21), it seems likely that the postprandial MyoPS was augmented following each of the leucine supplemented meals in the current study. This thesis is in agreement with a growing body of evidence indicating that the increase in intracellular leucine concentrations after dietary protein ingestion is 'sensed', likely by sestrin2, and drives the subsequent MPS response (9, 12, 50, 51). Leucine is known to be a particularly potent stimulator of translation initiation, in large part by activating the mTOR complex 1 (mTORC1) protein kinase (8, 12, 52). Although the exact mechanism(s) by which leucine stimulates mTORC1 signaling remains to be clearly established, recent *in vitro* work demonstrated that leucine disrupts the Sestrin2-GATOR2 interaction by binding to Sestrin2 and liberating GATOR2, a complex necessary for the activation of mTORC1 (9). As such, it is possible that in the present study an increase in intramuscular leucine concentration with leucine co-ingestion may have been 'sensed' by Sestrin2 resulting in an increase in mTORC1 signaling and an up-regulation of translation initiation. Although intramuscular leucine concentrations were not measured in the current study we observed that leucine co-ingestion induced robust elevations in leucinemia indicating an increase in systemic leucine availability (Table 5). As previous work has demonstrated that hyperleucinemia with leucine ingestion or infusion are accompanied by an increase in intramuscular leucine concentrations (12, 53, 54) we speculate that the per-meal dose of leucine provided in the current study was sufficient to raise intramuscular leucine

concentration above those that occurred in the placebo condition and served as the 'trigger' to enhance MyoPS.

That the leucine supplement may also have improved postabsorptive rates of MyoPS cannot be dismissed. Chronic leucine supplementation with meals ($\sim 4 - 4.5$ g leucine/meal) has been shown to augment postabsorptive MPS measured after 2 weeks in sedentary older adults consuming the RDA for protein (30) and in middle-aged adults subjected to bed rest and fed 1 g protein/kg/d (55). Whether postabsorptive rates of MyoPS would have been affected within the shorter timeframe of leucine supplementation in the current study (i.e. 3 d) is unknown. Nevertheless, Dickinson et al. (20) reported that postabsorptive MyoPS remained elevated 24 h after a session of resistance exercise in older men that consumed a leucine-enriched (3.5 g leucine) 10 g EAA mixture post-exercise, whereas MyoPS returned to pre-exercise levels among those that consumed a lower-leucine EAA dose (~ 1.9 g leucine). This suggests that an influence of leucine supplementation on postabsorptive MyoPS may occur quite rapidly, at least when combined with resistance exercise.

An unexpected observation of the current study was the lack of difference between the lower and higher protein groups with respect to MyoPS, in both the resting and resistance exercise conditions. The inadequacy of the current RDA for protein (0.8 g/kg/d) for older adults was demonstrated in a 14-week intervention in which healthy, ambulatory older adults fed the RDA for protein experienced a decrease in mid-thigh muscle area despite no change in body mass (56). These data are consistent with epidemiological evidence showing that older adults in the highest quintile for protein

intake (average intake 1.2 g/kg/d) lost less lean mass over 3 y compared to those in the lowest quintile (average intake of slightly less than 0.8 g/kg/d) (57). The current RDA for protein is derived from a meta-analysis of 19 nitrogen balance studies (58), among which only one studied older adults (59). While the limitations of the nitrogen balance method are well described (60, 61), more recent work has expanded the limited data on protein requirements in the elderly (40, 41). Using the indicator amino acid oxidation (IAAO) technique Rafii and colleagues (41) reported that the mean estimated average requirement (EAR) and upper 95% CI (approximating the RDA) protein requirement of women >65 y were 0.96 and 1.29 g/kg/d, respectively. These findings are in agreement with a study using the same technique in octogenarian women (estimated RDA 1.15 g/kg/d) (40). Thus, while the current RDA (0.8 g/kg/d) may be sufficient to maintain nitrogen balance in healthy people >65 y old (62), the protein requirement to support muscle mass and optimal physiological function and health is likely higher (16, 24). Indeed, a number of expert groups have recently advocated for higher protein intakes of 1.0 - 1.2 g/kg/d in healthy older adults, in particular, to support the preservation of muscle mass and potentially strength (16, 24). However, we did not observe a greater rate of MyoPS in the group consuming 1.2 g protein/kg/d compared to the group consuming 0.8 g protein/kg/d, but both groups saw a rise in MyoPS with LEU, suggesting that protein intakes even higher than 1.2 g/kg/d may be required to enhance MyoPS in healthy older adults. In support of this hypothesis, Kim et al. (63) reported a greater 24-h mixed MPS among older adults that consumed 1.5 g protein/kg/d compared to those that consumed 0.8 g/kg/d. Of particular note in the current study is that despite consuming $\sim 50\%$ more leucine per

day, postprandial plasma leucine concentrations in HP were only slightly higher than in LP and this did not reach statistical significance (Table 5). This is likely attributable to the blunted aminoacidemia that occurs with the ingestion of protein within solid, mixed meals (22, 23) and suggests that peripheral leucine availability may not have been sufficiently different between the two diets to alter the integrated MyoPS response. That we observed an increase in MyoPS when leucine was co-ingested with meals in the group consuming 1.2 g/kg/d further suggests that daily protein (or more specifically leucine) greater than the recent recommendations (16, 24) are required to enhance the MyoPS response and would, perhaps, confer a greater benefit in terms of muscle mass retention in older adults.

In conclusion, our results show that the co-ingestion of 5 g of leucine with daily meals enhanced the integrative MyoPS measured over a 3 d period in free-living older men and was equally effective among those consuming daily protein intakes above (1.2 g /kg/d) and at (0.8 g/kg/d) the protein RDA. Furthermore, we show that leucine co-ingestion augments integrative MyoPS under rested (non-exercised) conditions and further enhances the anabolic effect of resistance exercise. Although further work is required to determine whether the increase in MyoPS is maintained with prolonged leucine supplementation and translates into a long-term functional response, we propose that leucine co-ingestion has the potential to be a simple dietary strategy to mitigate muscle mass loss in older adults. Our data suggest that this could be an effective strategy among elderly individuals in whom protein intake is habitually low, or restricted due to comorbid conditions, and among those consuming apparently adequate protein intakes,

particularly when combined with resistance exercise training.

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CHAPTER 5:

GENERAL DISCUSSION

5.1. Introduction

The hyperaminoacidemia, and in particular the hyperleucinemia, following the ingestion of dietary protein is potent stimulus for a transient (~2-3 h) increase in muscle protein synthesis (MPS) (1, 2). The relationship between the quantity of protein consumed within a meal and the subsequent MPS response is a saturable dose-response process. The existence of this relationship would, it is proposed, mean that to optimize MPS protein intake should be considered on a *per-meal* basis over the day (3). This may be particularly important for older adults who are less anabolically sensitive to protein/leucine ingestion than their younger counterparts (3, 4) and are experiencing age-related muscle loss (i.e., sarcopenia). Acute studies have demonstrated that meal-based strategies, namely optimizing the protein dose within a meal and/or increasing the leucine content of a smaller suboptimal quantity of protein, can augment the muscle protein synthetic response over several hours (\sim 4-6 h) in older adults (3, 5-7). This has led to widespread speculation that implementation of these strategies at every meal consumed within a day would enhance the cumulative MPS response over time and, consequently, may offset sarcopenic muscle loss (8-10). Critically, however, the integrated MPS responses to these strategies over days/weeks remains largely unexplored, particularly in free-living settings.

The broad aim of the present thesis was to advance our knowledge and understanding of the potential for practical, meal-based protein intake strategies to augment MPS in older adults. Furthermore, we aimed to determine whether the MPS responses to these meal-based approaches could be further enhanced when combined with the performance of resistance exercise. An important aspect of the present thesis is that

we used novel methods to measure the longer-term, integrated MPS responses to these meal-focused strategies in free-living older men, thus improving the relevance of our findings to the real world. We also implemented, for only the second time in humans as far as we are aware, a proteomic analytical method that allowed the calculation of synthetic rates of individual skeletal muscle proteins. These data have not been reported before in older persons nor with the performance of RT.

In the first study (Chapter 2) we demonstrated that a balanced/even pattern of isolated protein over the day stimulated acute myofibrillar protein synthesis (MyoPS; %/h) more effectively than a traditional, skewed protein intake pattern following 2-wk of energy restriction (ER) and following 2-wk of energy restriction combined with resistance exercise training (ER + RT) in overweight/obese older men. Expanding on the acute (%/h), laboratory-based findings from Study 1, in Study 2 (Chapter 3) we examined the influence of daily protein intake pattern (balanced versus skewed) on the longer-term integrated response (%/d) of MyoPS and the synthesis individual skeletal muscle proteins over 2-wk of ER alone and 2-wk of ER + RT in the overweight/obese older men that took part in Study 1. In contrast to our acute findings from Study 1 we observed no influence of protein intake pattern on longer-term MyoPS during ER. Nonetheless, highlighting the potency of RT even under conditions of ER, we observed a stimulatory effective of RT on longer-term MyoPS in both groups and this increase was *qualitatively* greater in the balanced group. In Study 3 (Chapter 4) we demonstrated that leucine co-ingestion with the daily meals enhanced integrative MyoPS (%/d) over a 3-d period in older men and was equally effective among those consuming higher (1.2 g/kg/d) and lower (0.8 g/kg/d)

protein intakes. In agreement with Studies 1 and 2, we showed that the stimulatory effect of per-meal leucine supplementation on integrative MyoPS was potentiated by the performance of resistance exercise.

Collectively, these findings demonstrate the potential for meal-based strategies to augment MyoPS in older adults and emphasize the potency of resistance exercise to enhance the efficacy of these nutritional approaches. The present chapter attempts to integrate findings from all studies and highlight the collective contribution of the thesis to the larger field. Potential limitations and future directions are discussed.

5.2. Influence of protein intake pattern on acute measures of myofibrillar protein synthesis during energy restriction in older men

The saturable, dose-responsive relationship between the amount of protein consumed in a meal/food bolus and the postprandial MPS response (11, 12) has led to speculation that a balanced/even distribution of total protein intake over a person's daily meals, whereby a maximally stimulatory dose of protein is consumed at each meal, may augment the cumulative MPS response over the day (8). Such a strategy may be particularly important during weight loss when loss of muscle mass is accelerated (13). In support of this hypothesis in Study 1 we observed that during ER in overweight/obese older men, consumption of a balanced distribution of 75 g of whey protein (i.e. 3 x 25 g evenly spaced doses of protein) acutely stimulated MyoPS more effectively than a skewed distribution of the same amount of protein (i.e. 10 g at 'breakfast', 15 g at 'lunch', 50 g at 'dinner') (14).

In agreement with previous work in younger and middle-aged adults (15-17) we observed that short-term ER suppressed postabsorptive and postprandial MyoPS in older adults (14). Together these data suggest that a down-regulation of MyoPS may account for, or at least contribute to, the decline in muscle mass that typically accompanies prolonged energy restriction (18). This highlights the importance of maintaining the MyoPS response during ER, particularly among older adults in whom muscle mass loss may exacerbate sarcopenia. While we observed that the ER-induced reduction in MyoPS was less pronounced when protein intake was consumed in a balanced/even pattern compared to a traditional, skewed pattern during ER alone, the balanced pattern of protein consumption was even more effective when combined with RT, resulting in the restoration of MyoPS to levels observed during energy balance (EB). Based on these data it is tempting to suggest that the combination of RT and a balanced distribution of daily protein may represent an effective strategy to support weight loss with a high fat-tolean ratio in older adults. Nevertheless, the acute (13 h), laboratory-based nature of this study limits our ability to extrapolate these findings to the free-living setting.

The mechanism(s) underpinning the superior capacity of the balanced protein distribution to stimulate MyoPS over the day in ER cannot be determined from Study 1. Due to important ethical issues surrounding the number of skeletal muscle biopsies that could be obtained we were unable to measure postprandial MyoPS following each protein 'meal'. Nevertheless, given that protein ingestion has been shown to transiently stimulate MPS in a dose-responsive fashion, it appears likely that the acute MyoPS response was greater following breakfast and lunch in the balanced group when larger whey protein

doses were consumed (i.e., 25 g) compared to the skewed group (i.e., 10 g at breakfast, 15 g at lunch) (12, 19). Previous work has shown that hyperaminoacidemia and hyperleucinemia stimulate MPS and the increase in leucine availability, in particular, appears to play a central role directing the magnitude of the MPS response, especially in older adults (2, 7). As such, the larger leucinemia that we observed following breakfast and lunch in the balanced group compared to the skewed group could account for a greater stimulation of MyoPS following these meals.

In contrast to the ER and ER+RT conditions, we observed no influence of protein intake pattern under conditions of energy balance (EB). One possible explanation for this, and indeed a limitation of Study 1, is the dose of protein provided per meal. The decision to provide 25 g of whey protein/meal to the balanced group was based on the available evidence at the time the study was designed suggesting that 25 - 30 g of high quality protein/meal would be sufficient to maximize the postprandial MPS response in older adults (20, 21). Since, Moore et al. (3) have conducted a breakpoint analysis using MyoPS data from six published studies and have refined the maximally stimulatory protein dose to 0.40 g/kg body mass [BM]/meal or 0.61 protein/kg fat-free mass [FFM]/meal for healthy older adults. As the participants in Study 1 were overweight and obese (and consequently of high body mass), this recommendation would be equivalent to \sim 40 g/meal on average, with estimated optimal intakes for individual participants in our study ranging from 34 - 48 g/meal. As such, the per-meal protein dose of 25 g of whey protein consumed by the balanced group was likely insufficient to maximally stimulate postprandial MyoPS and it is therefore perhaps unsurprising that we did not observe a

greater MyoPS response in the balanced group compared to the skewed group during EB. It remains possible however, that a balanced protein distribution, with a maximally stimulatory protein dose per meal, would stimulate MyoPS to a greater extent than a skewed distribution under conditions of EB. In support of this hypothesis Mamerow et al. (22) reported that in young adults, in whom ~0.24 g protein/kg BM/meal has been reported to maximally stimulate MyoPS (3), the consumption of ~0.39 g protein/kg BM/meal stimulated 24-h mixed MPS to a greater extent than skewing protein intake toward the evening meal (i.e., 0.13 g/kg BM at breakfast, 0.20 g/kg BM at lunch, 0.85 g/kg BM at dinner) during EB (28). Adding further support to this contention, Norton et al. (23) recently reported that increasing protein intake to ≥ 0.4 g protein/kg BM at each of the three daily meals (by supplementing protein at the lower protein breakfast and lunch meals) resulted in a gain in appendicular skeletal muscle mass (ASMM) after 24 wk in weight stable older adults. As such, the suboptimal per-meal protein dose provided to the balanced group in our Study 1 may have hampered our ability to observe an enhancement of MyoPS above the skewed pattern during EB.

Intriguingly, the fact that we observed a greater acute MyoPS response with a balanced than a skewed protein distribution under conditions of ER and ER+RT in the Study 1, despite a potentially suboptimal per meal protein dose, indicates that the distribution of daily protein likely becomes increasingly important in weight loss situations in older adults. The potential mechanisms mediating an increased sensitivity of skeletal muscle to protein intake pattern during ER are unclear and cannot be ascertained from the present thesis. Nevertheless, this finding indicates that optimizing per-meal

protein intake during ER warrants further study as a potential strategy to maintain the MyoPS response during weight loss in older adults.

5.3. Influence of protein intake pattern on longer-term, integrated measures of myofibrillar protein synthesis during ER in older men

In Study 1 we obtained sensitive, acute measures of MyoPS in response to divergent patterns of protein intake (i.e., balanced versus skewed) under tightly controlled laboratory conditions. While this study provided important 'proof of principal' information these measurements do not account for all aspects of activity and diet that accompany day-to-day life, which limit our ability to extrapolate these findings to freeliving older adults. Study 2 built on the results of Study 1 by examining the impact of protein intake pattern on the integrated, longer-term MyoPS (%/d) and skeletal muscle proteome kinetics over 2-wk of ER alone and 2-wk of ER+RT in free-living overweight and obese older men. Contrary to our previous acute findings in Study 1 and to our hypothesis, we found no influence of protein intake pattern on integrative MyoPS or proteome kinetics.

There are a number of reasons that may account for this disparity between the acute MyoPS results from Study 1 and the longer-term MyoPS responses measured in Study 2. In Study 1 we measured MyoPS using the primed, continuous intravenous infusion of an isotopically labeled amino acid (i.e. L-[ring- $^{13}C_6$]phenylalanine), whereas in Study 2 we used the oral administration of deuterium oxide ($^{2}H_{2}O$ or D₂O). Recently, these two methods were simultaneously employed to acutely measure postabsorptive (3

h) and postprandial (3 h) MyoPS in the same individuals and were reported to yield comparable mean MyoPS rates (24). As such, it appears unlikely that differences in the validity of the two methods per se accounts for the inconsistency between our acute and longer-term findings. A more likely explanation for the discrepancy, as discussed in Chapter 3, is that fact that the conditions under which the acute and longer-term measurements were obtained were different. For example, during the acute study MyoPS (%/h) was measured under tightly controlled laboratory conditions in which participants rested quietly for the duration of each infusion trial (13-h) and consumed precisely timed, but differently sized, feedings of whey protein. As longer-term MyoPS (%/d) was measured over a 2-wk free-living period in Study 2 it follows that we did not have control over a number of variables that could potentially modify MyoPS such as activities of daily living, stress and sleep duration. Moreover, an important finding from our acute study (Study 1) was that the influence of protein intake pattern on MyoPS was specific to the fed and not the fasted rates of MyoPS (14). Since the longer-term measures of MyoPS integrate postabsorptive and postprandial periods it is likely that the postabsorptive periods, where MPS would ostensibly be similar in the skewed and balanced groups, particularly during the extended overnight fasting period would have 'diluted' feedingspecific effects on MyoPS.

Similar to Study 1, a limitation of Study 2 is the suboptimal dose provided per meal to the balanced group which may have hampered our ability to tease out an effect of protein intake pattern. As mentioned previously, at the time the study was designed the available evidence suggested that $\sim 25 - 30$ g of high quality protein/meal would be

sufficient to maximize the postprandial MPS response in older adults (20, 21). Since then evidence has emerged to indicate that protein doses equivalent to ~ 0.4 g protein/kg BM/meal or 0.6 g protein/kg FFM/meal are required to optimally stimulate MyoPS in older persons (3). Although we provided participants in the balanced group with ≥ 30 g protein/meal, this equated to 0.33 g/kg BM/meal (0.47 g/kg FFM/meal) which falls below maximally stimulatory dose for MPS (3). A further consideration is that the protein in Study 2 was consumed in meals that contained carbohydrate, fat, and fiber. These factors are associated with alterations in amino acid digestion and absorption kinetics leading to reduced plasma amino acid availability (25, 26). Differences in the pattern of aminoacidemia, and in particular leucinemia, have been shown to affect the subsequent MPS response with large, transient rises in plasma leucine concentration typically being associated with a greater postprandial MPS response (27, 28). As a result, the quantity of protein required to maximally stimulate MPS in a *mixed meal*, as opposed to recommendations based on consumption of *isolated* proteins, may be even higher than the optimal level determined by Moore et al. (3).

Notwithstanding that we likely provided a less-than-optimal per-meal protein dose, it is noteworthy that we observed a *qualitatively* greater rate of MyoPS in the balanced group compared to the skewed group during ER + RT. Indeed, although not statistically significant, the integrated MyoPS over the 2 wk of ER + RT was ~9% higher in the balanced group than the skewed group (p >0.05) a difference we may have been underpowered to detect. Consistent with Study 1, these data indicate that a balanced pattern of protein intake may be particularly effective for promoting MyoPS during ER

when combined with RT. In a 6-month weight loss intervention, McDonald et al. (29) recently examined the influence of enhanced per-meal protein intake on physical function and body composition in obese, functionally limited older adults. Specifically, 67 participants were randomized to consume either a balanced distribution of protein intake, with the consumption of an optimal quantity of protein at each of the 3 daily meals (protein intakes were 0.53, 0.69, 0.81 g/kg FFM at breakfast, lunch and dinner, respectively), or to consume a 'traditional' (0.8 g protein/kg/d, skewed distribution) diet. Despite similar amounts of weight loss in each group, the older adults that consumed optimal per-meal protein had greater improvements in physical function relative to the 'traditional diet' group. Although losses in lean mass at the end of the 6 month weight loss period were not statistically different between intervention diets ('traditional' diet: -1.8 kg, 'optimal per-meal protein' diet: -1.1 kg) the authors highlighted that sensitivity limitations associated with the method of body composition assessment (i.e. BOD POD) may have affected their power to detect a statistically significant difference between groups (29). Exercise was not included in the latter study but taken collectively with our findings from Studies 1 and 2 we speculate that a balanced pattern of optimal per-meal protein intake combined with RT during energy restriction may represent an effective strategy to mitigate muscle mass loss and optimize improvements physical function in obese older adults. This thesis clearly warrants further investigation in longer-term randomized controlled trials. Moving forward, it is of considerable clinical relevance to refine the optimal doses of protein required to maximally stimulate MPS for older adults in the context of food-based meals, both under conditions of energy balance and energy

restriction. These data would provide important information for the design of longer-term studies to elucidate the influence of protein distribution on clinical outcomes and, ultimately, allow for the development of protein distribution pattern guidelines for older adults.

5.4. Leucine supplementation with meals as a strategy to enhance integrated myofibrillar protein synthesis in older men

Older adults require higher doses of protein (~0.4 g/kg BM/meal) to maximally stimulate postprandial MyoPS compared to younger adults (3). As it may not be feasible for many elderly individuals to regularly consume such large per-meal protein doses (30), in Study 3 we examined the capacity for leucine co-ingestion with meals to augment the integrative MyoPS response without increasing the total amount of protein consumed. We observed that the ingestion of 5 g of crystalline leucine concomitant with the normal daily meals enhanced the integrated rate of MyoPS measured over 3 d, in both rested and resistance exercise conditions. Furthermore, we found that this strategy was equally effective among those consuming total protein intakes at the RDA (0.8 g/kg/d) and in line with optimal protein intake recommendations (1.2 g/kg/d) for in healthy older adults (10, 31).

The strategy of leucine supplementation as a potential countermeasure for sarcopenia is somewhat controversial in the literature. Acute MPS studies have demonstrated that older adults are less sensitive to the stimulatory effects of leucine ingestion and that higher doses of leucine may restore postprandial MPS rates to those
observed in the young (7). Indeed, the capacity for leucine supplementation to augment the *acute* MPS response to a suboptimal protein dose in older adults has been consistently reported in the literature (6, 32, 33). Nevertheless, longer-term (3 - 6 months) leucine supplementation of meals has not been associated with increases in muscle mass or strength in older adults (34, 35) suggesting that the acute observations may not translate into a longer-term enhancement of MPS and muscle mass. Indeed, comparison of our results from Studies 1 and 2 highlight that acute, laboratory-based responses of MyoPS cannot necessarily be directly translated into dietary strategies and expected to produce an equivalent longer-term response in free-living situations. Our data from Study 3 help to bridge a gap between the acute MPS studies and the long-term RCTs and indicate that leucine co-ingestion with normal, mixed meals does indeed lead to a cumulatively greater MyoPS response over several days in healthy older men. This suggests, as discussed in Chapter 4, that limitations associated with the longer-term trials (i.e., study duration, sensitivity of methods used to assess muscle mass, leucine dose) may account for the absence of benefits observed with chronic leucine supplementation (34, 35).

Given that the MyoPS data in Study 3 reflect an integrated rate of synthesis over 3 days it is not possible to determine whether leucine supplementation increased MyoPS during the postprandial or the postabsorptive period or both. Nevertheless, in view of a large body of previous work showing that increasing the leucine content of a suboptimal dose of protein can augment the postprandial MPS response (6, 7, 32), it seems likely that the postprandial MyoPS was enhanced following each of the leucine supplemented meals. A growing body of data indicates that the postprandial increase in leucine availability

plays a key role in 'triggering' translation initiation of MPS and may direct the magnitude of the response (2). Indeed, we observed that the higher integrated MyoPS rate in the leucine treatment was accompanied by a 3-fold increase in postprandial leucinemia compared to the placebo treatment. That we observed an increase in MyoPS with leucine supplementation of meals in the absence of an increase in protein intake is consistent with the notion that the 'dose response' of MyoPS to protein may not be driven by protein or even essential amino acid (EAA) content *per se* but instead by leucine content, provided sufficient quantities of other EAA are available to serve as precursors for the synthesis of new muscle proteins (33). This highlights that the per-meal leucine content is likely of critical importance when designing strategies to maximize MyoPS in older adults.

In addition to a potential increase in postprandial MyoPS with leucine supplementation of meals, it is also possible that the greater integrated response may relate to an increase in postabsorptive rates of MyoPS. Indeed, chronic leucine supplementation with meals (\sim 4 – 4.5 g leucine/meal) has been shown to augment postabsorptive MPS measured after 2 weeks in sedentary older adults consuming the RDA for protein (36) and in middle-aged adults subjected to bed rest and fed 1 g protein/kg/d (22). However, the potential mechanisms that may explain an increase in postabsorptive MyoPS with leucine supplementation of meals are less clear and require future study.

It is noteworthy that we observed that leucine supplementation of meals was equally effective among participants consuming higher (1.2 g/kg/d) and lower protein (0.8 g/kg/d) diets. This supports the possibility that leucine supplementation of meals

could be an effective strategy to enhance MyoPS in older adults consuming apparently adequate protein intakes as well as those in whom protein intake is low/restricted. The diets in Study 3 provided protein (and leucine) in a skewed pattern across the daily meals in order to emulate the manner in which protein is typically consumed over the course of the day in older adults (30). This meant that, even in the higher protein group, per-meal protein intakes were below the 0.4 g/kg BM dose reported to maximally stimulate MyoPS in older adults at every meal except for dinner (3). As such, it is possible that a more even distribution of daily protein (and leucine) across the meals in the higher protein group (resulting in the consumption of ~0.4 g protein/kg BM/meal, ~ 2.7 g leucine/meal) could have improved the integrated MyoPS response, with the result that leucine supplementation of meals may have no effect on MyoPS. Such a thesis, however, requires examination in future studies.

As discussed previously, the potential for fasted periods (particularly the overnight period) to 'dilute' feeding-specific effects when MyoPS is integrated over longer periods may have hampered our ability to detect a difference between the balanced and skewed protein intake patterns in Study 2. It is noteworthy that, despite the potential 'dilution' we observed a robust influence of leucine supplementation on integrative MyoPS, highlighting the potency of this strategy. Alternatively, as has been reported previously (36, 37), leucine supplementation may have improved postabsorptive MyoPS rates which would have mitigated a potential 'dilution' effect and improved our ability to detect an effect of the nutritional intervention in Study 3. In addition, our decision, which was based on our experiences with Study 2, to measure the integrative response to leucine

supplementation over a shorter time frame (i.e. 3 d versus 2 wk) allowed us greater control over some of the external factors that may have influenced the integrated response. For example compliance with the study diet was improved in Study 3 and daily activity was more rigorously controlled which may have reduced the variability in our integrated MyoPS measurements and improved our ability to measure an effect of the leucine supplementation.

One of the limitations of Study 3 is that all participants underwent placebo and leucine supplementation in the same order (i.e., 3-d of placebo treatment, followed by 3-d of leucine treatment). The order of the supplementation periods was designed to avoid any potential chronic influence of prior leucine supplementation on the MyoPS response, as has been reported previously (36), during the placebo period. While a washout period between treatments could have allowed us to counterbalance the order in which participants received the treatments this would have resulted in the requirement for additional muscle biopsies and participant burden. Furthermore, the duration of potential chronic effects of leucine supplementation are currently unknown. The alternative was a between group design which would have necessitated four separate groups (i.e., higher and lower protein, placebo and leucine) which would have increased between-subject variability. As we had no reason to think that the order of the treatments would affect the MyoPS response we opted for a repeated measured design. Another limitation of Study 3 is that we measured integrative MyoPS in response to a relatively short period (3-d) of leucine co-ingestion with meals. As such, it is unclear whether the higher rate of integrated MyoPS we observed would persist with prolonged supplementation. A possible concern with feeding high concentrations of leucine is branched-chain amino acid antagonism and prolonged leucine supplementation has been shown to result in a decline in postabsorptive plasma concentrations of the other branched-chain amino acids (BCAA), valine and isoleucine (34, 35). Although the physiological relevance of this is questionable given that concentrations do not fall below the normal range we cannot rule out that this could limit MyoPS.

5.5. Resistance exercise potentiates the effect of per-meal strategies on myofibrillar protein synthesis

A consistent finding throughout this thesis has been that resistance exercise potently enhanced the stimulatory effect of meal-focused protein intake strategies on MyoPS in older adults. This is likely accounted for by the fact that resistance exercise sensitizes the muscle protein synthetic machinery to protein feeding resulting in more of the ingested amino acids being incorporated into newly synthesized skeletal muscle protein (38). In Study 1 RT further enhanced the stimulatory effect of a balanced distribution of protein intake on acute MyoPS during ER, despite the last session of exercise having been performed 48 h before the isotope infusion trial. This indicates that the enhanced sensitivity of MyoPS to protein ingestion persists for at least 2 days following a resistance exercise session in older adults, which is consistent with findings of increased sensitivity to amino acid ingestion 24 h after exercise in young adults (39). The long-term stimulation of MPS would indicate that prior resistance exercise can augment the MyoPS response, not only to the post-exercise meal, but to every meal

consumed over a ~2 day period following the exercise. This likely explains why just one session (3 sets) of leg extension exercise was sufficient to further augment the influence of leucine supplementation on the integrated rate of MyoPS measured over a 3 d period in Study 3. Furthermore, although we did not observe an effect of protein intake pattern on integrated MyoPS during ER alone in Study 2, we observed a ~9% higher rate (albeit not statistically significant; p = 0.75) of longer-term MyoPS in the balanced versus the skewed group during ER + RT, suggesting that RT may have 'accentuated' a subtle anabolic effect of the balanced protein intake pattern.

Collectively, the studies in this thesis indicate that a combining regular RT with per-meal strategies may an effective approach to enhance MyoPS in older men. These studies demonstrate the potency of RT under conditions of ER (Studies 1 and 2) as well as EB (Study 3) and provide further support for the inclusion of RT as a strategy for muscle mass preservation/gain in the management of sarcopenia and sarcopenic obesity.

5.6. Conclusions

Sarcopenia and sarcopenic obesity are incongruent with active and healthy aging and have considerable personal, societal and economic consequences. As the number of older adults continues to increase globally (40), the need to identify strategies for the prevention and management of these conditions is becoming even more urgent. The current thesis examined the potential for meal-focused protein intake strategies to enhance acute and longer-term rates of MyoPS in older adults in the presence and absence of resistance exercise. Collectively, these studies support the potential for per-meal

strategies, namely optimizing the per-meal protein dose and leucine co-ingestion with lower protein-containing meals, to augment MyoPS, especially when combined with RT.

In the current thesis we employed stable isotope infusion techniques to obtain acute (several hours) measures of MyoPS, as well as the more novel oral D₂O ingestion method to obtain longer-term integrative rates of MyoPS (several days to weeks). While the acute infusion technique is most commonly used and provides excellent proof of principal data, longer-term measures of MPS in free-living individuals are more relevant to 'real life' and are expected to more accurately predict longer-term changes in muscle mass and function (41, 42). As such, the measurement of the longer-term MyoPS responses to the nutrition and exercise strategies examined in the current thesis improves the applicability and practical significance of our findings. Nonetheless, long-term investigations examining outcomes such as muscle mass, strength and function are necessary to determine the clinical relevance of meal-based protein intake strategies, with and without RT.

5.7. References

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